IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

U.S. Patent No. 8,822,148	§	
	§	
Issued: September 2, 2014	§	
	§	
Filed: July 31, 2012	§	Attorney Docket No.: P50033US00
	§	
For: METHOD OF PERFORMING PCR REACTION IN CONTINUOUSLY FLOWING MICROFLUIDIC PLUGS	§	
	§	

REQUEST FOR *EX PARTE* REEXAMINATION OF U.S. PATENT NO. 8,822,148

Mail Stop *Ex Parte* Reexam
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Commissioner:

Pursuant to the provisions of 35 U.S.C. § 302 and 37 C.F.R. § 1.510 et seq., the undersigned, on behalf of 10X Genomics, Inc., requests *ex parte* patent reexamination of claims 1-8 of U.S. Patent No. 8,822,148 ("the '148 patent," Exhibit 1001).

Ex Parte Patent Reexamination Filing Requirements

Pursuant to 37 C.F.R. § 1.510(b)(1), statements pointing out at least one substantial new question of patentability based on material, non-cumulative prior art patents for claims 1-8 of the '148 patent are provided in Section VI of this Request. Although some of these prior art references were previously cited in the record during the *ex parte* prosecution of the '148 patent, these references have not been considered in the new light demonstrated by the proposed substantial new questions of patentability.

Pursuant to 37 C.F.R. § 1.510(b)(2), reexamination of claims 1-8 of the '148 patent is requested, and a detailed explanation of the pertinence and manner of applying the cited prior art to claims 1-8 is provided in Section VII of this Request.

Pursuant to 37 C.F.R. § 1.510(b)(3), copies of every patent relied upon or referred to in the statement pointing out each substantial new question of patentability or in the detailed explanation of the pertinence and manner of applying the cited prior art are provided as Exhibits 1002-1114 of this Request.

Pursuant to 37 C.F.R. § 1.510(b)(4), a copy of the '148 patent is provided as Exhibit 1001 of this Request.

Pursuant to 37 C.F.R. § 1.510(b)(5), the attached Certificate of Service indicates that a copy of this Request, in its entirety, has been served on Patent Owner at the following address of record for Patent Owner, in accordance with 37 C.F.R. § 1.33(c):

Brown Rudnick LLP
One Financial Center
Boston, MA 02111

Also submitted herewith is the fee set forth in 37 C.F.R. § 1.20(c)(1).

Pursuant to 37 C.F.R. § 1.510(b)(6), Requester hereby certifies that neither the statutory estoppel provisions of 35 U.S.C. § 315(e)(1) nor 35 U.S.C. § 325(e)(1) prohibit Requester from filing this *ex parte* patent reexamination request.

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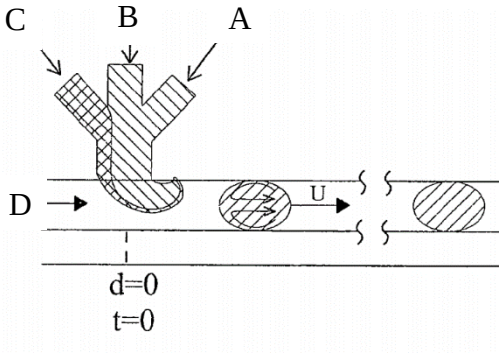
I. INTRODUCTION

This request for reexamination and the proposed grounds of rejection raised herein are supported by a declaration from Dr. Eric Shaqfeh, Chair of Stanford's Department of Chemical Engineering and author of one hundred journal articles relating to fluid mechanics. *See* Ex. 1004. Dr. Shaqfeh's declaration summarizes and reflects his knowledge, technical expertise, and understanding of the scope and content of the prior art applied in this request for reexamination. *Id.* at ¶¶ 2-3, 35-44, MPEP § 2258. The state of the art at the time of the filing of the '148 patent is presented by Dr. Shaqfeh in this request for reexamination.

The '148 patent is generally directed to microfabricated substrates and methods of conducting reactions within these substrates. Ex. 1001 at Abstract. As shown in Fig. 2A of the '148 patent, reproduced at right, the reactions occur in plugs transported in the flow of a carrier-fluid. *Id.* at 11:64-12-8, 17:37-41, Fig. 2A. In a preferred embodiment, two aqueous reagents form laminar streams that are separated by a "divider" aqueous stream. *Id.* at 17:37-41. The three streams enter a channel with flowing oil D, at which point plugs form and plug fluids mix. *Id.* at 17:41-43. The '148 patent claims a method for performing a polymerase chain reaction (PCR) in the droplets.



As discussed in Section IV.C, *infra*, the prosecution history taken as a whole indicates that the '148 patent was allowed primarily because the Examiner believed that the prior art failed to teach conducting reactions in droplets formed from two continuously flowing immiscible fluids. Significantly, the Examiner was not made aware that '148 patent's disclosure concerning

forming plugs from continuous streams of immiscible fluids was **copied almost verbatim from one of the prior art references of record**. See Sections III.A and IV.C, *infra*.

The PTAB denied institution of an *inter partes* review of U.S. Patent No. 8,822,148 because the petition failed to explain why a skilled artisan would have believed that the PCR technique of Haff could have been conducted on a microfluidic scale as taught in Quake. *See* Section IV.D, *infra*. Accordingly, the claims have been considered nonobvious by the Office because the **prior art was not believed to teach a technique for conducting PCR reactions in droplets formed from two continuously flowing immiscible fluids**.

As discussed in Section VI, *infra*, the prior art submitted herewith (much of which was not previously presented to the Office) shows that i) droplet micro reactors that formed plugs from continuous flowing immiscible fluids were well known, ii) microfluidic PCR devices were well known, and iii) there were compelling reasons to modify the known microfluidic droplet reactors to conduct the known continuous flow PCR reactions.

The Quake, Burns I and Shaw Stewart references show that droplet microreactors which formed plugs from continuous flowing immiscible fluids were well known. As noted by the Patent Trial and Appeal Board (PTAB), Quake (Ex. 1033) discloses a droplet reactor in which droplets are formed by continuous streams of immiscible fluids. Indeed, **Patent Owner copied this subject matter almost word-for-word from the Quake reference;** a fact about which the Office has not yet been made aware. Burns et al. also shows that plugs in microchannels also could be readily formed by continuous flow of fluids at an intersecting channel. Ex. 1007 (Burns I). The Shaw Stewart British Application (Ex. 1040) also teaches conducting reactions in droplets formed from two continuously flowing immiscible fluids. Significantly, the exclusive licensee of the ‘148 patent affirmatively (and correctly) took the position that Shaw Stewart

teaches plug or droplet formation combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid. More particularly, Bio-Rad (who recently acquired RainDance, the original exclusive licensee of the '148 patent, Ex. 1061, Ex. 1053 ¶71) explained that another party had mischaracterized the Shaw Stewart reference by failing to point out the portion of Shaw Stewart, in which Shaw Stewart specifically teaches that in certain embodiments droplets are formed from intersecting and continuously flowing streams. Thus, the exclusive licensee (Bio-Rad) expressly agreed that Shaw Stewart discloses forming plugs from intersecting streams of continuously flowing immiscible fluids.

As of the earliest claimed priority date, many groups had demonstrated that PCR could be successfully performed on a microfluidic scale, as demonstrated by Corbett, Burns II, Kopp, Lagally and Vogelstein. In 1991, eleven years prior to the earliest effective filing date, Corbett disclosed using a microchannel device to conduct DNA amplification reactions such as PCR by injecting plugs of reactants into a carrier stream that is passed through different temperature zones. Ex. 1010 (Corbitt). In 1996, Mark A. Burns from the University of Michigan reported the use of microfluidic devices to perform PCR. Ex. 1008 (Burns II). By 1998, Kopp et al. had successfully implemented continuous flow PCR on a chip. Ex. 1027 (Kopp). By 1999, Vogelstein et al. had reported various applications for single-molecule microfluidic PCR. Ex. 1044 (Vogelstein). By 2001, Lagally reported using microfluidic PCR to amplify single-molecule of DNA template to, among other things, detect rare genetic mutations. Ex. 1028 (Lagally). Corbett, Kopp, Burns II, and Vogelstein show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the combinations proposed herein, the microfluidic droplet reactor of Quake, Burns I and Shaw Stewart are relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[1] Ex 1001 at 78:22-24. In the proffered combinations, these limitations are met by using the microfluidic droplet reactors to perform PCR reactions, wherein each droplet or slug is created using continuous flow of two immiscible fluids and includes an aqueous mixture of DNA (the biological molecule) and reagents, *e.g.*, polymerase, primers, buffers and the like. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake, Burns I or Shaw Stewart to conduct microfluidic PCR as taught by Corbett, Burns II, Kopp, Lagally or Vogelstein. Ex. 1004 ⁋⁋109,111. Stated a different way, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic reactor of Quake, Burns I or Shaw Stewart to perform continuous flow droplet formation. *Id.* Moreover, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing, one would have a high expectation of success for the performance of PCR using the droplet reactors of Quake, Burns I or Shaw Stewart. *Id.*

A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic reactors of Quake, Burns I or Shaw Stewart because doing so has provided the substantial benefits known to be associated with such microfluidic reactors. *Id.* ¶109,111. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays

[1] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. In addition, decreasing the scale of PCR allows the reaction to be carried out **more efficiently**, producing more product in **less time** with **less side reactions**." Ex. 1013 at 192. It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Skilled artisans would also have recognized that performing Corbett's PCR reaction using two continuous immiscible fluids in the microfluidic reactors of Quake, Burns I or Shaw Stewart would **enhance the precision of the PCR reaction with a given primer** relative to then-traditional approaches. Ex. 1024 at 841. Moreover, using the microfluidic reactors for PCR reactions to create many droplets would have **substantially increased the tolerance of PCR reactions to primer non-specificity**. Ex. 1044 at 9236, 39; Ex. 1004 ¶83. This reduction in signal-to-noise ratio **enabled detection of rare mutations, dislocations and allelic imbalances**. *Id.*

Moreover, a skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Corbett, Kopp, Burns II, Lagally or Vogelstein) to incorporate a droplet reactor (Quake, Burns I or Shaw Stewart). Ex. 1004 ¶110. Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the microfluidic PCR devices taught in Kopp, Burns II, Lagally or Vogelstein. *Id*. ¶98-108. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent**

compared to the single fluid in microfluidic chip reactor (e.g., Burns II or Lagally) or droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Corbett or Kopp). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Further, a skilled artisan would have **fully expected these combinations to be successful**. Ex. 1004 ¶111. In 1998, four years prior to the earliest claimed priority date, Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, and continuous separations) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." Ex. 1027 at 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001, article Lagally *et al.* provided an overview of that evolution and further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR. Ex. 1028. Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date.

As further evidence that a skilled artisan would have expected to be successful in miniaturizing and modifying Corbett or modifying Kopp or Lagally to work in the microfluidic reactor of Quake, Burns I or Shaw Stewart, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex.

1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.

Section VI, *infra*, establishes that a reasonable Examiner consider the foregoing teachings relevant in deciding whether claims 1-8 of the '148 patent are patentable.

Section VII, *infra*, provides a detailed explanation of how the teachings apply to the claims of the '148 patent and demonstrates that the claims of the '148 patent should be rejected as obvious.

II. RELATED AND CO-PENDING PROCEEDINGS; ESTOPPEL

Neither 35 U.S.C. § 315(e)(1) nor 35 U.S.C. § 325(e)(1) prohibit Requester from filing this *ex parte* patent reexamination request. Only the former is relevant here, as the latter applies to Covered Business Method Review proceedings, which have not occurred relative to the '148 patent.

The '148 patent is currently the subject of *Bio-Rad Laboratories, Inc. et al v. 10X Genomics, Inc.*, Case No. 1-15-cv-00152 (D.Del.). Ex. 1053. RainDance Technologies and the University of Chicago brought suit against 10X Genomics, Inc. for infringement of the '148 patent in the District Court for the District of Delaware. *Id.* RainDance is the exclusive licensee under the '148 patent. Ex. 1053 at ¶71. RainDance was subsequently acquired by Bio-Rad Laboratories (Ex. 1061), and Bio-Rad Laboratories was officially substituted as the Plaintiff on May 25, 2017. The District Court has interpreted various claim terms appearing the '148 patent. Exhibits 1054-57. Dispositive motions are due by November 20, 2017. Trial is scheduled for April 16, 2018.

The ‘148 patent was the subject of IPR2015-01158 in which the Board declined to institute an *inter partes* review trial. Ex. 1052. Because the Board did not issue a final written decision, no estoppel can arise under 35 U.S.C. § 315(e)(1).

III. CITATION OF PRIOR ART PATENTS AND PRINTED PUBLICATIONS RELIED UPON IN REQUEST FOR REEXAMINATION

Reexamination of claims 1-8 of the ‘148 patent is requested in view of the following prior art patents and printed publications:

A. Quake (from which Key Passages Were Copied Almost Verbatim by Patent Owner)

WO 02/23163 A1 to Quake et al. (“Quake PCT”) is attached as Exhibit 1034. Quake PCT was filed on September 14, 2001, the WIPO publication was in the English language, and the PCT application designated the United States. Accordingly, the Quake PCT has a § 102(e) date of September 14, 2001 and qualifies as prior art under that section.

The Quake PCT corresponds to U.S. Published Application 2002/0058332 to Quake *et al.*, which is attached as Exhibit 1033 (“Quake”).

The Quake PCT is relied upon herein in lieu of the published U.S. application because the Quake PCT demonstrates that key portions of the ‘148 patent specification were copied almost verbatim from the Quake PCT. As shown in the table below, the supposed point of novelty of the ‘148 patent (i.e., forming droplets from two continuous streams of immiscible fluids) was copied almost verbatim from the Quake PCT. The Patent Owner copied verbatim the underlined portions from the Quake PCT.

Quake PCT (published March 21, 2002) (Ex. 1034)	**Ismagilov provisional application (filed May 9, 2002) (Ex. 1003) and ‘148 patent (Ex. 1001)**
34:32-35:7 The pressure at the droplet extrusion region can also be regulated by adjusting the pressure on the main and sample inlets, for	Provisional at 22:12-19, ‘148 patent at 21:56-65 The pressure at the plug-forming region can also be regulated by adjusting the pressure

example, with pressurized syringes feeding into those inlets. By controlling the pressure difference between the oil and water sources at the droplet extrusion region, the size and periodicity of the droplets generated may be regulated. Alternatively, a valve may be placed at or coincident to either the droplet extrusion region or the sample inlet connected thereto to control the flow of solution into the droplet extrusion region, thereby controlling the size and periodicity of the droplets.	on the main and sample inlets, for example with pressurized syringes feeding into those inlets. By controlling the difference between the oil and water flow rates at the plug-forming region, the size and periodicity of the plugs generated may be regulated. Alternatively, a valve may be placed at or coincident to either the plug forming region or the sample inlet connected thereto to control the flow of solution into the plug-forming region, thereby controlling the size and periodicity of the plugs.

Attached as Ex. 1035 is a table showing the numerous passages which Ismagilov copied key disclosures verbatim or nearly verbatim from the Quake PCT.

As discussed in sections VI and VII, below, notwithstanding statements to the contrary during *ex parte* prosecution of the '148 patent, it is clear that Quake does in fact teach forming plugs from continuous streams of immiscible fluids. Indeed, the Patent Trial and Appeal Board expressly found that Quake disclosed such a technique. Ex. 1050 at 9-10. However, neither the examiner nor the PTAB appear to have been made aware that Ismagilov's disclosure concerning forming plugs from continuous streams of immiscible fluids was copied almost word-for-word from Quake.

1. Potential applicability of Dynamic Drinkware

According to the Federal Circuit, claims of an issued U.S. patent must be supported by the disclosure of the application to which it claims priority for the patent to qualify as prior art under 35 U.S.C. §102(e) as of the priority date. *Dynamic Drinkware, LLC v. National Graphics, Inc.*, 800 F.3d 1375 (Fed. Cir. 2015). The court cited *In re Wertheim*, 646 F.2d 527, 537 (CCPA 1981), and found that because Petitioner failed to compare the claims of the issued prior art patent to the disclosures of its provisional, Petitioner could not establish an earlier prior art date

for the patent. *Id.* However, it appears that this holding has not been extended to U.S. published applications or WIPO publications by any court. It should not be so extended, as explained below.

The rationale of *In re Wertheim* and *Dynamic Drinkware* applies at most to alleged prior art issued patents under 35 U.S.C. § 102(e)(2) and not to prior art publications under 35 U.S.C. § 102(e)(1). *Dynamic Drinkware* referred to the status of an "issued patent" as prior art under Section 102(e)(2). *Dynamic Drinkware*, Slip Op. at 11, 1. Indeed, the rationale of *In re Wertheim* was that "in a situation where there are continuation-in-part applications," the "type of new matter added must be inquired into" because "if a patent could not theoretically have issued the day the application was filed, it is not entitled to be used against another . . . ." *In re Wertheim*, 646 F.2d 527, 536-37 (CCPA 1981). By contrast, as the BPAI has explained, "[w]hen a patent application is published . . . the PTO has yet to have made a final determination as to whether the claimed subject matter conforms to the patentability requirements of Chapter 35 . . . . Yet, the published application makes its disclosure, and that of any parent applications, available to the public. Such disclosure . . . is explicit evidence of the activity of another as of the earliest claimed filing date. . . . [Thus] a published patent application . . . constitutes prior art for all that it discloses on its earliest filing date." *Ex parte Jo Anne Robbins*, No. 2009-001866, 2009 WL 3490271, *4 (BPAI Oct. 27, 2009); *see also Ex parte Yamaguchi*, 88 U.S.P.Q.2d 1606 (BPAI 2008) (declining to apply *Wertheim* to, *inter alia*, publications and holding "a provisional application is considered prior art for all that it teaches"). As noted in the legislative history of the AIA: "Wertheim…was almost completely overruled by the American Inventors Protection Act of 1999. . . which, by making any published application prior art [under §102(e)(1)], effectively displaced Wertheim's requirement that the application have been capable of becoming

a patent on the day that it was filed." *See* 157 Cong. Rec. S1360-02, 2011 WL 797877 (Mar. 8, 2011). In short, *In re Wertheim* and *Dynamic Drinkware* have no application to published applications under Section 102(e)(1).

Moreover, the provisional application at issue in *Dynamic Drinkware* was not incorporated by reference into the alleged prior art patent (as is the case with the Quake PCT). The rationale of *In re Wertheim* and *Dynamic Drinkware* applies when the content of a provisional application is not published, but is simply referred to by a purported prior art patent. In such circumstances, courts have held that the disclosure of the provisional must provide written description for the later issued patent. However, where, as here, the entire disclosure of the provisional is incorporated by reference, and thereby effectively published on the publication date, this rationale does not apply. *See Telemac Cellular Corp. v. Topp Telecom, Inc.*,247 F.3d 1316, 1329 (Fed. Cir. 2001) (document "'incorporated by reference' . . .becomes effectively part of the host document"); MPEP § 2127(I) (subject matter of application may be relied on under Section 102(e) if it is "actually included or incorporated by reference").

Accordingly, the *Dynamic Drinkware* rule should not apply to the WIPO publications such as the Quake PCT.

2. Chart Showing Compliance with *Dynamic Drinkware*

However, in the event *Dynamic Drinkware* is deemed to apply to WIPO publications, the chart below shows that the claims of the WIPO publication are indeed supported by the priority document.

WO 02/23163 A1	**U.S. Pat. App. No. 60/233,037**
1. A microfluidic device comprising	This invention relates **to microfluidic devices** and methods, including microfabricated, multi-layered elastomeric devices with active pumps and valves. P. 1, lines 11-12.

a main channel, and	The devices and methods of the invention comprise a **main channel,** through which a pressurized stream of oil is passed, and at least one sample inlet channel, through which a pressurized stream of aqueous solution is passed. p.1, lines 14-17.
at least one inlet region in communication with the main channel at a droplet extrusion region.	A junction or **"droplet extrusion region" joins the sample inlet channel to the main channel** such that the aqueous solution can be introduced to the main channel, *e.g.*, at an angle that is perpendicular to the stream of oil. p. 1, lines 17-19.
28. A device for sorting biological material comprising:	The invention also provides **a device for sorting biological material comprising**: p.8, line 6.
(a) a microfabricated substrate having at least one main channel, an inlet region which meets the main channel at a droplet extrusion region, and at least two branch channels meeting at a junction downstream from the droplet extrusion region;	The invention also provides a device for sorting biological material comprising: a microfabricated substrate…In more detail, **the microfabricated substrate has at least one main channel, an inlet which meets the main channel at a droplet extrusion region, and at least two branch channels meeting at a junction downstream from the droplet extrusion region**. p. 8, lines 6-10.
(b) a detection region within or coincident with at least a portion of the main channel and associated with a detector; and	**The detection region of the device is within or coincident with at least a portion of the main channel, and is also associated with a detector.** p. 8, lines 10-12.
38. A method for sorting biological material, said method comprising:	The invention also **provides a method for sorting biological material.** p. 9, line 3.
(a) furnishing an extrusion fluid to a main channel of a microfabricated substrate;	The method, which is preferably implemented using a microfabricated device of the invention…p. 9. lines 7-9 An **extrusion fluid,** which is incompatible with the sample fluid, **flows through the main channel** so that the droplets of the sample fluid are within **the flow of the extrusion fluid in the main channel**. p. 9, lines 14-16.
(b) providing droplets of a sample fluid containing the biological material to the main channel, wherein the sample fluid is	(a) **providing droplets of a sample fluid containing the biological material to the**

incompatible with the extrusion fluid;	**main channel** of a microfabricated substrate; p. 9. lines 9-10 **An extrusion fluid, which is incompatible with the sample fluid, flows through the main channel** so that the droplets of the sample fluid are within the flow of the extrusion fluid in the main channel. p. 9, lines 14-16.
(c) interrogating the biological material in each droplet for a predetermined characteristic as it passes through a detection region associated with the main channel; and	(b) **interrogating** each droplet (or **the biological material within each droplet**) **for a predetermined characteristic as it passes through a detection region associated with the main channel**; p. 9. lines 10-12.
(d) directing the flow of each droplet into a selected branch channel according to the results of the interrogation.	(c) **directing the flow of each droplet into a selected branch channel according to the results of the interrogation.** p. 12-14,

B. Burns I (Cited but Not Discussed or Applied by the Examiner)

Burns, J. R. et al., *The Intensification of Rapid Reactions in Multiphase Systems Using Slug Flow in Capillaries*, Lab on a Chip, vol. 1, 2001, pp. 10-15 ("Burns I") is attached as Exhibit 1007. Burns I indicates on its face that it was published on the web August 9, 2001. Burns I is thus available as prior art under 35 U.S.C. § 102(a). Burns I was among the hundreds of references cited but was not applied or discussed by the Examiner during *ex parte* prosecution of the '148 patent. Requester notes that another reference by a *different* Burns (*i.e.*, Mark A. Burns) was cited by the Examiner in an office action for a related application. (Ex. 1109 at July 11, 2011 rejection p 7). That article is referenced herein as Exhibit 1008 "Burns II."

C. Burns II (Cited but Not Previously Applied to the Independent Claim)

Burns, M.A. *et al*., *Microfabricated structures for integrated DNA analysis*, Proc. Natl. Acad. Sci. USA, May 1996, vol. 93, pp. 5556-5561 (Burns II) is attached as Exhibit 1008. Burns

II was published in 1996 and is available as prior art under 35 U.S.C. § 102(b). Burns II was cited by the Examiner only against dependent claims in a related application reciting autocatalytic reactions, enzymes or DNA. Ex. 1109 at July 11, 2011 rejection p 7. Burns II was not applied against any independent claim.

D. Shaw Stewart British Application (Cited but Not Previously Applied to the Independent Claim)

UK Patent Application GB 2,097,692A to Shaw Stewart (the "Shaw Stewart British Application") is attached as Exhibit 1040. The Shaw Stewart British Application published in 1982 is available as prior art under 35 U.S.C. § 102(b). The British Application was specifically considered in a related application only for teachings concerning the surfactants recited in pending dependent claim 33 (which issued as dependent claim 9). Ex. 1109 at February 15, 2012 rejection p. 7.

E. Corbett (Not Previously Considered)

U.S. Patent No. 5,270,183 to Corbett et al. ("Corbett") is attached as Exhibit 1010. Corbett issued in 1993 and is available as prior art under 35 U.S.C. § 102(b). Corbett was not previously disclosed or considered in connection with *ex parte* prosecution or any post-grant proceeding involving the '148 patent.

F. Kopp (Not Previously Considered)

Kopp, M.U. et al., Chemical Amplification: Continuous-Flow PCR on a Chip, Science, 1998, vol. 280, pp. 1046-48 ("Kopp") is attached as Exhibit 1027. Kopp published in 1998 and is available as prior art under 35 U.S.C. § 102(b). Kopp was not previously disclosed or considered in connection with *ex parte* prosecution or any post-grant proceeding involving the '148 patent.

G. Lagally (Not Previously Considered)

Lagally, E.T. et al., Single-Molecule DNA Amplification and Analysis in an Integrated Microfluidic Device, Anal. Chem., 2001, vol. 73, pp. 565-570 ("Lagally") is attached as Exhibit 1028. Lagally indicates on its face that it was published on the web on January 3, 2001. Lagally is available as prior art under 35 U.S.C. § 102(b). Lagally was not previously disclosed or considered in connection with *ex parte* prosecution or any post-grant proceeding involving the '148 patent.

H. Vogelstein (Cited but Not Discussed or Applied by the Examiner)

Vogelstein, B. et al., *Digital PCR*, Proc. Natl. Acad. Sci., August 1999, Vol. 96, pp. 9236–9241 ("Vogelstein") is attached as Exhibit 1044. Vogelstein was published in August 1999 and is thus available as prior art under 35 U.S.C. § 102(b). Vogelstein was among the hundreds of references cited but was not applied or discussed by the Examiner during *ex parte* prosecution of the '148 patent.

I. Additional References

Requester further relies on various of the other references cited in the Table of Exhibits to support the proposed grounds of rejection, as explained below, in the claim charts appended hereto, and in the declaration of Dr. Shaqfeh (Ex. 1004). For example, various additional references are relied upon to establish the state of the art and the level of skill in the art (Section IV.A) and motivations to combine the references as set forth in the proposed grounds set forth in Sections VI and VII.

IV. OVERVIEW OF THE '148 PATENT

A. State of the Art and Level of Skill in the Art[2]

In the years leading up to the earliest claimed priority date (May 9, 2002), there was a substantial body of literature describing performing continuous flow biochemical assays, such as polymerase chain reaction, and other reactions on a microfabricated chip.

In 1996, Brody, J. P. et al. ("Brody") (not previously presented to the USPTO) published an article, *Biotechnology* at *Low Reynolds Numbers*, *Biophysical Journal*, vol. 71, December 1996, pp. 3430-3441 (Ex. 1006). Brody, in summarizing the existing state of the art in microfluidics, stated

> "[t]here has been a **surge of interest in the 'lab-on-a-chip' concept**, which involves the miniaturization of many chemical processes onto a single silicon chip. [ ] Because these systems allow one to manipulate single cells, and even single macromolecules, there is great interest in the biotechnology community in using microfluid systems for analytical tests. For example, in the **polymerase chain reaction, amplification of DNA** in a microenvironment is attractive both because the temperature can be rapidly cycled and because the sample volume is extremely small."

Ex. 1006 at 3430 [emphasis added].

Brody explained that "[a]t [micrometer] scales, viscous forces dominate over inertial forces, turbulence is nonexistent, surface tension can be a powerful force, diffusion becomes the basic method for mixing, and evaporation acts quickly on exposed liquid surfaces." Ex. 1006 at 3430. Brody noted that in microfluidic devices "[m]ixing must be chiefly done by diffusion, and in fact diffusion cannot be neglected in the design of these devices." *Id.* at 3440.

[2] *See Ex. 1004 (Shaqfeh Decl.)* ¶¶ 26-56 for full Background discussion, including evidentiary support.

In 1996, Mark A. Burns from the University of Michigan reported the use of microfluidic devices to perform PCR. *Microfabricated structures for integrated DNA analysis*, Proc. Natl. Acad. Sci. USA, May 1996, vol. 93, pp. 5556-5561. (Ex. 1008, Burns II).[3] Burns II reported that "[n]o new biochemistries or DNA detection methods are required for their use; the components simply reproduce current laboratory procedures in a micron-sized environment." *Id*. at 5560. In other words, the PCR chemistry did not need to be modified – it worked without modification on the microfluidic scale. In the method of Burns II, a sample containing a DNA plasmid was loaded into one of the reaction wells (A) and a mixture of Taq enzyme and buffer was loaded into the other. *Id.* at 5558. Each formed droplets of about 60 nl in the parallel 500 x 25 µm channels (B) came together at the Y junction to form a single larger droplet (about 120 nl). *Id.* at 5558-59; Ex. 1004 ¶40.

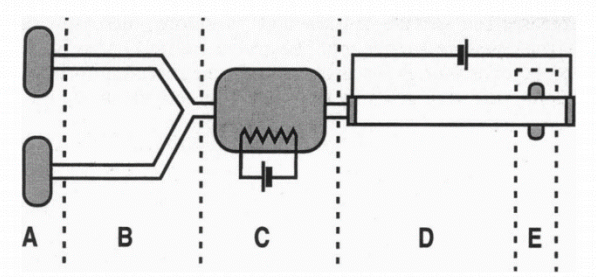


FIG. 1. Example of an integrated DNA analysis system, represented schematically. The individual components of the system are injection entry ports (*A*), liquid pumping channels (*B*), thermally controlled reaction chamber (*C*), electrophoresis channel (*D*), and DNA band migration detector (*E*). Each component would have associated sensors, control circuitry, and external connections.

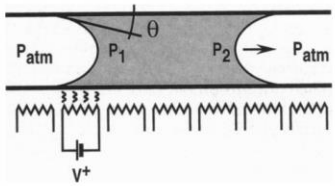


FIG. 2. Schematic drawing showing the principle of thermally induced drop motion in a closed channel. The case of a single aqueous drop in a hydrophilic channel is presented, where V is an applied voltage, P_{atm} is atmospheric pressure, P_1 is the receding-edge internal pressure, P_2 is the advancing-edge internal pressure, and θ is the contact angle of the liquid–gas–solid interface. The contact angle will depend on the surface characteristics of the channel and the constituents of the drop, with a hydrophilic interaction giving θ between 0 and 90 degrees, and a hydrophobic surface giving θ between 90 and 180 degrees. Surface treatments can also reduce contact angle hysteresis and, therefore, reduce the temperature difference necessary for drop motion.

The droplet then passed through the resistive heater portion (C) in which PCR amplification occurred. *Id.* at 5556. Afterwards the amplification results were optically detected in zone D via gel electrophoresis *Id.* at 5559. Burns II noted that "[t]he use of common fabrication methods

[3] This author (**Mark** Burns) reference should not be confused with **J.R.** Burns of the University of Newcastle. The latter published a different article relevant to this reexamination request: Burns, J. R. et al., *The Intensification of Rapid Reactions in Multiphase Systems Using Slug Flow in Capillaries*, Lab on a Chip, vol. 1, 2001, pp. 10-15. ("Burns I", Ex. 1007)

allows the assembly of increasingly complex, multicomponent, integrated systems from a small, defined set of standardized elements" and that "[f]uture design efforts have a strong impetus to reduce component size and improve efficiency." *Id.* at 5560-61.

In 1997, Knapp *et al*. described microchannel reactors in which multiple reactants were continuously or intermittently flowed into and reacted within a microchannel. In U.S. Patent No. 6,444,461 to Knapp (Ex. 1025, priority 1997, not presented to the USPTO), Knapp described the system as follows:

> The use of an integrated microfluidic system as herein before described, wherein said biochemical system flows through one of said channels substantially **continuously**, enabling sequential testing of said plurality of test compounds. . . .
>
> Finally, in a screening run, streams of antibody, antigen and sample are **flowed continuously** into the reaction channel 404a and into waste well 428. A slug of this mixture is then injected into the separation channel 404b. . . .
>
> Modulating voltages are then concomitantly applied to the various reservoirs to affect a **desired fluid flow characteristic, e.g., continuous or discontinuous** (e.g., a regularly pulsed field causing the flow to oscillate direction of travel) flow of receptor/enzyme, ligand/substrate toward the waste reservoir with the periodic introduction of test compounds.

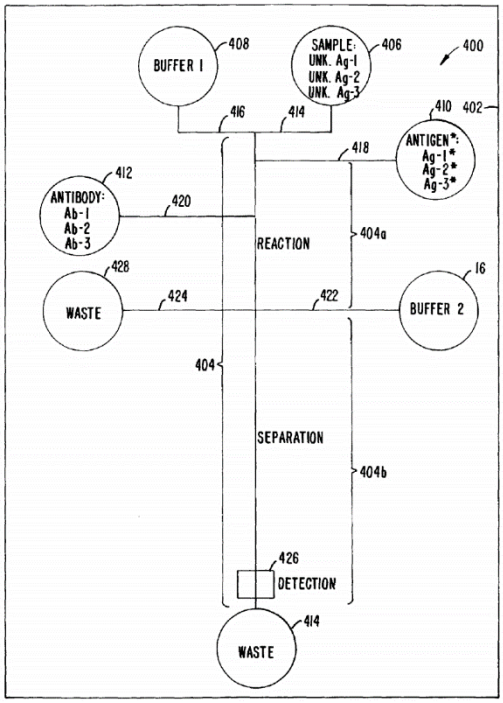
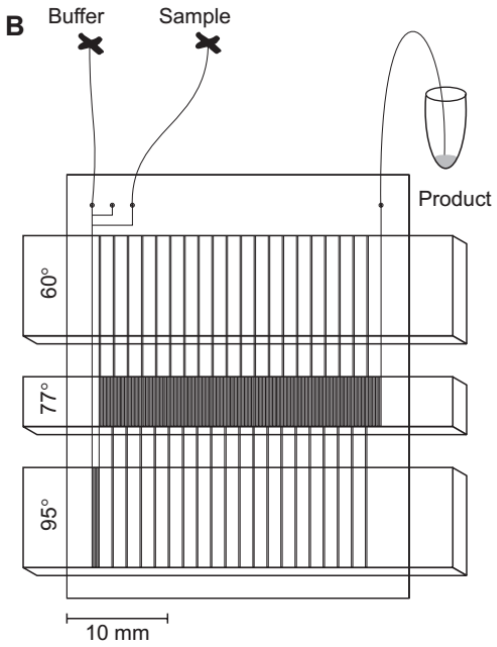


FIG. 4A.

Ex. 1025 at 59:21-25, 29:43-46, 51:64-52:2 [emphasis added].

By 1998, Kopp *et al.* had successfully implemented continuous flow PCR on a chip. In his 1988 article (not previously presented to the USPTO), Kopp explained that "[a] wide range of microreactors, microcapillary electrophoresis devices, and microcell manipulation devices have been described in recent years." Ex. 1027 at 1046. Building on that body of work, Kopp's group developed

> [a] micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in **continuous flow** at high speed. The device is analogous to an electronic amplifier and relies on the movement of sample through thermostated temperature zones on a glass microchip. Input and output of material (DNA) is **continuous**, and amplification is independent of input concentration. . . .
>
> The results demonstrate that with a very simple device and virtually no optimization, PCR can be performed in **continuous flow**, yielding product quality and quantity comparable to standard thermal cycling methods (Fig. 2B). . . .
>
> We suggest that cross-contamination in a **continuous-flow** format may be much less of a problem than in a stationary-tube PCR format. Thus, a flow-through PCR chip can be reused for extended periods without major cleaning. . . .

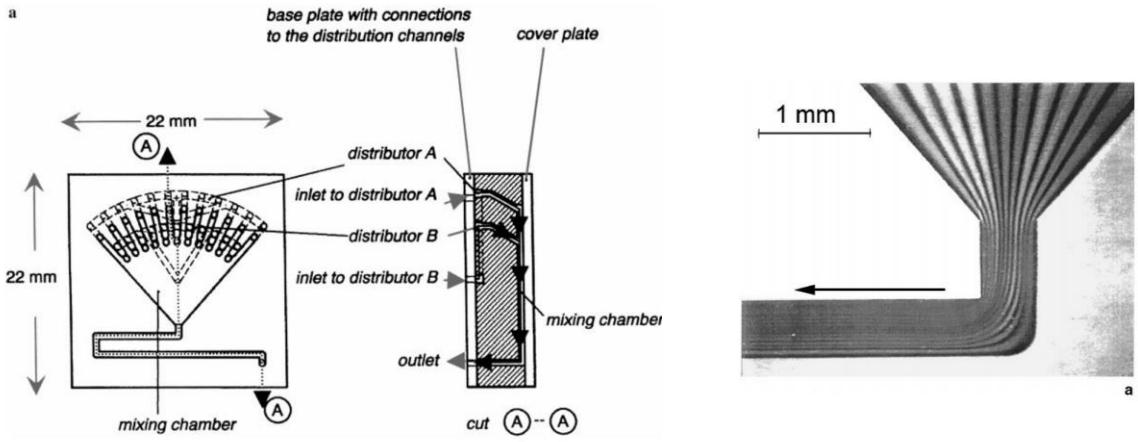


Ex. 1027 at 1046-1047 [emphasis added]. Kopp et al. also explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications:

> To build a complex system analogous to an electronic integrated circuit, it is necessary to integrate a variety of other continuously working parts. A cursory glance at developments in miniaturized total analysis systems (uTAS) during the past 5 years reveals that most of the relevant components, such as **continuous-flow mixers** (8), **continuous-flow microreactors** (9), **continuous separations** (10), and high-speed capillary gel electrophoresis (as a conventional batch process), have already been presented (11). Accordingly, the **continuous-flow microamplifier** should allow the creation of highly elaborate analysis and synthesis systems.
>
> Although chemical amplification reactors will obviously not be applicable to all reactions, neither will they be limited to PCR. Devices of this kind open up whole new areas of application in medical diagnostics. For example, the online amplification and monitoring of a specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy.

Ex. 1027 at 1047 [emphasis added].

Starting in 1999, consistent with Kopp's prediction, other groups reported conducting various other types of reactions in continuous flow microchannel devices. Also in 1999, Erbacher et al., reported in Erbacher *et al., Towards Integrated Continuous-Flow Chemical Reactors*, Mikrochim. Acta, 1999, vol. 131, pp. 19-24, Ex. 1012, ("Erbacher") (another article not previously presented to the USPTO) on their development of a continuous flow microchannel reactor in which complete mixing of the reactants occurred within just a few seconds. Ex. 1012 at 19. The figures of Erbarcher's article, reproduced below, illustrate the mixing manifold and the manner in which the fluids mix after they are introduced into the same conduit.



In 2000, Kenis *et al.* reported (in another article not previously disclosed to the USPTO) that a similar continuous flow microchannel reactor could be used for various fabrication reactions. Ex. 1024 at 841; Ex. 1004 ¶46. Kenis *et al.* explained that the laminar flow which predominates in microchannel devices makes them well suited as microreactors:

> Channels that are 0.02-2 mm wide -- channels which, in this Account, we call almost interchangeably "microchannels" or "capillaries" – can be considered as reaction vessels with two unusual characteristics. First, their

> interior volumes are small, but readily and rapidly accessible from the outside by pumping fluids or gases into them. Second, fluids moving in them at low to moderate velocities flow laminarly, that is, without turbulence. This laminar flow can be used to deliver reagents spatially inside capillaries with **remarkable precision**. These characteristics, combined with the ease with which microchannels can be assembled and disassembled using soft lithography and other techniques, suggest them as a new system of reactors with which to carry out microfabrication.

Id. at 841[emphasis added]. Kenis *et al*. explained that a wide range of technologies had already been used to accomplish chemical reactions in microchannels or capillaries, including deposition of proteins and cells.

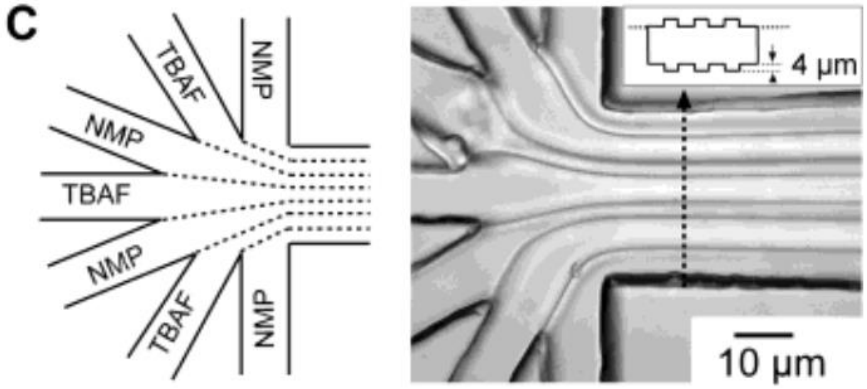
Table 1. Techniques Used for Microfabrication inside Microchannels

Strategy for Microfabrication Process or Technology	*Reference*
Multilayer Photolithography	2
Single-Stream Liquid Flow	
Chemical Vapor Deposition	3
Light Tweezers assisted Deposition	4
Polymer Deposition	8
Electroless Deposition	9
Electrochemical Fiber Growth	10
Etching of Optical Fibers	11,12
Micromolding in capillaries (MIMIC)	13-17
Deposition of Proteins	18
Multiple-Stream Liquid Flow	
Electroless Deposition	20
Etching	20
Nucleation and Crystallization ($CaCO_3$)	20
Deposition of Proteins	24
Deposition of Cells	24

Id. at 842, 846. Kenis et al. reported that "[t]he occurrence of laminar flow on the microscopic scale allows liquid streams to be maintained as entities that are separate and capable of

delivering different reagents" and that "[f]luid flows have clear potential to pattern delicate biological structures" such as those shown in Fig. 4C, reproduced below. *Id.* at 845.

.



Also in the year 2000, Floyd, *et al., "Novel Liquid Phase Microreactors for Safe Production of Hazardous Specialty Chemicals*," Microreaction Technology: Industrial Prospects, 2000 ("Floyd"), reported the development of "liquid-phase microreactor with thin film temperature sensing, good thermal management, and fast mixing has been fabricated for the production of hazardous specialty chemicals, specifically for organic peroxides." Ex. 1014 at 171 (also not previously presented to the USPTO). In Floyd's microreactor, multiple two different reactants are **continuously** injected in a fan of converging channels. *Id.* at 177, Fig. 1 (reproduced in part below). The convergence and fast mixing of acid and base reactants is shown in Floyd's Fig. 9 (reproduced in part below). *Id.* at 173, 178.

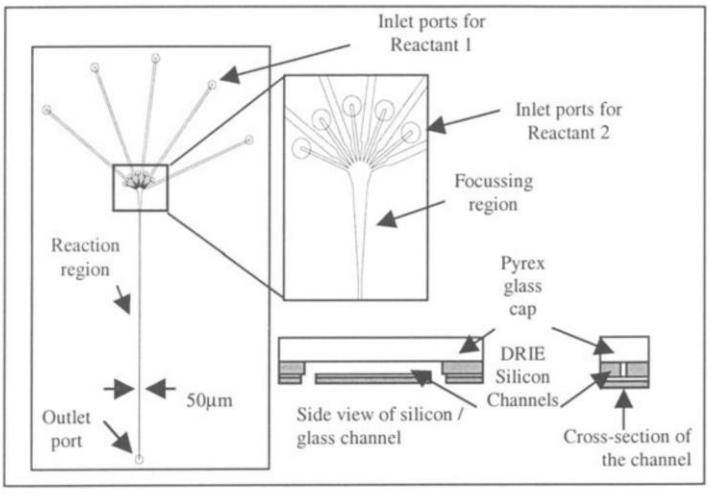


Fig. 1. First generation reactor design

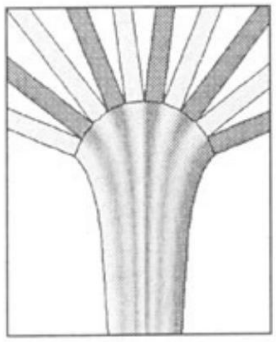


Fig. 9. Laminating

Also in 2000, Ferrance, *et al.* published a book chapter (not previously considered by the Office) which provided an overview of known techniques and apparatus for performing continuous flow **PCR in microfabricated chips**. Ex. 1013.

> To utilize this sequence most efficiently there **should be a continuous flow** from sample collection to diagnosis. . . .
>
> The **same advantages of reduced time, sample, and reagents** brought to the separations field by miniaturization also apply to low volume PCR in capillaries. Microchip formats have also been developed for PCR where the reactions are carried out in reservoirs or microreaction chambers formed in glass, silicon, or plastic microchips. In addition, decreasing the scale of PCR allows the reaction to be carried out **more efficiently**, producing more product in **less time** with **less side reactions**. Both capillaries and microchip devices have reduced the time needed for PCR but cycle times concomitant with the fast separations now possible are still being developed. . .
>
> Although capillaries have proven useful for small scale PCR, true **integration of the PCR and fast separation steps will require a microchip device where continuous flow of the PCR products** to the separation channels is achievable on a single coordinated platform (see Note 2). With this type of platform, integration not only of the last two

> steps shown in Fig. 1 is possible, but total integration of the complete process. . . .

Ex. 1013 at 191, 192 [emphasis added]. Ferrance noted that several groups had reported successful PCR reactions with sample volumes on the nano-liter scale.

> Kalinina et al. (12) have **reduced the volume of reaction by using capillaries as small as 20 pm** in diameter to hold a total volume of 10 nL, allowing single copies of template DNA to be amplified and detected after 30 cycles using fluorescent energy transfer (FET). . . .
>
> The system of Oda et al. (8) has been further modified by Huhmer and Landers (13) to carry out reactions in 150-pm id capillaries, which hold volumes as low as 100 nL. . . . The submicroliter volumes and noncontact, direct heating of the PCR solution by the IR radiation **allowed cycle times to be reduced to less than 3 s** in this system.

Id. at 196 [emphasis added].

In 2001, in another article not previously presented to the USPTO, Lagally *et al.* reported using a microfluidic reactor to conduct single-molecule DNA amplification by PCR. Lagally summarized the state of the art as of February 2001, explaining **continuous flow PCR microreactors had already undergone substantial evolution**:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use

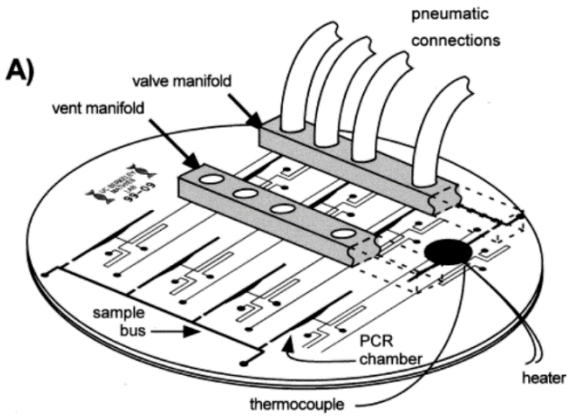
> resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

Lagally *et al.* developed a further improvement – a device and method for achieving single molecule amplification in a microchannel environment.

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1

> template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> Microfluidic PCR-CE device. The PCR chambers are connected to a common sample bus through a set of valves. Hydrophobic vents at the other end of the PCR chambers are used to locate the sample and to eliminate gas. The PCR chambers are directly connected to the cross channel of the CE system for product injection and analysis. Two aluminum manifolds, one each for the vents and valves, are placed onto the respective ports and clamped in place using vacuum. The manifolds connect to external solenoid valves for pressure and vacuum actuation. Thermal cycling is accomplished using a resistive heater and a miniature thermocouple below the 280-nL chamber.



Ex. 1028 at 566 (see figure legend).

In the same year, Whitesides published an article (also not previously considered by the USPTO) which surveyed the state of the art and known applications for microchannel reactors. Whitesides explained that "[f]or the relatively large feature sizes used in biology (>50 um), production of prototype patterns and structures is convenient, inexpensive, and rapid." Ex. 1047 at 335. The table of contents in Whitesides shows that microfabricated reactors were widely used in PCR, DNA detection and analysis, biochemical analysis, and other applications.

Id. at 336. Whitesides explained that as of 2001 "[a] variety of companies and academic research groups have developed systems for nucleic acid amplification and/or detection." *Id.* at 353. Among the work specifically cited by Whitesides was Kopp (affiliated with Zeneca/SmithKline Beecham), Lagally (University of California Berkeley), and Burns II (University of Michigan).

Also in 2001, the University of Newcastle in the United Kingdom published its findings concerning the suitability of microchannel slug flow reactors for industrial, high-throughput chemical reactions like nitrations, hydrogenations, sulfonations and oxidations. Ex. 1007. In the article "*The intensification of rapid reactions in multiphase systems using slug flow in capillaries*," Lab on a Chip, 2001, vol. 1, pp. 10-15 (Burns I), Burns reported that circulation within the plugs caused by shear forces against the wall may greatly enhance the mass transfer or mixing and hence the rate of reaction. *Id.* Burns taught that "[i]n practice, as shown in Fig. 1, a **circulation is generated within the slugs which is stimulated by the shear** between the stationary fluid at the capillary

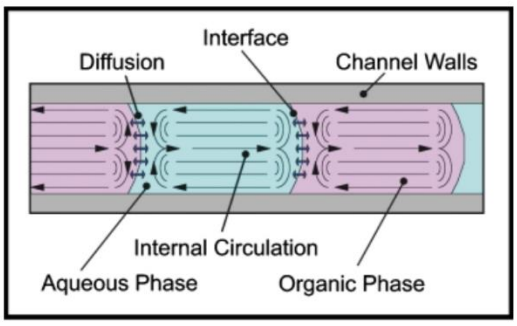


Fig. 1 Illustration of internal circulation generated within immiscible slug flow.

wall and the slug axis. This convects fluid from the slug ends and establishes a concentration gradient in the radial direction. The resulting mass transfer between the wall flow and that along the axis is enhanced because: (i) the path length for diffusion is approximately equal to the tube radius—generally much shorter than the slug half length; (ii) diffusion occurs across an area which approaches the cylindrical slug area— generally larger than the capillary cross-section area. **Thus circulation can provide a powerful boost to the inter-phase mass transfer**." *Id.* at 10 [emphasis added].

Burns I also taught that the slugs may be formed from the continuous flow of fluids at an intersecting channel. Burns I explained that "various methods may be used to generate slugs of liquid within a microreactor." *Id.* at 10. Burns I selected a method in which "the **continuous flow** of both phases through T or cross-shaped intersections" and "[s]lugs are generated by the action of one phase flowing into the channel **whilst the other phase moves into the intersection, eventually cutting off the flow of the first phase** into the channel and reversing the process." Ex. 1007 at 11 [emphasis added]. "Fig. 3 shows the typical pattern of slug generation within this device when an organic phase, with a room temperature viscosity of 27 cP, composed of 67% silicone oil (1000 cP) and 33% kerosene (dyed blue) was used in conjunction with water. The slug lengths produced were around 1.5 mm long with a flow velocity of 5.6 mm s^{-1}." *Id.* at 11.

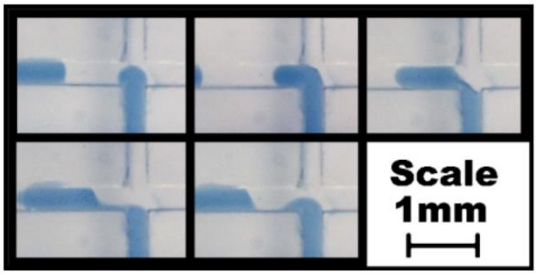


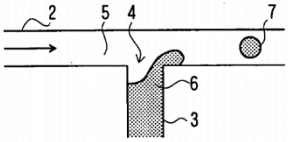
Fig. 3 Typical slug generation in glass device with 380 μm wide channels.

In early 2001, a group from the University of Tokyo developed a droplet reactor that appears to fall within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and

III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are relevant to the state of the art at the time of filing.[4]

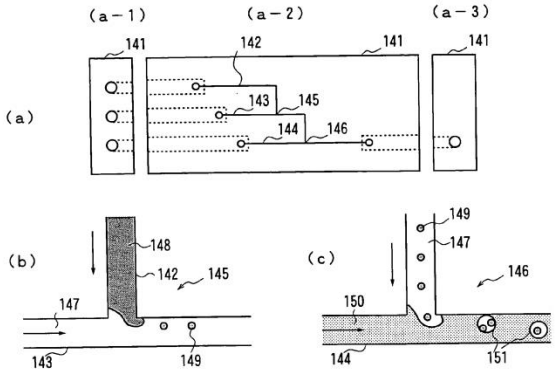
Higuchi I discloses a microreactor in which a dispersion phase (6) is ejected from a dispersion phase feeding port (4) toward a continuous phase (5) flowing in a microchannel (2) in such a manner that flows of the dispersion phase (6) and the continuous phase (5) cross each other, thereby obtaining microdroplets (7), formed by the shear force of the continuous phase (5), having a size smaller than the width of the channel for feeding the dispersion phase (6). Ex. 1018 at Fig. 2 (reproduced at right). Higuchi teaches many variations of the embodiment of Figure 2 and one representative variation is shown in Fig. 19.

FIG. 2



In [Figures 19a-c], reference numeral 141 represents a main body of the microcapsule-forming apparatus, reference numeral 142 represents a microchannel in which a dispersion phase (for example, water) flows, reference numeral 143 represents a microchannel in which a first continuous phase (for example, oil) flows, reference numeral

FIG. 19



[4] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

> 144 represents a microchannel in which a second continuous phase (for example, water) flows, reference numeral 145 represents the first junction at which flows of the dispersion phase and the first continuous phase are joined together, reference numeral 146 represents the second junction at which flows of the dispersion phase, the first continuous phase, and the second continuous phase are joined together, reference numeral 147 represents the first continuous phase, reference numeral 148 represents the dispersion phase, reference numeral 149 represents an emulsion (for example, water), reference numeral 150 represents the second continuous phase, and reference numeral 151 represents formed microcapsules. The microcapsules 151 can contain one or more emulsions 149. Numeral 143 represents a microchannel in which a first continuous phase (for example, oil) flows, reference numeral 144 represents a microchannel in which a second continuous phase (for example, water) flows, reference numeral 145 represents the first junction at which flows of the dispersion phase and the first continuous phase are joined together, reference numeral 146 represents the second junction at which flows of the dispersion phase, the first continuous phase, and the second continuous phase are joined together, reference numeral 147 represents the first continuous phase, reference numeral 148 represents the dispersion phase, reference numeral 149 represents an emulsion (for example, water), reference numeral 150 represents the second continuous phase, and reference numeral 151 represents formed microcapsules. The microcapsules 151 can contain one or more emulsions 149.

Ex. 1018 ¶100.

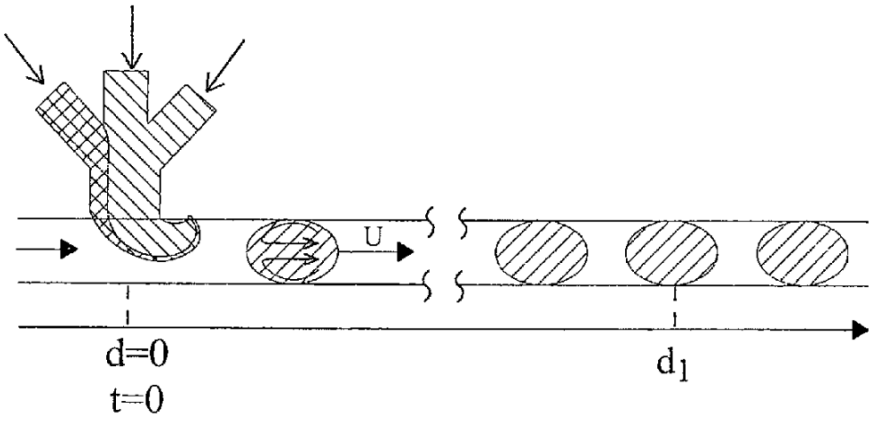
The microchannel device of Higuchi I-III was specifically intended to be used to perform chemical reactions; indeed, that is typically why emulsions are created. Ex. 1043 (A contemporaneous article by Higuchi entitled *Chemical reactions in microdroplets by electrostatic manipulation of droplets in liquid media*). Higuchi I-III militate in favor of a finding that show that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was actually within the level of ordinarily skill in the art at the time of filing.

In light of the foregoing references, which demonstrate the state of the art, a person having ordinary skill in the art would have had knowledge of the scientific literature concerning microfluidic devices and the methods of using such devices before May 9, 2002. A skilled artisan would have had a **Ph.D. in chemistry, biochemistry, mechanical engineering, or a related discipline, with two years of experience in using, designing or creating microfluidic devices.** *Id.* A person of ordinary skill in the art would have known how to research the scientific literature in fields relating to microfluidics, including fluid dynamics, microscale reactions, chemistry, biochemistry, and mechanical engineering, and to consult with team members having specialized skills in these fields. Ex. 1004 at ¶29. The Patent Trial and Appeal Board has adopted this definition in an *inter partes* review of another microfluidic device patent owned assigned to RainDance Technologies with a priority date of 2011. Exhibit 1046 (Final written decision in IPR2015-01558 cancelling the claims of U.S. Patent No. 8,658,430) at p. 7.

B. Summary of the '148 Patent

Against this backdrop, Ismagilov et al. filed the patent application that issued as the '148 patent. The '148 patent claims priority to May 9, 2002, and is assigned to the University of Chicago ("Patent Owner"), according to the Office's electronic-assignment records. The '148 patent claims methods of performing a reaction in plugs of an aqueous fluid flowing in a carrier-fluid in a microfluidic device. Ex. 1001 at 78:18-42. The methods claimed in the '148 patent recite continuously flowing an aqueous fluid through a first channel into a main channel in which a continuously flowing immiscible carrier fluid comprising an oil is present. The aqueous fluid comprises at least one biological molecule and at least one reagent for conducting the reaction between the biological molecule and the at least one reagent. And upon introduction of the

aqueous fluid from the first inlet into the main channel, the immiscible carrier fluid partitions the aqueous fluid into a plug that is substantially surrounded by the immiscible carrier fluid and that then flows through the main channel. *Id.* at 78:66-79:8. Figure 2A of the '148 patent illustrates this process:



Independent claim 1 of the '148 patent reads as follows:

> 1. A method for conducting a reaction in plugs in a microfluidic system, comprising the steps of:
>
> providing a microfluidic system comprising one or more channels;
>
> providing within the one or more channels a continuously flowing carrier fluid comprising an oil and a continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule in the fluid that can react with the target DNA or RNA molecules under conditions in which the target DNA or RNA molecules and the other molecules in the fluid do not react with each other;
>
> controlling flow rates of said aqueous fluid and said carrier fluid to partition the continuously flowing aqueous fluid with the continuously flowing carrier fluid to form a plurality of plugs of the aqueous fluid, each having a substantially uniform size of about 200 μm or less, wherein the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule; and
>
> providing conditions suitable for a polymerase-chain reaction in at least one plug of the plurality of plugs such that the target DNA or RNA is amplified.

C. Prosecution History of the '148 Patent

1. Prosecution of the Immediate Parent Application

In U.S. Patent Application Serial No. 13/024,155 Patent Owner presented a single independent claim:

> 1. A method comprising the steps of:
>
> providing a microfluidic system comprising one or more channels;
>
> providing within the one or more channels a carrier fluid comprising an oil and at least one plug substantially surrounded by the carrier fluid, wherein the plug comprises reagents sufficient for an autocatalytic reaction including a first species of molecule in a concentration such that the plug contains no more than a single molecule of the first species; and
>
> conducting an autocatalytic reaction such that the single molecule is amplified.

Ex. 1114 at originally filed claims February 9, 2011.

Examiner Gakh rejected the pending claims as indefinite and either anticipated or rendered obvious by Beer et al. (Anal. Chem., 2007). *Id.* at July 11, 2011 Non-final Office Action, 3-7. Examiner Gakh made the following findings:

> Beer teaches "On-Chip, Real-Time, Single-Copy Polymerase Chain Reaction in Picoliter Droplets" (Title) as the following:
>
> "The first lab-on-chip system for picoliter droplet generation and PCR amplification with real-time fluorescence detection has performed PCR in isolated droplets at volumes 10^6 smaller than commercial real-time PCR instruments. The system utilized a shearing T-junction in a silicon device to generate a stream of monodisperse picoliter droplets that were isolated from the microfluidic channel walls and each other by the oil-phase carrier. An off-chip valving system stopped the droplets on-chip, allowing them to be thermally cycled through the PCR protocol without droplet motion. With this system, a 10-pL droplet, encapsulating less than one copy of viral genomic DNA through Poisson statistics, showed realtime PCR amplification curves with a cycle threshold of ~18, 20 cycles earlier than commercial instruments. This combination of the established real-time PCR assay with digital microfluidics is ideal for isolating single-copy nucleic acids in a complex environment." (Abstract)
>
> The method comprises heating (see page 8473).

Ex. 1002 at July 11, 2011 Non-final Office Action, p. 5.

In response, the Applicants amended independent claim 1 as follows:

> 1. (Currently amended) A method comprising the steps of:
>
> providing a microfluidic system comprising one or more channels;
>
> ~~providing within the one or more channels~~ forming a plug of aqueous fluid by partitioning the aqueous fluid as it continuously flows through a first of the one or more channels, the ~~a carrier fluid comprising an oil and at least one~~ plug being substantially surrounded by ~~the carrier fluid~~ an oil, wherein the plug comprises a single substrate molecule and reagents ~~sufficient~~ for conducting an autocatalytic reaction with the single substrate molecule ~~including a first species of molecule in a concentration such that the plug contains no more than a single molecule of the first species~~; and
>
> conducting an autocatalytic reaction in the plug such that the single substrate molecule is amplified.

Id. at Amendment After Non-Final Rejection of December 16, 2011, p. 2.

The Applicants argued that the pending claims were allowable for because Beer was not available as prior art:

> Beer is not prior art and cannot be used to reject the present claims. Beer is a journal article that published in 2007, and thus the date that Beer is available as a prior art reference under 35 U.S.C. §102 and 35 U.S.C. §103(a) is 2007.
>
> Applicants have shown above that the subject matter of the pending claims is adequately described in each of U.S. patent application serial number 12/777,099 and U.S. patent application serial number 10/765,718. Since claims of the present application are adequately described and enabled in each of 12/777,099 and U.S. patent application serial number 10/765,718, the present claims are entitled to at least the priority date of January 26, 2004.

Id. at December 16, 2011 Applicant Arguments/Remarks Made in an amendment p. 78.

Examiner Gakh found that the new claims were not supported in the priority documents because the latter did not describe single molecule reactions:

> Even for this reaction and even for femtoliter volumes of the plugs the concentration of the molecules statistically is almost two molecules (1.8) per plug. Nowhere is it shown that single molecules were obtained in the plugs. PCR is mentioned only as another example of possible autocatalytic reaction. However, the Applicants did not show the possession of the claimed invention at the time of filing the priority document. Therefore, the instant case does not obtain the priority date of the parent application.

Id. at January 31, 2012 Final Rejection, pp. 3-4 [emphasis added].

The Examiner also rejected the claims as being indefinite. For instance, the Examiner found that the claims did not clearly set forth how the partitioning occurs:

> Claim 1 recites providing a microfluidic system comprising one or more channels and forming a plug of aqueous fluid by partitioning the aqueous fluids as it continuously flows through a first of one of the one or more channels. First of all, it is not clear, how the plugs can be formed just within one channel with continuously flowing water. It requires at least two channels – for flowing water and for flowing the carrier fluid. Also, it is not clear, how the plug is formed with only a single molecule of the substrate and reagents? How is it possible? The recitation of the claims differs from the disclosure of the specification.

Id. at 2012-01-31 Final Rejection p. 6 [emphasis added]. Lastly, the Examiner maintained that the claims were anticipated by or rendered obvious by Beer et al. (Anal. Chem., 2007). *Id.* pp. 7-8.

The Applicants next conducted an in-person interview with Examiner Gakh and tentatively agreed on amended claim language that would address the Examiner's enablement, written description and art-based rejections. *Id.* at July 27, 2012 Interview Summary. The Applicants also persuaded the Examiner that the amended claims were entitled to the asserted priority date. *Id.*

The Applicants filed the amended claims without a further explanation of the substantive reasons underlying the Examiner's change in position. Independent claim 1 was amended as follows:

> 1. (Currently amended) A method for conducting an autocatalytic reaction, comprising the steps of:
>
> providing a microfluidic system comprising one or more channels;
>
> forming ~~a~~ at least one plug of aqueous fluid containing at least one substrate molecule and reagents for conducting an autocatalytic reaction by partitioning the aqueous fluid as it continuously flows through a first of the one or more channels, the plug being substantially surrounded by an oil flowing through the channel, wherein the at least one plug comprises ~~a~~ ~~single~~ at least one substrate molecule and reagents for conducting an autocatalytic reaction with the ~~single~~ at least one substrate molecule; and
>
> ~~conducting~~ providing conditions suitable for an autocatalytic reaction in the at least one plug such that the ~~single~~ at least one substrate molecule is amplified.

Id. at July 27, 2012 Response After Final Action, p. 3. In their remarks, the Applicants took the position that the foregoing amendments did not narrow the scope of the claims but provided no disclosure of the reasons the Examiner agreed to withdraw her rejections:

> Applicants and their counsel thank Examiner Gakh for comments the Examiner made on claims submitted in a facsimile from Applicant's counsel dated June 28, 2012. The amendment submitted herewith is to clarify the claims and is not intended to, and does not, change the scope of the claims. The amended claims in this Amendment and Response retain the amendments previously made in the facsimile response submitted on June 28, 2012, as that response has not officially been entered at this time. As agreed with the Examiner, these claims are in condition for allowance pending a supplemental prior art search by the Examiner. If there are any further issues for discussion, the Examiner is kindly invited to contact the undersigned.

Id. p. 5. [emphasis added]

After the Examiner's supplemental search, the Examiner required additional amendments to the claims. The Examiner made the following amendment to independent claim 1 with the Applicants' permission:

> 1. (Currently amended) A method for conducting an autocatalytic reaction in plugs in a microfluidic system, comprising the steps of:
>
> providing ~~a~~ the microfluidic system comprising ~~one or more~~ at least two channels having at least one junction;
>
> flowing an aqueous fluid containing at least one substrate molecule and reagents for conducting an autocatalytic reaction through a first channel of the at least two channels;
>
> flowing an oil through the second channel of the at least two channels;
>
> forming at least one plug of the aqueous fluid containing the at least one substrate molecule and reagents ~~for conducting an autocatalytic reaction~~ by partitioning the aqueous fluid with the flowing oil at the junction of the at least two channels ~~as it continuously flows through a first of the one or more channels~~, the plug being substantially surrounded by an oil flowing through the channel, wherein the at least one plug comprises at least one substrate molecule and reagents for conducting an autocatalytic reaction with the at least one substrate molecule; and
>
> providing conditions suitable for ~~an~~ the autocatalytic reaction in the at least one plug such that the at least one substrate molecule is amplified.
>
> Claim 16 (New). The method of claim 1, further comprising a mixing step, wherein the mixing step occurs via a special design of the at least one channel of the at least two channels below the junction.

Id. at August 7, 2012 Notice of Allowance and Fees Due (PTOL-85), pp. 2-3. Examiner Gakh stated that she was allowing the further amended claims because the Thorson and Quake prior art

did not appear to disclose performing chemical reactions in droplets formed at the intersection of two channels.

> The following is an examiner's statement of reasons for allowance: the claims recite the subject matter which was disclosed in the parent case having priority over all relevant art found by the examiner. In particular, *Takahashi and Inoue* disclose analogous reaction in *US 2006/0257893*; however this is not the prior art, as its filing date is February 17, 2006.
>
> The review by *Gu et al. "Droplets Formation and Merging in Two-Phase Flow Microfluidics" (Int. J. Mol. Sci., 2011)* refers to the formation of the droplets from continuous flows of two liquids with the first publication of such method by *Thorsen et al. "Dynamic Pattern Formation in a Vesicle-Generating Microfluidic Device" (Phys. Rev. Lett. 2001)*. The publication by Thorsen et al. is devoted to the study of the physical-chemical processes of droplet formation in microfluidic systems and the effects of different conditions and channel designs for such formation. The paper does not mention any application of the systems for the chemical reactions in droplets formed at the junction of two channels by two constantly flowing immiscible fluids. The review of *Atencia and Beebe "Controlled microfluidic interfaces" (Nature, 2005)* refers to the microfluidic platform for performing a two-step reaction, which is disclosed in the publication from Ismagilov's group, *Shestopalov, I., Tice, J. D. and Ismagilov, R. F. "Multi-step synthesis of nanoparticles performed on millisecond time scale in a microfluidic droplet-based system (Lab Chip, 2004, IDS).*
>
> *Quake and Thorsen in US 2002/0058332 (IDS)* disclose a system with two channels and the method of forming plugs (droplets) with reagents in immiscible oil; however, the droplets in the oil are not formed from two continuously flowing immiscible fluids, but rather by a discrete dispensing the solution with biological material into the carrier fluid. This is a different approach from the claimed invention.

Id. at 2012-08-07 Notice of Allowance and Fees Due (PTOL-85), pp. 3-4 [emphasis added]. The '155 application issued as U.S. Patent No. 8,304,193, the parent of the '148 patent (Ex. 1001) which is the subject of the instant Request.

Significantly, the examiner was not made aware that '193 patent's disclosure concerning forming plugs from continuous streams of immiscible fluids was plagiarized from Quake. As shown in the table below, the supposed point of novelty of the '193 patent (i.e., forming droplets from two continuous streams of immiscible fluids) was copied almost verbatim from the Quake PCT.

Quake PCT (published March 21, 2002) (Ex. 1034)	**Ismagilov provisional application (filed May 9, 2002) (Ex. 1003) and '148 patent (Ex. 1001)**
34:32-35:7 The pressure at the droplet extrusion region can also be regulated by adjusting the pressure on the main and sample inlets, for example, with pressurized syringes feeding into those inlets. By controlling the pressure difference between the oil and water sources at the droplet extrusion region, the size and periodicity of the droplets generated may be regulated. Alternatively, a valve may be placed at or coincident to either the droplet extrusion region or the sample inlet connected thereto to control the flow of solution into the droplet extrusion region, thereby controlling the size and periodicity of the droplets.	Provisional at 22:12-19, '148 patent at 21:56-65 The pressure at the plug-forming region can also be regulated by adjusting the pressure on the main and sample inlets, for example with pressurized syringes feeding into those inlets. By controlling the difference between the oil and water flow rates at the plug-forming region, the size and periodicity of the plugs generated may be regulated. Alternatively, a valve may be placed at or coincident to either the plug forming region or the sample inlet connected thereto to control the flow of solution into the plug-forming region, thereby controlling the size and periodicity of the plugs.

Attached as Ex. 1035 is a table showing the numerous passages which Ismagilov copied key disclosures verbatim or nearly verbatim from the Quake PCT.

2. Prosecution of the '347 Continuation Application, which Matured into the '148 Patent

Shortly after issuing the notice of allowance for the '155 application, Examiner Gakh turned to application no. 13/563,347 (the '347 application) a continuation of the '155 application. Applicants amended the claim in a similar manner and Examiner ultimately allowed the '347 application for similar reasons as the '155 parent application.

In the '347 application Patent Owner presented a single independent claim which was similar in scope to the independent claim originally presented in the '155 parent application.

> 1. A method comprising the steps of:
>
> providing a microfluidic system comprising one or more channels;
>
> providing within the one or more channels a carrier fluid comprising an oil and at least one plug substantially surrounded by the carrier fluid, wherein the plug comprises reagents sufficient for an autocatalytic reaction including a first species of molecule in a concentration such that the plug contains no more than a single molecule of the first species; and
>
> conducting an autocatalytic reaction such that the single molecule is amplified.

Ex. 1002 at originally filed claims July 31, 2012.

Examiner Gakh rejected the pending claims as being indefinite, lacking written descriptive support and being rendered obvious by Stewart (WO 84/02000) in view of Kalinina (Nucleic Acids Research 1997). *Id.* at December 27, 2012 Non-final Office Action, pp. 3-7. Examiner Gakh made the following findings:

> Stewart teaches a method comprising:
>
> providing a microfluidic system comprising one or more channels;
>
> providing within the one or more channels a carrier fluid comprising an oil and at least one plug substantially surrounded by the carrier fluid, wherein the plug comprises reagents for performing reactions, see Abstract and Description of the Invention, specifically pages 3 and 4 (the suitable carrier liquids are mineral oils and fluorinated hydrocarbons).
>
> Stewart does not specifically disclose autocatalytic reaction, and especially PCR.
>
> Kalinina teaches "Nanoliter scale PCR with TaqMan detection", which comprises fluorescence detection of the TaqMan labeled DNA when performing PCR of a single DNA.
>
> The labeled DNA and corresponding reagents for performing PCR are placed in a droplet and into a capillary tube for monitoring PCR.
>
> It would have been obvious for a person of ordinary skill in the art to apply Kalinina's teaching in Stewart's method, since Stewart's method provides exact conditions which are required for fluorescence monitoring of PCR of a single labeled DNA molecule in a small volume, which is easily controllable.

Id. at 6-7.

In response, the Applicants amended independent claim 1 as follows:

> 1. (Currently amended) A method comprising the steps of:
>
> providing a microfluidic system comprising one or more channels;
>
> providing within the one or more channels a continuously flowing carrier fluid comprising an oil and a continuously flowing aqueous fluid comprising biological molecules and at least one other molecule that reacts with the biological molecules under conditions in which the biological molecules and the other molecules do not react with each other ~~at least one plug substantially surrounded by the carrier fluid, wherein the plug comprises reagents sufficient for an autocatalytic reaction including a first species of molecule in a concentration such that the plug contains no more than a single molecule of the first species; and~~
>
> partitioning the continuously flowing aqueous fluid with the continuously flowing carrier fluid to form a plug of the aqueous fluid, the plug comprising only a single biological molecule and at least one of the other molecules that reacts with the biological molecules; and
>
> providing conditions suitable for ~~conducting~~ an autocatalytic reaction in the plug such that the single biological molecule is amplified.

Id. at Amendment After Non-Final Rejection of June 27, 2013, p. 2.

The Applicants argued that the pending claims were allowable for because Beer was not available as prior art:

> Stewart (WO 84/02000) reports a droplet forming system that involves closed conduits, with each movement or set of movements occurring after the previous movement is complete (Stewart, 1st paragraph of the Summary of the Invention). Stewart specifically states that his system is not a continuous flow system and that his system does not operate with a continuous stream (Stewart, 1st paragraph of the Summary of the Invention).
>
> Kalinina reports conducting PCR in capillary tubes (p. 2000, section entitled "PCR apparatus"). In Kalinina's apparatus, drop of aqueous fluid is sucked into a capillary, the capillary is sealed, and PCR is conducted in the capillary (p. 2000, section entitled "PCR apparatus"). Analysis reveals that Kalinina's device does not even form droplets, rather a discrete amount of fluid is sucked into a capillary.
>
> Therefore, the combination of Stewart and Kalinina does not teach or suggest the elements of amended claim 1 of partitioning continuously flowing aqueous fluid with continuously flowing carrier fluid to form a plug of the aqueous fluid, and providing conditions suitable for an autocatalytic reaction in the plug such that the single biological molecule is amplified. Accordingly, amended claim 1, and claims that depend from amended claim 1, are not obvious in view of the combination of Stewart and Kalinina.

Id. at p. 6-7.

Significantly, Applicants did not point out that the corresponding UK Patent Application, GB 2,097,692A to Shaw Stewart (the "Shaw Stewart British Application") does in fact disclose a

continuous flow system. See Section VI.A.1, *infra*. The British Application was brought to the Applicants' attention in connection with a sibling application (serial no. 13/024145 at February 15, 2012 rejection p. 7.) Requester submits that Applicants' omission was quite intentional. Nowhere in the family of applications do the Applicants directly take the position that the Shaw Stewart British Application fails to disclose continuous flows. Indeed, the exclusive licensee of the '148 patent argued that the Applicants had "misdirected" Examiner Gakh with respet to these teachings of Shaw Stewart. Ex. 1059 at 2-3; See discussion in Section VI.A.1., *infra*.

In the next action, Examiner Gakh accepted the Applicants' misleading argument with respect to Shaw Stewart and found that "Stewart does not specifically disclose forming plugs by two immiscible flowing fluids." *Id.* at August 29, 2013 Final Office Action, p. 7. Examiner Gakh issued a new rejection based on the combination of Stewart, Thorsen and Nakana as well as various written description and indefiniteness rejections. *Id.* at 6-7.

Applicants next amended the independent claims to address the §112 rejection and argued that rejection should be withdrawn because the Nakano reference did not qualify as prior art. The amendment to the independent claim was as follows:

> 1. (Currently amended) A method comprising the steps of:
>
> providing a microfluidic system comprising one or more channels;
>
> providing within the one or more channels a continuously flowing carrier fluid comprising an oil and a continuously flowing aqueous fluid comprising biological molecules and at least one other molecule that can react ~~reacts~~ with the biological molecules under conditions in which the biological molecules and the other molecules do not react with each other;
>
> partitioning the continuously flowing aqueous fluid with the continuously flowing carrier fluid to form a plurality of plugs of the aqueous fluid, where a subset of the plurality of plugs comprises ~~the plug comprising only~~ a single biological molecule and at least one of the other molecules that can react ~~reacts~~ with the biological molecule[[s]]; and
>
> providing conditions suitable for a polymerase-chain ~~an autocatalytic~~ reaction in the subset of the plurality of plugs such that the single biological molecule is amplified.

Id. at November 27, 2013 response at 2. The Applicants argued that Nakano reference was not prior art because the claims were entitled to the priority date of Provisional Application No. 60/394,544, field July 8, 2002. *Id*. at 5. The Applicant asked that the obviousness rejection be withdrawn on this basis.

In the next action Examiner Gakh found that the amendments introduced new matter and that the claims were otherwise not entitled to the asserted priority date. *Id.* at December 16, 2013 non-final rejection, pp. 3-4. Examiner Gahk also rejected the claims as nonenabling:

> 8. Claims 1, 4-11 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, because the specification, while being enabling for biological molecules which are DNA or RNA, does not reasonably provide enablement for any other biological molecules. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to practice the invention commensurate in scope with these claims. A polymerase-chain reaction is a specific reaction of amplifying DNA, and therefore no other biological molecules can undergo this reaction. It would have been impossible for a routineer in the art to perform PCR reaction with any other biomolecules besides DNA or RNA.
>
> 9. Claims 6 and 7 are further rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, because the specification, while being enabling for PCR reaction in which the molecules are labeled with fluorophores, does not reasonably provide enablement for the reaction in which the molecules are unlabeled. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to practice the invention commensurate in scope with these claims. It would have been an undue experimentation for a practitioner in the art to find conditions for PCR reaction which would emit light without fluorophore labels, because PCR reaction itself does not generate any light.

Id. at p. 6. Examiner Gahk also maintained various indefiniteness and written descriptive support rejections. *Id.* at 7-8. Lastly, the Examiner maintained the obviousness rejection in light of the finding that the claims were not entitled to the claimed priority date. *Id.* at 7-8.

In response Applicants then amended the independent claim as follows:

> 1. (Currently Amended) A method comprising the steps of:
>
> providing a microfluidic system comprising one or more channels;
>
> providing within the one or more channels a continuously flowing carrier fluid comprising an oil and a continuously flowing aqueous fluid comprising target DNA or RNA ~~biological~~ molecules and at least one other molecule in the fluid that can react with the target DNA or RNA ~~biological~~ molecules under conditions in which the target DNA or RNA ~~biological~~ molecules and the other molecules in the fluid do not react with each other;
>
> controlling flow rates of said aqueous fluid and said carrier fluid to partition ~~partitioning~~ the continuously flowing aqueous fluid with the continuously flowing carrier fluid to form a plurality of plugs of the aqueous fluid, each having a substantially uniform size of about 200µm or less, ~~where~~ wherein the target DNA or RNA in said ~~subset of the~~ plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA ~~biological~~ molecule and at least one ~~of the~~ other molecules that can react with the target DNA or RNA ~~biological~~ molecule; and
>
> providing conditions suitable for a polymerase-chain reaction in ~~the~~ at least one plug ~~subset~~ of the plurality of plugs such that the target DNA or RNA ~~single biological molecule~~ is amplified.

Id. at February 14, 2014 p. 2. Applicants also cancelled the claim language which drew the enablement rejections. *Id.* p. 5. Lastly, Applicants argued that the claims as amended were entitled to the claimed priority date, thus removing Nakano as a reference. *Id.* pp. 7-9.

Examiner Gahk then issued a notice of allowance. The reasons for allowance were as follows:

> The following is an examiner's statement of reasons for allowance: the Applicants' representatives and the examiner had numerous interviews and discussions on the prior art rejections and priority documents for this and co-pending applications.
>
> As a result of these discussions the examiner allows the claims of the instant application, because the Applicants convinced the examiner that they pioneered performing PCR reactions in flowing droplets in the high-competitive field of microfluidics related to biochemical applications.

Id. at May 9, 2014 Notice of Allowance p. 2. Based on the foregoing, it appears that Applicants never brought to Examiner Gakh's attention that the Shaw Stewart British Application disclosed forming plugs by flowing two continuous fluid streams together. See also discussion at VI.A.1,

infra. The Applicants also appear to have neglected to bring to Examiner Gakh's attention that the Applicants copied the claimed subject matter almost verbatim from another prior art reference, Quake. See discussion *supra*.

In sum, the prosecution history taken as a whole indicates that the '193 patent was allowed primarily because the Examiner believed that the prior art failed to teach **conducting PCR reactions in droplets formed from two flowing immiscible fluids, despite the fact that Patent Owner copied this subject matter almost verbatim from Quake**.

D. The *Inter Partes* Review Proceedings

Requester filed an *inter partes* review petition (Ex. 1050) asserting that claims 1-8 of the '148 patent were rendered obvious by US 6,033,880, issued Mar. 7, 2000 to Haff (Ex. 1017) in view of Quake (Ex. 1033, "Quake '332"); and claim 6 was rendered obvious by Haff in view of Quake and further in view of US 6,524,456 B1, issued Feb. 25, 2003 to Ramsey (Ex. 1037, "Ramsey II").

Haff discloses an automated machine for performing polymerase chain reaction ("PCR"). Ex. 1017, 1:12–15. Haff describes capillary tube PCR instruments use various mechanisms to heat and cool the reaction mixture and various fluid handling mechanisms to move the reaction mixture. As shown in Figure 2, reproduced at right, capillary tubing 50 defines a fluid path that alternately passes through hot and cold zones 52, 54. *Id*. at 9:58–65. Syringe 51 is used to dispense the PCR reaction mixture 53. Syringe 55 optionally contains an immiscible carrier fluid. *Id*. at 10:20–32. Syringe 55 is used to intermittently inject the immiscible fluid into the flow stream of reaction mixture 53. *Id.* at 10:32–35. This creates

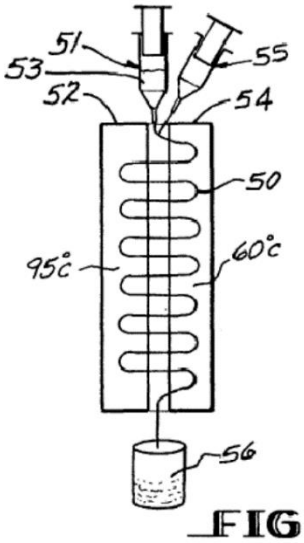


FIG-2

"slugs" of PCR reaction mixture separated by the immiscible carrier fluid. *Id*. at 9:50–52. The PCR amplification is controlled by the size of the tube, the flow rate and the temperatures of the hot and cold zones. *Id.* at 10:15–19.

Turning to Quake, the petition relied primarily on the embodiment shown in Figure 16A, reproduced below. *See, e.g.*, Ex. 1050 at pp. 20, 25-26. Quake teaches that a "junction or 'droplet extrusion region' joins the sample inlet channel to the main channel such that the aqueous solution can be introduced to the main channel, e.g., at an angle that is perpendicular to the stream of oil. By adjusting the pressure of the oil and/or the aqueous solution, a pressure difference can be established between the two channels such that the stream of aqueous solution is sheared off at a regular frequency as it enters the oil stream, thereby forming droplets." Ex. 1033 ¶ 3. The droplets of the sample fluid "contain [] the biological material for analysis, reaction or sorting" *Id.* ¶ 20. Optionally, the droplets of the sample fluid each "contain, on average, no more than one particle of the biological material." *Id*. In Figure 16A, channel 1601 contains the aqueous solution and intersects with main channel 1602, which contains the oil. *Id.* ¶ 292.

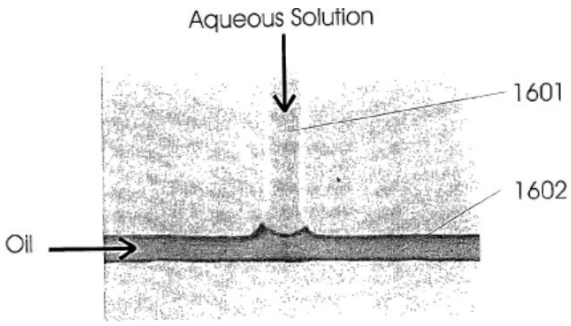


FIG. 16A

The Board denied institution on the basis that the petition did not sufficiently explain why a person of skill in the art would combine Haff and Quake. Ex. 1052. In particular, the Board held that

> [W]e determine that Petitioner has not explained why a person of ordinary skill in the art would have had a reasonable expectation of success combining Haff's capillary tube PCR device with Quake's microfluidic system to amplify a substrate in droplets. Simply stating that it would have

> been "routine" is insufficient. To the extent Petitioner relies on the background information to support a reasonable expectation of success, Petitioner fails to explain any connection between the two. For example, if conducting PCR in a stream of reaction mixture in a microfluidic device was known, Petitioner has not explained why, with that knowledge, it would have been routine to conduct PCR in droplets in a microfluidic device. Moreover, even though Haff describes conducting PCR in a capillary tube device, neither Petitioner nor its declarant explains whether the conditions for conducting PCR in microfluidic droplets would differ, or why a person of ordinary skill in the art would consider conducting PCR in microfluidic droplets to be "routine."

Ex. 1052 at 16-17.

The Board was not presented, and the decision did not consider, the teachings of Burns I, Burns II, Shaw Stewart, Corbett, Kopp, Lagally or Vogelstein. Nor did the Board consider any testimony that is comparable to that of Dr. Shaqfeh. (Ex. 1004).

When the PTAB proceedings are view in light of the *ex parte* prosecution, it appears that the '148 claims have been considered nonobvious by the Office because the **prior art was not believed to teach a technique for conducting polymerase chain reaction (PCR) in droplets formed from two continuously flowing immiscible fluids**.

V. CLAIM CONSTRUCTION

Claim terms are generally given their ordinary and customary meaning as would be understood by one of ordinary skill in the art. *Phillips v. AWH Corp.*, 415 F.3d 1303, 1312-13 (Fed. Cir. 2005) (*en banc*). In an *ex parte* reexamination, a claim in an unexpired patent is "given the broadest reasonable interpretation consistent with the specification." MPEP § 2258(I)(G).

A. "Microfluidic system" (Claim 1)

Claim 1 recites "providing a microfluidic system comprising one or more channels." The Board did not find it necessary to interpret this term for purposes of its decision. Ex. 1052 at 9. The District Court adopted the following definition under *Phillips*:

> The term "microfluidic system" (as used in the '193, '148, '083, and '407 patents) means a "system comprised of at least one substrate having a network of channels of micrometer dimension through which fluid may be transported." A "microfluidic system" is not limited to or the equivalent of a "substrate."

Ex. 1055 at 1. Consistent with this definition, the expert retained by the exclusive licensee of the '148 patent (Bio-Rad), averred in a declaration that the term "[m]icrofluidics" is "understood by those of ordinary skill in the art, like myself, to encompass devices and methods for control and manipulation of fluids that contain geometric features that are on the sub-millimeter scale (i.e., less than 1000 microns)." Ex. 1060 at 19.

B. "Plug of the Aqueous Fluid" (Claim 1)

Independent claim 1 recites the step of "to form a plurality of plugs of the aqueous fluid." Ex. 1001 at 78:31-32. The '148 patent includes two paragraphs specifically addressing the meaning of the term "plug":

> "Plugs" in accordance with the present invention are formed in a substrate when a stream of at least one plug-fluid is introduced into the flow of a carrier-fluid in which it is substantially immiscible. The flow of the fluids in the device is induced by a driving force or stimulus that

> arises, directly or indirectly, from the presence or application of, for example, pressure, radiation, heat, vibration, sound waves, an electric field, or a magnetic field. Plugs in accordance with the present invention may vary in size but when formed, their cross-section **should be** substantially similar to the cross-section of the channels in which they are formed. When plugs merge or get trapped inside plug traps, the cross-section of the plugs may change. For example, when a plug enters a wider channel, its cross-section typically increases.
>
> Further, plugs in accordance with the present invention may vary in shape, and for example **may be** spherical or non-spherical. The shape of the plug may be independent of the shape of the channel (e.g., a plug may be a deformed sphere traveling in a rectangular channel). The plugs **may be** in the form of plugs comprising an aqueous plug-fluid containing one or more reagents and/or one or more products formed from a reaction of the reagents, wherein the aqueous plug-fluid is surrounded by a non-polar or hydrophobic fluid such as an oil. The plugs **may also be** in the form of plugs comprising mainly a non-polar or hydrophobic fluid which is surrounded by an aqueous fluid. The plugs may be encased by one or more layers of molecules that comprise both hydrophobic and hydrophilic groups or moieties. The term "plugs" also includes plugs comprising one or more smaller plugs, that is, plugs-within-plugs. The relative amounts of reagents and reaction products contained in the plugs at any given time depend on factors such as the extent of a reaction occurring within the plugs. **Preferably**, plugs contain a mixture of at least two plug fluids.

Ex. 1001 at 9:27-60. [emphasis added].

Consistent with the foregoing, the Board broadly interpreted the term "plug" as to mean "volumes of aqueous fluid formed when a stream of aqueous fluid is introduced into the flow of a substantially immiscible carrier-fluid." Ex. 1052 at 7-8. The Board noted that this did not

incorporate various features of the preferred embodiments such as plug size and its separation from the channel wall. *Id*.

The District Court did not specifically construe the term "plug" but noted that preamble of claim 1 ("A method for conducting an autocatalytic reaction in plugs in a microfluidic system") was nonlimiting. Ex. 1054 at 12.

C. "Conditions Suitable for a Polymerase-Chain Reaction in at Least One Plug of the Plurality of Plugs such that the Target DNA or RNA is Amplified" (Claim 1)

Claim 1 recites "providing conditions suitable for a polymerase-chain reaction in at least one plug of the plurality of plugs such that the target DNA or RNA is amplified." The Board noted that the "specification sets forth certain conditions that must be addressed when conducting PCR in a microfluidic droplet, including unwanted adsorption of proteins on the surface of the droplet." Ex. 1052 at 9. The Board interpreted this term as requiring "conditions that allow the substrate molecule to be amplified in a microfluidic system." *Id.*

VI. STATEMENT POINTING OUT EACH SUBSTANTIAL NEW QUESTION OF PATENTABILITY FOR THE CHALLENGED CLAIMS

A. Summary of the Significant New Questions of Patentability

As discussed in Section IV.C, above, the prosecution history taken as a whole indicates that the '148 patent was allowed primarily because the Examiner believed that the prior art failed to teach conducting PCR reactions in droplets formed from two continuously flowing immiscible fluids.

As discussed in Section IV.D, above, the PTAB denied institution of an *inter partes* review of U.S. Patent No. 8,822,148 because the petition failed to explain why a skilled artisan

would have believed that the PCR technique of Haff could have been conducted on a microfluidic scale as taught in Quake.

Accordingly, the claims have been considered nonobvious by the Office because the **prior art was not believed to teach a technique for conducting polymerase chain reaction (PCR) in droplets formed from two continuously flowing immiscible fluidsat**

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuous flowing immiscible fluids were well known, ii) microfluidic PCR reactions were well known, and iii) there were compelling reasons to modify the microfluidic droplet reactors to conduct continuous flow PCR.

1. Quake, Burns I and Shaw Stewart Show that Droplet Microreactors which Formed Plugs from Continuous Flowing Immiscible Fluids Were Well known

As noted by the Patent Trial and Appeal Board (PTAB), Quake (Ex. 1033) discloses a droplet reactor in which droplets are formed by continuous streams of immiscible fluids. "Quake relates to microfluidic devices designed to compartmentalize small droplets of aqueous solution within microfluidic channels filled with oil." Ex. 1052 at 11, citing Quake (Ex. 1033) ¶ 3. "The devices comprise a main channel, through which a pressurized stream of oil is passed, and at least one sample inlet channel, through which a pressurized stream of aqueous solution is passed." *Id.* "A junction joins the main channel with the sample inlet channel." *Id. "*By adjusting the pressure of the oil and/or the aqueous solution, a pressure difference can be established such that the stream of aqueous solution is sheared off at a regular frequency as it enters the oil stream, thereby forming droplets." *Id.* at 12. Figure 16A of Quake is reproduced below:

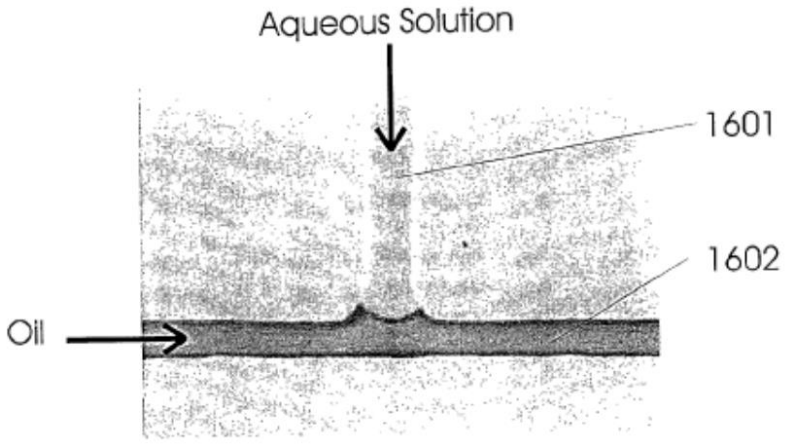


FIG. 16A

"Figure 16A depicts the channel architecture for the droplet extrusion region. Channel 1601 containing an aqueous solution intersects with main channel 1602 containing oil." *Id.*, citing Quake (Ex. 1033) ¶ 292. The Petitioner asserted that this disclosure in Quake met the plug-formed-by-continuous-streams limitations of the '148 patent and neither the Patent Owner nor the Board contested that premise. Ex. 1051 *passim*; Ex. 1052, *passim*. Moreover, Quake himself during prosecution characterized his invention as involving continuous streams. When distinguishing his invention over one of the embodiments of Shaw Stewart in which reagent is periodically injected, Quake noted that his "invention relates, in part, to the discovery that combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid (e.g., decane) it is possible to produce small, discrete, monodisperse droplets of the aqueous solution flowing in the second fluid." Ex. 1058 at September 25, 2006 response, p. 15. Accordingly, notwithstanding statements to the contrary during *ex parte* prosecution of the '148 patent (discussed in Section IV.C above) it is clear that Quake does in fact teach forming plugs from continuous streams of immiscible fluids.

Another body of work from the University of Newcastle shows that plugs in microchannels also could be readily formed by continuous flow of fluids at an intersecting channel. Burns explained that "various methods may be used to generate slugs of liquid within a microreactor." Ex. 1007 at 10 ("Burns I"). Burns I chose a method in which "the continuous flow of both phases through T or cross-shaped intersections" and "[s]lugs are generated by the action of one phase flowing into the channel whilst the other phase moves into the intersection, eventually cutting off the flow of the first phase into the channel and reversing the process." Ex. 1007 at 11 [emphasis added]. Fig. 3 shows oil-in-water slug generation and Fig. 4 shows water-in-oil slug generation. *Id.* at 11. In Burns' Fig. 4 embodiment, an aqueous plug of reagents is formed in a continuous oil phase. The continuous flow plug formation used by Burns was a well-known alternative to periodically injecting a measured slug of reactant into the main channel. See Ex. 1010; 1027; Ex. 1025 at 23:2-8. Burns I demonstrates that making slugs or plugs with continuous flows of immiscible fluids was a mere design choice as of 2001.

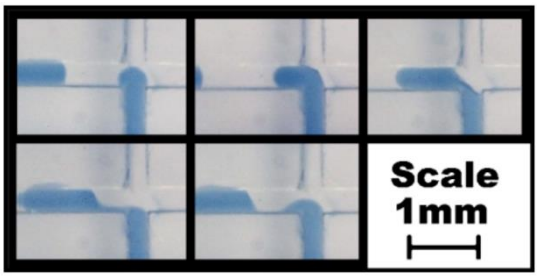


Fig. 3 Typical slug generation in glass device with 380 µm wide channels.

The Shaw Stewart British Application also teaches conducting reactions in droplets formed from two continuously flowing immiscible fluids. The Shaw Stewart British Application discloses a "droplet reactor" and illustrates at Figure 1 a carrier phase 1 flowing along a conduit into which reagent 2 is injected, thereby forming droplet 4. Ex. 1040 at 1:1-20, 70-90. "[I]f large numbers of droplets are required, a continuous flow of reagent through the opening will be produced, while a continuous current of

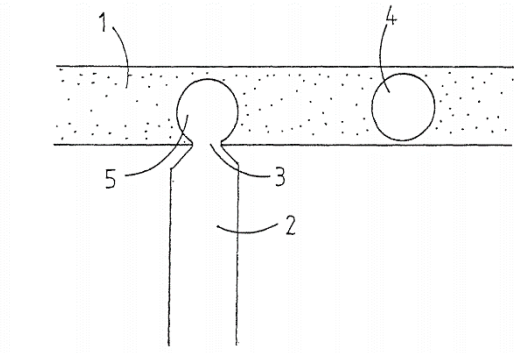


Figure 1.

carrier phase flows down the tube. When each droplet almost spans the tube, it will be broken off. The exact size of the droplets produced will depend on the relative and absolute magnitude of the two currents." *Id.* at 1:83-90.

Significantly, the exclusive licensee of the '148 patent affirmatively (and correctly) took the position that Shaw Stewart teaches plug or droplet formation combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid. More particularly, Bio-Rad (who recently acquired RainDance, the original exclusive licensee of the '148 patent, Ex. 1061, Ex. 1053 ¶71) explained that another party had mischaracterized the Shaw Stewart reference by failing to point out the above-quoted portion of Shaw Stewart, in which Shaw Stewart specifically teaches that in certain embodiments droplets are formed from intersecting and continuously flowing streams.

> To overcome Stewart I and secure allowance of their patent, the Patent Owner ("PO") mischaracterized the device disclosed in Stewart I (and hence Stewart II), misdirecting the Examiner to irrelevant portions of Stewart I while ignoring other portions that directly contradicted PO's arguments. *See, e.g.*, Ex. 1003 at 174-180. For example, one of PO's primary arguments was that Stewart I only disclosed introducing fluid in a "stepwise fashion," whereas in the '503 Patent droplets are formed by "combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid." *Id.* at 174. In truth, however, Stewart I also discloses combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid:
>
> > If large numbers of droplets are required, a continuous flow of reagent through the opening will be produced, while a continuous current of carrier phase flows down the tube. When each droplet almost spans the tube, it will be broken off.
>
> Ex. 1008, pg. 1, col. 2, ll. 83-88.

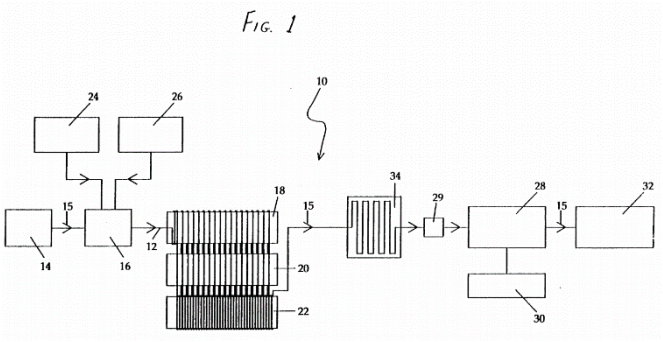
Ex. 1059 at 2-3.

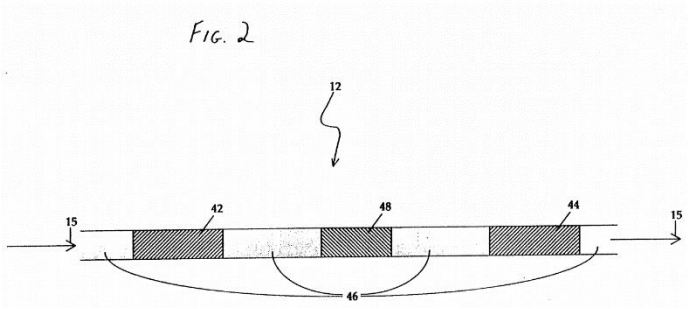
Thus the exclusive licensee (Bio-Rad) expressly agreed that Shaw Stewart discloses forming plugs from intersecting streams of continuously flowing immiscible fluids.

To summarize on the first point, the Shaw Stewart British Application, (Ex. 1040), Quake (Ex. 1034) and Burns I (Ex. 1007) each provide the teaching that the *ex parte* Examiner believed was missing from the prior art: conducting reactions in droplets formed from two continuously flowing immiscible fluids.

2. Corbett, Kopp, Burns II, Lagally and Vogelstein Show that Microfluidic PCR Devices Were Well Known

As of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Corbett, Kopp, Burns II, Lagally and Vogelstein. In 1991, about eleven years prior to the earliest effective filing date, Corbett disclosed using a microchannel or capillary device to conduct DNA amplification reactions such as PCR by injecting plugs of reactants into a carrier stream that is passed through different temperature zones. Corbett explained that as of 1991 "the most critical problem in applying nucleic acid amplification procedures, particularly in repetitive diagnostic assays in a clinical laboratory, is contamination of the reaction mixture by small amounts of DNA which contain sequences capable of being amplified under the conditions of assay." Ex. 1010 at 3:7-12. "Automation of procedures minimize[s] the opportunity for such contamination and allows the implementation of stringent quality control." *Id.* 3:15-19 *"*As is shown in FIG. 1, the apparatus 10 comprises tube 12 through which a fluid stream passes. As is more clearly shown in FIG. 2, the fluid stream includes pockets of reaction mixtures 42 and 44 and purging solution 48 separated by carrier fluid 46." *Id*. at 7:58-62.





"The pump is actuated and the reaction mixture of 20 μl or less is injected into the mineral oil or silicone oil carrier fluid and specific DNA sequences (whose limits are defined by the oligonucleotide primers) present in the sample is amplified as it passes cyclically through the temperature zones." Ex. 1010 at 9:35-40. Corbett thus demonstrates that it was known at least as of 1991 that reduction of contamination risk in PCR could be achieved by performing the PCR on microliter sized plugs of reactant in capillary tube which passes through various temperature zones. Ex. 1004 ¶77.

In 1996, Mark A. Burns from the University of Michigan reported the use of microfluidic devices to perform PCR. *Microfabricated structures for integrated DNA analysis*, Proc. Natl. Acad. Sci. USA, May 1996, vol. 93, pp. 5556-5561. (Ex. 1008, Burns II).[5] Burns II reported that "[n]o new biochemistries or DNA detection methods are required for their use; the components

[5] This author (Mark Burns) reference should not be confused with J.R. Burns of the University of Newcastle. The latter published a different article relevant to this reexamination request: Burns, J. R. et al., *The Intensification of Rapid Reactions in Multiphase Systems Using Slug Flow in Capillaries*, Lab on a Chip, vol. 1, 2001, pp. 10-15. ("Burns I", Ex. 1007)

simply reproduce current laboratory procedures in a micron-sized environment." *Id*. at 5560. In other words, the PCR chemistry did not need to be modified – it worked without modification on the microfluidic scale. In the method of Burns II, a sample containing a DNA plasmid was loaded into one of the reaction wells (A) and a mixture of Taq enzyme and buffer was loaded into the other. *Id.* at 5558. Each formed droplets of about 60 nl in the parallel 500 x 25 µm channels (B) which came together at the Y junction to form a single larger droplet (about 120 nl). *Id*. at 5558-59; Ex. 1004 ¶40.

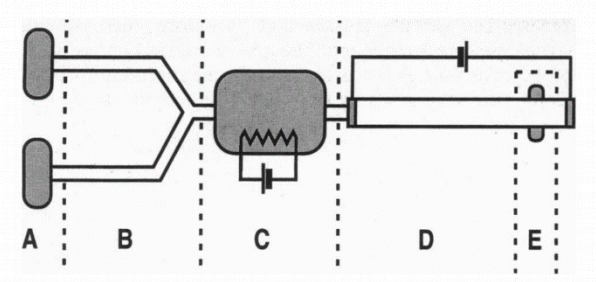


FIG. 1. Example of an integrated DNA analysis system, represented schematically. The individual components of the system are injection entry ports (*A*), liquid pumping channels (*B*), thermally controlled reaction chamber (*C*), electrophoresis channel (*D*), and DNA band migration detector (*E*). Each component would have associated sensors, control circuitry, and external connections.

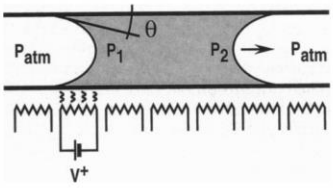


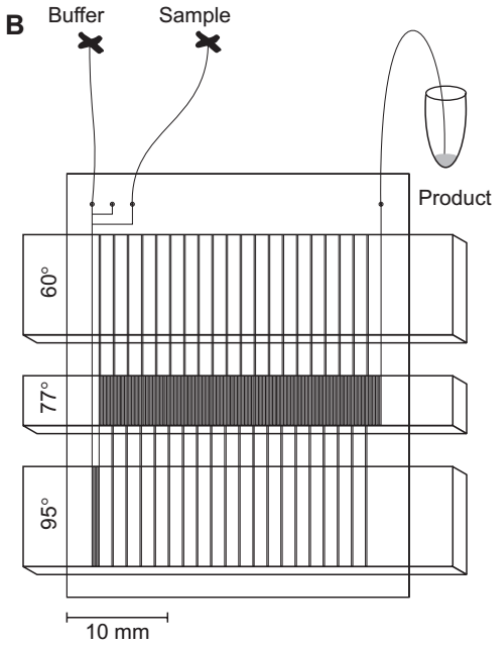
FIG. 2. Schematic drawing showing the principle of thermally induced drop motion in a closed channel. The case of a single aqueous drop in a hydrophilic channel is presented, where V is an applied voltage, P_{atm} is atmospheric pressure, P_1 is the receding-edge internal pressure, P_2 is the advancing-edge internal pressure, and θ is the contact angle of the liquid–gas–solid interface. The contact angle will depend on the surface characteristics of the channel and the constituents of the drop, with a hydrophilic interaction giving θ between 0 and 90 degrees, and a hydrophobic surface giving θ between 90 and 180 degrees. Surface treatments can also reduce contact angle hysteresis and, therefore, reduce the temperature difference necessary for drop motion.

The droplet then passed through the resistive heater portion (C) in which PCR amplification occurred. Ex. 1008 at 5556. Afterwards the amplification results were optically detected in zone D via gel electrophoresis *Id*. at 5559. Burns II noted that "[t]he use of common fabrication methods allows the assembly of increasingly complex, multicomponent, integrated systems from a small, defined set of standardized elements" and that "[f]uture design efforts have a strong impetus to reduce component size and improve efficiency." *Id.* at 5560-61.

By 1998 Kopp *et al*. had successfully implemented continuous flow PCR on a chip. In his 1998 article (not previously presented to the USPTO), Kopp explained that "[a] wide range of microreactors, microcapillary electrophoresis devices, and microcell manipulation devices have

been described in recent years." Ex. 1027 at 1046. Building on that body of work, Kopp's group developed

> [a] micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in **continuous flow** at high speed. The device is analogous to an electronic amplifier and relies on the movement of sample through thermostated temperature zones on a glass microchip. Input and output of material (DNA) is **continuous**, and amplification is independent of input concentration. . . .
>
> The results demonstrate that with a very simple device and virtually no optimization, PCR can be performed in **continuous flow**, yielding product quality and quantity comparable to standard thermal cycling methods (Fig. 2B). .. .
>
> We suggest that cross-contamination in a **continuous-flow** format may be much less of a problem than in a stationary-tube PCR format. Thus, a flow-through PCR chip can be reused for extended periods without major cleaning. . . .



Ex. 1027 at 1046 [emphasis added]. Kopp et al. also explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications:

> To build a complex system analogous to an electronic integrated circuit, it is necessary to integrate a variety of other continuously working parts. A cursory glance at developments in miniaturized total analysis systems (uTAS) during the past 5 years reveals that most of the relevant components, such as **continuous-flow mixers** (8), **continuous-flow microreactors** (9), **continuous separations** (10), and high-speed capillary gel electrophoresis (as a conventional batch process), have already been presented (11). Accordingly, the **continuous-flow microamplifier** should allow the creation of highly elaborate analysis and synthesis systems.
>
> Although chemical amplification reactors will obviously not be applicable to all reactions, neither will they be limited to PCR. Devices of this kind open up whole new areas of application in medical diagnostics. For example, the online amplification and monitoring of a specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy.

Ex. 1027 at 1047 [emphasis added].

In 2001, in another article not previously considered by the Office, Lagally reported using a microfluidic reactor to conduct single-molecule DNA amplification by PCR. Lagally summarized the state of the art as of February 2001, explaining continuous flow PCR microreactors had recently undergone substantial evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array

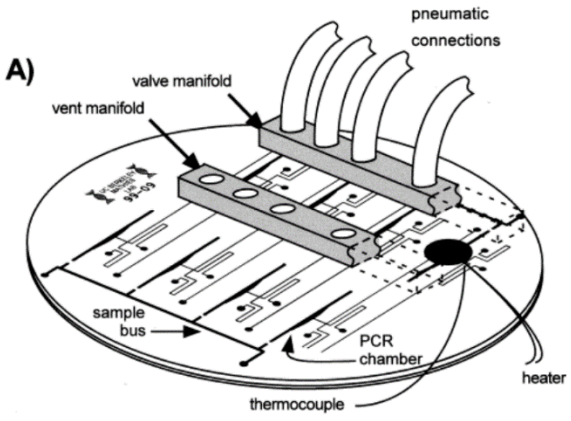
electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.

Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems.[22-24] An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

Lagally *et al.* developed a further improvement – a device and method for achieving single molecule amplification in a microchannel environment.

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> Microfluidic PCR-CE device. The PCR chambers are connected to a common sample bus through a set of valves. Hydrophobic vents at the other end of the PCR chambers are used to locate the sample and to eliminate gas. The PCR chambers are directly connected to the cross channel of the CE system for product injection and analysis. Two aluminum manifolds, one each for the vents and valves, are placed onto the respective ports and clamped in place using vacuum. The manifolds connect to external solenoid valves for pressure and vacuum actuation. Thermal cycling is accomplished using a resistive heater and a miniature thermocouple below the 280-nL chamber.



Ex. 1028 at 565 (see figure legend).

By 1999, Vogelstein had reported **various other applications for single-molecule microfluidic PCR.** Ex. 1044 at 9241. In a seminal article which has been cited by over 1,000 other publications, Vogelstein explained that PCR amplification of small aliquots or droplets containing single molecules was effective for "detection of a small number of mutant-containing cells among a large excess of normal cells." *Id.* at 9236. In Vogelstein's method, the DNA was diluted into 7 µl volumes such that, on average, there was one template molecule for every two wells (i.e., approximately half of the wells contained no DNA template molecule and approximately half contained a single DNA template molecule). *Id.* at 9236, 9237. This avoided the problem of background noise impairing the determination of whether each volume contained the mutant sequence. *Id.* By counting the number of volumes which indicated wild type or mutant templates, the presence or absence of the mutation (e.g., a sequence associated with colorectal cancer) can be determined. *Id.* at 9238. The accuracy of the test is a function of the number of discrete volumes into which the sample is diluted. *Id*. at 9239. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. *Id.* at 9239. ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude.") *Id.* 9239. The article explained that the same technique would be useful for **detecting a wide array of mutations, dislocations and allelic imbalances**, as summarized in Table 1. *Id.* at 9241. Vogelstein thus taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby overcoming the signal-to-noise limitations associated with certain other PCR

techniques and enabling the detection of relatively rare mutations, dislocations and allelic imbalances. Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84.

In sum, Corbett, Kopp, Burns II, Lagally and Vogelstein show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

3. There Were Strong Motivations to Modify the Microfluidic Droplet Reactors of Quake, Burns I or Shaw Stewart to Conduct Microfluidic PCR as Taught by Corbett, Kopp, Burns II, Lagally or Vogelstein

In the combinations proffered herein, the microfluidic droplet reactor of Quake, Burns I and Shaw Stewart are relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[6] Ex 1001 at 78:22-24. In the proffered combinations, these limitations are met by using the microfluidic droplet reactors that utilize two continuously flowing immiscible fluids to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake, Burns I or Shaw Stewart to conduct microfluidic PCR as taught by Corbett, Kopp, Burns II, Lagally or Vogelstein. Stated a different way, a skilled artisan would have considered it obvious to miniaturize and modify the PCR apparatus of Corbett by modifying it to incorporate the microfluidic reactor using two continuously flowing immiscible fluids of Quake, Burns I or Shaw Stewart, as evidenced by Kopp, Burns II, Lagally and Vogelstein's

[6] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing.

Quake **specifically suggests that his microfluidic device could be used to perform PCR**. Quake explained that "[m]icrofabrication permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR…." Ex. 1034 at 23:26-30. Quake noted that "PCR is [] generally the method of choice to detect viral DNA or RNA directly in clinical specimens. The advantage of PCR for viral diagnostics is its high sensitivity; PCR can detect very low numbers of viruses in a small clinical specimen." *Id.* at 2:31-3:2.

Further, at the time of filing it was **well-known that PCR could be performed in plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the

polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

More generally, a skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic reactors of Quake, Burns I or Shaw Stewart because doing so would have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. "In addition, decreasing the scale of PCR allows the reaction to be carried out **more efficiently**, producing more product in **less time** with **less side reactions**." Ex. 1013 at 192, Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions, and using two continuous streams as opposed to injecting the aqueous fluid into the immiscible oil, would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing**).** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could

demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Quake, Burns I or Shaw Stewart to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing a PCR reaction in the microfluidic droplet reactor of Quake, Burns I or Shaw Stewart would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶ 91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id*. These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004

¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241.

A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Quake, Burns I and Shaw Stewart to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Corbett, Kopp, Burns II, Lagally or Vogelstein) to incorporate a droplet reactor (Quake, Burns I or Shaw Stewart). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Kopp, Burns II, Lagally or Vogelstein. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the single fluid in microfluidic chip reactor (e.g., Burns II or Lagally) or droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Corbett or Kopp). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow

PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or continuous flow through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.

> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides

> an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an

autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

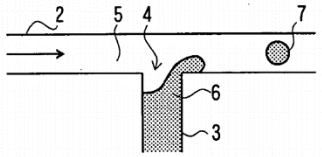
Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Quake could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful in miniaturizing and modifying Corbett or modifying Kopp or Lagally to work in the microfluidic droplet reactor of Quake, Burns I or Shaw Stewart, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[7]

[7] "In *Monarch Knitting Machinery Corp. v. Sulzer Mo*rat *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

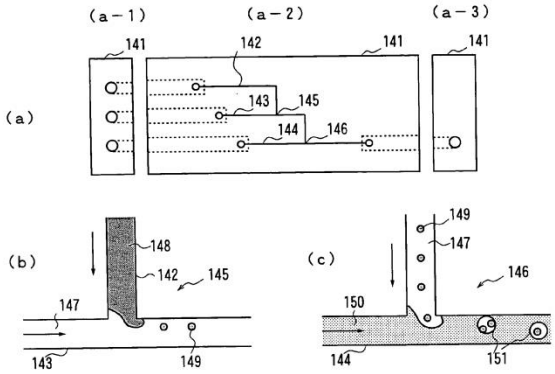
Higuchi I discloses a microreactor in which a dispersion phase (6) is ejected from a dispersion phase feeding port (4) toward a continuous phase (5) flowing in a microchannel (2) in such a manner that flows of the dispersion phase (6) and the continuous phase (5) cross each other, thereby obtaining microdroplets (7), formed by the shear force of the continuous phase (5), having a size smaller than the width of the channel for feeding the dispersion phase (6). Ex. 1018 at Fig. 2 (reproduced at right). Higuchi teaches many variations of the embodiment of Figure 2 and one representative variation is shown in Fig. 19.

FIG. 2



> In [Figures 19a-c], reference numeral 141 represents a main body of the microcapsule-forming apparatus, reference numeral 142 represents a microchannel in which a dispersion phase (for example, water) flows, reference numeral 143 represents a microchannel in which a first continuous phase (for example, oil) flows, reference numeral 144 represents a microchannel in which a second continuous phase (for example, water) flows, reference numeral 145 represents the first junction at which flows of the dispersion phase and the first continuous phase are joined together, reference numeral 146 represents the second junction at which flows of the dispersion phase, the first continuous phase, and the second continuous phase are joined together, reference numeral 147 represents the first continuous phase, reference numeral 148 represents the dispersion phase, reference numeral 149 represents an emulsion (for example, water), reference numeral 150 represents the second continuous phase, and reference numeral 151 represents formed microcapsules. The microcapsules 151 can contain one or more emulsions 149. Numeral 143 represents a microchannel in which a first continuous phase (for example, oil) flows, reference numeral 144 represents a microchannel in which a second

FIG. 19



> continuous phase (for example, water) flows, reference numeral 145 represents the first junction at which flows of the dispersion phase and the first continuous phase are joined together, reference numeral 146 represents the second junction at which flows of the dispersion phase, the first continuous phase, and the second continuous phase are joined together, reference numeral 147 represents the first continuous phase, reference numeral 148 represents the dispersion phase, reference numeral 149 represents an emulsion (for example, water), reference numeral 150 represents the second continuous phase, and reference numeral 151 represents formed microcapsules. The microcapsules 151 can contain one or more emulsions 149.

Ex. 1018 ¶100.

The microchannel device of Higuchi I-III was specifically intended to be used to perform emulsion-based chemical reactions. Ex. 1043 (contemporaneous article by Higuchi entitled *Chemical reactions in microdroplets by electrostatic manipulation of droplets in liquid media*). Higuchi I-III thus demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail in the following sections, the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake, Burns I or Shaw Stewart to conduct microfluidic PCR as taught by Corbett, Kopp, Burns II or Lagally. Ex. 1004 ⁋⁋109,111. Alternatively, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic droplet reactor of Quake, Burns I or Shaw Stewart, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing. *Id.*

B. The Combination of Quake, Corbett and Lagally Presents a Substantial New Question of Patentability for Claims 1-8 of the '148 Patent

As discussed in Section IV.C, above, the prosecution history taken as a whole indicates that the '148 patent was allowed primarily because the Examiner believed that the prior art failed to teach conducting PCR reactions in droplets formed from two continuously flowing immiscible fluids.

As discussed in Section IV.D, above, the PTAB denied institution of an *inter partes* review of U.S. Patent No. 8,882,148 because the petition failed to explain why a skilled artisan would have believed that the PCR technique of Haff could have been conducted on a microfluidic scale as taught in Quake.

Accordingly, the claims have been considered nonobvious by the Office because the **prior art was not believed to teach a technique for conducting PCR reactions in droplets formed from two continuously flowing immiscible fluids**.

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Quake), ii) microfluidic PCR devices were well known (Corbett and Lagally), and iii) there were compelling reasons to use the microfluidic droplet reactors to conduct microfluidic PCR (Lagally, Kopp, Burns II and Vogelstein).

As to the first feature – forming plugs from continuously flowing immiscible fluids – the '148 patent's teachings in this regard were copied almost verbatim from the Quake PCT (Ex. 1034). The table below compares the Quake PCT publication with the Ismagilov specification, which was filed about two months later.

Quake PCT (published March 21, 2002) (Ex. 1034)	**Ismagilov provisional application (filed May 9, 2002) (Ex. 1003) and '148 patent (Ex. 1001)**

34:32-35:7 The pressure at the droplet extrusion region can also be regulated by adjusting the pressure on the main and sample inlets, for example, with pressurized syringes feeding into those inlets. By controlling the pressure difference between the oil and water sources at the droplet extrusion region, the size and periodicity of the droplets generated may be regulated. Alternatively, a valve may be placed at or coincident to either the droplet extrusion region or the sample inlet connected thereto to control the flow of solution into the droplet extrusion region, thereby controlling the size and periodicity of the droplets.	Provisional at 22:12-19, '148 patent at 21:56-65 The pressure at the plug-forming region can also be regulated by adjusting the pressure on the main and sample inlets, for example with pressurized syringes feeding into those inlets. By controlling the difference between the oil and water flow rates at the plug-forming region, the size and periodicity of the plugs generated may be regulated. Alternatively, a valve may be placed at or coincident to either the plug forming region or the sample inlet connected thereto to control the flow of solution into the plug-forming region, thereby controlling the size and periodicity of the plugs.

Attached as Ex. 1035 is a table showing the numerous passages which Ismagilov copied key disclosures verbatim or nearly verbatim from the Quake PCT. These were not merely background or "state of the art" descriptions; rather, they go to the heart of the claimed subject matter.

The disclosure of Quake was summarized by the Patent Trial and Appeal Board in its November 15, 2015 decision. Therein the Board found that Quake discloses a droplet reactor in which droplets are formed by continuous streams of immiscible fluids:

> Quake relates to microfluidic devices and methods for analyzing and/or sorting biological materials. Ex. 1004, Abstract [which corresponds to Ex. 1033]….
> The devices comprise a main channel, through which a pressurized stream of oil is passed, and at least one sample inlet channel, through which a pressurized stream of aqueous solution is passed. *Id*. A junction joins the main channel with the sample inlet channel. *Id*. By adjusting the pressure of the oil and/or the aqueous solution, a pressure difference can be established such that the stream of aqueous solution is sheared off at a regular frequency as it enters the oil stream, thereby forming droplets. *Id*. Figure 16A of Quake is reproduced below:

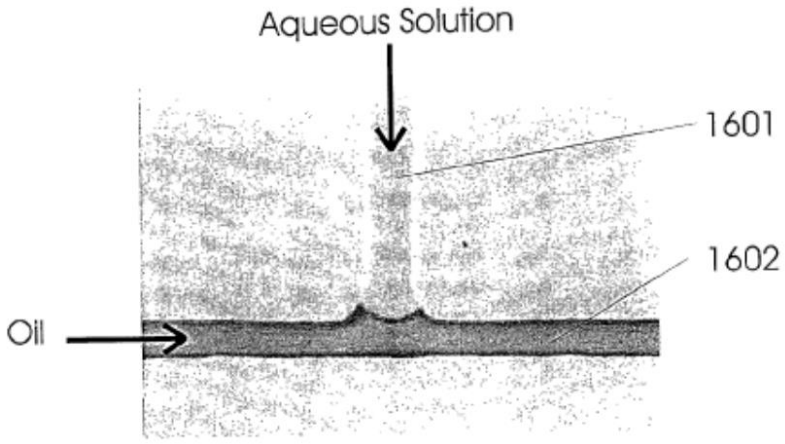


FIG. 16A

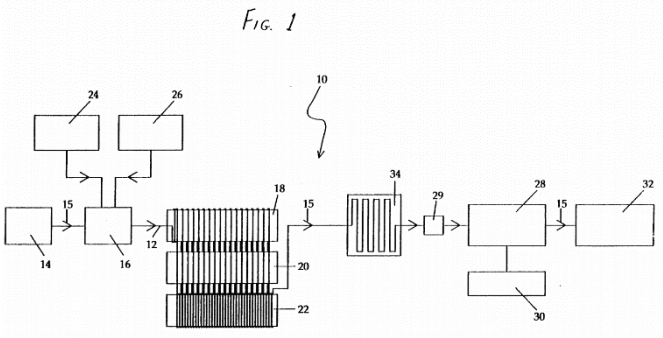
> Figure 16A depicts the channel architecture for the droplet extrusion region. Channel 1601 containing an aqueous solution intersects with the main channel 1602 containing oil. [*Id.*] ¶ 292.
>
> In preferred embodiments, the droplets have a volume of approximately 0.1 to 100 picoliters. *Id.* According to Quake, microfabrication of the device "permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." *Id.* ¶ 80.

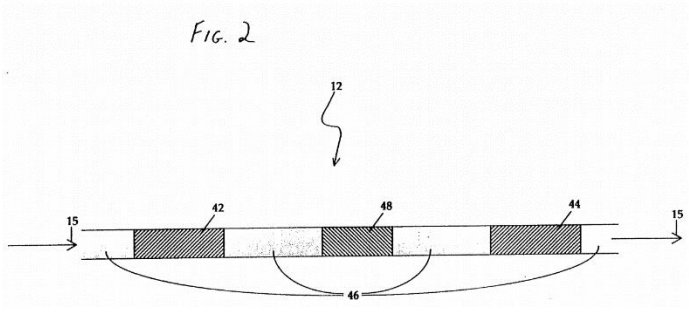
Ex. 1052 at 11-12.

The Patent Owner did not contest in the *inter partes* review proceeding that Quake met the plug-formed-by-continuous-streams limitations of the '148 patent. Ex. 1051 *passim*; Ex. 1052, *passim*. Moreover, Quake himself during prosecution characterized his invention as involving continuous streams.[8] Accordingly, notwithstanding statements to the contrary during *ex parte* prosecution of the '148 patent (discussed in Section IV.C above) it is clear that Quake does in fact teach forming plugs from continuous streams of immiscible fluids.

[8] When distinguishing his invention over one of the embodiments of Shaw Stewart in which reagent is periodically injected, Quake noted that his "invention relates, in part, to the discovery that combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid (e.g., decane) it is possible to produce small, discrete, monodisperse droplets of the aqueous solution flowing in the second fluid." Ex. 1058 at September 25, 2006 response, p. 15.

Turning the second issue, as of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Corbett and Lagally. In 1991, about eleven years prior to the earliest effective filing date, Corbett disclosed using a microchannel or capillary device to conduct DNA amplification reactions such as PCR by injecting plugs of reactants into a carrier stream that is passed through different temperature zones. Corbett explained that as of 1991 "the most critical problem in applying nucleic acid amplification procedures, particularly in repetitive diagnostic assays in a clinical laboratory, is contamination of the reaction mixture by small amounts of DNA which contain sequences capable of being amplified under the conditions of assay." Ex. 1010 at 3:7-12. "Automation of procedures minimize[s] the opportunity for such contamination and allows the implementation of stringent quality control." *Id.* at 3:15-19. "As is shown in FIG. 1, the apparatus 10 comprises tube 12 through which a fluid stream passes. As is more clearly shown in FIG. 2, the fluid stream includes pockets of reaction mixtures 42 and 44 and purging solution 48 separated by carrier fluid 46." *Id*. at 7:58-62.





"The pump is actuated and the reaction mixture of 20 µl or less is injected into the mineral oil or silicone oil carrier fluid and specific DNA sequences (whose limits are defined by the oligonucleotide primers) present in the sample is amplified as it passes cyclically through the temperature zones." Ex. 1010 at 9:35-40. Corbett thus demonstrates that it was known at least as of 1991 that reduction of contamination risk in PCR could be achieved by performing the PCR on microliter sized plugs of reactant in capillary tube which passes through various temperature zones. Ex. 1004 ¶77.

In 2001, in another article not previously considered by the Office, Lagally reported using a single fluid microfluidic reactor to conduct single-molecule DNA amplification by PCR. Lagally summarized the state of the art as of February 2001, explaining continuous flow PCR microreactors had recently undergone substantial evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/µL, in volumes down to 1 µL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample

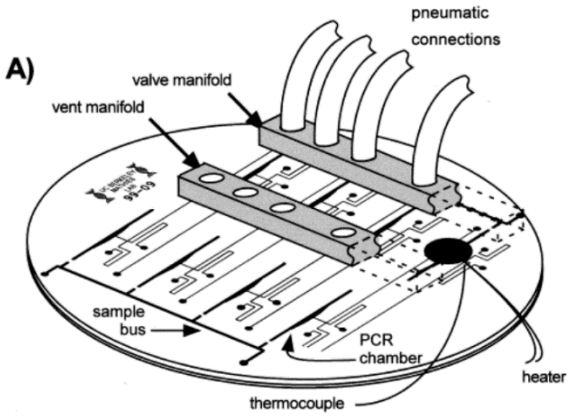
> volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 566.

Lagally *et al.* developed a further improvement – a device and method for achieving single molecule amplification in a microchannel environment.

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced

> microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> Microfluidic PCR-CE device. The PCR chambers are connected to a common sample bus through a set of valves. Hydrophobic vents at the other end of the PCR chambers are used to locate the sample and to eliminate gas. The PCR chambers are directly connected to the cross channel of the CE system for product injection and analysis. Two aluminum manifolds, one each for the vents and valves, are placed onto the respective ports and clamped in place using vacuum. The manifolds connect to external solenoid valves for pressure and vacuum actuation. Thermal cycling is accomplished using a resistive heater and a miniature thermocouple below the 280-nL chamber.



Ex. 1028 at 567 (see figure legend).

In sum, Corbett and Lagally show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Quake is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule"), Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Quake) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake to conduct microfluidic PCR in droplets as taught by Corbett, or to modify the reactions of Lagally to be performed in microfluidic droplets instead of continuous flow of a single fluid in a microfluidic channel. Stated a different way, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to use two continuous streams of immiscible fluid as used in a microfluidic droplet reactor of Quake, as evidenced by Lagally's contemporaneous report that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing.

Quake **specifically suggests that his microfluidic device could be used to perform PCR**. Quake explained that "[m]icrofabrication permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." Ex. 1034 at 23:26-30. Quake noted that "PCR is [] generally the method of choice to detect viral DNA or RNA directly in clinical specimens. The advantage of PCR for viral diagnostics is its high sensitivity; PCR can detect very low numbers of viruses in a small clinical specimen." *Id.* at 2:31-3:2.

More generally, a skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic reactors of Quake because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to

microfluidic dimensions and the use of two continuous streams of immiscible fluids would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. Likewise, the reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Quake to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Quake would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as

the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id*. These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241;

Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Quake to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Lagally) to incorporate a droplet reactor (Quake). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Lagally. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the single fluid in microfluidic chip reactor (e.g., Lagally) or droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Corbett). Droplet reactors also **decrease contamination and increase the**

number of reactors that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed." Ex. 1027 at Abstract). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* at 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or continuous flow through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling

> profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced

> microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic**

droplet (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Quake could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful in miniaturizing and modifying Corbett or modifying Lagally to work in the microfluidic droplet reactor of Quake, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[9] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail in the following sections, the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake to conduct microfluidic PCR as taught by Corbett, Kopp, Burns II or Lagally. Ex. 1004 ¶¶109,111. Alternatively, a skilled artisan would

[9] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic droplet reactor of Quake, as evidenced by Lagally's contemporaneous report that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing. *Id*.

Not only would a reasonable Examiner consider these teachings relevant in deciding whether claims 1-8 of the '148 patent are patentable, but would have rejected the claims of the '148 patent in view of these teachings. See MPEP § 2242. The correspondence between the claims of the '148 patent and the combined teachings of the Quake, Corbett and Lagally references are set forth in Section VII.A, below.

C. The Combination of Quake, Corbett and Burns II Presents a Substantial New Question of Patentability for Claims 1-8 of the '148 Patent

As discussed in Section IV.C and IV.D above, the claims have been considered nonobvious by the Office because the **prior art was not believed to teach a technique for conducting PCR reactions in droplets formed from two continuously flowing immiscible fluids**.

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Quake), ii) microfluidic PCR devices were well known (Corbett and Burns II), and iii) there were compelling reasons to use the microfluidic droplet reactors to conduct microfluidic PCR (Lagally, Kopp, Burns II and Vogelstein).

As to the first feature – forming plugs from continuously flowing immiscible fluids – the '148 patent's teachings in this regard were copied almost verbatim from the Quake PCT (Ex.

1034). The table below compares the Quake PCT publication with the Ismagilov specification, which was filed about two months later.

Quake PCT (published March 21, 2002) (Ex. 1034)	**Ismagilov provisional application (filed May 9, 2002) (Ex. 1003) and '148 patent (Ex. 1001)**
34:32-35:7 The pressure at the droplet extrusion region can also be regulated by adjusting the pressure on the main and sample inlets, for example, with pressurized syringes feeding into those inlets. By controlling the pressure difference between the oil and water sources at the droplet extrusion region, the size and periodicity of the droplets generated may be regulated. Alternatively, a valve may be placed at or coincident to either the droplet extrusion region or the sample inlet connected thereto to control the flow of solution into the droplet extrusion region, thereby controlling the size and periodicity of the droplets.	Provisional at 22:12-19, '148 patent at 21:56-65 The pressure at the plug-forming region can also be regulated by adjusting the pressure on the main and sample inlets, for example with pressurized syringes feeding into those inlets. By controlling the difference between the oil and water flow rates at the plug-forming region, the size and periodicity of the plugs generated may be regulated. Alternatively, a valve may be placed at or coincident to either the plug forming region or the sample inlet connected thereto to control the flow of solution into the plug-forming region, thereby controlling the size and periodicity of the plugs.

Attached as Ex. 1035 is a table showing the numerous passages which Ismagilov copied key disclosures verbatim or nearly verbatim from the Quake PCT. These were not merely background or "state of the art" descriptions; rather, they go to the heart of the claimed subject matter.

The disclosure of Quake was summarized by the Patent Trial and Appeal Board in its November 15, 2015 decision. Therein the Board found that Quake discloses a droplet reactor in which droplets are formed by continuous streams of immiscible fluids:

> Quake relates to microfluidic devices and methods for analyzing and/or sorting biological materials. Ex. 1004, Abstract [which corresponds to Ex. 1033]….
> The devices comprise a main channel, through which a pressurized stream of oil is passed, and at least one sample inlet channel, through which a pressurized

stream of aqueous solution is passed. *Id*. A junction joins the main channel with the sample inlet channel. *Id*. By adjusting the pressure of the oil and/or the aqueous solution, a pressure difference can be established such that the stream of aqueous solution is sheared off at a regular frequency as it enters the oil stream, thereby forming droplets. *Id*. Figure 16A of Quake is reproduced below:

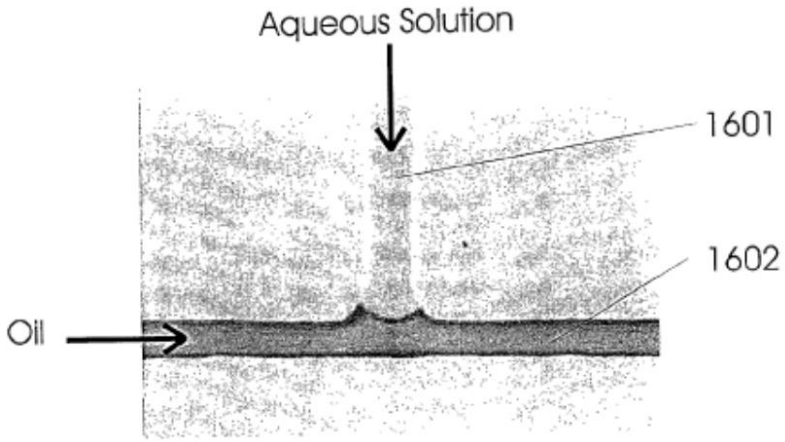


FIG. 16A

Figure 16A depicts the channel architecture for the droplet extrusion region. Channel 1601 containing an aqueous solution intersects with the main channel 1602 containing oil. [*Id.*] ¶ 292.

In preferred embodiments, the droplets have a volume of approximately 0.1 to 100 picoliters. *Id*. According to Quake, microfabrication of the device "permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." *Id*. ¶ 80.

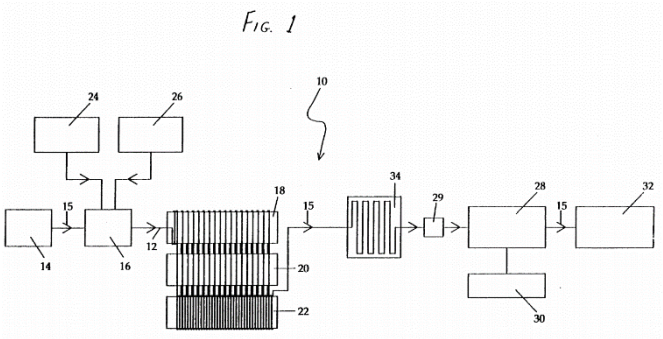
Ex. 1052 at 11-12.

The Patent Owner did not contest in the *inter partes* review proceeding that Quake met the plug-formed-by-continuous-streams limitations of the '148 patent. Ex. 1051 *passim*; Ex. 1052, *passim*. Moreover, Quake himself during prosecution characterized his invention as involving continuous streams.[10] Accordingly, notwithstanding statements to the contrary during

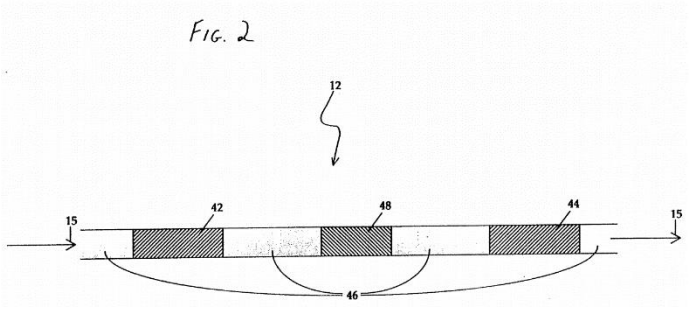
[10] When distinguishing his invention over one of the embodiments of Shaw Stewart in which reagent is periodically injected, Quake noted that his "invention relates, in part, to the discovery that combining a flowing stream of an

ex parte prosecution of the '148 patent (discussed in Section IV.C above) it is clear that Quake does in fact teach forming plugs from continuous streams of immiscible fluids.

Turning the second issue, as of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Corbett and Burns II. In 1991, about eleven years prior to the earliest effective filing date, Corbett disclosed using a microchannel or capillary device to conduct DNA amplification reactions such as PCR by injecting plugs of reactants into a carrier stream that is passed through different temperature zones. Corbett explained that as of 1991 "the most critical problem in applying nucleic acid amplification procedures, particularly in repetitive diagnostic assays in a clinical laboratory, is contamination of the reaction mixture by small amounts of DNA which contain sequences capable of being amplified under the conditions of assay." Ex. 1010 at 3:7-12. "Automation of procedures minimize[s] the opportunity for such contamination and allows the implementation of stringent quality control." *Id.* at 3:15-19. "As is shown in FIG. 1, the apparatus 10 comprises tube 12 through which a fluid stream passes. As is more clearly shown in FIG. 2, the fluid stream includes pockets of reaction mixtures 42 and 44 and purging solution 48 separated by carrier fluid 46." *Id*. at 7:58-62.



aqueous solution and a flowing stream of an immiscible fluid (e.g., decane) it is possible to produce small, discrete, monodisperse droplets of the aqueous solution flowing in the second fluid." Ex. 1058 at September 25, 2006 response, p. 15.



"The pump is actuated and the reaction mixture of 20 µl or less is injected into the mineral oil or silicone oil carrier fluid and specific DNA sequences (whose limits are defined by the oligonucleotide primers) present in the sample is amplified as it passes cyclically through the temperature zones." Ex. 1010 at 9:35-40. Corbett thus demonstrates that it was known at least as of 1991 that reduction of contamination risk in PCR could be achieved by performing the PCR on microliter sized plugs of reactant in capillary tube which passes through various temperature zones. Ex. 1004 ¶77.

In 1996, Burns II reported the use of microfluidic devices to perform PCR. Ex. 1008. Burns II explained that "[n]o new biochemistries or DNA detection methods are required for their use; the components simply reproduce current laboratory procedures in a micron-sized environment." *Id*. at 5560. In other words, the PCR chemistry did not need to be modified – it worked without modification on the microfluidic scale. In the method of Burns II, a sample containing a DNA plasmid was loaded into one of the reaction wells (A) and a mixture of Taq enzyme and buffer was loaded into the other. *Id.* at 5558. Each formed droplets of about 60 nl in the parallel 500 x 25 µm channels (B) which came together at the Y junction to form a single larger droplet (about 120 nl). *Id.* at 5558-59; Ex. 1004 ¶40.

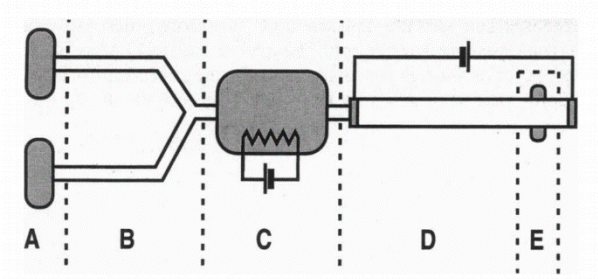


FIG. 1. Example of an integrated DNA analysis system, represented schematically. The individual components of the system are injection entry ports (*A*), liquid pumping channels (*B*), thermally controlled reaction chamber (*C*), electrophoresis channel (*D*), and DNA band migration detector (*E*). Each component would have associated sensors, control circuitry, and external connections.

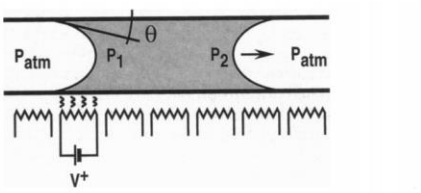


FIG. 2. Schematic drawing showing the principle of thermally induced drop motion in a closed channel. The case of a single aqueous drop in a hydrophilic channel is presented, where V is an applied voltage, P_{atm} is atmospheric pressure, P_1 is the receding-edge internal pressure, P_2 is the advancing-edge internal pressure, and θ is the contact angle of the liquid–gas–solid interface. The contact angle will depend on the surface characteristics of the channel and the constituents of the drop, with a hydrophilic interaction giving θ between 0 and 90 degrees, and a hydrophobic surface giving θ between 90 and 180 degrees. Surface treatments can also reduce contact angle hysteresis and, therefore, reduce the temperature difference necessary for drop motion.

The droplet then passed through the resistive heater portion (C) in which PCR amplification occurred. *Id.* at 5556. Afterwards the amplification results were optically detected in zone D via gel electrophoresis *Id.* at 5559. Burns II noted that "[t]he use of common fabrication methods allows the assembly of increasingly complex, multicomponent, integrated systems from a small, defined set of standardized elements" and that "[f]uture design efforts have a strong impetus to reduce component size and improve efficiency." *Id.* at 5560-61.

In sum, Corbett and Burns II show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Quake is relied upon to meet all of the limitations of the independent claims of the '148 patent (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[11] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Quake) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

[11] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake to conduct microfluidic PCR as taught by Corbett and Burns II. Ex. 1004 ⁋⁋109,111. Stated a different way, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic droplet reactor of Quake, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing. *Id.*

Quake **specifically suggests that his microfluidic device could be used to perform PCR**. Quake explained that "[m]icrofabrication permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR..." Ex. 1034 at 23:26-27. Quake noted that "PCR is [] generally the method of choice to detect viral DNA or RNA directly in clinical specimens. The advantage of PCR for viral diagnostics is its high sensitivity; PCR can detect very low numbers of viruses in a small clinical specimen." *Id.* at 2:31-3:2.

More generally, a skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic droplet reactor of Quake because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts of reagents used, which would in turn

decrease operating costs. Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Quake to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Quake or Shaw Stewart would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient

time for nonspecific binding. *Id*. These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the

assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Quake, Burns I and Shaw Stewart to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Burns II) to incorporate a droplet reactor (Quake). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Burns II. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the single fluid in microfluidic chip reactor (e.g., Burns II) or droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Corbett or Kopp). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed." Ex. 1027 at Abstract). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* at 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or continuous flow through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually

> transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .

> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an

autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Quake could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful in modifying Burns II to work in the microfluidic droplet reactor of Quake, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[12] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of microdroplet

[12] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail in the following sections, the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake to conduct microfluidic PCR as taught by Corbett and Burns II. Ex. 1004 ¶¶109,111. Alternatively, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic droplet reactor of Quake, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing. *Id*.

Not only would a reasonable Examiner consider these teachings relevant in deciding whether claims 1-8 of the '148 patent are patentable, but would have rejected the claims of the '148 patent in view of these teachings. See MPEP § 2242. The correspondence between the claims of the '148 patent and the combined teachings of the Quake, Corbett and Burns II references are set forth in Section VII.B, below.

D. The Combination of Quake, Corbett and Vogelstein Presents a Substantial New Question of Patentability for Claims 1-8 of the '148 Patent

As discussed in Section IV.C and IV.D above, the claims have been considered nonobvious by the Office because the **prior art was not believed to teach a technique for conducting PCR reactions in droplets formed from two continuously flowing immiscible fluids**.

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Quake), ii) microfluidic PCR

devices were well known (Corbett and Vogelstein), and iii) there were compelling reasons to use the microfluidic droplet reactors to conduct microfluidic PCR (Lagally, Kopp and Vogelstein).

As to the first feature – forming plugs from continuously flowing immiscible fluids – the '148 patent's teachings in this regard were copied almost verbatim from the Quake PCT (Ex. 1034). The table below compares the Quake PCT publication with the Ismagilov specification, which was filed about two months later.

Quake PCT (published March 21, 2002) (Ex. 1034)	**Ismagilov provisional application (filed May 9, 2002) (Ex. 1003) and '148 patent (Ex. 1001)**
34:32-35:7 The pressure at the droplet extrusion region can also be regulated by adjusting the pressure on the main and sample inlets, for example, with pressurized syringes feeding into those inlets. By controlling the pressure difference between the oil and water sources at the droplet extrusion region, the size and periodicity of the droplets generated may be regulated. Alternatively, a valve may be placed at or coincident to either the droplet extrusion region or the sample inlet connected thereto to control the flow of solution into the droplet extrusion region, thereby controlling the size and periodicity of the droplets.	Provisional at 22:12-19, '148 patent at 21:56-65 The pressure at the plug-forming region can also be regulated by adjusting the pressure on the main and sample inlets, for example with pressurized syringes feeding into those inlets. By controlling the difference between the oil and water flow rates at the plug-forming region, the size and periodicity of the plugs generated may be regulated. Alternatively, a valve may be placed at or coincident to either the plug forming region or the sample inlet connected thereto to control the flow of solution into the plug-forming region, thereby controlling the size and periodicity of the plugs.

Attached as Ex. 1035 is a table showing the numerous passages which Ismagilov copied key disclosures verbatim or nearly verbatim from the Quake PCT. These were not merely background or "state of the art" descriptions; rather, they go to the heart of the claimed subject matter.

The disclosure of Quake was summarized by the Patent Trial and Appeal Board in its November 15, 2015 decision. Therein the Board found that Quake (Ex. 1033) discloses a droplet reactor in which droplets are formed by continuous streams of immiscible fluids:

> Quake relates to microfluidic devices and methods for analyzing and/or sorting biological materials. Ex. 1004, Abstract [which corresponds to Ex. 1033]….
> The devices comprise a main channel, through which a pressurized stream of oil is passed, and at least one sample inlet channel, through which a pressurized stream of aqueous solution is passed. *Id*. A junction joins the main channel with the sample inlet channel. *Id*. By adjusting the pressure of the oil and/or the aqueous solution, a pressure difference can be established such that the stream of aqueous solution is sheared off at a regular frequency as it enters the oil stream, thereby forming droplets. *Id*. Figure 16A of Quake is reproduced below:

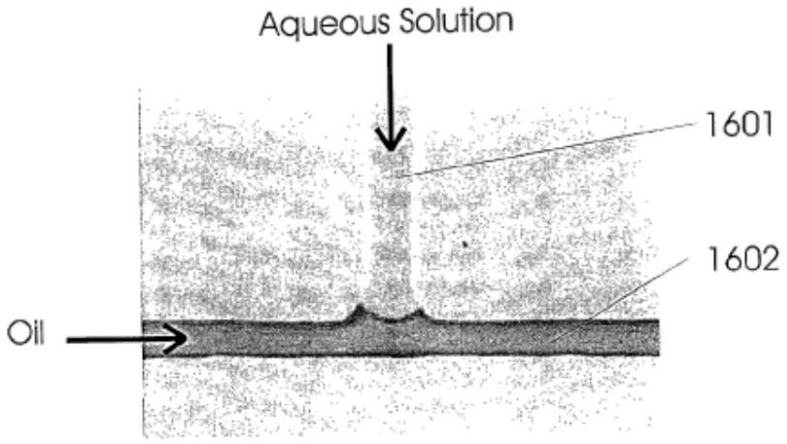


FIG. 16A

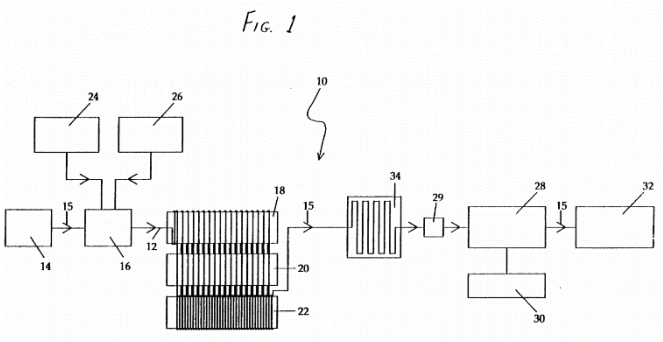
> Figure 16A depicts the channel architecture for the droplet extrusion region. Channel 1601 containing an aqueous solution intersects with the main channel 1602 containing oil. [*Id.*] ¶ 292.
> In preferred embodiments, the droplets have a volume of approximately 0.1 to 100 picoliters. *Id.* According to Quake, microfabrication of the device "permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." *Id*. ¶ 80.

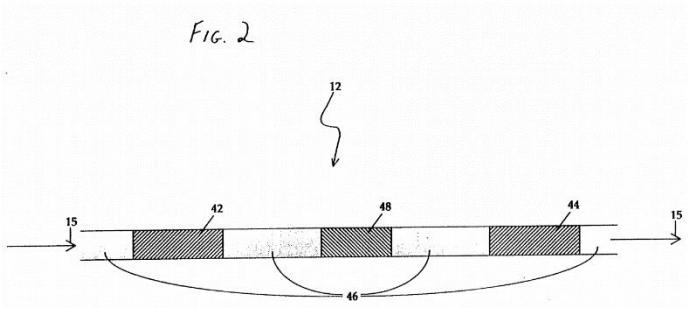
Ex. 1052 at 11-14.

The Patent Owner did not contest in the *inter partes* review proceeding that Quake met the plug-formed-by-continuous-streams limitations of the '148 patent. Ex. 1051 *passim*; Ex. 1052, *passim*. Moreover, Quake himself during prosecution characterized his invention as involving continuous streams.[13] Accordingly, notwithstanding statements to the contrary during *ex parte* prosecution of the '148 patent (discussed in Section IV.C above) it is clear that Quake does in fact teach forming plugs from continuous streams of immiscible fluids.

Turning the second issue, as of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Corbett and Vogelstein. In 1991, about eleven years prior to the earliest effective filing date, Corbett disclosed using a microchannel or capillary device to conduct DNA amplification reactions such as PCR by injecting plugs of reactants into a carrier stream that is passed through different temperature zones. Corbett explained that as of 1991 "[t]he most critical problem in applying nucleic acid amplification procedures, particularly in repetitive diagnostic assays in a clinical laboratory, is contamination of the reaction mixture by small amounts of DNA which contain sequences capable of being amplified under the conditions of assay." Ex. 1010 at 3:7-12. "Automation of procedures minimize[s] the opportunity for such contamination and allows the implementation of stringent quality control." *Id.* at 3:15-19. *"*As is shown in FIG. 1, the apparatus 10 comprises tube 12 through which a fluid stream passes. As is more clearly shown in FIG. 2, the fluid stream includes pockets of reaction mixtures 42 and 44 and purging solution 48 separated by carrier fluid 46." *Id*. at 7:58-62.

[13] When distinguishing his invention over one of the embodiments of Shaw Stewart in which reagent is periodically injected, Quake noted that his "invention relates, in part, to the discovery that combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid (e.g., decane) it is possible to produce small, discrete, monodisperse droplets of the aqueous solution flowing in the second fluid." Ex. 1058 at September 25, 2006 response, p. 15.





"The pump is actuated and the reaction mixture of 20 µl or less is injected into the mineral oil or silicone oil carrier fluid and specific DNA sequences (whose limits are defined by the oligonucleotide primers) present in the sample is amplified as it passes cyclically through the temperature zones." Ex. 1010 at 9:35-40. Corbett thus demonstrates that it was known at least as of 1991 that reduction of contamination risk in PCR could be achieved by performing the PCR on microliter sized plugs of reactant in capillary tube which passes through various temperature zones. Ex. 1004 ¶77.

By 1999, Vogelstein had reported **various applications for single-molecule microfluidic PCR**. Ex. 1044 at 9241. In a seminal article which has been cited over by over 1,000 other publications, Vogelstein explained that PCR amplification of small aliquots or droplets containing single molecules was effective for "detection of a small number of mutant-containing cells among a large excess of normal cells." *Id.* at 9236. In Vogelstein's method, the DNA was diluted into 7 µl volumes such that, on average, there was one template molecule for every two wells (i.e., approximately half of the wells contained no DNA template molecule and

approximately half contained a single DNA template molecule). *Id.* at 9236, 9237. This avoided the problem of background noise impairing the determination of whether each volume contained the mutant sequence. *Id.* By counting the number of volumes which indicated wild type or mutant templates, the presence or absence of the mutation (e.g., a sequence associated with colorectal cancer) can be determined. *Id.* at 9238. The accuracy of the test is a function of the number of discrete volumes into which the sample is diluted. *Id*. at 9239. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. *Id.* at 9239. ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude.") The article explained that the same technique would be useful for **detecting a wide array of mutations, dislocations and allelic imbalances**, as summarized in Table 1. *Id.* at 9241. Vogelstein thus taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby overcoming the signal-to-noise limitations associated with certain other PCR techniques and enabling the detection of relatively rare mutations, dislocations and allelic imbalances. Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84.

In sum, Corbett and Vogelstein show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Quake is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules

and at least one other molecule").[14] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Quake) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake to conduct microfluidic PCR as taught by Corbett and Vogelstein. Stated a different way, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic droplet reactor of Quake, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing.

Quake **specifically suggests that his microfluidic device could be used to perform PCR**. Quake explained that "[m]icrofabrication permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." Ex. 1034 at 23:26-30. Quake noted that "PCR is [] generally the method of choice to detect viral DNA or RNA directly in clinical specimens. The advantage of PCR for viral diagnostics is its high sensitivity; PCR can detect very low numbers of viruses in a small clinical specimen." *Id.* at 2:31-3:2.

More generally, skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic droplet reactor of Quake because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays

[14] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Quake to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Quake would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from

the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each

contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Quake to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Vogelstein) to incorporate a droplet reactor (Quake). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Vogelstein. The use of two continuously flowing, immiscible fluids create droplets which in turn **further reduces the volumes of reagent** compared to droplets produced

using injection of an aqueous fluid into an immiscible fluid (e.g., Corbett). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize

> noncontact methods[16] or continuous flow through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson

> distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at

1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Quake could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful in miniaturizing and modifying Corbett or modifying Vogelstein to work in the microfluidic droplet reactor of Quake, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[15] As discussed in Section VI.A.3, above, Higuchi I-III

[15] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the

demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail in the following sections, the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake to conduct microfluidic PCR as taught by Corbett and Vogelstein. Ex. 1004 ¶¶109-112. Alternatively, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic droplet reactor of Quake, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing. *Id.*

Not only would a reasonable Examiner consider these teachings relevant in deciding whether claims 1-8 of the '148 patent are patentable, but would have rejected the claims of the '148 patent in view of these teachings. See MPEP § 2242. The correspondence between the claims of the '148 patent and the combined teachings of the Quake, Corbett and Vogelstein references are set forth in Section VII.B, below.

references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

E. The Combination of Quake, Kopp and Lagally Presents a Substantial New Question of Patentability for Claims 1-8 of the '148 Patent

As discussed in Sections IV.C and IV.D, above, the claims have been considered nonobvious by the Office because the **prior art was not believed to teach a technique for conducting PCR reactions in droplets formed from two continuously flowing immiscible fluids**.

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Quake), ii) microfluidic PCR devices were well known (Kopp and Lagally), and iii) there were compelling reasons to use the microfluidic droplet reactors to conduct microfluidic PCR (Kopp, Burns II, Lagally and Vogelstein).

As to the first feature – forming plugs from continuously flowing immiscible fluids – the '148 patent's teachings in this regard were copied almost verbatim from the Quake PCT (Ex. 1034). The table below compares the Quake PCT publication with the Ismagilov specification, which was filed about two months later.

Quake PCT (published March 21, 2002) (Ex. 1034)	**Ismagilov provisional application (filed May 9, 2002) (Ex. 1003) and '148 patent (Ex. 1001)**
34:32-35:7 The pressure at the droplet extrusion region can also be regulated by adjusting the pressure on the main and sample inlets, for example, with pressurized syringes feeding into those inlets. By controlling the pressure difference between the oil and water sources at the droplet extrusion region, the size and periodicity of the droplets generated may be regulated. Alternatively, a valve may be placed at or coincident to either the droplet extrusion region or the sample inlet connected thereto to control the flow of solution into the	Provisional at 22:12-19, '148 patent at 21:56-65 The pressure at the plug-forming region can also be regulated by adjusting the pressure on the main and sample inlets, for example with pressurized syringes feeding into those inlets. By controlling the difference between the oil and water flow rates at the plug-forming region, the size and periodicity of the plugs generated may be regulated. Alternatively, a valve may be placed at or coincident to either the plug forming region or the sample inlet connected thereto to control the flow of solution into the plug-

droplet extrusion region, thereby controlling the size and periodicity of the droplets.	forming region, thereby controlling the size and periodicity of the plugs.

Attached as Ex. 1035 is a table showing the numerous passages which Ismagilov copied key disclosures verbatim or nearly verbatim from the Quake PCT. These were not merely background or "state of the art" descriptions; rather, they go to the heart of the claimed subject matter.

The disclosure of Quake was summarized by the Patent Trial and Appeal Board in its November 15, 2015 decision. Therein the Board found that Quake discloses a droplet reactor in which droplets are formed by continuous streams of immiscible fluids:

> Quake relates to microfluidic devices and methods for analyzing and/or sorting biological materials. Ex. 1004, Abstract [which corresponds to Ex. 1033]….
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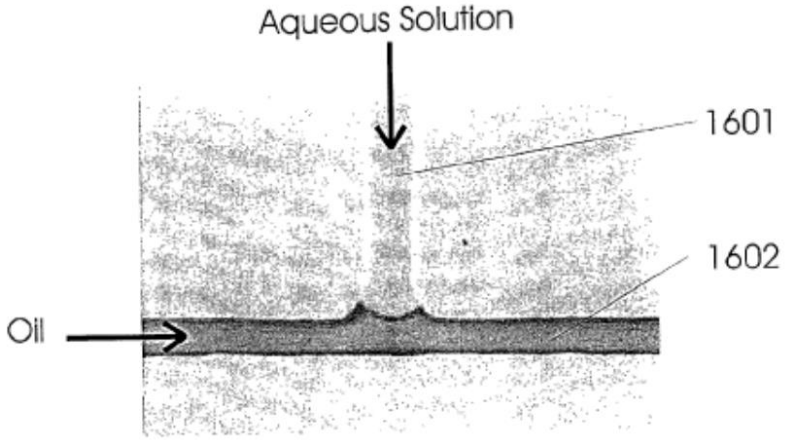


FIG. 16A

> Figure 16A depicts the channel architecture for the droplet extrusion region. Channel 1601 containing an aqueous solution intersects with the main channel 1602 containing oil. [*Id.*] ¶ 292.
>
> In preferred embodiments, the droplets have a volume of approximately 0.1 to 100 picoliters. *Id.* According to Quake, microfabrication of the device "permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." *Id.* ¶ 80.

Ex. 1052 at 11-12.

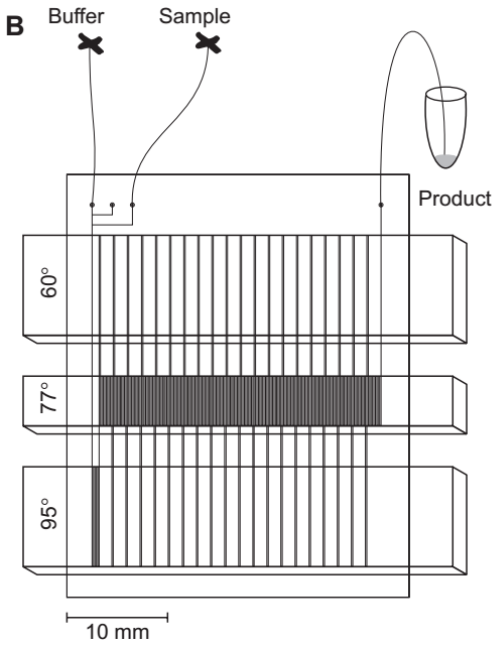
The Patent Owner did not contest in the *inter partes* review proceeding that Quake met the plug-formed-by-continuous-streams limitations of the '148 patent. Ex. 1051 *passim*; Ex. 1052, *passim*. Moreover, Quake himself during prosecution characterized his invention as involving continuous streams.[16] Accordingly, notwithstanding statements to the contrary during *ex parte* prosecution of the '148 patent (discussed in Section IV.C above) it is clear that Quake does in fact teach forming plugs from continuous streams of immiscible fluids.

Turning the second issue, as of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Kopp and Lagally. By 1998 Kopp et al. had successfully implemented continuous flow PCR on a chip. In his 1988 article (not previously presented to the USPTO), Kopp explained that "[a] wide range of microreactors, microcapillary electrophoresis devices, and microcell manipulation devices have been described in recent years." Ex. 1027 at 1046. Building on that body of work, Kopp's group developed

> [a] micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in **continuous flow** at high speed.

[16] When distinguishing his invention over one of the embodiments of Shaw Stewart in which reagent is periodically injected, Quake noted that his "invention relates, in part, to the discovery that combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid (e.g., decane) it is possible to produce small, discrete, monodisperse droplets of the aqueous solution flowing in the second fluid." Ex. 1058 at September 25, 2006 response, p. 15.

> The device is analogous to an electronic amplifier and relies on the movement of sample through thermostated temperature zones on a glass microchip. Input and output of material (DNA) is **continuous**, and amplification is independent of input concentration. . . .
>
> The results demonstrate that with a very simple device and virtually no optimization, PCR can be performed in **continuous flow**, yielding product quality and quantity comparable to standard thermal cycling methods (Fig. 2B). . . .
>
> We suggest that cross-contamination in a **continuous-flow** format may be much less of a problem than in a stationary-tube PCR format. Thus, a flow-through PCR chip can be reused for extended periods without major cleaning. . . .



Ex. 1027 at 1046-1047 [emphasis added]. Kopp et al. also explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications:

> To build a complex system analogous to an electronic integrated circuit, it is necessary to integrate a variety of other continuously working parts. A cursory glance at developments in miniaturized total analysis

> systems (uTAS) during the past 5 years reveals that most of the relevant components, such as **continuous-flow mixers** (8), **continuous-flow microreactors** (9), **continuous separations** (10), and high-speed capillary gel electrophoresis (as a conventional batch process), have already been presented (11). Accordingly, the **continuous-flow microamplifier** should allow the creation of highly elaborate analysis and synthesis systems.
>
> Although chemical amplification reactors will obviously not be applicable to all reactions, neither will they be limited to PCR. Devices of this kind open up whole new areas of application in medical diagnostics. For example, the online amplification and monitoring of a specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy.

Ex. 1027 at 1047 [emphasis added].

In 2001, in another article not previously considered by the Office, Lagally reported using a microfluidic reactor to conduct single-molecule DNA amplification by PCR. Lagally summarized the state of the art as of February 2001, explaining continuous flow PCR microreactors had recently undergone substantial evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize

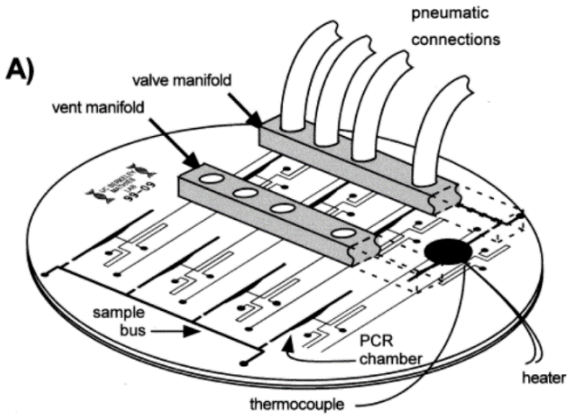
> noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

Lagally *et al.* developed a further improvement – a device and method for achieving single molecule amplification in a microchannel environment.

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson

> distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> Microfluidic PCR-CE device. The PCR chambers are connected to a common sample bus through a set of valves. Hydrophobic vents at the other end of the PCR chambers are used to locate the sample and to eliminate gas. The PCR chambers are directly connected to the cross channel of the CE system for product injection and analysis. Two aluminum manifolds, one each for the vents and valves, are placed onto the respective ports and clamped in place using vacuum. The manifolds connect to external solenoid valves for pressure and vacuum actuation. Thermal cycling is accomplished using a resistive heater and a miniature thermocouple below the 280-nL chamber.



Ex. 1028 at 566 (see figure legend).

In sum, Kopp and Lagally show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Quake is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[17] Ex 1001 at 78:22-24. In the instant combination, these

[17] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Quake) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

Quake **specifically suggests that his microfluidic device could be used to perform PCR**. Quake explained that "[m]icrofabrication permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." Ex. 1034 at 23:26-30. Quake noted that "PCR is [] generally the method of choice to detect viral DNA or RNA directly in clinical specimens. The advantage of PCR for viral diagnostics is its high sensitivity; PCR can detect very low numbers of viruses in a small clinical specimen." *Id.* at 2:31-3:2.

More generally, a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake to conduct microfluidic PCR as taught by Kopp and Lagally. A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic droplet reactor of Quake because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the

droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Quake to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Quake or Shaw Stewart would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-

target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 (“It is also possible that Dig-PCR can be performed in

microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Quake to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Kopp or Lagally) to incorporate a droplet reactor (Quake). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Kopp or Lagally. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the single fluid in microfluidic chip reactor (e.g., Lagally) or droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Kopp). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually

> transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .

> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an

autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Quake could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful modifying Kopp or Lagally to work in the microfluidic droplet reactor of Quake, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[18] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of microdroplet

[18] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail in the following sections, the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake to conduct microfluidic PCR as taught by Kopp and Lagally.

Not only would a reasonable Examiner consider these teachings relevant in deciding whether claims 1-8 of the '148 patent are patentable, but would have rejected the claims of the '148 patent in view of these teachings. See MPEP § 2242. The correspondence between the claims of the '148 patent and the combined teachings of the Quake, Kopp and Lagally references are set forth in Section VII.C, below.

F. The Combination of Quake, Kopp and Burns II Presents a Substantial New Question of Patentability for Claims 1-8 of the '148 Patent

As discussed in Sections IV.C and IV.D, above, the claims have been considered nonobvious by the Office because the **prior art was not believed to teach a technique for conducting PCR reactions in droplets formed from two continuously flowing immiscible fluids**.

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Quake), ii) microfluidic PCR devices were well known (Kopp and Burns II), and iii) there were compelling reasons to use the microfluidic droplet reactors to conduct microfluidic PCR (Kopp, Burns II, Lagally and Vogelstein).

As to the first feature – forming plugs from continuously flowing immiscible fluids – the '148 patent's teachings in this regard were copied almost verbatim from the Quake PCT (Ex.

1034). The table below compares the Quake PCT publication with the Ismagilov specification, which was filed about two months later.

Quake PCT (published March 21, 2002) (Ex. 1034)	**Ismagilov provisional application (filed May 9, 2002) (Ex. 1003) and '148 patent (Ex. 1001)**
34:32-35:7 The pressure at the droplet extrusion region can also be regulated by adjusting the pressure on the main and sample inlets, for example, with pressurized syringes feeding into those inlets. By controlling the pressure difference between the oil and water sources at the droplet extrusion region, the size and periodicity of the droplets generated may be regulated. Alternatively, a valve may be placed at or coincident to either the droplet extrusion region or the sample inlet connected thereto to control the flow of solution into the droplet extrusion region, thereby controlling the size and periodicity of the droplets.	Provisional at 22:12-19, '148 patent at 21:56-65 The pressure at the plug-forming region can also be regulated by adjusting the pressure on the main and sample inlets, for example with pressurized syringes feeding into those inlets. By controlling the difference between the oil and water flow rates at the plug-forming region, the size and periodicity of the plugs generated may be regulated. Alternatively, a valve may be placed at or coincident to either the plug forming region or the sample inlet connected thereto to control the flow of solution into the plug-forming region, thereby controlling the size and periodicity of the plugs.

Attached as Ex. 1035 is a table showing the numerous passages which Ismagilov copied key disclosures verbatim or nearly verbatim from the Quake PCT. These were not merely background or "state of the art" descriptions; rather, they go to the heart of the claimed subject matter.

The disclosure of Quake was summarized by the Patent Trial and Appeal Board in its November 15, 2015 decision. Therein the Board found that Quake discloses a droplet reactor in which droplets are formed by continuous streams of immiscible fluids:

> Quake relates to microfluidic devices and methods for analyzing and/or sorting biological materials. Ex. 1004, Abstract [which corresponds to Ex. 1033]….
> The devices comprise a main channel, through which a pressurized stream of oil is passed, and at least one sample inlet channel, through which a pressurized

stream of aqueous solution is passed. *Id*. A junction joins the main channel with the sample inlet channel. *Id*. By adjusting the pressure of the oil and/or the aqueous solution, a pressure difference can be established such that the stream of aqueous solution is sheared off at a regular frequency as it enters the oil stream, thereby forming droplets. *Id*. Figure 16A of Quake is reproduced below:

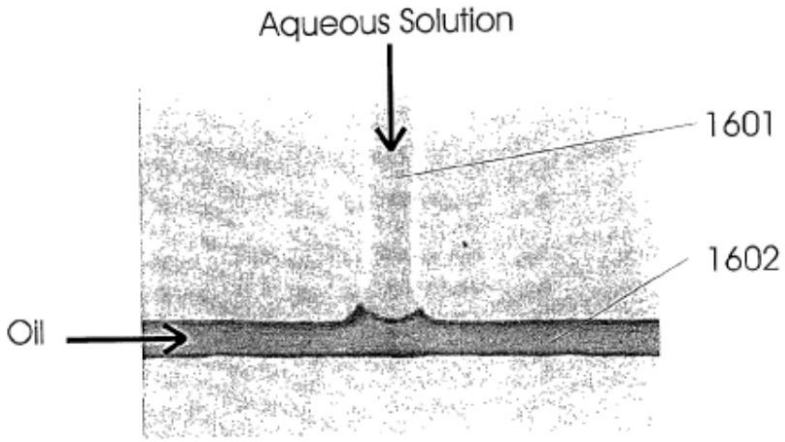


FIG. 16A

Figure 16A depicts the channel architecture for the droplet extrusion region. Channel 1601 containing an aqueous solution intersects with the main channel 1602 containing oil. [*Id.*] ¶ 292.

In preferred embodiments, the droplets have a volume of approximately 0.1 to 100 picoliters. *Id.* According to Quake, microfabrication of the device "permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." *Id*. ¶ 80.

Ex. 1052 at 11-12.

The Patent Owner did not contest in the *inter partes* review proceeding that Quake met the plug-formed-by-continuous-streams limitations of the '148 patent. Ex. 1051 *passim*; Ex. 1052, *passim*. Moreover, Quake himself during prosecution characterized his invention as involving continuous streams.[19] Accordingly, notwithstanding statements to the contrary during

[19] When distinguishing his invention over one of the embodiments of Shaw Stewart in which reagent is periodically injected, Quake noted that his "invention relates, in part, to the discovery that combining a flowing stream of an

ex parte prosecution of the '148 patent (discussed in Section IV.C above) it is clear that Quake does in fact teach forming plugs from continuous streams of immiscible fluids.

Turning the second issue, as of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Kopp and Burns II. In 1996, Burns II reported the use of microfluidic devices to perform PCR. Ex. 1008. Burns II explained that "[n]o new biochemistries or DNA detection methods are required for their use; the components simply reproduce current laboratory procedures in a micron-sized environment." *Id.* at 5560. In other words, the PCR chemistry did not need to be modified – it worked without modification on the microfluidic scale. In the method of Burns II, a sample containing a DNA plasmid was loaded into one of the reaction wells (A) and a mixture of Taq enzyme and buffer was loaded into the other. *Id.* at 5558. Each formed droplets of about 60 nl in the parallel 500 x 25 μm channels (B) which came together at the Y junction to form a single larger droplet (about 120 nl). *Id.* at 5558-59; Ex. 1004 ¶40.

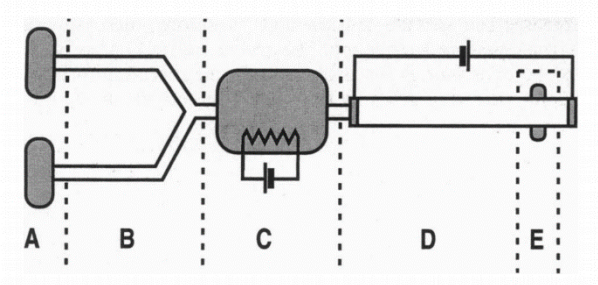


FIG. 1. Example of an integrated DNA analysis system, represented schematically. The individual components of the system are injection entry ports (*A*), liquid pumping channels (*B*), thermally controlled reaction chamber (*C*), electrophoresis channel (*D*), and DNA band migration detector (*E*). Each component would have associated sensors, control circuitry, and external connections.

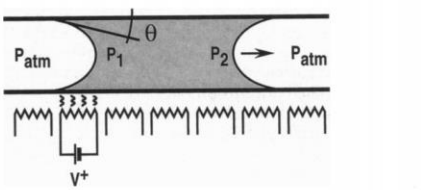


FIG. 2. Schematic drawing showing the principle of thermally induced drop motion in a closed channel. The case of a single aqueous drop in a hydrophilic channel is presented, where V is an applied voltage, P_{atm} is atmospheric pressure, P_1 is the receding-edge internal pressure, P_2 is the advancing-edge internal pressure, and θ is the contact angle of the liquid–gas–solid interface. The contact angle will depend on the surface characteristics of the channel and the constituents of the drop, with a hydrophilic interaction giving θ between 0 and 90 degrees, and a hydrophobic surface giving θ between 90 and 180 degrees. Surface treatments can also reduce contact angle hysteresis and, therefore, reduce the temperature difference necessary for drop motion.

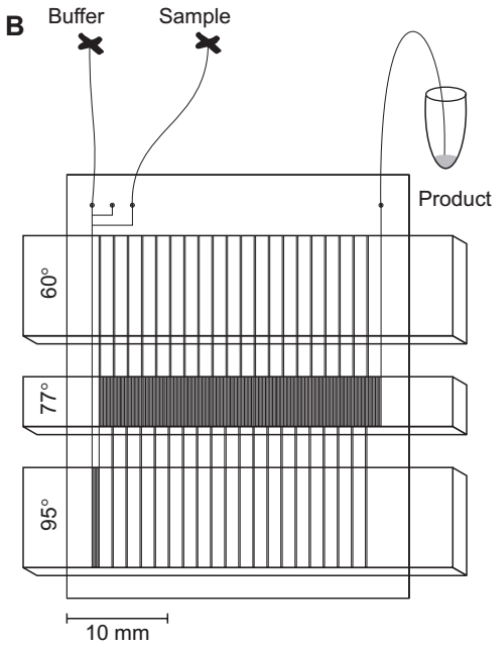
The droplet then passed through the resistive heater portion (C) in which PCR amplification occurred. *Id.* at 5556. Afterwards the amplification results were optically detected in zone D via

aqueous solution and a flowing stream of an immiscible fluid (e.g., decane) it is possible to produce small, discrete, monodisperse droplets of the aqueous solution flowing in the second fluid." Ex. 1058 at September 25, 2006 response, p. 15.

gel electrophoresis *Id.* at 5559. Burns II noted that "[t]he use of common fabrication methods allows the assembly of increasingly complex, multicomponent, integrated systems from a small, defined set of standardized elements" and that "[f]uture design efforts have a strong impetus to reduce component size and improve efficiency." *Id.* at 5560-61.

By the late 1990s Kopp et al. had successfully implemented continuous flow PCR on a chip. In his 1988 article (not previously presented to the USPTO), Kopp explained that "[a] wide range of microreactors, microcapillary electrophoresis devices, and microcell manipulation devices have been described in recent years." Ex. 1027 at 1046. Building on that body of work, Kopp's group developed

> [a] micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in **continuous flow** at high speed. The device is analogous to an electronic amplifier and relies on the movement of sample through thermostated temperature zones on a glass microchip. Input and output of material (DNA) is **continuous**, and amplification is independent of input concentration. . . .
>
> The results demonstrate that with a very simple device and virtually no optimization, PCR can be performed in **continuous flow**, yielding product quality and quantity comparable to standard thermal cycling methods (Fig. 2B). . . .
>
> We suggest that cross-contamination in a **continuous-flow** format may be much less of a problem than in a stationary-tube PCR format. Thus, a flow-through PCR chip can be reused for extended periods without major cleaning. . . .



Ex. 1027 at 1046-1047 [emphasis added]. Kopp et al. also explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications:

> To build a complex system analogous to an electronic integrated circuit, it is necessary to integrate a variety of other continuously working parts. A cursory glance at developments in miniaturized total analysis systems (uTAS) during the past 5 years reveals that most of the relevant components, such as **continuous-flow mixers** (8), **continuous-flow microreactors** (9), **continuous separations** (10), and high-speed capillary gel electrophoresis (as a conventional batch process), have already been presented (11). Accordingly, the **continuous-flow microamplifier** should allow the creation of highly elaborate analysis and synthesis systems.
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> Although chemical amplification reactors will obviously not be applicable to all reactions, neither will they be limited to PCR. Devices of this kind open up whole new areas of application in medical diagnostics. For example, the online amplification and monitoring of a specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy.

Ex. 1027 at 1047 [emphasis added].

In sum, Kopp and Burns II show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Quake is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[20] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Quake) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

Quake **specifically suggests that his microfluidic device could be used to perform PCR**. Quake explained that "[m]icrofabrication permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." Ex. 1034 at 23:26-30. Quake noted that "PCR is [] generally the method of choice to detect viral DNA or RNA directly in clinical specimens. The advantage of PCR for viral diagnostics is its high sensitivity; PCR can detect very low numbers of viruses in a small clinical specimen." *Id.* at 2:31-3:2.

More generally, a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake, to conduct microfluidic PCR as taught by Kopp and Burns II. Ex. 1004 ⁋⁋109,111. A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic droplet reactor of Quake because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by

[20] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Quake to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Quake would **enhance the precision of the PCR reaction**

with a given primer and reduce the production of non-specific products relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Quake to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Kopp or Lagally) to incorporate a droplet reactor (Quake). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR

devices taught in Kopp or Lagally. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the single fluid in microfluidic chip reactor (e.g., Lagally) or droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Kopp). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template

> concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials

> are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority

date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Quake could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful modifying Kopp or Lagally to work in the microfluidic droplet reactor of Quake, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e).

Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[21] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail in the following sections, the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake to conduct microfluidic PCR as taught by Kopp and Burns II.

Not only would a reasonable Examiner consider these teachings relevant in deciding whether claims 1-8 of the '148 patent are patentable, but would have rejected the claims of the '148 patent in view of these teachings. See MPEP § 2242. The correspondence between the claims of the '148 patent and the combined teachings of the Quake, Kopp and Burns II references are set forth in Section VII.D, below.

G. The Combination of Quake, Kopp and Vogelstein Presents a Substantial New Question of Patentability for Claims 1-8 of the '148 Patent

As discussed in Sections IV.C and IV.D, above, the claims have been considered nonobvious by the Office because the **prior art was not believed to teach a technique for**

[21] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

conducting PCR reactions in droplets formed from two continuously flowing immiscible fluids.

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Quake), ii) microfluidic PCR devices were well known (Kopp and Vogelstein), and iii) there were compelling reasons to use the microfluidic droplet reactors to conduct microfluidic PCR (Kopp, Burns II, Lagally and Vogelstein).

As to the first feature – forming plugs from continuously flowing immiscible fluids – the '148 patent's teachings in this regard were copied almost verbatim from the Quake PCT (Ex. 1034). The table below compares the Quake PCT publication with the Ismagilov specification, which was filed about two months later.

Quake PCT (published March 21, 2002) (Ex. 1034)	**Ismagilov provisional application (filed May 9, 2002) (Ex. 1003) and '148 patent (Ex. 1001)**
34:32-35:7 The pressure at the droplet extrusion region can also be regulated by adjusting the pressure on the main and sample inlets, for example, with pressurized syringes feeding into those inlets. By controlling the pressure difference between the oil and water sources at the droplet extrusion region, the size and periodicity of the droplets generated may be regulated. Alternatively, a valve may be placed at or coincident to either the droplet extrusion region or the sample inlet connected thereto to control the flow of solution into the droplet extrusion region, thereby controlling the size and periodicity of the droplets.	Provisional at 22:12-19, '148 patent at 21:56-65 The pressure at the plug-forming region can also be regulated by adjusting the pressure on the main and sample inlets, for example with pressurized syringes feeding into those inlets. By controlling the difference between the oil and water flow rates at the plug-forming region, the size and periodicity of the plugs generated may be regulated. Alternatively, a valve may be placed at or coincident to either the plug forming region or the sample inlet connected thereto to control the flow of solution into the plug-forming region, thereby controlling the size and periodicity of the plugs.

Attached as Ex. 1035 is a table showing the numerous passages which Ismagilov copied key disclosures verbatim or nearly verbatim from the Quake PCT. These were not merely background or "state of the art" descriptions; rather, they go to the heart of the claimed subject matter.

The disclosure of Quake was summarized by the Patent Trial and Appeal Board in its November 15, 2015 decision. Therein the Board found that Quake discloses a droplet reactor in which droplets are formed by continuous streams of immiscible fluids:

> Quake relates to microfluidic devices and methods for analyzing and/or sorting biological materials. Ex. 1004, Abstract [which corresponds to Ex. 1033]…. The devices comprise a main channel, through which a pressurized stream of oil is passed, and at least one sample inlet channel, through which a pressurized stream of aqueous solution is passed. *Id.* A junction joins the main channel with the sample inlet channel. *Id*. By adjusting the pressure of the oil and/or the aqueous solution, a pressure difference can be established such that the stream of aqueous solution is sheared off at a regular frequency as it enters the oil stream, thereby forming droplets. *Id*. Figure 16A of Quake is reproduced below:

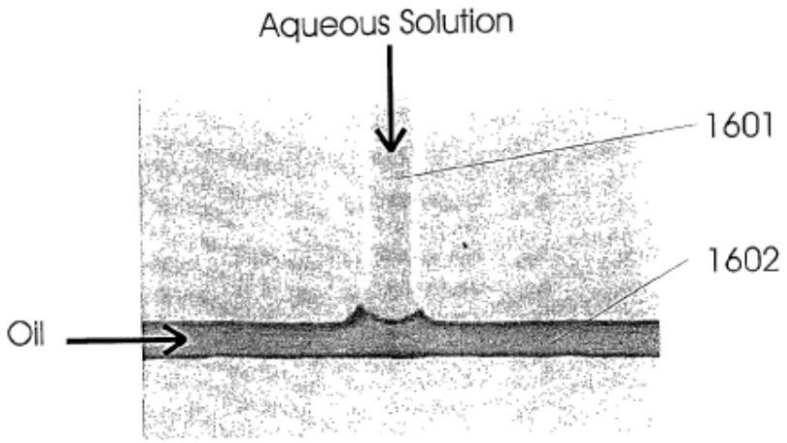


FIG. 16A

> Figure 16A depicts the channel architecture for the droplet extrusion region. Channel 1601 containing an aqueous solution intersects with the main channel 1602 containing oil. [*Id.*] ¶ 292.

> In preferred embodiments, the droplets have a volume of approximately 0.1 to 100 picoliters. *Id*. According to Quake, microfabrication of the device "permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." *Id*. ¶ 80.

Ex. 1052 at 11-12.

The Patent Owner did not contest in the *inter partes* review proceeding that Quake met the plug-formed-by-continuous-streams limitations of the '148 patent. Ex. 1051 *passim*; Ex. 1052, *passim*. Moreover, Quake himself during prosecution characterized his invention as involving continuous streams.[22] Accordingly, notwithstanding statements to the contrary during *ex parte* prosecution of the '148 patent (discussed in Section IV.C above) it is clear that Quake does in fact teach forming plugs from continuous streams of immiscible fluids.

Turning the second issue, as of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Kopp and Vogelstein. By the late 1990s Kopp et al. had successfully implemented continuous flow PCR on a chip. In his 1988 article (not previously presented to the USPTO), Kopp explained that "[a] wide range of microreactors, microcapillary electrophoresis devices, and microcell manipulation devices have been described in recent years." Ex. 1027 at 1046. Building on that body of work, Kopp's group developed

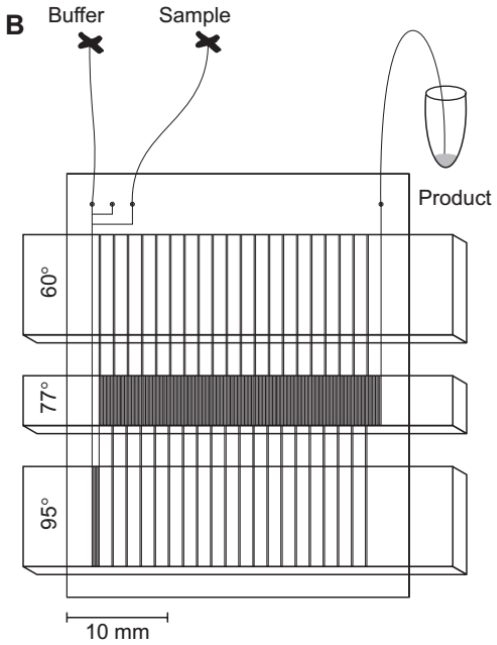
> [a] micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in **continuous flow** at high speed. The device is analogous to an electronic amplifier and relies on the movement of sample through thermostated temperature zones on a glass

[22] When distinguishing his invention over one of the embodiments of Shaw Stewart in which reagent is periodically injected, Quake noted that his "invention relates, in part, to the discovery that combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid (e.g., decane) it is possible to produce small, discrete, monodisperse droplets of the aqueous solution flowing in the second fluid." Ex. 1058 at September 25, 2006 response, p. 15.

microchip. Input and output of material (DNA) is **continuous**, and amplification is independent of input concentration. . . .

The results demonstrate that with a very simple device and virtually no optimization, PCR can be performed in **continuous flow**, yielding product quality and quantity comparable to standard thermal cycling methods (Fig. 2B). . . .

We suggest that cross-contamination in a **continuous-flow** format may be much less of a problem than in a stationary-tube PCR format. Thus, a flow-through PCR chip can be reused for extended periods without major cleaning. . . .



Ex. 1027 at 1046-1047 [emphasis added]. Kopp et al. also explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications:

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> Although chemical amplification reactors will obviously not be applicable to all reactions, neither will they be limited to PCR. Devices of this kind open up whole new areas of application in medical diagnostics. For example, the online amplification and monitoring of a specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy.

Ex. 1027 at 1047 [emphasis added].

By 1999, Vogelstein had reported **various applications for single-molecule microfluidic PCR**. Ex. 1044 at 9241. In a seminal article which has been cited over by over 1,000 other publications, Vogelstein explained that PCR amplification of small aliquots or droplets containing single molecules was effective for "detection of a small number of mutant-containing cells among a large excess of normal cells." *Id.* at 9236. In Vogelstein's method, the DNA was diluted into 7 µl volumes such that, on average, there was one template molecule for every two wells (i.e., approximately half of the wells contained no DNA template molecule and approximately half contained a single DNA template molecule). *Id.* at 9236, 9237. This avoided the problem of background noise impairing the determination of whether each volume contained the mutant sequence. *Id.* By counting the number of volumes which indicated wild type or mutant templates, the presence or absence of the mutation (e.g., a sequence associated with colorectal cancer) can be determined. *Id.* at 9238. The accuracy of the test is a function of the number of discrete volumes into which the sample is diluted. *Id*. at 9239. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. *Id.* at 9239. ("It is also

possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude.") *Id.* 9239. The article explained that the same technique would be useful for **detecting a wide array of mutations, dislocations and allelic imbalances**, as summarized in Table 1. *Id.* at 9241. Vogelstein thus taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby overcoming the signal-to-noise limitations associated with certain other PCR techniques and enabling the detection of relatively rare mutations, dislocations and allelic imbalances. Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84.

In sum, Kopp and Vogelstein show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Quake is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[23] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Quake) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

Quake **specifically suggests that his microfluidic device could be used to perform PCR**. Quake explained that "[m]icrofabrication permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." Ex. 1034 at 23:26-30. Quake

[23] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

noted that "PCR is [] generally the method of choice to detect viral DNA or RNA directly in clinical specimens. The advantage of PCR for viral diagnostics is its high sensitivity; PCR can detect very low numbers of viruses in a small clinical specimen." *Id.* at 2:31-3:2.

More generally, a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake, to conduct microfluidic PCR as taught by Kopp and Vogelstein. Ex. 1004 ¶110. A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic droplet reactor of Quake because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex.

1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Quake to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Quake would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241.A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Quake to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Kopp or Vogelstein) to incorporate a droplet reactor (Quake). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Kopp or Vogelstein. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Kopp). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow

PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant

> time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Quake could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful modifying Kopp or Lagally to work in the microfluidic droplet reactor of Quake, the new teachings set forth

above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[24] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail in the following sections, the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake to conduct microfluidic PCR as taught by Kopp and Vogelstein.

Not only would a reasonable Examiner consider these teachings relevant in deciding whether claims 1-8 of the '148 patent are patentable, but would have rejected the claims of the '148 patent in view of these teachings. See MPEP § 2242. The correspondence between the

[24] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

claims of the ‘148 patent and the combined teachings of the Quake, Kopp and Vogelstein references are set forth in Section VII.D, below.

H. The Combination of Shaw Stewart, Corbett and Lagally Presents a Substantial New Question of Patentability for Claims 1-8 of the ‘148 Patent

As discussed in Sections IV.C and IV.D, above, the claims have been considered nonobvious by the Office because the **prior art was not believed to teach a technique for conducting PCR reactions in droplets formed from two continuously flowing immiscible fluids**.

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Shaw Stewart), ii) microfluidic PCR devices were well known (Corbett and Lagally), and iii) there were compelling reasons to use the microfluidic droplet reactors to conduct microfluidic PCR (Kopp, Burns II, Lagally and Vogelstein).

The Shaw Stewart British Application teaches conducting reactions in microdroplets formed from two continuously flowing immiscible fluids. The Shaw Stewart British Application discloses a “droplet reactor” and illustrates at Figure 1 a carrier phase 1 flowing along a conduit into which reagent 2 is injected, thereby forming droplet 4. Ex. 1040 at 1:1-20, 70-90. “[I]f large numbers of droplets are required, a continuous flow of reagent through the opening will be produced, while a continuous current of carrier phase flows down the tube. When each droplet almost spans the tube, it will be broken off. The exact size of the droplets produced will depend on the relative and absolute magnitude of the two currents.” *Id.* at 1:83-90.

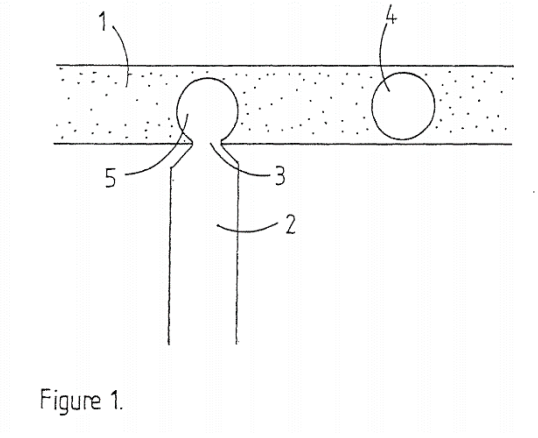


Figure 1.

Significantly, the exclusive licensee of the '148 patent affirmatively (and correctly) took the position that Shaw Stewart teaches plug or droplet formation combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid. More particularly, Bio-Rad (who recently acquired RainDance, the original exclusive licensee of the '148 patent, Ex. 1061, Ex. 1053 ¶71) explained that another party had mischaracterized the Shaw Stewart reference by failing to point out the above-quoted portion of Shaw Stewart, in which Shaw Stewart specifically teaches that in certain embodiments droplets are formed from intersecting and continuously flowing streams.

> To overcome Stewart I and secure allowance of their patent, the Patent Owner ("PO") mischaracterized the device disclosed in Stewart I (and hence Stewart II), misdirecting the Examiner to irrelevant portions of Stewart I while ignoring other portions that directly contradicted PO's arguments. *See, e.g.*, Ex. 1003 at 174-180. For example, one of PO's primary arguments was that Stewart I
>
> only disclosed introducing fluid in a "stepwise fashion," whereas in the '503 Patent droplets are formed by "combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid." *Id.* at 174. In truth, however, Stewart I also discloses combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid:
>
> > If large numbers of droplets are required, a continuous flow of reagent through the opening will be produced, while a continuous current of carrier phase flows down the tube. When each droplet almost spans the tube, it will be broken off.
>
> Ex. 1008, pg. 1, col. 2, ll. 83-88.

Ex. 1059 at 2-3.

Thus the exclusive licensee (Bio-Rad) expressly agreed that Shaw Stewart discloses forming plugs from intersecting streams of continuously flowing immiscible fluids.

As of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Corbett and Lagally. In 1991, about eleven years prior to the earliest effective filing date, Corbett disclosed using a microchannel or capillary device to conduct DNA amplification reactions such as PCR by injecting plugs of reactants into a carrier stream that is passed through different temperature zones. Corbett explained that as of 1991 "the most critical problem in applying nucleic acid amplification procedures, particularly in repetitive diagnostic assays in a clinical laboratory, is contamination of the reaction mixture by small amounts of DNA which contain sequences capable of being amplified under the conditions of assay." Ex. 1010 at 3:7-12. "Automation of procedures minimize[s] the opportunity for such contamination and allows the implementation of stringent quality control." *Id.* at 3:15-19. "As is shown in FIG. 1, the apparatus 10 comprises tube 12 through which a fluid stream passes. As is more clearly shown in FIG. 2, the fluid stream includes pockets of reaction mixtures 42 and 44 and purging solution 48 separated by carrier fluid 46." *Id*. at 7:58-62.

FIG. 1

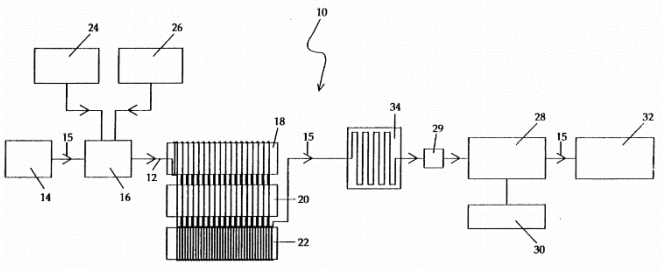
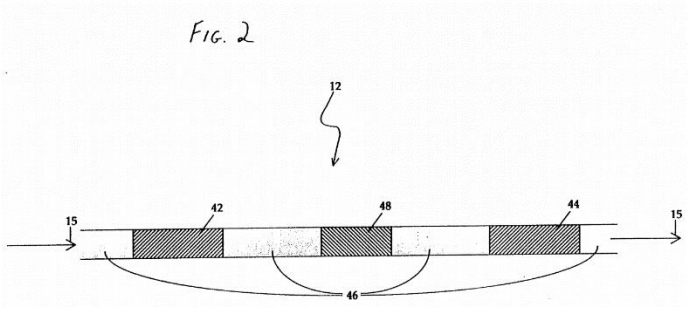


FIG. 2



"The pump is actuated and the reaction mixture of 20 µl or less is injected into the mineral oil or silicone oil carrier fluid and specific DNA sequences (whose limits are defined by the oligonucleotide primers) present in the sample is amplified as it passes cyclically through the temperature zones." Ex. 1010 at 9:35-40. Corbett thus demonstrates that it was known at least as of 1991 that reduction of contamination risk in PCR could be achieved by performing the PCR on microliter sized plugs of reactant in capillary tube which passes through various temperature zones. Ex. 1004 ¶77.

In 2001, in another article not previously considered by the Office, Lagally reported using a microfluidic droplet reactor to conduct single-molecule DNA amplification by PCR. Lagally summarized the state of the art as of February 2001, explaining continuous flow PCR microreactors had recently undergone substantial evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/µL, in volumes down to 1 µL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample

> volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
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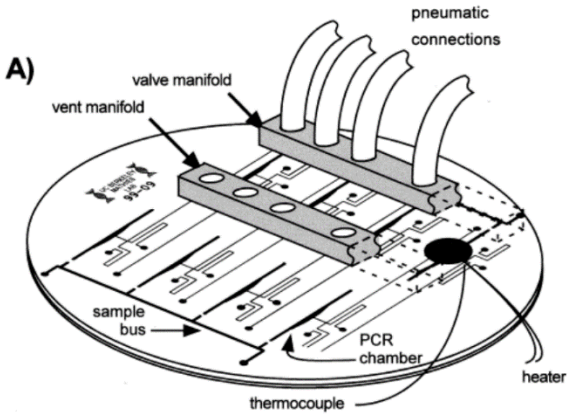
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

Lagally *et al.* developed a further improvement – a device and method for achieving single molecule amplification in a microchannel environment.

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced

> microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> Microfluidic PCR-CE device. The PCR chambers are connected to a common sample bus through a set of valves. Hydrophobic vents at the other end of the PCR chambers are used to locate the sample and to eliminate gas. The PCR chambers are directly connected to the cross channel of the CE system for product injection and analysis. Two aluminum manifolds, one each for the vents and valves, are placed onto the respective ports and clamped in place using vacuum. The manifolds connect to external solenoid valves for pressure and vacuum actuation. Thermal cycling is accomplished using a resistive heater and a miniature thermocouple below the 280-nL chamber.



Ex. 1028 at 566 (see figure legend).

In sum, Corbett and Lagally show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Shaw Stewart are relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[25] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Shaw Stewart) to perform PCR reactions, wherein each

[25] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Shaw Stewart to conduct microfluidic PCR as taught by Corbett and Lagally. Ex. 1004 ⁋⁋109,111. Stated a different way, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic droplet reactor of Shaw Stewart, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing.

A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic droplet reactor of Shaw Stewart because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Shaw Stewart to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Shaw Stewart would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product

specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude.").

Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Shaw Stewart to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Lagally) to incorporate a droplet reactor (Shaw Stewart). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Lagally. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the single fluid in microfluidic chip reactor (e.g., Lagally) or droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Corbett). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow

PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.

> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides

> an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

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Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an

autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactors Shaw Stewart could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful in miniaturizing and modifying Corbett or modifying Lagally to work in the microfluidic droplet reactor of Shaw Stewart, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[26] As discussed in Section VI.A.3, above, Higuchi I-III

[26] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail in the following sections, the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Shaw Stewart to conduct microfluidic PCR as taught by Corbett and Lagally. Ex. 1004 ⁋⁋109,111. Alternatively, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic droplet reactor of Shaw Stewart, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing. *Id.*

Not only would a reasonable Examiner consider these teachings relevant in deciding whether claims 1-8 of the '148 patent are patentable, but would have rejected the claims of the '148 patent in view of these teachings. See MPEP § 2242. The correspondence between the claims of the '148 patent and the combined teachings of the Shaw Stewart, Corbett and Lagally references are set forth in Section VII.A, below.

I. The Combination of Shaw Stewart, Corbett and Burns II Presents a Substantial New Question of Patentability for Claims 1-8 of the '148 Patent

As discussed in Section IV.C and IV.D above, the claims have been considered nonobvious by the Office because the **prior art was not believed to teach a technique for conducting PCR reactions in droplets formed from two continuously flowing immiscible fluids**.

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Shaw Stewart), ii) microfluidic

PCR devices were well known (Corbett and Burns II), and iii) there were compelling reasons to use the microfluidic droplet reactors to conduct microfluidic PCR (Lagally, Kopp, Burns II and Vogelstein).

The Shaw Stewart British Application teaches conducting reactions in microdroplets formed from two continuously flowing immiscible fluids. The Shaw Stewart British Application discloses a "droplet reactor" and illustrates at Figure 1 a carrier phase 1 flowing along a conduit into which reagent 2 is injected, thereby forming droplet 4. Ex. 1040 at 1:1-20, 70-90. "[I]f large numbers of droplets are required, a continuous flow of reagent through the opening will be produced, while a continuous current of carrier phase flows down the tube. When each droplet almost spans the tube, it will be broken off. The exact size of the droplets produced will depend on the relative and absolute magnitude of the two currents." *Id.* at 1:83-90.

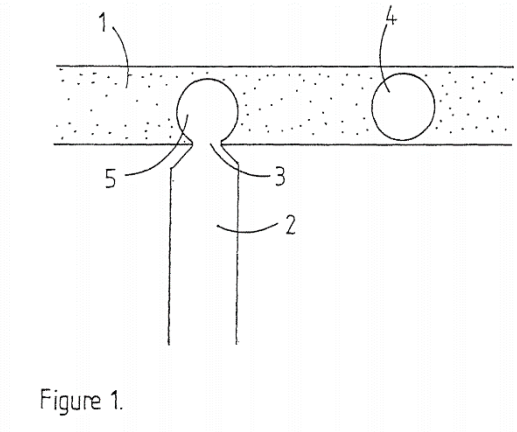


Figure 1.

Significantly, the exclusive licensee of the '148 patent affirmatively (and correctly) took the position that Shaw Stewart teaches plug or droplet formation combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid. More particularly, Bio-Rad (who recently acquired RainDance, the original exclusive licensee of the '148 patent, Ex. 1061, Ex. 1053 ¶71) explained that another party had mischaracterized the Shaw Stewart reference by failing to point out the above-quoted portion of Shaw Stewart, in which Shaw Stewart specifically teaches that in certain embodiments droplets are formed from intersecting and continuously flowing streams.

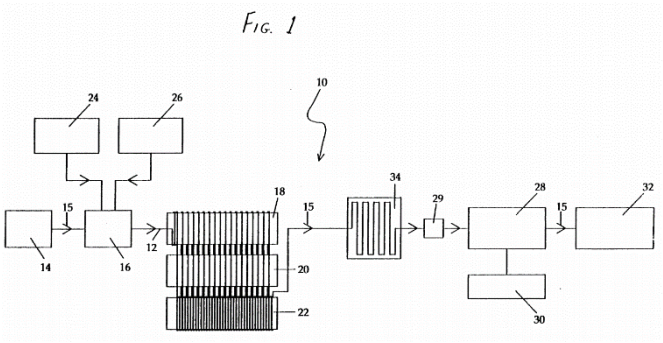
> To overcome Stewart I and secure allowance of their patent, the Patent Owner ("PO") mischaracterized the device disclosed in Stewart I (and hence Stewart II), misdirecting the Examiner to irrelevant portions of Stewart I while ignoring other portions that directly contradicted PO's arguments. *See, e.g.*, Ex. 1003 at 174-180. For example, one of PO's primary arguments was that Stewart I only disclosed introducing fluid in a "stepwise fashion," whereas in the '503 Patent droplets are formed by "combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid." *Id.* at 174. In truth, however, Stewart I also discloses combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid:
>
> > If large numbers of droplets are required, a continuous flow of reagent through the opening will be produced, while a continuous current of carrier phase flows down the tube. When each droplet almost spans the tube, it will be broken off.
>
> Ex. 1008, pg. 1, col. 2, ll. 83-88.

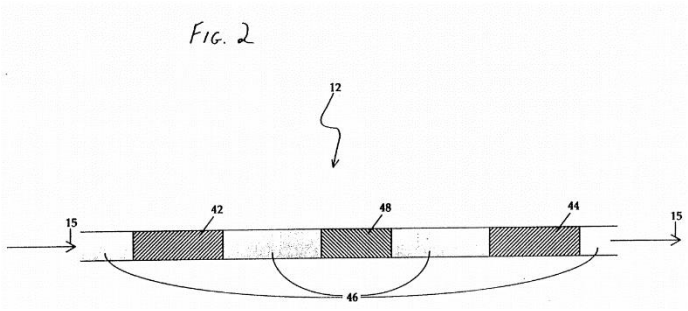
Ex. 1059 at 2-3.

Thus the exclusive licensee (Bio-Rad) expressly agreed that Shaw Stewart discloses forming plugs from intersecting streams of continuously flowing immiscible fluids.

As of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Corbett and Burns II. In 1991, about eleven years prior to the earliest effective filing date, Corbett disclosed using a microchannel or capillary device to conduct DNA amplification reactions such as PCR by injecting plugs of reactants into a carrier stream that is passed through different temperature zones. Corbett explained that as of 1991 "the most critical problem in applying nucleic acid amplification procedures, particularly in repetitive diagnostic assays in a clinical laboratory, is contamination of the reaction mixture by small amounts of DNA which contain sequences capable of being

amplified under the conditions of assay." Ex. 1010 at 3:7-12. "Automation of procedures minimize[s] the opportunity for such contamination and allows the implementation of stringent quality control." *Id.* at 3:15-19. "As is shown in FIG. 1, the apparatus 10 comprises tube 12 through which a fluid stream passes. As is more clearly shown in FIG. 2, the fluid stream includes pockets of reaction mixtures 42 and 44 and purging solution 48 separated by carrier fluid 46." *Id*. at 7:58-62.





"The pump is actuated and the reaction mixture of 20 μl or less is injected into the mineral oil or silicone oil carrier fluid and specific DNA sequences (whose limits are defined by the oligonucleotide primers) present in the sample is amplified as it passes cyclically through the temperature zones." Ex. 1010 at 9:35-40. Corbett thus demonstrates that it was known at least as of 1991 that reduction of contamination risk in PCR could be achieved by performing the PCR on microliter sized plugs of reactant in capillary tube which passes through various temperature zones. Ex. 1004 ¶77.

In 1996, Burns II reported the use of microfluidic devices to perform PCR. Ex. 1008. Burns II explained that "[n]o new biochemistries or DNA detection methods are required for their use; the components simply reproduce current laboratory procedures in a micron-sized environment." *Id.* at 5560. In other words, the PCR chemistry did not need to be modified – it worked without modification on the microfluidic scale. In the method of Burns II, a sample containing a DNA plasmid was loaded into one of the reaction wells (A) and a mixture of Taq enzyme and buffer was loaded into the other. *Id.* at 5558. Each formed droplets of about 60 nl in the parallel 500 x 25 µm channels (B) which came together at the Y junction to form a single larger droplet (about 120 nl). *Id.* at 5558-59; Ex. 1004 ¶40.

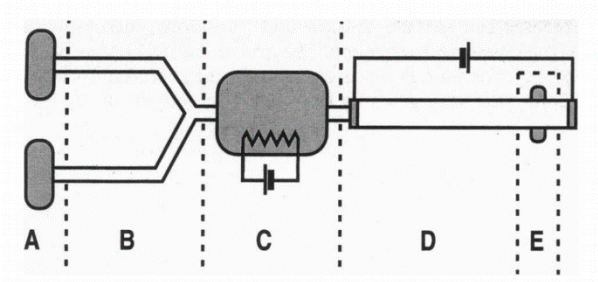


FIG. 1. Example of an integrated DNA analysis system, represented schematically. The individual components of the system are injection entry ports (*A*), liquid pumping channels (*B*), thermally controlled reaction chamber (*C*), electrophoresis channel (*D*), and DNA band migration detector (*E*). Each component would have associated sensors, control circuitry, and external connections.

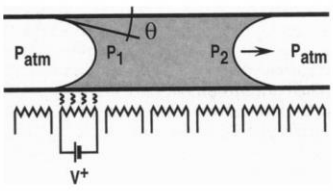


FIG. 2. Schematic drawing showing the principle of thermally induced drop motion in a closed channel. The case of a single aqueous drop in a hydrophilic channel is presented, where V is an applied voltage, P_{atm} is atmospheric pressure, P_1 is the receding-edge internal pressure, P_2 is the advancing-edge internal pressure, and θ is the contact angle of the liquid–gas–solid interface. The contact angle will depend on the surface characteristics of the channel and the constituents of the drop, with a hydrophilic interaction giving θ between 0 and 90 degrees, and a hydrophobic surface giving θ between 90 and 180 degrees. Surface treatments can also reduce contact angle hysteresis and, therefore, reduce the temperature difference necessary for drop motion.

The droplet then passed through the resistive heater portion (C) in which PCR amplification occurred. *Id.* at 5556. Afterwards the amplification results were optically detected in zone D via gel electrophoresis *Id.* at 5559. Burns II noted that "[t]he use of common fabrication methods allows the assembly of increasingly complex, multicomponent, integrated systems from a small, defined set of standardized elements" and that "[f]uture design efforts have a strong impetus to reduce component size and improve efficiency." *Id.* at 5560-61.

In sum, Corbett and Burns II show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Shaw Stewart is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule") Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Shaw Stewart) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Shaw Stewart to conduct PCR as taught by Corbett and Burns II. Stated a different way, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic droplet reactor of Shaw Stewart, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing.

A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic droplet reactor of Shaw Stewart because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have

reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Shaw Stewart to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Shaw Stewart would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is

too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed

sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Shaw Stewart to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Burns II) to incorporate a droplet reactor (Shaw Stewart). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Burns II. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the single fluid in microfluidic chip reactor (e.g., Burns II) or droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Corbett). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller

reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

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> microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
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> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

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droplet (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Shaw Stewart could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful in miniaturizing and modifying Corbett or modifying Burns II to work in the microfluidic droplet reactor of Shaw Stewart, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[27] As discussed in Section VI.A.3, above, Higuchi I-III

[27] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations

demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail in the following sections, the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Shaw Stewart to conduct microfluidic PCR as taught by Corbett and Burns II. Ex. 1004 ⁋⁋109,111. Alternatively, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic droplet reactor of Shaw Stewart, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing. *Id.*

Not only would a reasonable Examiner consider these teachings relevant in deciding whether claims 1-8 of the '148 patent are patentable, but would have rejected the claims of the '148 patent in view of these teachings. See MPEP § 2242. The correspondence between the claims of the '148 patent and the combined teachings of the Shaw Stewart, Corbett and Burns II references are set forth in Section VII.F, below.

J. The Combination of Shaw Stewart, Corbett and Vogelstein Presents a Substantial New Question of Patentability for Claims 1-8 of the '148 Patent

As discussed in Section IV.C and IV.D above, the claims have been considered nonobvious by the Office because the **prior art was not believed to teach a technique for**

omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

conducting PCR reactions in droplets formed from two continuously flowing immiscible fluids.

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Shaw Stewart), ii) microfluidic PCR devices were well known (Corbett and Vogelstein), and iii) there were compelling reasons to use the microfluidic droplet reactors to conduct microfluidic PCR (Lagally, Kopp, Burns II and Vogelstein).

The Shaw Stewart British Application teaches conducting reactions in microdroplets formed from two continuously flowing immiscible fluids. The Shaw Stewart British Application discloses a "droplet reactor" and illustrates at Figure 1 a carrier phase 1 flowing along a conduit into which reagent 2 is injected, thereby forming droplet 4. Ex. 1040 at 1:1-20, 70-90. "[I]f large numbers of droplets are required, a continuous flow of reagent through the opening will be produced, while a continuous current of carrier phase flows down the tube. When each droplet almost spans the tube, it will be broken off. The exact size of the droplets produced will depend on the relative and absolute magnitude of the two currents." *Id.* at 1:83-90.

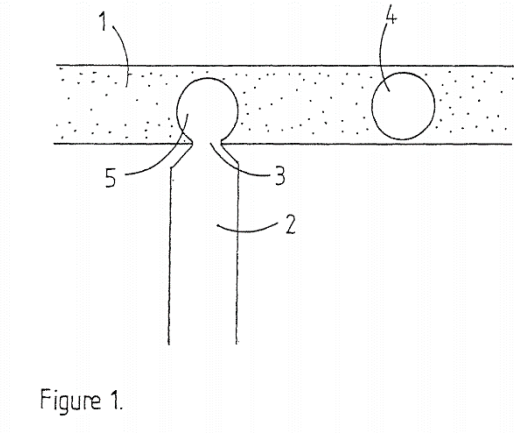


Figure 1.

Significantly, the exclusive licensee of the '148 patent affirmatively (and correctly) took the position that Shaw Stewart teaches plug or droplet formation combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid. More particularly, Bio-Rad (who recently acquired RainDance, the original exclusive licensee of the '148 patent, Ex. 1061, Ex. 1053 ¶71) explained that another party had mischaracterized the Shaw Stewart reference by

failing to point out the above-quoted portion of Shaw Stewart, in which Shaw Stewart specifically teaches that in certain embodiments droplets are formed from intersecting and continuously flowing streams.

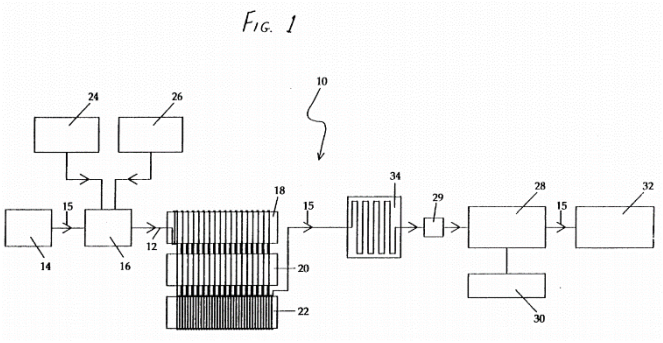
> To overcome Stewart I and secure allowance of their patent, the Patent Owner ("PO") mischaracterized the device disclosed in Stewart I (and hence Stewart II), misdirecting the Examiner to irrelevant portions of Stewart I while ignoring other portions that directly contradicted PO's arguments. *See, e.g.*, Ex. 1003 at 174-180. For example, one of PO's primary arguments was that Stewart I
>
> only disclosed introducing fluid in a "stepwise fashion," whereas in the '503 Patent droplets are formed by "combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid." *Id.* at 174. In truth, however, Stewart I also discloses combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid:
>
> > If large numbers of droplets are required, a continuous flow of reagent through the opening will be produced, while a continuous current of carrier phase flows down the tube. When each droplet almost spans the tube, it will be broken off.
>
> Ex. 1008, pg. 1, col. 2, ll. 83-88.

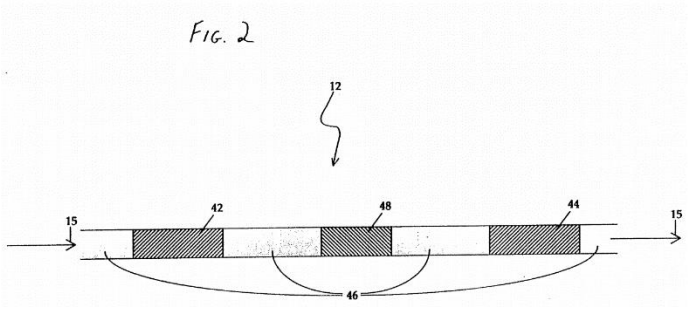
Ex. 1059 at 2-3.

Thus the exclusive licensee (Bio-Rad) expressly agreed that Shaw Stewart discloses forming plugs from intersecting streams of continuously flowing immiscible fluids.

As of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Corbett and Vogelstein. In 1991, about eleven years prior to the earliest effective filing date, Corbett disclosed using a microchannel or capillary device to conduct DNA amplification reactions such as PCR by injecting plugs of reactants into a carrier stream that is passed through different temperature zones. Corbett

explained that as of 1991 "the most critical problem in applying nucleic acid amplification procedures, particularly in repetitive diagnostic assays in a clinical laboratory, is contamination of the reaction mixture by small amounts of DNA which contain sequences capable of being amplified under the conditions of assay." Ex. 1010 at 3:7-12. "Automation of procedures minimize[s] the opportunity for such contamination and allows the implementation of stringent quality control." *Id.* at 3:15-19. "As is shown in FIG. 1, the apparatus 10 comprises tube 12 through which a fluid stream passes. As is more clearly shown in FIG. 2, the fluid stream includes pockets of reaction mixtures 42 and 44 and purging solution 48 separated by carrier fluid 46." *Id*. at 7:58-62.





"The pump is actuated and the reaction mixture of 20 μl or less is injected into the mineral oil or silicone oil carrier fluid and specific DNA sequences (whose limits are defined by the oligonucleotide primers) present in the sample is amplified as it passes cyclically through the temperature zones." Ex. 1010 at 9:35-40. Corbett thus demonstrates that it was known at least as of 1991 that reduction of contamination risk in PCR could be achieved by performing the PCR

on microliter sized plugs of reactant in capillary tube which passes through various temperature zones. Ex. 1004 ¶77.

By 1999, Vogelstein had reported **various applications for single-molecule microfluidic PCR**. Ex. 1044 at 9241. In a seminal article which has been cited over by over 1,000 other publications, Vogelstein explained that PCR amplification of small aliquots or droplets containing single molecules was effective for "detection of a small number of mutant-containing cells among a large excess of normal cells." *Id.* at 9236. In Vogelstein's method, the DNA was diluted into 7 µl volumes such that, on average, there was one template molecule for every two wells (i.e., approximately half of the wells contained no DNA template molecule and approximately half contained a single DNA template molecule). *Id.* at 9236, 9237. This avoided the problem of background noise impairing the determination of whether each volume contained the mutant sequence. *Id.* By counting the number of volumes which indicated wild type or mutant templates, the presence or absence of the mutation (e.g., a sequence associated with colorectal cancer) can be determined. *Id.* at 9238. The accuracy of the test is a function of the number of discrete volumes into which the sample is diluted. *Id.* at 9239. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. *Id.* at 9239. ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude.") *Id.* 9239. The article explained that the same technique would be useful for **detecting a wide array of mutations, dislocations and allelic imbalances**, as summarized in Table 1. *Id.* at 9241. Vogelstein thus taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template

molecule, thereby overcoming the signal-to-noise limitations associated with certain other PCR techniques and enabling the detection of relatively rare mutations, dislocations and allelic imbalances. Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84.

In sum, Corbett and Vogelstein show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Shaw Stewart is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule") Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Shaw Stewart) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Shaw Stewart to conduct microfluidic PCR as taught by Corbett and Vogelstein. Ex. 1004 ¶109-112. Stated a different way, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic droplet reactor of Shaw Stewart, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing. *Id.*

A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic droplet reactor of Shaw Stewart because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he

advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Shaw Stewart to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Shaw Stewart would **enhance the precision of the PCR**

reaction with a given primer and reduce the production of non-specific products relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Shaw Stewart to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Vogelstein) to incorporate a droplet reactor (Shaw Stewart). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic

PCR devices taught in Vogelstein. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Corbett). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in

> silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control.

> The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-

chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Shaw Stewart could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful in miniaturizing and modifying Corbett or modifying Vogelstein to work in the microfluidic droplet reactor of Shaw Stewart, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered

as evidence of the level of skill in the art.[28] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail in the following sections, the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Shaw Stewart to conduct microfluidic PCR as taught by Corbett and Vogelstein. Ex. 1004 ¶¶109-112. Alternatively, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic droplet reactor of Shaw Stewart, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing. *Id.*

Not only would a reasonable Examiner consider these teachings relevant in deciding whether claims 1-8 of the '148 patent are patentable, but would have rejected the claims of the '148 patent in view of these teachings. See MPEP § 2242. The correspondence between the claims of the '148 patent and the combined teachings of the Shaw Stewart, Corbett and Vogelstein references are set forth in Section VII.F, below.

[28] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

K. The Combination of Shaw Stewart, Kopp and Lagally Presents a Substantial New Question of Patentability for Claims 1-8 of the '148 Patent

As discussed in Sections IV.C and IV.D, above, the claims have been considered nonobvious by the Office because the **prior art was not believed to teach a technique for conducting PCR reactions in droplets formed from two continuously flowing immiscible fluids**.

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Shaw Stewart), ii) microfluidic PCR devices were well known (Kopp and Lagally), and iii) there were compelling reasons to use the microfluidic droplet reactors to conduct microfluidic PCR (Kopp, Burns II, Lagally and Vogelstein).

The Shaw Stewart British Application teaches conducting reactions in microdroplets formed from two continuously flowing immiscible fluids. The Shaw Stewart British Application discloses a "droplet reactor" and illustrates at Figure 1 a carrier phase 1 flowing along a conduit into which reagent 2 is injected, thereby forming droplet 4. Ex. 1040 at 1:1-20, 70-90. "[I]f large numbers of droplets are required, a continuous flow of reagent through the opening will be produced, while a continuous current of carrier phase flows down the tube. When each droplet almost spans the tube, it will be broken off. The exact size of the droplets produced will depend on the relative and absolute magnitude of the two currents." *Id.* at 1:83-90.

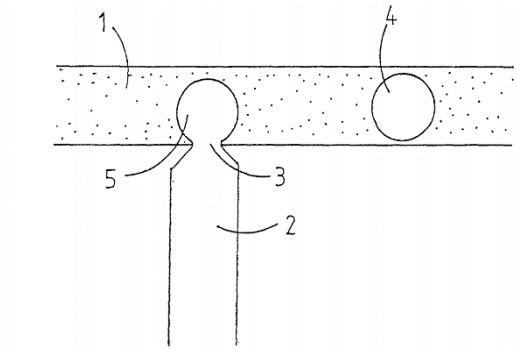


Figure 1.

Significantly, the exclusive licensee of the '148 patent affirmatively (and correctly) took the position that Shaw Stewart teaches plug or droplet formation combining a flowing stream of

an aqueous solution and a flowing stream of an immiscible fluid. More particularly, Bio-Rad (who recently acquired RainDance, the original exclusive licensee of the '148 patent, Ex. 1061, Ex. 1053 ¶71) explained that another party had mischaracterized the Shaw Stewart reference by failing to point out the above-quoted portion of Shaw Stewart, in which Shaw Stewart specifically teaches that in certain embodiments droplets are formed from intersecting and continuously flowing streams.

> To overcome Stewart I and secure allowance of their patent, the Patent Owner ("PO") mischaracterized the device disclosed in Stewart I (and hence Stewart II), misdirecting the Examiner to irrelevant portions of Stewart I while ignoring other portions that directly contradicted PO's arguments. *See, e.g.*, Ex. 1003 at 174-180. For example, one of PO's primary arguments was that Stewart I only disclosed introducing fluid in a "stepwise fashion," whereas in the '503 Patent droplets are formed by "combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid." *Id.* at 174. In truth, however, Stewart I also discloses combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid:
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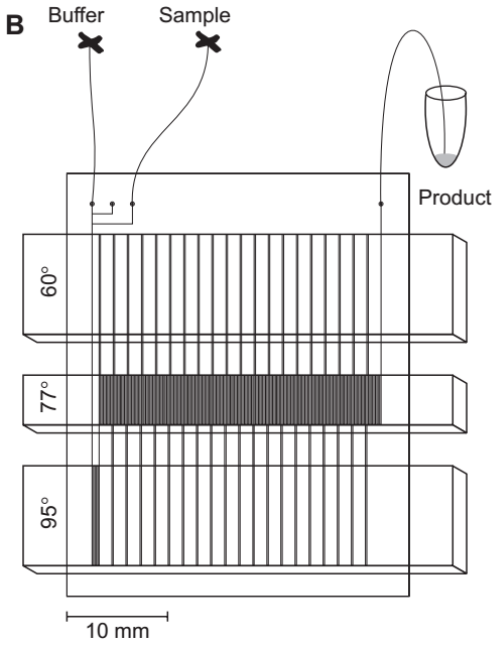
Ex. 1059 at 2-3.

Thus the exclusive licensee (Bio-Rad) expressly agreed that Shaw Stewart discloses forming plugs from intersecting streams of continuously flowing immiscible fluids.

As of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Kopp and Lagally. By 1998 Kopp et al. had

successfully implemented continuous flow PCR on a chip. In his 1988 article (not previously presented to the USPTO), Kopp explained that "[a] wide range of microreactors, microcapillary electrophoresis devices, and microcell manipulation devices have been described in recent years." Ex. 1027 at 1046. Building on that body of work, Kopp's group developed

> [a] micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in **continuous flow** at high speed. The device is analogous to an electronic amplifier and relies on the movement of sample through thermostated temperature zones on a glass microchip. Input and output of material (DNA) is **continuous**, and amplification is independent of input concentration. . . .
>
> The results demonstrate that with a very simple device and virtually no optimization, PCR can be performed in **continuous flow**, yielding product quality and quantity comparable to standard thermal cycling methods (Fig. 2B). . . .
>
> We suggest that cross-contamination in a **continuous-flow** format may be much less of a problem than in a stationary-tube PCR format. Thus, a flow-through PCR chip can be reused for extended periods without major cleaning. . . .



Ex. 1027 at 1046-1047 [emphasis added]. Kopp et al. also explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications:

> To build a complex system analogous to an electronic integrated circuit, it is necessary to integrate a variety of other continuously working parts. A cursory glance at developments in miniaturized total analysis systems (uTAS) during the past 5 years reveals that most of the relevant components, such as **continuous-flow mixers** (8), **continuous-flow microreactors** (9), **continuous separations** (10), and high-speed capillary gel electrophoresis (as a conventional batch process), have already been presented (11). Accordingly, the **continuous-flow microamplifier** should allow the creation of highly elaborate analysis and synthesis systems.
>
> Although chemical amplification reactors will obviously not be applicable to all reactions, neither will they be limited to PCR. Devices of this kind open up whole new areas of application in medical diagnostics. For example, the online amplification and monitoring of a specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy.

Ex. 1027 at 1047 [emphasis added].

In 2001, in another article not previously considered by the Office, Lagally reported using a microfluidic reactor to conduct single-molecule DNA amplification by PCR. Lagally summarized the state of the art as of February 2001, explaining continuous flow PCR microreactors had recently undergone substantial evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array

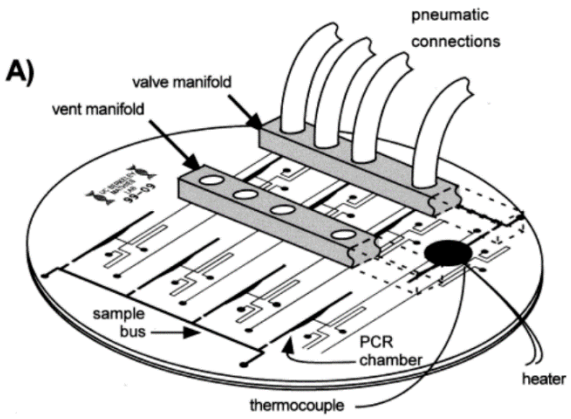
> electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

Lagally *et al.* developed a further improvement – a device and method for achieving single molecule amplification in a microchannel environment.

Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .

Microfluidic PCR-CE device. The PCR chambers are connected to a common sample bus through a set of valves. Hydrophobic vents at the other end of the PCR chambers are used to locate the sample and to eliminate gas. The PCR chambers are directly connected to the cross channel of the CE system for product injection and analysis. Two aluminum manifolds, one each for the vents and valves, are placed onto the respective ports and clamped in place using vacuum. The manifolds connect to external solenoid valves for pressure and vacuum actuation. Thermal cycling is accomplished using a resistive heater and a miniature thermocouple below the 280-nL chamber.



Ex. 1028 at 566 (see figure legend).

In sum, Kopp and Lagally show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Shaw Stewart is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule") Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Shaw Stewart) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Shaw Stewart to conduct microfluidic PCR as taught by Kopp and Lagally. Ex. 1004 ⁋⁋109,111. A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic reactors of Shaw Stewart because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Shaw Stewart to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Shaw Stewart would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product

specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude.").

Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Shaw Stewart to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Kopp or Lagally) to incorporate a droplet reactor (Shaw Stewart). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Kopp or Lagally. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the single fluid in microfluidic chip reactor (e.g., Lagally) or droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Kopp). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow

PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.

> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides

> an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an

autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Shaw Stewart could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful modifying Kopp or Lagally to work in the microfluidic reactor of Quake, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[29] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of microdroplet

[29] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail in the following sections, the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Shaw Stewart to conduct microfluidic PCR as taught by Kopp and Lagally.

Not only would a reasonable Examiner consider these teachings relevant in deciding whether claims 1-8 of the '148 patent are patentable, but would have rejected the claims of the '148 patent in view of these teachings. See MPEP § 2242. The correspondence between the claims of the '148 patent and the combined teachings of the Shaw Stewart, Kopp and Lagally references are set forth in Section VII.G, below.

L. The Combination of Shaw Stewart, Kopp and Burns II Presents a Substantial New Question of Patentability for Claims 1-8 of the '148 Patent

As discussed in Sections IV.C and IV.D, above, the claims have been considered nonobvious by the Office because the **prior art was not believed to teach a technique for conducting PCR reactions in droplets formed from two continuously flowing immiscible fluids**.

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Shaw Stewart), ii) microfluidic PCR devices were well known (Kopp and Burns II), and iii) there were compelling reasons to use the microfluidic droplet reactors to conduct microfluidic PCR (Kopp, Burns II, Lagally and Vogelstein).

The Shaw Stewart British Application teaches conducting reactions in microdroplets formed from two continuously flowing immiscible fluids. The Shaw Stewart British Application discloses a "droplet reactor" and illustrates at Figure 1 a carrier phase 1 flowing along a conduit into which reagent 2 is injected, thereby forming droplet 4. Ex. 1040 at 1:1-20, 70-90. "[I]f large numbers of droplets are required, a continuous flow of reagent through the opening will be produced, while a continuous current of carrier phase flows down the tube. When each droplet almost spans the tube, it will be broken off. The exact size of the droplets produced will depend on the relative and absolute magnitude of the two currents." *Id.* at 1:83-90.

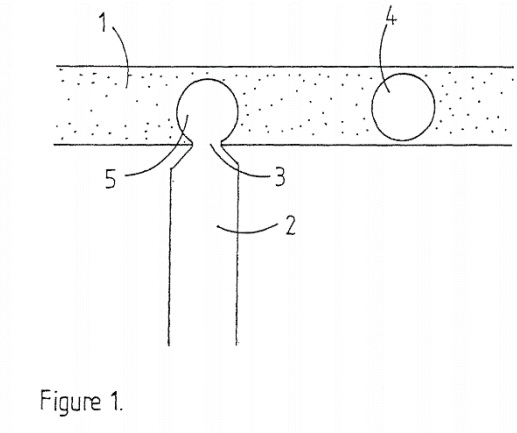


Figure 1.

Significantly, the exclusive licensee of the '148 patent affirmatively (and correctly) took the position that Shaw Stewart teaches plug or droplet formation combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid. More particularly, Bio-Rad (who recently acquired RainDance, the original exclusive licensee of the '148 patent, Ex. 1061, Ex. 1053 ¶71) explained that another party had mischaracterized the Shaw Stewart reference by failing to point out the above-quoted portion of Shaw Stewart, in which Shaw Stewart specifically teaches that in certain embodiments droplets are formed from intersecting and continuously flowing streams.

> To overcome Stewart I and secure allowance of their patent, the Patent Owner ("PO") mischaracterized the device disclosed in Stewart I (and hence Stewart II), misdirecting the Examiner to irrelevant portions of Stewart I while ignoring other portions that directly contradicted PO's arguments. *See, e.g.*, Ex. 1003 at 174-180. For example, one of PO's primary arguments was that Stewart I

> only disclosed introducing fluid in a "stepwise fashion," whereas in the '503 Patent droplets are formed by "combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid." *Id.* at 174. In truth, however, Stewart I also discloses combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid:
>
> > If large numbers of droplets are required, a continuous flow of reagent through the opening will be produced, while a continuous current of carrier phase flows down the tube. When each droplet almost spans the tube, it will be broken off.
>
> Ex. 1008, pg. 1, col. 2, ll. 83-88.

Ex. 1059 at 2-3.

Thus the exclusive licensee (Bio-Rad) expressly agreed that Shaw Stewart discloses forming plugs from intersecting streams of continuously flowing immiscible fluids.

As of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Kopp and Burns II. In 1996, Burns II reported the use of microfluidic devices to perform PCR. Ex. 1008. Burns II explained that "[n]o new biochemistries or DNA detection methods are required for their use; the components simply reproduce current laboratory procedures in a micron-sized environment." *Id.* at 5560. In other words, the PCR chemistry did not need to be modified – it worked without modification on the microfluidic scale. In the method of Burns II, a sample containing a DNA plasmid was loaded into one of the reaction wells (A) and a mixture of Taq enzyme and buffer was loaded into the other. *Id.* at 5558. Each formed droplets of about 60 nl in the parallel 500 x 25 µm channels (B) which came together at the Y junction to form a single larger droplet (about 120 nl). *Id.* at 5558-59; Ex. 1004 ¶40.

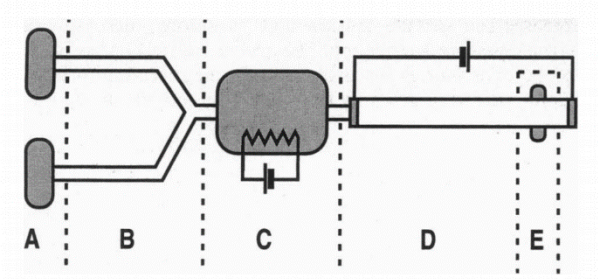


FIG. 1. Example of an integrated DNA analysis system, represented schematically. The individual components of the system are injection entry ports (*A*), liquid pumping channels (*B*), thermally controlled reaction chamber (*C*), electrophoresis channel (*D*), and DNA band migration detector (*E*). Each component would have associated sensors, control circuitry, and external connections.

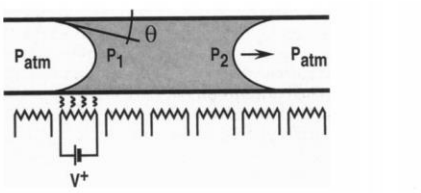


FIG. 2. Schematic drawing showing the principle of thermally induced drop motion in a closed channel. The case of a single aqueous drop in a hydrophilic channel is presented, where V is an applied voltage, P_{atm} is atmospheric pressure, P_1 is the receding-edge internal pressure, P_2 is the advancing-edge internal pressure, and θ is the contact angle of the liquid–gas–solid interface. The contact angle will depend on the surface characteristics of the channel and the constituents of the drop, with a hydrophilic interaction giving θ between 0 and 90 degrees, and a hydrophobic surface giving θ between 90 and 180 degrees. Surface treatments can also reduce contact angle hysteresis and, therefore, reduce the temperature difference necessary for drop motion.

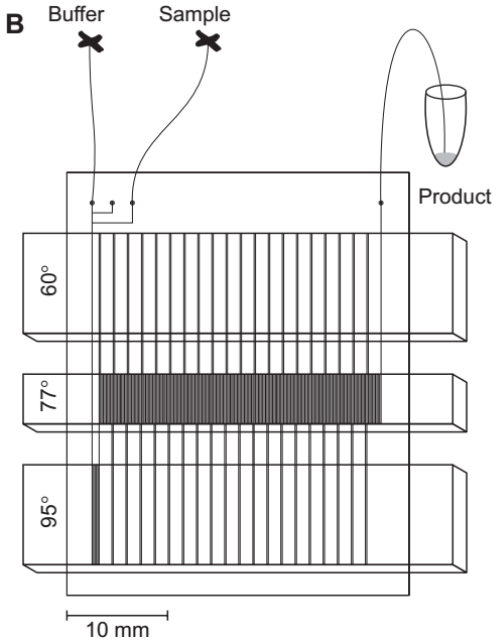
The droplet then passed through the resistive heater portion (C) in which PCR amplification occurred. *Id.* at 5556. Afterwards the amplification results were optically detected in zone D via gel electrophoresis *Id.* at 5559. Burns II noted that "[t]he use of common fabrication methods allows the assembly of increasingly complex, multicomponent, integrated systems from a small, defined set of standardized elements" and that "[f]uture design efforts have a strong impetus to reduce component size and improve efficiency." *Id.* at 5560-61.

By the late 1990s Kopp et al. had successfully implemented continuous flow PCR on a chip. In his 1988 article (not previously presented to the USPTO), Kopp explained that "[a] wide range of microreactors, microcapillary electrophoresis devices, and microcell manipulation devices have been described in recent years." Ex. 1027 at 1046. Building on that body of work, Kopp's group developed

> [a] micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in **continuous flow** at high speed. The device is analogous to an electronic amplifier and relies on the movement of sample through thermostated temperature zones on a glass microchip. Input and output of material (DNA) is **continuous**, and amplification is independent of input concentration. . . .
>
> The results demonstrate that with a very simple device and virtually no optimization, PCR can be performed in **continuous flow**, yielding product

quality and quantity comparable to standard thermal cycling methods (Fig. 2B). . . .

We suggest that cross-contamination in a **continuous-flow** format may be much less of a problem than in a stationary-tube PCR format. Thus, a flow-through PCR chip can be reused for extended periods without major cleaning. . . .



Ex. 1027 at 1046-1047 [emphasis added]. Kopp et al. also explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications:

To build a complex system analogous to an electronic integrated circuit, it is necessary to integrate a variety of other continuously working parts. A cursory glance at developments in miniaturized total analysis systems (uTAS) during the past 5 years reveals that most of the relevant components, such as **continuous-flow mixers** (8), **continuous-flow microreactors** (9), **continuous separations** (10), and high-speed capillary gel electrophoresis (as a conventional batch process), have already been presented (11). Accordingly, the **continuous-flow microamplifier** should allow the creation of highly elaborate analysis and synthesis systems.

> Although chemical amplification reactors will obviously not be applicable to all reactions, neither will they be limited to PCR. Devices of this kind open up whole new areas of application in medical diagnostics. For example, the online amplification and monitoring of a specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy.

Ex. 1027 at 1047 [emphasis added].

In sum, Kopp and Burns II show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Shaw Stewart is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule") Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Shaw Stewart) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Shaw Stewart, to conduct microfluidic PCR as taught by Kopp and Burns II. Ex. 1004 ⁋⁋109,111. A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic droplet reactor of Shaw Stewart because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis

added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Shaw Stewart to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

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Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise**

limitations associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Shaw Stewart to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Kopp or Burns II) to incorporate a droplet reactor (Shaw Stewart). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Kopp or Burns II. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the single fluid in microfluidic chip reactor (e.g., Burns II) or droplets produced using injection of an

aqueous fluid into an immiscible fluid (e.g., Kopp). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize

> noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson

> distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at

1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Shaw Stewart could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful modifying Kopp or Lagally to work in the microfluidic droplet reactor of Shaw Stewart, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[30] As

[30] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the

discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail in the following sections, the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Shaw Stewart to conduct microfluidic PCR as taught by Kopp and Burns II.

Not only would a reasonable Examiner consider these teachings relevant in deciding whether claims 1-8 of the '148 patent are patentable, but would have rejected the claims of the '148 patent in view of these teachings. See MPEP § 2242. The correspondence between the claims of the '148 patent and the combined teachings of the Shaw Stewart, Kopp and Burns II references are set forth in Section VII.H, below.

M. The Combination of Shaw Stewart, Kopp and Vogelstein Presents a Substantial New Question of Patentability for Claims 1-8 of the '148 Patent

As discussed in Sections IV.C and IV.D, above, the claims have been considered nonobvious by the Office because the **prior art was not believed to teach a technique for conducting PCR reactions in droplets formed from two continuously flowing immiscible fluids**.

references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Shaw Stewart), ii) microfluidic PCR devices were well known (Kopp and Vogelstein), and iii) there were compelling reasons to use the microfluidic droplet reactors to conduct microfluidic PCR (Kopp, Burns II, Lagally and Vogelstein).

The Shaw Stewart British Application teaches conducting reactions in microdroplets formed from two continuously flowing immiscible fluids. The Shaw Stewart British Application discloses a "droplet reactor" and illustrates at Figure 1 a carrier phase 1 flowing along a conduit into which reagent 2 is injected, thereby forming droplet 4. Ex. 1040 at 1:1-20, 70-90. "[I]f large numbers of droplets are required, a continuous flow of reagent through the opening will be produced, while a continuous current of carrier phase flows down the tube. When each droplet almost spans the tube, it will be broken off. The exact size of the droplets produced will depend on the relative and absolute magnitude of the two currents." *Id.* at 1:83-90.

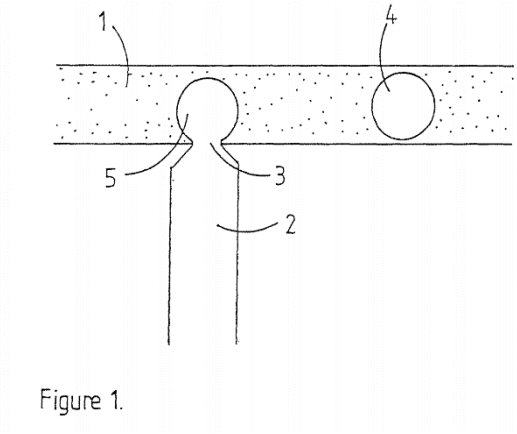


Figure 1.

Significantly, the exclusive licensee of the '148 patent affirmatively (and correctly) took the position that Shaw Stewart teaches plug or droplet formation combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid. More particularly, Bio-Rad (who recently acquired RainDance, the original exclusive licensee of the '148 patent, Ex. 1061, Ex. 1053 ¶71) explained that another party had mischaracterized the Shaw Stewart reference by failing to point out the above-quoted portion of Shaw Stewart, in which Shaw Stewart

specifically teaches that in certain embodiments droplets are formed from intersecting and continuously flowing streams.

> To overcome Stewart I and secure allowance of their patent, the Patent Owner ("PO") mischaracterized the device disclosed in Stewart I (and hence Stewart II), misdirecting the Examiner to irrelevant portions of Stewart I while ignoring other portions that directly contradicted PO's arguments. *See, e.g.*, Ex. 1003 at 174-180. For example, one of PO's primary arguments was that Stewart I only disclosed introducing fluid in a "stepwise fashion," whereas in the '503 Patent droplets are formed by "combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid." *Id.* at 174. In truth, however, Stewart I also discloses combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid:
>
> > If large numbers of droplets are required, a continuous flow of reagent through the opening will be produced, while a continuous current of carrier phase flows down the tube. When each droplet almost spans the tube, it will be broken off.
>
> Ex. 1008, pg. 1, col. 2, ll. 83-88.

Ex. 1059 at 2-3.

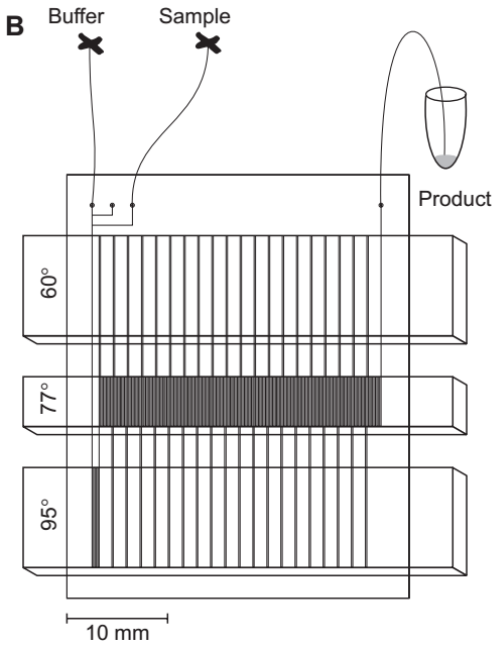
Thus the exclusive licensee (Bio-Rad) expressly agreed that Shaw Stewart discloses forming plugs from intersecting streams of continuously flowing immiscible fluids.

As of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Kopp and Vogelstein. By the late 1990s Kopp et al. had successfully implemented continuous flow PCR on a chip. In his 1988 article (not previously presented to the USPTO), Kopp explained that "[a] wide range of microreactors, microcapillary electrophoresis devices, and microcell manipulation devices have been described in recent years." Ex. 1027 at 1046. Building on that body of work, Kopp's group developed

[a] micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in **continuous flow** at high speed. The device is analogous to an electronic amplifier and relies on the movement of sample through thermostated temperature zones on a glass microchip. Input and output of material (DNA) is **continuous**, and amplification is independent of input concentration. . . .

The results demonstrate that with a very simple device and virtually no optimization, PCR can be performed in **continuous flow**, yielding product quality and quantity comparable to standard thermal cycling methods (Fig. 2B). . . .

We suggest that cross-contamination in a **continuous-flow** format may be much less of a problem than in a stationary-tube PCR format. Thus, a flow-through PCR chip can be reused for extended periods without major cleaning. . . .



Ex. 1027 at 1046-1047 [emphasis added]. Kopp et al. also explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications:

> To build a complex system analogous to an electronic integrated circuit, it is necessary to integrate a variety of other continuously working parts. A cursory glance at developments in miniaturized total analysis systems (uTAS) during the past 5 years reveals that most of the relevant components, such as **continuous-flow mixers** (8), **continuous-flow microreactors** (9), **continuous separations** (10), and high-speed capillary gel electrophoresis (as a conventional batch process), have already been presented (11). Accordingly, the **continuous-flow microamplifier** should allow the creation of highly elaborate analysis and synthesis systems.
>
> Although chemical amplification reactors will obviously not be applicable to all reactions, neither will they be limited to PCR. Devices of this kind open up whole new areas of application in medical diagnostics. For example, the online amplification and monitoring of a specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy.

Ex. 1025 at 1047 [emphasis added].

By 1999, Vogelstein had reported **various applications for single-molecule microfluidic PCR**. Ex. 1044 at 9241. In a seminal article which has been cited over by over 1,000 other publications, Vogelstein explained that PCR amplification of small aliquots or droplets containing single molecules was effective for "detection of a small number of mutant-containing cells among a large excess of normal cells." *Id.* at 9236. In Vogelstein's method, the DNA was diluted into 7 µl volumes such that, on average, there was one template molecule for every two wells (i.e., approximately half of the wells contained no DNA template molecule and approximately half contained a single DNA template molecule). *Id.* at 9236, 9237. This avoided the problem of background noise impairing the determination of whether each volume contained the mutant sequence. *Id.* By counting the number of volumes which indicated wild type or mutant templates, the presence or absence of the mutation (e.g., a sequence associated with

colorectal cancer) can be determined. *Id.* at 9238. The accuracy of the test is a function of the number of discrete volumes into which the sample is diluted. *Id.* at 9239. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. *Id.* at 9239. ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude.") *Id.* 9239. The article explained that the same technique would be useful for **detecting a wide array of mutations, dislocations and allelic imbalances**, as summarized in Table 1. *Id.* at 9241. Vogelstein thus taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby overcoming the signal-to-noise limitations associated with certain other PCR techniques and enabling the detection of relatively rare mutations, dislocations and allelic imbalances. Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84.

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Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely

reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

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Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

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speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or continuous flow through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.

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Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
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> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In

1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Shaw Stewart could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful modifying Kopp or Lagally to work in the microfluidic droplet reactor of Shaw Stewart, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148

patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[31] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail in the following sections, the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Shaw Stewart to conduct microfluidic PCR as taught by Kopp and Vogelstein.

Not only would a reasonable Examiner consider these teachings relevant in deciding whether claims 1-8 of the '148 patent are patentable, but would have rejected the claims of the '148 patent in view of these teachings. See MPEP § 2242. The correspondence between the claims of the '148 patent and the combined teachings of the Shaw Stewart, Kopp and Vogelstein references are set forth in Section VII.H, below.

[31] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

N. The Combination of Burns I, Corbett and Lagally Presents a Substantial New Question of Patentability for Claims 1-5 of the '148 Patent

As discussed in Sections IV.C and IV.D, above, the claims have been considered nonobvious by the Office because the **prior art was not believed to teach a technique for conducting PCR reactions in droplets formed from two continuously flowing immiscible fluids**.

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Burns I), ii) microfluidic PCR devices were well known (Corbett and Lagally), and iii) there were compelling reasons to use the microfluidic droplet reactors to conduct microfluidic PCR (Kopp, Burns II, Lagally and Vogelstein).

Burns I teaches that plugs in the channels of a microreactor by the continuous flow of fluids at an intersecting channel. Burns I explained that "various methods may be used to generate slugs of liquid within a microreactor." Ex. 1007 at 10. Burns I chose a method in which "the continuous flow of both phases through T or cross-shaped intersections" and "[s]lugs are generated by the action of one phase flowing into the channel whilst the other phase moves into the intersection, eventually cutting off the flow of the first phase into the channel and reversing the process." Ex. 1007 at 11 [emphasis added]. Fig. 3 shows oil-in-water slug generation and Fig. 4 shows water-in-oil slug generation. *Id.* at 11. In Burns' Fig. 4 embodiment, an aqueous plug of reagents is formed in a continuous oil phase. The continuous flow plug formation used by Burns was a well-known alternative to periodically injecting a measured slug of reactant into the main channel. See Ex. 1010; 1027; Ex. 1025 at 23:2-8. Burns I

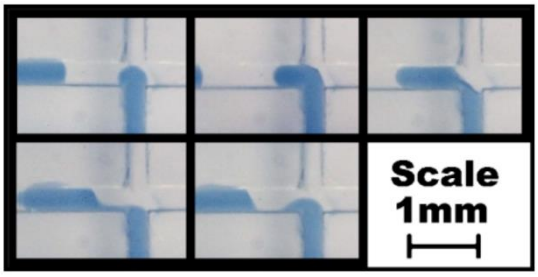
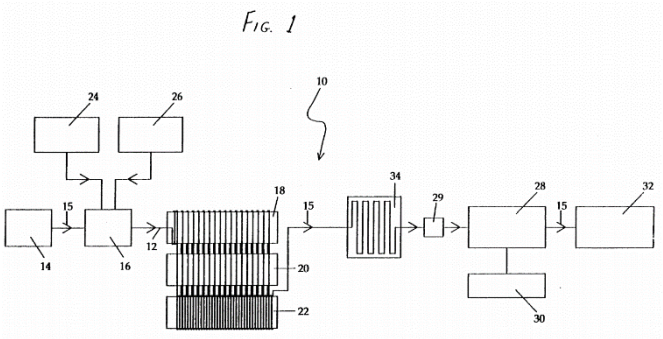
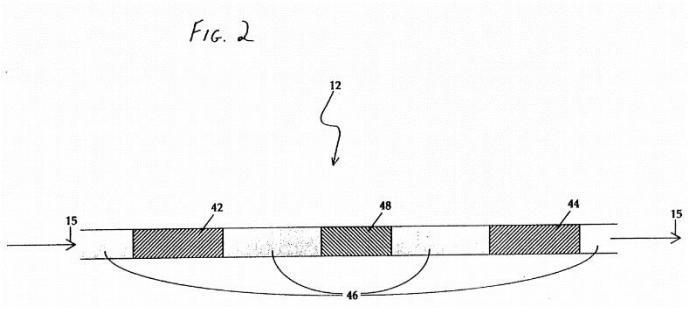


Fig. 3 Typical slug generation in glass device with 380 µm wide channels.

demonstrates that making slugs or plugs with continuous flows of immiscible fluids was a mere design choice as of 2001.

As of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Corbett and Lagally. In 1991, about eleven years prior to the earliest effective filing date, Corbett disclosed using a microchannel or capillary device to conduct DNA amplification reactions such as PCR by injecting plugs of reactants into a carrier stream that is passed through different temperature zones. Corbett explained that as of 1991 "the most critical problem in applying nucleic acid amplification procedures, particularly in repetitive diagnostic assays in a clinical laboratory, is contamination of the reaction mixture by small amounts of DNA which contain sequences capable of being amplified under the conditions of assay." Ex. 1010 at 3:7-12. "Automation of procedures minimize[s] the opportunity for such contamination and allows the implementation of stringent quality control." *Id.* at 3:15-19. "As is shown in FIG. 1, the apparatus 10 comprises tube 12 through which a fluid stream passes. As is more clearly shown in FIG. 2, the fluid stream includes pockets of reaction mixtures 42 and 44 and purging solution 48 separated by carrier fluid 46." *Id.* at 7:58-62.





"The pump is actuated and the reaction mixture of 20 µl or less is injected into the mineral oil or silicone oil carrier fluid and specific DNA sequences (whose limits are defined by the oligonucleotide primers) present in the sample is amplified as it passes cyclically through the temperature zones." Ex. 1010 at 9:35-40. Corbett thus demonstrates that it was known at least as of 1991 that reduction of contamination risk in PCR could be achieved by performing the PCR on microliter sized plugs of reactant in capillary tube which passes through various temperature zones. Ex. 1004 ¶77.

In 2001, in another article not previously considered by the Office, Lagally reported using a microfluidic reactor to conduct single-molecule DNA amplification by PCR. Lagally summarized the state of the art as of February 2001, explaining continuous flow PCR microreactors had recently undergone substantial evolution:

> The advent of biological microdevices [6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/µL, in volumes down to 1 µL.[11] Since then, stand-alone PCR reactors have been constructed in

> silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
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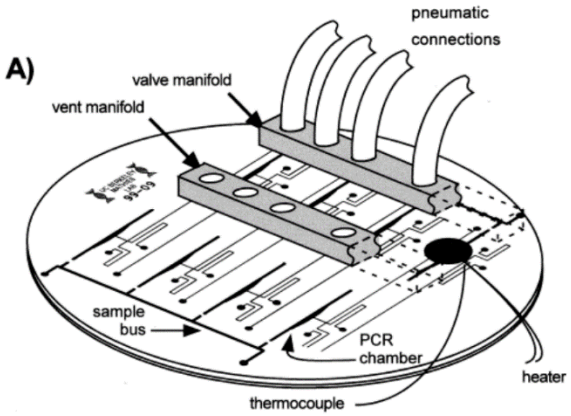
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

Lagally *et al.* developed a further improvement – a device and method for achieving single molecule amplification in a microchannel environment.

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control.

> The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> Microfluidic PCR-CE device. The PCR chambers are connected to a common sample bus through a set of valves. Hydrophobic vents at the other end of the PCR chambers are used to locate the sample and to eliminate gas. The PCR chambers are directly connected to the cross channel of the CE system for product injection and analysis. Two aluminum manifolds, one each for the vents and valves, are placed onto the respective ports and clamped in place using vacuum. The manifolds connect to external solenoid valves for pressure and vacuum actuation. Thermal cycling is accomplished using a resistive heater and a miniature thermocouple below the 280-nL chamber.



Ex. 1028 at 566 (see figure legend).

In sum, Corbett and Lagally show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Burns I is relied upon to meet all of the limitations of the independent claims of the ‘148 patent except the PCR-related limitations (e.g., “continuously flowing aqueous fluid comprising target DNA or RNA molecules

and at least one other molecule").[32] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Burns I) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I to conduct microfluidic PCR as taught by Corbett and Lagally. Ex. 1004 ¶¶109,111. Stated a different way, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic reactor of Burns I, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing.

A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic reactors of Burns I because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004

[32] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

¶105, 107. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Burns I to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the droplet reactors of Burns I would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific

binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the

assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Burns I to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Lagally) to incorporate a droplet reactor (Burns I). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Lagally. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the single fluid in microfluidic chip reactor (e.g., Lagally) or droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Corbett). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

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autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

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streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail in the following sections, the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I to conduct microfluidic PCR as taught by Corbett and Lagally. Ex. 1004 ¶¶109,111. Alternatively, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic reactor of Burns I, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing. *Id*.

Not only would a reasonable Examiner consider these teachings relevant in deciding whether claims 1-5 of the '148 patent are patentable, but would have rejected the claims of the '148 patent in view of these teachings. See MPEP § 2242. The correspondence between the claims of the '148 patent and the combined teachings of the Burns I, Corbett and Lagally references are set forth in Section VII.I, below.

O. The Combination of Burns I, Corbett and Burns II Presents a Substantial New Question of Patentability for Claims 1-5 of the '148 Patent

As discussed in Section IV.C and IV.D above, the claims have been considered nonobvious by the Office because the **prior art was not believed to teach a technique for conducting PCR reactions in droplets formed from two continuously flowing immiscible fluids**.

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Burns I), ii) microfluidic PCR devices were well known (Corbett and Burns II), and iii) there were compelling reasons to use

the microfluidic droplet reactors to conduct microfluidic PCR (Lagally, Kopp, Burns II and Vogelstein).

Burns I teaches that plugs in the channels of a microreactor by the continuous flow of fluids at an intersecting channel. Burns I explained that "various methods may be used to generate slugs of liquid within a microreactor." Ex. 1007 at 10. Burns I chose a method in which "the continuous flow of both phases through T or cross-shaped intersections" and "[s]lugs are generated by the action of one phase flowing into the channel whilst the other phase moves into the intersection, eventually cutting off the flow of the first phase into the channel and reversing the process." Ex. 1007 at 11 [emphasis added]. Fig. 3 shows oil-in-water slug generation and Fig. 4 shows water-in-oil slug generation. *Id.* at 11. In Burns' Fig. 4 embodiment, an aqueous plug of reagents is formed in a continuous oil phase. The continuous flow plug formation used by Burns was a well-known alternative to periodically injecting a measured slug of reactant into the main channel. See Ex. 1010; 1027; Ex. 1025 at 23:2-8. Burns I demonstrates that making slugs or plugs with continuous flows of immiscible fluids was a mere design choice as of 2001.

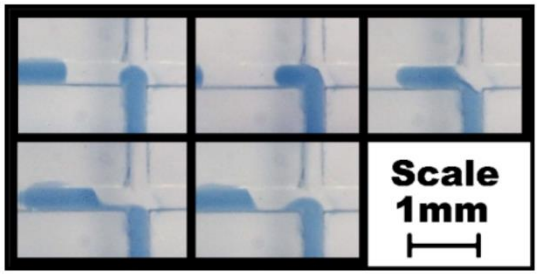
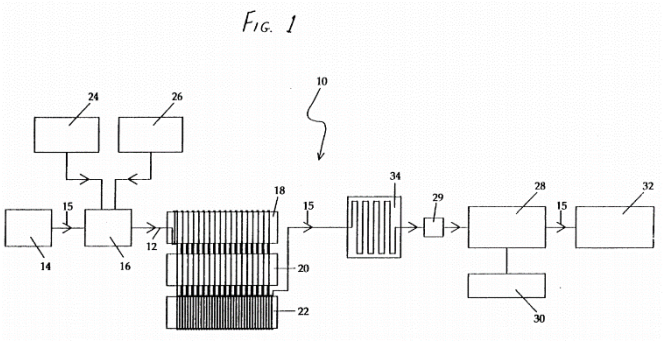
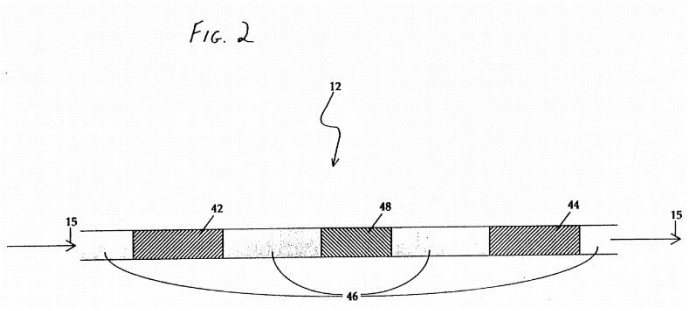


Fig. 3 Typical slug generation in glass device with 380 μm wide channels.

As of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Corbett and Burns II. In 1991, about eleven years prior to the earliest effective filing date, Corbett disclosed using a microchannel or capillary device to conduct DNA amplification reactions such as PCR by injecting plugs of reactants into a carrier stream that is passed through different temperature zones. Corbett explained that as of 1991 "the most critical problem in applying nucleic acid amplification

procedures, particularly in repetitive diagnostic assays in a clinical laboratory, is contamination of the reaction mixture by small amounts of DNA which contain sequences capable of being amplified under the conditions of assay." Ex. 1010 at 3:7-12. "Automation of procedures minimize[s] the opportunity for such contamination and allows the implementation of stringent quality control." *Id.* at 3:15-19. "As is shown in FIG. 1, the apparatus 10 comprises tube 12 through which a fluid stream passes. As is more clearly shown in FIG. 2, the fluid stream includes pockets of reaction mixtures 42 and 44 and purging solution 48 separated by carrier fluid 46." *Id.* at 7:58-62.





"The pump is actuated and the reaction mixture of 20 µl or less is injected into the mineral oil or silicone oil carrier fluid and specific DNA sequences (whose limits are defined by the oligonucleotide primers) present in the sample is amplified as it passes cyclically through the temperature zones." Ex. 1010 at 9:35-40. Corbett thus demonstrates that it was known at least as of 1991 that reduction of contamination risk in PCR could be achieved by performing the PCR

on microliter sized plugs of reactant in capillary tube which passes through various temperature zones. Ex. 1004 ¶77.

In 1996, Burns II reported the use of microfluidic devices to perform PCR. Ex. 1008. Burns II explained that "[n]o new biochemistries or DNA detection methods are required for their use; the components simply reproduce current laboratory procedures in a micron-sized environment." *Id.* at 5560. In other words, the PCR chemistry did not need to be modified – it worked without modification on the microfluidic scale. In the method of Burns II, a sample containing a DNA plasmid was loaded into one of the reaction wells (A) and a mixture of Taq enzyme and buffer was loaded into the other. *Id.* at 5558. Each formed droplets of about 60 nl in the parallel 500 x 25 µm channels (B) which came together at the Y junction to form a single larger droplet (about 120 nl). *Id.* at 5558-59; Ex. 1004 ¶40.

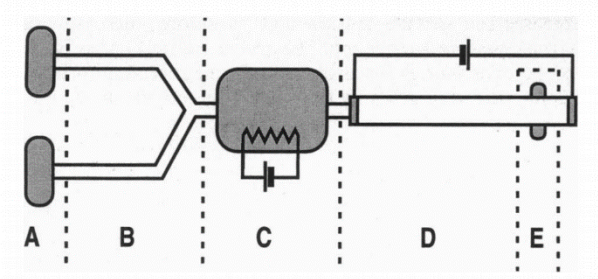


FIG. 1. Example of an integrated DNA analysis system, represented schematically. The individual components of the system are injection entry ports (*A*), liquid pumping channels (*B*), thermally controlled reaction chamber (*C*), electrophoresis channel (*D*), and DNA band migration detector (*E*). Each component would have associated sensors, control circuitry, and external connections.

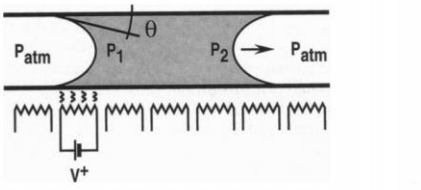


FIG. 2. Schematic drawing showing the principle of thermally induced drop motion in a closed channel. The case of a single aqueous drop in a hydrophilic channel is presented, where V is an applied voltage, P_{atm} is atmospheric pressure, P_1 is the receding-edge internal pressure, P_2 is the advancing-edge internal pressure, and θ is the contact angle of the liquid–gas–solid interface. The contact angle will depend on the surface characteristics of the channel and the constituents of the drop, with a hydrophilic interaction giving θ between 0 and 90 degrees, and a hydrophobic surface giving θ between 90 and 180 degrees. Surface treatments can also reduce contact angle hysteresis and, therefore, reduce the temperature difference necessary for drop motion.

The droplet then passed through the resistive heater portion (C) in which PCR amplification occurred. *Id.* at 5556. Afterwards the amplification results were optically detected in zone D via gel electrophoresis *Id.* at 5559. Burns II noted that "[t]he use of common fabrication methods allows the assembly of increasingly complex, multicomponent, integrated systems from a small, defined set of standardized elements" and that "[f]uture design efforts have a strong impetus to reduce component size and improve efficiency." *Id.* at 5560-61.

In sum, Corbett and Burns II show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Burns I is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[34] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Burns I) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I to conduct microfluidic PCR as taught by Corbett and Burns II. Stated a different way, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic reactor of Burns I, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing.

A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic reactor of Burns I because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that

[34] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

"[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Burns I to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the droplet reactors of Burns I would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to

production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise**

limitations associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Burns I to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Burns II) to incorporate a droplet reactor (Burns I). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Burns II. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the single fluid in microfluidic chip reactor (e.g., Burns II) or droplets produced using injection of an aqueous fluid into an

immiscible fluid (e.g., Corbett). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or continuous flow through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk

> heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with

> microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching**

whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Burns I could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful in miniaturizing and modifying Corbett or modifying Burns II to work in the microfluidic reactor of Burns I, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[35] As discussed in Section VI.A.3, above, Higuchi I-III

[35] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations

demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail in the following sections, the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I to conduct microfluidic PCR as taught by Corbett and Burns II. Ex. 1004 ¶¶109,111. Alternatively, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic reactor of Burns I, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing. *Id*.

Not only would a reasonable Examiner consider these teachings relevant in deciding whether claims 1-5 of the '148 patent are patentable, but would have rejected the claims of the '148 patent in view of these teachings. See MPEP § 2242. The correspondence between the claims of the '148 patent and the combined teachings of the Burns I, Corbett and Burns II references are set forth in Section VII.J, below.

P. The Combination of Burns I, Corbett and Vogelstein Presents a Substantial New Question of Patentability for Claims 1-5 of the '148 Patent

As discussed in Section IV.C and IV.D above, the claims have been considered nonobvious by the Office because the **prior art was not believed to teach a technique for**

omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

conducting PCR reactions in droplets formed from two continuously flowing immiscible fluids.

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Burns I), ii) microfluidic PCR devices were well known (Corbett and Vogelstein), and iii) there were compelling reasons to use the microfluidic droplet reactors to conduct microfluidic PCR (Lagally, Kopp, Burns II and Vogelstein).

Burns I teaches that plugs in the channels of a microreactor by the continuous flow of fluids at an intersecting channel. Burns I explained that "various methods may be used to generate slugs of liquid within a microreactor." Ex. 1007 at 10. Burns I chose a method in which "the continuous flow of both phases through T or cross-shaped intersections" and "[s]lugs are generated by the action of one phase flowing into the channel whilst the other phase moves into the intersection, eventually cutting off the flow of the first phase into the channel and reversing the process." Ex. 1007 at 11 [emphasis added]. Fig. 3 shows oil-in-water slug generation and Fig. 4 shows water-in-oil slug generation. *Id.* at 11. In Burns' Fig. 4 embodiment, an aqueous plug of reagents is formed in a continuous oil phase. The continuous flow plug formation used by Burns was a well-known alternative to periodically injecting a measured slug of reactant into the main channel. See Ex. 1010; 1027; Ex. 1025 at 23:2-8. Burns I demonstrates that making slugs or plugs with continuous flows of immiscible fluids was a mere design choice as of 2001.

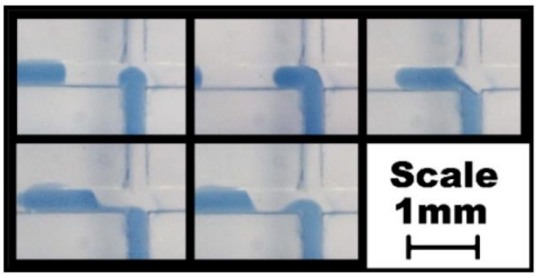


Fig. 3 Typical slug generation in glass device with 380 μm wide channels.

As of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Corbett and Vogelstein. In 1991, about eleven years prior to the earliest effective filing date, Corbett disclosed using a microchannel or capillary device to conduct DNA amplification reactions such as PCR by injecting plugs of reactants into a carrier stream that is passed through different temperature zones. Corbett explained that as of 1991 "the most critical problem in applying nucleic acid amplification procedures, particularly in repetitive diagnostic assays in a clinical laboratory, is contamination of the reaction mixture by small amounts of DNA which contain sequences capable of being amplified under the conditions of assay." Ex. 1010 at 3:7-12. "Automation of procedures minimize[s] the opportunity for such contamination and allows the implementation of stringent quality control." *Id.* at 3:15-19. "As is shown in FIG. 1, the apparatus 10 comprises tube 12 through which a fluid stream passes. As is more clearly shown in FIG. 2, the fluid stream includes pockets of reaction mixtures 42 and 44 and purging solution 48 separated by carrier fluid 46." *Id.* at 7:58-62.

FIG. 1

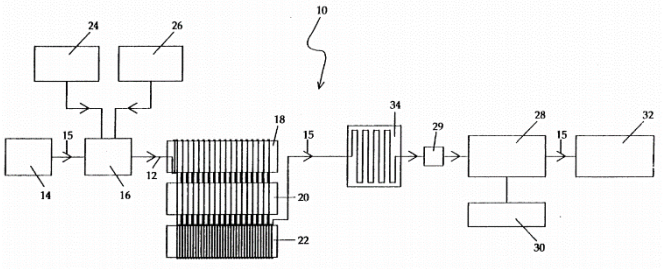
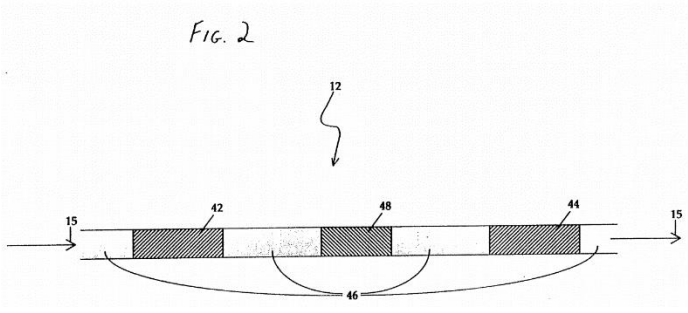


FIG. 2



"The pump is actuated and the reaction mixture of 20 µl or less is injected into the mineral oil or silicone oil carrier fluid and specific DNA sequences (whose limits are defined by the oligonucleotide primers) present in the sample is amplified as it passes cyclically through the temperature zones." Ex. 1010 at 9:35-40. Corbett thus demonstrates that it was known at least as of 1991 that reduction of contamination risk in PCR could be achieved by performing the PCR on microliter sized plugs of reactant in capillary tube which passes through various temperature zones. Ex. 1004 ¶77.

By 1999, Vogelstein had reported **various applications for single-molecule microfluidic PCR**. Ex. 1044 at 9241. In a seminal article which has been cited over by over 1,000 other publications, Vogelstein explained that PCR amplification of small aliquots or droplets containing single molecules was effective for "detection of a small number of mutant-containing cells among a large excess of normal cells." *Id.* at 9236. In Vogelstein's method, the DNA was diluted into 7 µl volumes such that, on average, there was one template molecule for every two wells (i.e., approximately half of the wells contained no DNA template molecule and approximately half contained a single DNA template molecule). *Id.* at 9236, 9237. This avoided the problem of background noise impairing the determination of whether each volume contained the mutant sequence. *Id.* By counting the number of volumes which indicated wild type or mutant templates, the presence or absence of the mutation (e.g., a sequence associated with colorectal cancer) can be determined. *Id.* at 9238. The accuracy of the test is a function of the number of discrete volumes into which the sample is diluted. *Id.* at 9239. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. *Id.* at 9239. ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the

sensitivity by another order of magnitude.") *Id.* 9239. The article explained that the same technique would be useful for **detecting a wide array of mutations, dislocations and allelic imbalances**, as summarized in Table 1. *Id.* at 9241. Vogelstein thus taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby overcoming the signal-to-noise limitations associated with certain other PCR techniques and enabling the detection of relatively rare mutations, dislocations and allelic imbalances. Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84.

In sum, Corbett and Vogelstein show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Burns I is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule") Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Burns I) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I to conduct microfluidic PCR as taught by Corbett and Vogelstein. Ex. 1004 ¶109-112. Stated a different way, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic reactor of Burns I, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that

PCR had been widely and successfully implemented at the microfluidic scale at the time of filing.

A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic reactor of Burns I because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as

to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Burns I to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the droplet reactors of Burns I would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely

reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Burns I to dilute the samples and thereby enable the detection of

mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Vogelstein) to incorporate a droplet reactor (Burns I). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Vogelstein. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Corbett). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the

speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or continuous flow through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.

Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.20,21 The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.21 This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In

1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Burns I could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful in miniaturizing and modifying Corbett or modifying Vogelstein to work in the microfluidic reactor of Burns I, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year

prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[36] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail in the following sections, the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I to conduct microfluidic PCR as taught by Corbett and Vogelstein. Ex. 1004 ¶109-112. Alternatively, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic reactor of Burns I, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing. *Id*.

Not only would a reasonable Examiner consider these teachings relevant in deciding whether claims 1-5 of the '148 patent are patentable, but would have rejected the claims of the

[36] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

'148 patent in view of these teachings. See MPEP § 2242. The correspondence between the claims of the '148 patent and the combined teachings of the Burns I, Corbett and Vogelstein references are set forth in Section VII.J, below.

Q. The Combination of Burns I, Kopp and Lagally Presents a Substantial New Question of Patentability for Claims 1-5 of the '148 Patent

As discussed in Sections IV.C and IV.D, above, the claims have been considered nonobvious by the Office because the **prior art was not believed to teach a technique for conducting PCR reactions in droplets formed from two continuously flowing immiscible fluids**.

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Burns I), ii) microfluidic PCR devices were well known (Kopp and Lagally), and iii) there were compelling reasons to use the microfluidic droplet reactors to conduct microfluidic PCR (Kopp, Burns II, Lagally and Vogelstein).

Burns I teaches that plugs in the channels of a microreactor by the continuous flow of fluids at an intersecting channel. Burns I explained that "various methods may be used to generate slugs of liquid within a microreactor." Ex. 1007 at 10. Burns I chose a method in which "the continuous flow of both phases through T or cross-shaped intersections" and "[s]lugs are generated by the action of one phase flowing into the channel whilst the other phase moves into the intersection, eventually cutting off the flow of the first phase into the channel and reversing the process." Ex. 1007 at 11 [emphasis added]. Fig. 3 shows oil-in-water slug generation and Fig. 4 shows water-in-oil slug generation. *Id.* at 11. In

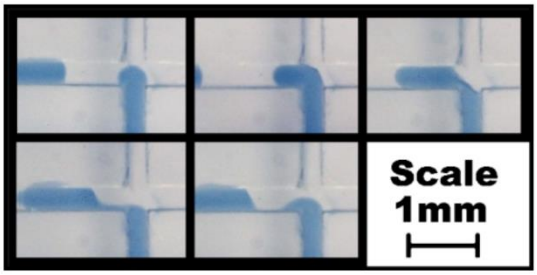
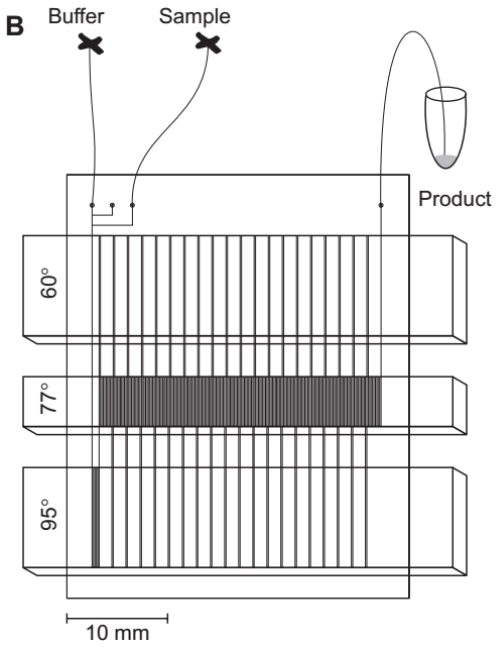


Fig. 3 Typical slug generation in glass device with 380 μm wide channels.

Burns' Fig. 4 embodiment, an aqueous plug of reagents is formed in a continuous oil phase. The continuous flow plug formation used by Burns was a well-known alternative to periodically injecting a measured slug of reactant into the main channel. See Ex. 1010; 1027; Ex. 1025 at 23:2-8. Burns I demonstrates that making slugs or plugs with continuous flows of immiscible fluids was a mere design choice as of 2001.

As of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Kopp and Lagally. By 1998 Kopp et al. had successfully implemented continuous flow PCR on a chip. In his 1988 article (not previously presented to the USPTO), Kopp explained that "[a] wide range of microreactors, microcapillary electrophoresis devices, and microcell manipulation devices have been described in recent years." Ex. 1027 at 1046. Building on that body of work, Kopp's group developed

> [a] micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in **continuous flow** at high speed. The device is analogous to an electronic amplifier and relies on the movement of sample through thermostated temperature zones on a glass microchip. Input and output of material (DNA) is **continuous**, and amplification is independent of input concentration. . . .
>
> The results demonstrate that with a very simple device and virtually no optimization, PCR can be performed in **continuous flow**, yielding product quality and quantity comparable to standard thermal cycling methods (Fig. 2B). . . .
>
> We suggest that cross-contamination in a **continuous-flow** format may be much less of a problem than in a stationary-tube PCR format. Thus, a flow-through PCR chip can be reused for extended periods without major cleaning. . . .



Ex. 1027 at 1046-1047 [emphasis added]. Kopp et al. also explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications:

> To build a complex system analogous to an electronic integrated circuit, it is necessary to integrate a variety of other continuously working parts. A cursory glance at developments in miniaturized total analysis systems (uTAS) during the past 5 years reveals that most of the relevant components, such as **continuous-flow mixers** (8), **continuous-flow microreactors** (9), **continuous separations** (10), and high-speed capillary gel electrophoresis (as a conventional batch process), have already been presented (11). Accordingly, the **continuous-flow microamplifier** should allow the creation of highly elaborate analysis and synthesis systems.
>
> Although chemical amplification reactors will obviously not be applicable to all reactions, neither will they be limited to PCR. Devices of this kind open up whole new areas of application in medical diagnostics. For example, the online amplification and monitoring of a specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy.

Ex. 1027 at 1047 [emphasis added].

In 2001, in another article not previously considered by the Office, Lagally reported using a microfluidic reactor to conduct single-molecule DNA amplification by PCR. Lagally summarized the state of the art as of February 2001, explaining continuous flow PCR microreactors had recently undergone substantial evolution:

> The advent of biological microdevices [6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with

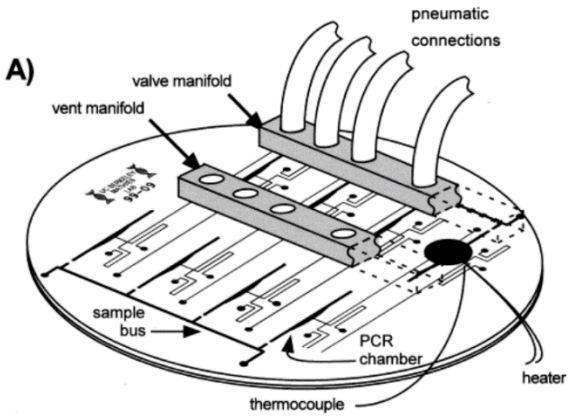
> polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

Lagally *et al.* developed a further improvement – a device and method for achieving single molecule amplification in a microchannel environment.

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ∼1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> Microfluidic PCR-CE device. The PCR chambers are connected to a common sample bus through a set of valves. Hydrophobic

vents at the other end of the PCR chambers are used to locate the sample and to eliminate gas. The PCR chambers are directly connected to the cross channel of the CE system for product injection and analysis. Two aluminum manifolds, one each for the vents and valves, are placed onto the respective ports and clamped in place using vacuum. The manifolds connect to external solenoid valves for pressure and vacuum actuation. Thermal cycling is accomplished using a resistive heater and a miniature thermocouple below the 280-nL chamber.



Ex. 1028 at 566 (see figure legend).

In sum, Kopp and Lagally show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Burns I is relied upon to meet all of the limitations of the independent claims of the ‘148 patent except the PCR-related limitations (e.g., “continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule”) Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Burns I) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I to conduct microfluidic PCR as taught by Kopp and Lagally. A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic reactors

of Burns I because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Burns I to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the droplet reactors of Burns I would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026

(Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
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Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Burns I to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Kopp or Lagally) to

incorporate a droplet reactor (Burns I). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Kopp or Lagally. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the single fluid in microfluidic chip reactor (e.g., Lagally) or droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Kopp). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally et al. provided an overview of that evolution:

> The advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid

> chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or continuous flow through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
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> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.20,21 The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.21 This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that

"the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Burns I could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful modifying Kopp or Lagally to work in the microfluidic reactor of Quake, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact

that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[37] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail in the following sections, the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I to conduct microfluidic PCR as taught by Kopp and Lagally.

Not only would a reasonable Examiner consider these teachings relevant in deciding whether claims 1-5 of the '148 patent are patentable, but would have rejected the claims of the '148 patent in view of these teachings. See MPEP § 2242. The correspondence between the claims of the '148 patent and the combined teachings of the Shaw Stewart, Kopp and Lagally references are set forth in Section VII.K, below.

R. The Combination of Burns I, Kopp and Burns II Presents a Substantial New Question of Patentability for Claims 1-5 of the '148 Patent

As discussed in Sections IV.C and IV.D, above, the claims have been considered nonobvious by the Office because the **prior art was not believed to teach a technique for**

[37] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

conducting PCR reactions in droplets formed from two continuously flowing immiscible fluids.

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Burns I), ii) microfluidic PCR devices were well known (Kopp and Burns II), and iii) there were compelling reasons to use the microfluidic droplet reactors to conduct microfluidic PCR (Kopp, Burns II, Lagally and Vogelstein).

Burns I teaches that plugs in the channels of a microreactor by the continuous flow of fluids at an intersecting channel. Burns I explained that "various methods may be used to generate slugs of liquid within a microreactor." Ex. 1007 at 10. Burns I chose a method in which "the continuous flow of both phases through T or cross-shaped intersections" and "[s]lugs are generated by the action of one phase flowing into the channel whilst the other phase moves into the intersection, eventually cutting off the flow of the first phase into the channel and reversing the process." Ex. 1007 at 11 [emphasis added]. Fig. 3 shows oil-in-water slug generation and Fig. 4 shows water-in-oil slug generation. *Id.* at 11. In Burns' Fig. 4 embodiment, an aqueous plug of reagents is formed in a continuous oil phase. The continuous flow plug formation used by Burns was a well-known alternative to periodically injecting a measured slug of reactant into the main channel. See Ex. 1010; 1027; Ex. 1025 at 23:2-8. Burns I demonstrates that making slugs or plugs with continuous flows of immiscible fluids was a mere design choice as of 2001.

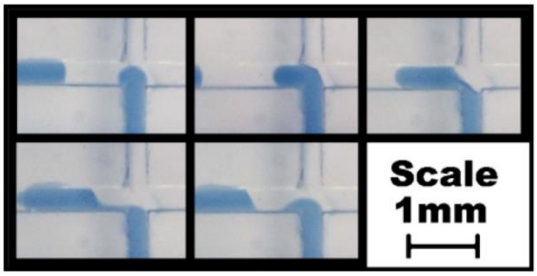


Fig. 3 Typical slug generation in glass device with 380 μm wide channels.

As of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Kopp and Burns II. In 1996, Burns II reported the use of microfluidic devices to perform PCR. Ex. 1008. Burns II explained that "[n]o new biochemistries or DNA detection methods are required for their use; the components simply reproduce current laboratory procedures in a micron-sized environment." *Id.* at 5560. In other words, the PCR chemistry did not need to be modified – it worked without modification on the microfluidic scale. In the method of Burns II, a sample containing a DNA plasmid was loaded into one of the reaction wells (A) and a mixture of Taq enzyme and buffer was loaded into the other. *Id.* at 5558. Each formed droplets of about 60 nl in the parallel 500 x 25 µm channels (B) which came together at the Y junction to form a single larger droplet (about 120 nl). *Id.* at 5558-59; Ex. 1004 ¶40.

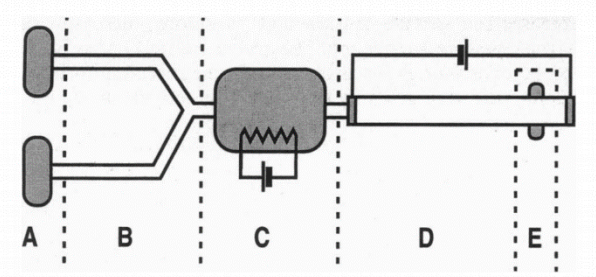


FIG. 1. Example of an integrated DNA analysis system, represented schematically. The individual components of the system are injection entry ports (*A*), liquid pumping channels (*B*), thermally controlled reaction chamber (*C*), electrophoresis channel (*D*), and DNA band migration detector (*E*). Each component would have associated sensors, control circuitry, and external connections.

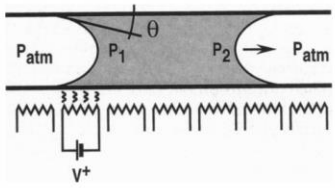
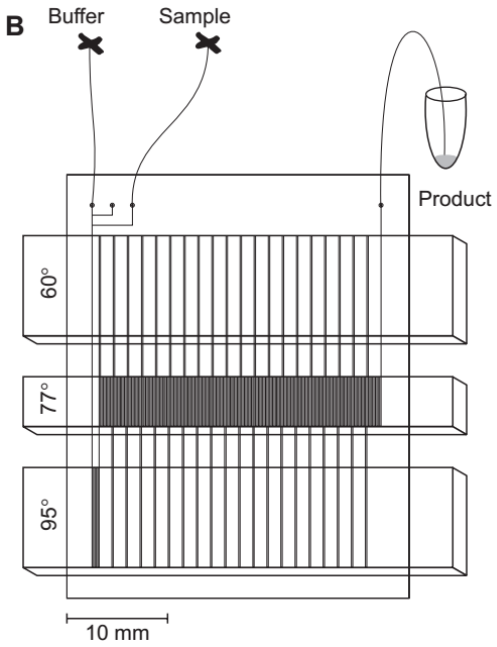


FIG. 2. Schematic drawing showing the principle of thermally induced drop motion in a closed channel. The case of a single aqueous drop in a hydrophilic channel is presented, where V is an applied voltage, P_{atm} is atmospheric pressure, P_1 is the receding-edge internal pressure, P_2 is the advancing-edge internal pressure, and θ is the contact angle of the liquid–gas–solid interface. The contact angle will depend on the surface characteristics of the channel and the constituents of the drop, with a hydrophilic interaction giving θ between 0 and 90 degrees, and a hydrophobic surface giving θ between 90 and 180 degrees. Surface treatments can also reduce contact angle hysteresis and, therefore, reduce the temperature difference necessary for drop motion.

The droplet then passed through the resistive heater portion (C) in which PCR amplification occurred. *Id.* at 5556. Afterwards the amplification results were optically detected in zone D via gel electrophoresis *Id.* at 5559. Burns II noted that "[t]he use of common fabrication methods allows the assembly of increasingly complex, multicomponent, integrated systems from a small, defined set of standardized elements" and that "[f]uture design efforts have a strong impetus to reduce component size and improve efficiency." *Id.* at 5560-61.

By the late 1990s Kopp et al. had successfully implemented continuous flow PCR on a chip. In his 1988 article (not previously presented to the USPTO), Kopp explained that "[a] wide range of microreactors, microcapillary electrophoresis devices, and microcell manipulation devices have been described in recent years." Ex. 1027 at 1046. Building on that body of work, Kopp's group developed

> [a] micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in **continuous flow** at high speed. The device is analogous to an electronic amplifier and relies on the movement of sample through thermostated temperature zones on a glass microchip. Input and output of material (DNA) is **continuous**, and amplification is independent of input concentration. . . .
>
> The results demonstrate that with a very simple device and virtually no optimization, PCR can be performed in **continuous flow**, yielding product quality and quantity comparable to standard thermal cycling methods (Fig. 2B). . . .
>
> We suggest that cross-contamination in a **continuous-flow** format may be much less of a problem than in a stationary-tube PCR format. Thus, a flow-through PCR chip can be reused for extended periods without major cleaning. . . .



Ex. 1027 at 1046-1047 [emphasis added]. Kopp et al. also explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications:

> To build a complex system analogous to an electronic integrated circuit, it is necessary to integrate a variety of other continuously working parts. A cursory glance at developments in miniaturized total analysis systems (uTAS) during the past 5 years reveals that most of the relevant components, such as **continuous-flow mixers** (8), **continuous-flow microreactors** (9), **continuous separations** (10), and high-speed capillary gel electrophoresis (as a conventional batch process), have already been presented (11). Accordingly, the **continuous-flow microamplifier** should allow the creation of highly elaborate analysis and synthesis systems.
>
> Although chemical amplification reactors will obviously not be applicable to all reactions, neither will they be limited to PCR. Devices of this kind open up whole new areas of application in medical diagnostics. For example, the online amplification and monitoring of a specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy.

Ex. 1027 at 1047 [emphasis added].

In sum, Kopp and Burns II show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Burns I is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[38] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Burns I) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I, to conduct microfluidic PCR as taught by Kopp and Burns II. A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic reactor of Burns I because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts

[38] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Burns I to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

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Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

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Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Burns I to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

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Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

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>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ∼1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .

> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an

autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Burns I could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful modifying Kopp or Lagally to work in the microfluidic reactor of Burns I, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[39] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of microdroplet

[39] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail in the following sections, the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I to conduct microfluidic PCR as taught by Kopp and Burns II.

Not only would a reasonable Examiner consider these teachings relevant in deciding whether claims 1-5 of the '148 patent are patentable, but would have rejected the claims of the '148 patent in view of these teachings. See MPEP § 2242. The correspondence between the claims of the '148 patent and the combined teachings of the Burns I, Kopp and Burns II references are set forth in Section VII.L, below.

S. The Combination of Burns I, Kopp and Vogelstein Presents a Substantial New Question of Patentability for Claims 1-5 of the '148 Patent

As discussed in Sections IV.C and IV.D, above, the claims have been considered nonobvious by the Office because the **prior art was not believed to teach a technique for conducting PCR reactions in droplets formed from two continuously flowing immiscible fluids**.

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Burns I), ii) microfluidic PCR devices were well known (Kopp and Vogelstein), and iii) there were compelling reasons to use the microfluidic droplet reactors to conduct microfluidic PCR (Kopp, Burns II, Lagally and Vogelstein).

Burns I teaches that plugs in the channels of a microreactor by the continuous flow of fluids at an intersecting channel. Burns I explained that "various methods may be used to

generate slugs of liquid within a microreactor." Ex. 1007 at 10. Burns I chose a method in which "the continuous flow of both phases through T or cross-shaped intersections" and "[s]lugs are generated by the action of one phase flowing into the channel whilst the other phase moves into the intersection, eventually cutting off the flow of the first phase into the channel and reversing the process." Ex. 1007 at 11 [emphasis added]. Fig. 3 shows oil-in-water slug generation and Fig. 4 shows water-in-oil slug generation. *Id.* at 11. In Burns' Fig. 4 embodiment, an aqueous plug of reagents is formed in a continuous oil phase. The continuous flow plug formation used by Burns was a well-known alternative to periodically injecting a measured slug of reactant into the main channel. See Ex. 1010; 1027; Ex. 1025 at 23:2-8. Burns I demonstrates that making slugs or plugs with continuous flows of immiscible fluids was a mere design choice as of 2001.

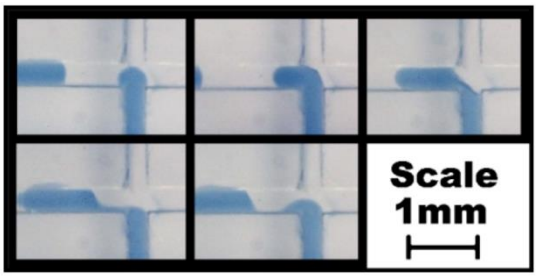
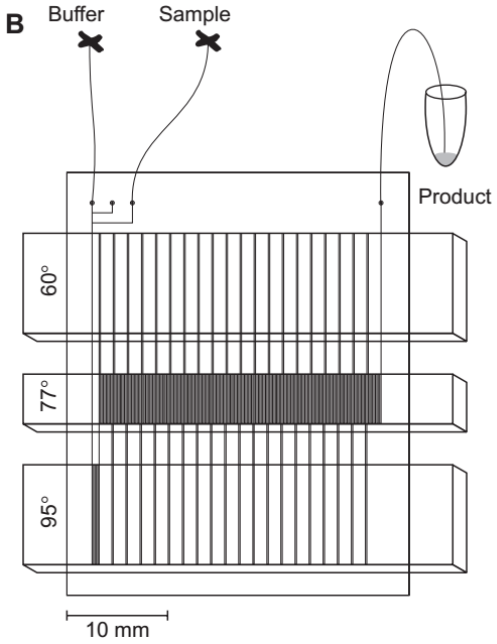


Fig. 3 Typical slug generation in glass device with 380 μm wide channels.

As of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Kopp and Vogelstein. By the late 1990s Kopp *et al.* had successfully implemented continuous flow PCR on a chip. In his 1988 article (not previously presented to the USPTO), Kopp explained that "[a] wide range of microreactors, microcapillary electrophoresis devices, and microcell manipulation devices have been described in recent years." Ex. 1027 at 1046. Building on that body of work, Kopp's group developed

> [a] micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in **continuous flow** at high speed. The device is analogous to an electronic amplifier and relies on the movement of sample through thermostated temperature zones on a glass microchip. Input and output of material (DNA) is **continuous**, and amplification is independent of input concentration. . . .

> The results demonstrate that with a very simple device and virtually no optimization, PCR can be performed in **continuous flow**, yielding product quality and quantity comparable to standard thermal cycling methods (Fig. 2B). . . .
>
> We suggest that cross-contamination in a **continuous-flow** format may be much less of a problem than in a stationary-tube PCR format. Thus, a flow-through PCR chip can be reused for extended periods without major cleaning. . . .



Ex. 1027 at 1046-1047 [emphasis added]. Kopp et al. also explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications:

> To build a complex system analogous to an electronic integrated circuit, it is necessary to integrate a variety of other continuously working parts. A cursory glance at developments in miniaturized total analysis systems (uTAS) during the past 5 years reveals that most of the relevant components, such as **continuous-flow mixers** (8), **continuous-flow microreactors** (9), **continuous separations** (10), and high-speed capillary gel electrophoresis (as a conventional batch process), have already been

> presented (11). Accordingly, the **continuous-flow microamplifier** should allow the creation of highly elaborate analysis and synthesis systems.
>
> Although chemical amplification reactors will obviously not be applicable to all reactions, neither will they be limited to PCR. Devices of this kind open up whole new areas of application in medical diagnostics. For example, the online amplification and monitoring of a specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy.

Ex. 1027 at 1047 [emphasis added].

By 1999, Vogelstein had reported **various applications for single-molecule microfluidic PCR**. Ex. 1044 at 9241. In a seminal article which has been cited over by over 1,000 other publications, Vogelstein explained that PCR amplification of small aliquots or droplets containing single molecules was effective for "detection of a small number of mutant-containing cells among a large excess of normal cells." *Id.* at 9236. In Vogelstein's method, the DNA was diluted into 7 µl volumes such that, on average, there was one template molecule for every two wells (i.e., approximately half of the wells contained no DNA template molecule and approximately half contained a single DNA template molecule). *Id.* at 9236, 9237. This avoided the problem of background noise impairing the determination of whether each volume contained the mutant sequence. *Id.* By counting the number of volumes which indicated wild type or mutant templates, the presence or absence of the mutation (e.g., a sequence associated with colorectal cancer) can be determined. *Id.* at 9238. The accuracy of the test is a function of the number of discrete volumes into which the sample is diluted. *Id.* at 9239. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. *Id.* at 9239. ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the

sensitivity by another order of magnitude.") *Id.* 9239. The article explained that the same technique would be useful for **detecting a wide array of mutations, dislocations and allelic imbalances**, as summarized in Table 1. *Id.* at 9241. Vogelstein thus taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby overcoming the signal-to-noise limitations associated with certain other PCR techniques and enabling the detection of relatively rare mutations, dislocations and allelic imbalances. Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84.

In sum, Kopp and Vogelstein show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Burns I is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[40] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Burns I) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I, to conduct microfluidic PCR as taught by Kopp and Vogelstein. A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic reactor of Burns I because doing so have provided the substantial benefits known to be

[40] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Burns I to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the droplet reactors of Burns I would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026

(Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Burns I to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Kopp or Vogelstein) to

incorporate a droplet reactor (Burns I). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Kopp or Vogelstein. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Kopp). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were

> constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or continuous flow through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of

> single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority

date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Burns I could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful modifying Kopp or Lagally to work in the microfluidic reactor of Burns I, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e).

Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[41] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail in the following sections, the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I to conduct microfluidic PCR as taught by Kopp and Vogelstein.

Not only would a reasonable Examiner consider these teachings relevant in deciding whether claims 1-5 of the '148 patent are patentable, but would have rejected the claims of the '148 patent in view of these teachings. See MPEP § 2242. The correspondence between the claims of the '148 patent and the combined teachings of the Burns I, Kopp and Vogelstein references are set forth in Section VII.L, below.

VII. DETAILED EXPLANATION OF THE PERTINENCE AND MANNER OF APPLYING THE PRIOR ART REFERENCES TO EVERY CLAIM FOR WHICH REEXAMINATION IS REQUESTED

In accordance with 37 C.F.R. § 1.510(b)(2), Requester provides the following detailed explanation of the pertinence and manner of applying the prior art to claims 1-8 of the '148 patent, for which reexamination is requested.

[41] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

A. The Combination of Quake, Corbett and Lagally Renders Obvious Claims 1-8 of the '148 Patent Under (pre-AIA) 35 U.S.C. § 103(a)

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Quake), ii) microfluidic PCR devices were well known (Corbett and Lagally), and iii) there were compelling reasons to use the microfluidic droplet reactors to conduct microfluidic PCR (Lagally, Kopp, Burns II and Vogelstein).

As to the first feature – forming plugs from continuously flowing immiscible fluids – the '148 patent's teachings in this regard were copied almost verbatim from the Quake PCT (Ex. 1034). The table below compares the Quake PCT publication with the Ismagilov specification, which was filed about two months later.

Quake PCT (published March 21, 2002) (Ex. 1034)	**Ismagilov provisional application (filed May 9, 2002) (Ex. 1003) and '148 patent (Ex. 1001)**
34:32-35:7 The pressure at the droplet extrusion region can also be regulated by adjusting the pressure on the main and sample inlets, for example, with pressurized syringes feeding into those inlets. By controlling the pressure difference between the oil and water sources at the droplet extrusion region, the size and periodicity of the droplets generated may be regulated. Alternatively, a valve may be placed at or coincident to either the droplet extrusion region or the sample inlet connected thereto to control the flow of solution into the droplet extrusion region, thereby controlling the size and periodicity of the droplets.	Provisional at 22:12-19, '148 patent at 21:56-65 The pressure at the plug-forming region can also be regulated by adjusting the pressure on the main and sample inlets, for example with pressurized syringes feeding into those inlets. By controlling the difference between the oil and water flow rates at the plug-forming region, the size and periodicity of the plugs generated may be regulated. Alternatively, a valve may be placed at or coincident to either the plug forming region or the sample inlet connected thereto to control the flow of solution into the plug-forming region, thereby controlling the size and periodicity of the plugs.

Attached as Ex. 1035 is a table showing the numerous passages which Ismagilov copied key disclosures verbatim or nearly verbatim from the Quake PCT. These were not merely

background or "state of the art" descriptions; rather, they go to the heart of the claimed subject matter.

The disclosure of Quake was summarized by the Patent Trial and Appeal Board in its November 15, 2015 decision. Therein the Board found that Quake discloses a droplet reactor in which droplets are formed by continuous streams of immiscible fluids:

> Quake relates to microfluidic devices and methods for analyzing and/or sorting biological materials. Ex. 1004, Abstract [which corresponds to Ex. 1033]….
> The devices comprise a main channel, through which a pressurized stream of oil is passed, and at least one sample inlet channel, through which a pressurized stream of aqueous solution is passed. *Id.* A junction joins the main channel with the sample inlet channel. *Id.* By adjusting the pressure of the oil and/or the aqueous solution, a pressure difference can be established such that the stream of aqueous solution is sheared off at a regular frequency as it enters the oil stream, thereby forming droplets. *Id.* Figure 16A of Quake is reproduced below:

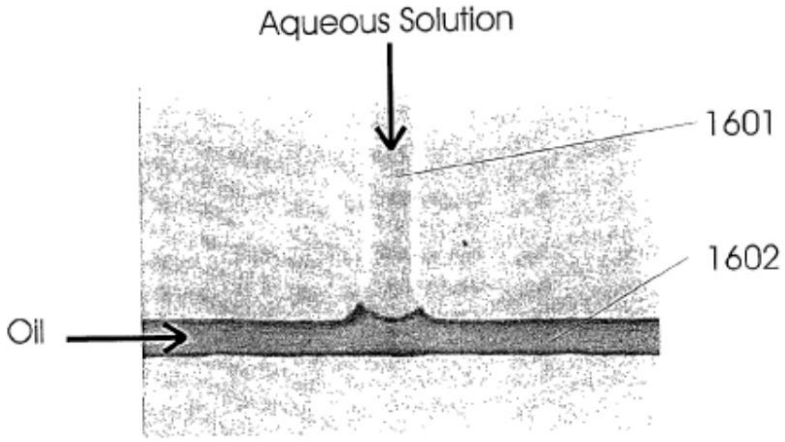


FIG. 16A

> Figure 16A depicts the channel architecture for the droplet extrusion region. Channel 1601 containing an aqueous solution intersects with the main channel 1602 containing oil. [*Id.*] ¶ 292.
> In preferred embodiments, the droplets have a volume of approximately 0.1 to 100 picoliters. *Id.* According to Quake, microfabrication of the device "permits

> other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." *Id.* ¶ 80.

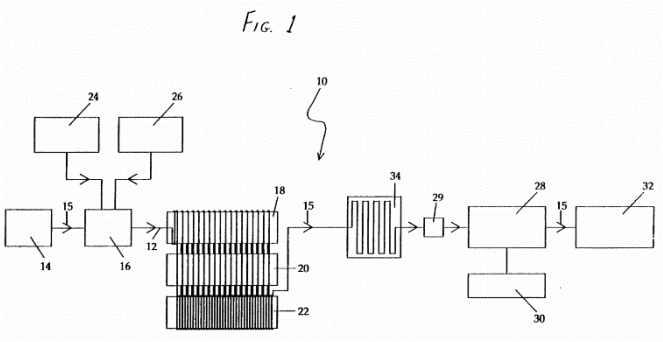
Ex. 1052 at 11-12.

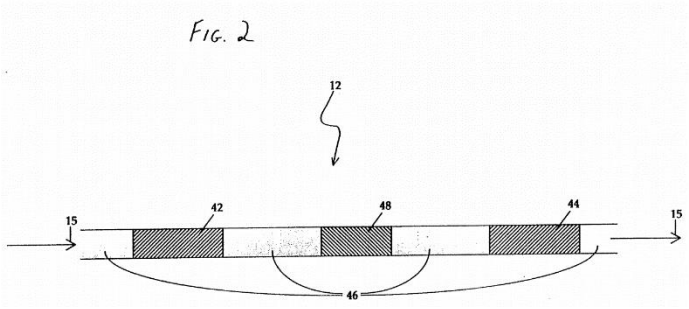
The Patent Owner did not contest in the *inter partes* review proceeding that Quake met the plug-formed-by-continuous-streams limitations of the '148 patent. Ex. 1051 *passim*; Ex. 1052, *passim*. Moreover, Quake himself during prosecution characterized his invention as involving continuous streams.[42] Accordingly, notwithstanding statements to the contrary during *ex parte* prosecution of the '148 patent (discussed in Section IV.C above) it is clear that Quake does in fact teach forming plugs from continuous streams of immiscible fluids.

Turning to the second issue, as of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Corbett and Lagally. In 1991, about eleven years prior to the earliest effective filing date, Corbett disclosed using a microchannel or capillary device to conduct DNA amplification reactions such as PCR by injecting plugs of reactants into a carrier stream that is passed through different temperature zones. Corbett explained that as of 1991 "the most critical problem in applying nucleic acid amplification procedures, particularly in repetitive diagnostic assays in a clinical laboratory, is contamination of the reaction mixture by small amounts of DNA which contain sequences capable of being amplified under the conditions of assay." Ex. 1010 at 3:7-12. "Automation of procedures minimize[s] the opportunity for such contamination and allows the implementation of stringent quality control." *Id.* at 3:15-19. *"*As is shown in FIG. 1, the apparatus 10 comprises tube 12 through which a fluid stream passes. As is more clearly shown in FIG. 2, the fluid stream

[42] When distinguishing his invention over one of the embodiments of Shaw Stewart in which reagent is periodically injected, Quake noted that his "invention relates, in part, to the discovery that combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid (e.g., decane) it is possible to produce small, discrete, monodisperse droplets of the aqueous solution flowing in the second fluid." Ex. 1058 at September 25, 2006 response, p. 15.

includes pockets of reaction mixtures 42 and 44 and purging solution 48 separated by carrier fluid 46." *Id.* at 7:58-62.





"The pump is actuated and the reaction mixture of 20 μl or less is injected into the mineral oil or silicone oil carrier fluid and specific DNA sequences (whose limits are defined by the oligonucleotide primers) present in the sample is amplified as it passes cyclically through the temperature zones." Ex. 1010 at 9:35-40. Corbett thus demonstrates that it was known at least as of 1991 that reduction of contamination risk in PCR could be achieved by performing the PCR on microliter sized plugs of reactant in capillary tube which passes through various temperature zones. Ex. 1004 ¶77.

In 2001, in another article not previously considered by the Office, Lagally reported using a single fluid microfluidic reactor to conduct single-molecule DNA amplification by PCR. Lagally summarized the state of the art as of February 2001, explaining continuous flow PCR microreactors had recently undergone substantial evolution:

The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.

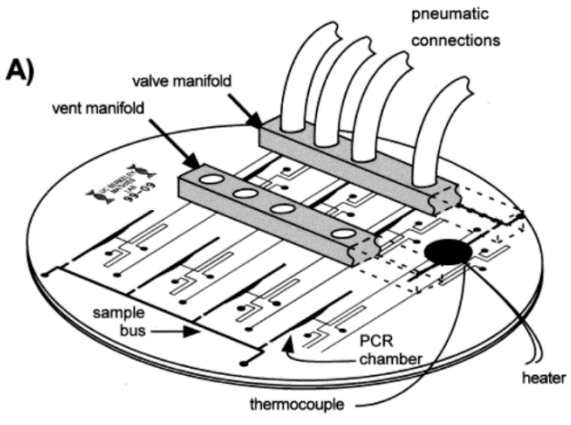
Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with

> sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

Lagally *et al.* developed a further improvement – a device and method for achieving single molecule amplification in a microchannel environment.

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> Microfluidic PCR-CE device. The PCR chambers are connected to a common sample bus through a set of valves. Hydrophobic vents at the other end of the PCR chambers are used to locate the sample and to eliminate gas. The PCR chambers are directly connected to the cross channel of the CE system for product injection and analysis. Two aluminum manifolds, one each for the vents and valves, are placed onto the respective ports and clamped in place using vacuum. The manifolds connect to external solenoid valves for pressure and vacuum



> actuation. Thermal cycling is accomplished using a resistive heater and a miniature thermocouple below the 280-nL chamber.

Ex. 1028 at 566 (see figure legend).

In sum, Corbett and Lagally show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Quake is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[43] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Quake) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake to conduct microfluidic PCR in droplets as taught by Corbett, or to modify the reactions of Lagally to be performed in microfluidic droplets instead of continuous flow of a single fluid in a microfluidic channel. Stated a different way, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to use two continuous streams of immiscible fluid as used in a microfluidic droplet reactor of Quake, as evidenced by Lagally's contemporaneous report that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing.

[43] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

Quake **specifically suggests that his microfluidic device could be used to perform PCR**. Quake explained that "[m]icrofabrication permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." Ex. 1034 at 23:26-30. Quake noted that "PCR is [] generally the method of choice to detect viral DNA or RNA directly in clinical specimens. The advantage of PCR for viral diagnostics is its high sensitivity; PCR can detect very low numbers of viruses in a small clinical specimen." *Id.* at 2:31-3:2.

More generally, a skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic droplet reactor of Quake because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions and the use of two continuous streams of immiscible fluids would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. Likewise, the reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such

portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Quake to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Quake would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004

¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Quake to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Lagally) to incorporate a droplet reactor (Quake). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Lagally. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the single fluid in microfluidic chip reactor (e.g., Lagally) or droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Corbett). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers,

continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with

> polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ∼1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic

> diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent

with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Quake could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful in miniaturizing and modifying Corbett or modifying Lagally to work in the microfluidic droplet reactor of Quake, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[44] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail below, in the accompanying claim chart (Ex. 1062), and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith

[44] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake to conduct microfluidic PCR as taught by Corbett and Lagally. Ex. 1004 ¶¶109-112. Alternatively, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic droplet reactor of Quake, as evidenced by Lagally's contemporaneous report that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing. *Id*.

Independent Claim 1

Claim 1[a]: A method comprising the steps of: providing a microfluidic system comprising one or more channels

Quake PCT discloses "[a] microfluidic device for analyzing and/or sorting biological materials (e.g., molecules such as polynucleotides and polypeptides, including proteins and enzymes; viruses and cells) and methods for its use are provide[d]. . . . Ex. 1034 Abstract. For instance, **in those embodiments where aqueous droplets are used as microreactors for chemical reactions (including biochemical reactions) or are used to analyze and/or sort biomaterials**, a water soluble surfactant such as SDS may denature or inactivate the contents of the droplet." Ex. 1034 at 28:20-23 [emphasis added].

Quake's device comprises a main channel, through which a pressurized stream of oil is passed, and at least one sample inlet channel, through which a pressurized stream of aqueous solution is passed: "The channel architecture for the droplet extrusion region of the first device is shown in FIG. 16A. In this device, the inlet channel 1601 (inner diameter 30 μm) intersects the main channel 1602 (inner diameter 30 μm) at a T-intersection (i.e., an angle perpendicular to the main channel). Other intersections and angles may be used. The walls of the inlet and main channels were not tapered in this device." Ex. 1034 at 79:23-28.

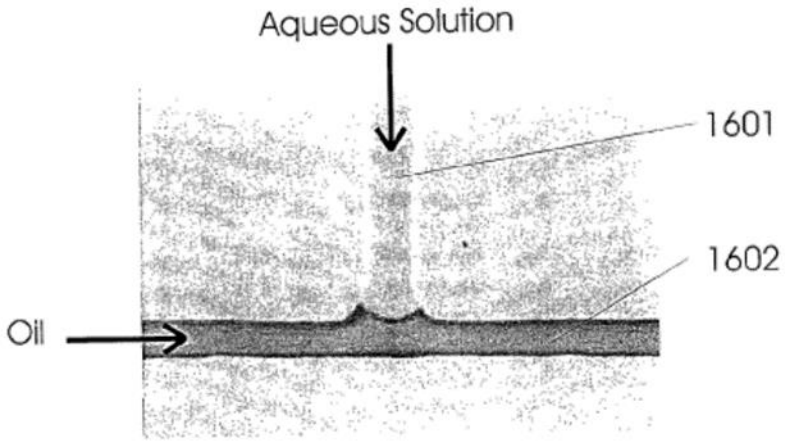


FIG. 16A

During the *inter partes* review proceeding, Patent Owner did not contest that Quake met this limitation. Ex. 1051, *passim*. The PTAB appears to have concluded that this limitation was met by Quake. Ex. 1052, *passim*.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1062.

> **Claim 1[b]: providing within the one or more channels a continuously flowing carrier fluid comprising an oil and a continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule in the fluid that can react with the target DNA or RNA molecules under conditions in which the target DNA or RNA molecules and the other molecules in the fluid do not react with each other**

Quake teaches that "[a] first fluid flows through the main channel, and a second fluid, which is incompatible or immiscible with the second fluid, passes through the inlet region so that droplets of the second fluid are sheared into the main channel. … The second phase or fluid which passes through the inlet region can be an **aqueous solution**, for example ultra pure water, TE buffer, phosphate buffer saline and acetate buffer. The second fluid may also contain a **biological sample** (e.g., molecules of an enzyme or a substrate, or one or more cells, or one or more viral particles) for analysis or sorting in the device. In preferred embodiments the second

fluid includes a biological sample that comprises one or more **molecules**, cells, virions or particles." Ex. 1034 at 6:6-16 [emphasis added]. Quake further teaches that "[i]n preferred embodiments, a first fluid, which may be referred to as an 'extrusion' or 'barrier' fluid, passes (i.e., flows) through the main channel of the device and a second fluid, referred to as a "sample" or "droplet" fluid, passes or flows through the inlet region. ... Thus, droplets of the sample fluid containing the biological material for analysis, reaction or sorting are sheared at the droplet extrusion region into the flow of the extrusion fluid in the main channel." *Id.* at 7:30-8:5.

Regarding Quake's disclosure of the "continuous" limitation, see the discussion set forth at element 1[c], below.

In the instant combination, the microfluidic droplet reactor of Quake is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[45] These limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Quake) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake to conduct microfluidic PCR in droplets as taught by Corbett, or to modify the reactions of Lagally to be performed in microfluidic droplets instead of continuous flow of a single fluid in a microfluidic channel. Ex. 1004 ¶¶109-112. Stated a different way, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to use two continuous streams of immiscible fluid as used in a microfluidic droplet

[45] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

reactor of Quake, as evidenced by Lagally's contemporaneous report that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing. *Id.*

Quake **specifically suggests that his microfluidic device could be used to perform PCR**. Quake explained that "[m]icrofabrication permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." Ex. 1034 at 23:26-30. Quake noted that "PCR is [] generally the method of choice to detect viral DNA or RNA directly in clinical specimens. The advantage of PCR for viral diagnostics is its high sensitivity; PCR can detect very low numbers of viruses in a small clinical specimen." *Id.* at 2:31-3:2.

More generally, a skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic droplet reactor of Quake because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions and the use of two continuous streams of immiscible fluids would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. Likewise, the reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Quake to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Quake would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product

specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 (“It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude.”).

Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Quake to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Lagally) to incorporate a droplet reactor (Quake). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Lagally. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the single fluid in microfluidic chip reactor (e.g., Bu Lagally) or droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Corbett). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was

successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-

> volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the

> development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent

with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Quake could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful in miniaturizing and modifying Corbett or modifying Lagally to work in the microfluidic droplet reactor of Quake, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[46] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained further in the accompanying claim chart (Ex. 1062) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled

[46] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake to conduct microfluidic PCR as taught by Corbett and Lagally. Ex. 1004 ¶¶109-112. Alternatively, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic droplet reactor of Quake, as evidenced by Corbett and Lagally's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing. *Id*.

With regard to the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other," this is merely a description of a PCR reaction. Ex. 1004 ¶31,32. As explained in Corbett, Kopp, Burns II, and Lagally, in a PCR reaction the sample DNA or RNA is admixed with various reagents including polymerase under conditions in which no reaction occurs; only when heat is applied does the PCR reaction occur. (Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68.) In the PCR of the combined method, as in virtually any PCR method, the DNA and polymermase do not react until mixture of reagents is heated. *Id*.; see also Ex. 1004 ¶32. The combined method thus meets the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other" this is merely a description of a PCR reaction. Ex. 1004 ¶31,32.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1062.

Claim 1[c]: controlling flow rates of said aqueous fluid and said carrier fluid to partition the continuously flowing aqueous fluid with the continuously flowing carrier fluid to form a plurality of plugs of the aqueous fluid, each having a substantially uniform size of about 200 µm or less, wherein the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member

of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule

As discussed above, the written descriptive support in the '148 patent for this claim element (which was the supposed point of novelty) was copied almost verbatim from Quake. The table below shows the correspondence between the relevant disclosures in the Quake PCT and the Ismagilov provisional application and '148 patent.

Quake PCT (published March 21, 2002) (Ex. 1034)	**Ismagilov provisional application (filed May 9, 2002) (Ex. 1003) and '148 patent (Ex. 1001)**
34:32-35:7 The pressure at the droplet extrusion region can also be regulated by adjusting the pressure on the main and sample inlets, for example, with pressurized syringes feeding into those inlets. By controlling the pressure difference between the oil and water sources at the droplet extrusion region, the size and periodicity of the droplets generated may be regulated. Alternatively, a valve may be placed at or coincident to either the droplet extrusion region or the sample inlet connected thereto to control the flow of solution into the droplet extrusion region, thereby controlling the size and periodicity of the droplets.	Provisional at 22:12-19, '148 patent at 21:56-65 The pressure at the plug-forming region can also be regulated by adjusting the pressure on the main and sample inlets, for example with pressurized syringes feeding into those inlets. By controlling the difference between the oil and water flow rates at the plug-forming region, the size and periodicity of the plugs generated may be regulated. Alternatively, a valve may be placed at or coincident to either the plug forming region or the sample inlet connected thereto to control the flow of solution into the plug-forming region, thereby controlling the size and periodicity of the plugs.

The disclosure of Quake was summarized by the Patent Trial and Appeal Board in its November 15, 2015 decision. Therein the Board found that Quake discloses a droplet reactor in which droplets are formed by continuous streams of immiscible fluids:

> Quake relates to microfluidic devices and methods for analyzing and/or sorting biological materials. Ex. 1004, Abstract [which corresponds to Ex. 1033]….
> The devices comprise a main channel, through which a pressurized stream of oil is passed, and at least one sample inlet channel, through which a pressurized stream of aqueous solution is passed. *Id.* A junction joins the main channel with

the sample inlet channel. *Id.* By adjusting the pressure of the oil and/or the aqueous solution, a pressure difference can be established such that the stream of aqueous solution is sheared off at a regular frequency as it enters the oil stream, thereby forming droplets. *Id.* Figure 16A of Quake is reproduced below:

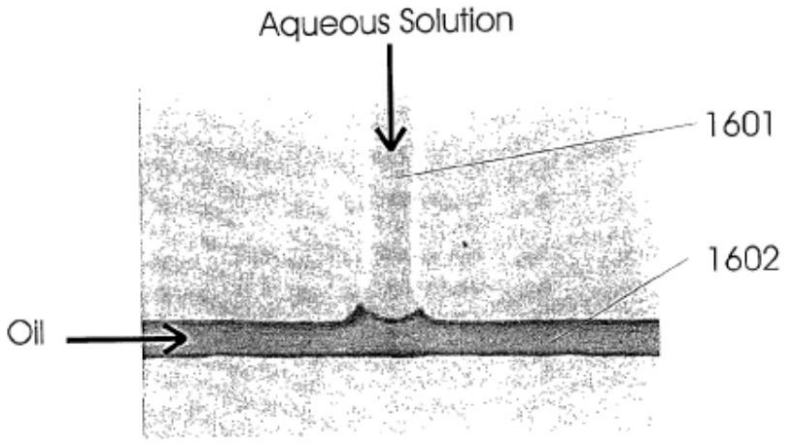


FIG. 16A

Figure 16A depicts the channel architecture for the droplet extrusion region. Channel 1601 containing an aqueous solution intersects with the main channel 1602 containing oil. [*Id.*] ¶ 292.

In preferred embodiments, the droplets have a volume of approximately 0.1 to 100 picoliters. *Id.* According to Quake, microfabrication of the device "permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." *Id.* ¶ 80.

Ex. 1052 at 11-12.

The Patent Owner did not contest in the *inter partes* review proceeding that Quake met the plug-formed-by-continuous-streams limitations of the '148 patent. Ex. 1051 *passim*; Ex. 1052, *passim*. Moreover, Quake himself during prosecution characterized his invention as involving continuous streams.[47] Accordingly, notwithstanding statements to the contrary during

[47] When distinguishing his invention over one of the embodiments of Shaw Stewart in which reagent is periodically injected, Quake noted that his "invention relates, in part, to the discovery that combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid (e.g., decane) it is possible to produce small, discrete, monodisperse droplets of the aqueous solution flowing in the second fluid." Ex. 1058 at September 25, 2006 response, p. 15.

ex parte prosecution of the '148 patent (discussed in Section IV.C above) it is clear that Quake does in fact teach forming plugs from continuous streams of immiscible fluids.

Turning to the recitation "each having a substantially uniform size of about 200 μm or less," Quake teaches that "the droplets have a diameter that is smaller than the diameter of the microchannel; i.e., preferably less than 60 μm." Ex. 1034 at 27:29-30. Fig. 21 of Quake shows the droplets sizes generated by the Quake droplet generator were all less than 60 μm.

This leaves the recitation that "the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule." As discussed above, in the combined method of Quake, Corbett and Lagally, the samples are separated into a number of discrete droplets. That has the advantages of i) overcoming the signal-to-noise limitations associated with certain other PCR techniques and ii) enabling the detection of relatively rare mutations, dislocations and allelic imbalances. Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Lagally expressly teaches that "[t]he event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a **Poisson** distribution, demonstrating that we are operating at the single-molecule level." Ex. 1028 at 566 [emphasis added]. Vogelstein likewise teaches that the aforementioned advantages are realized by **reducing the number of sample molecules per droplet to zero or one.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶82**.** That ensures that any amplification that occurs in a given droplet can be readily identified as containing the target sequence. *Id.* Thus, in the combined method the droplets have either one or zero template molecules. *Id.*

As suggested by Lagally, any process which is generating droplets containing about one template molecule per droplet is expected to conform to a random, or Poisson, distribution. Ex. 1028 at 566; Ex. 1004 ¶48. It was known in the art that "[i]f a solution containing x molecules of enzyme per ml is dispersed into droplets of v ml volume, the average number of enzyme molecules per droplet n is equal to vx. Because of statistical fluctuations each droplet will not have exactly n enzyme molecules. The probability p(r) of finding 0, 1, 2, 3, . .. , r enzyme molecules in a droplet **is given by the Poisson law**." Ex. 1112 at 1985 [emphasis added]; see also Ex. 1111 at 1667 ("Spectra observed in a time sequence for an average of 0.6 dye molecule in the probed volume exhibited the expected Poisson distribution for actually measuring 0, 1, 2, or 3 molecules.").

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1062.

Claim 1[d]: providing conditions suitable for a polymerase-chain reaction in at least one plug of the plurality of plugs such that the target DNA or RNA is amplified.

Quake **specifically suggests that his microfluidic device could be used to perform PCR**. Quake explained that "[m]icrofabrication permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." Ex. 1034 at 23:26-30. Quake noted that "PCR is [] generally the method of choice to detect viral DNA or RNA directly in clinical specimens. The advantage of PCR for viral diagnostics is its high sensitivity; PCR can detect very low numbers of viruses in a small clinical specimen." *Id.* at 2:31-3:2.

As discussed above in connection with element 1[b], in the instant combination Quake is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or

RNA molecules and at least one other molecule").[48] These limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Quake) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1062.

Thus, claim 1 is shown to be rendered obvious by the combination of Quake, Corbett and Lagally.

Dependent Claim 2

Claim 2 of the '148 patent recites "wherein the step of providing conditions includes heating."

As discussed above in connection claim elements 1[b] and 1[c], in the combined method (and in virtually any other PCR reaction) the DNA and polymerase undergo polymerase chain reaction when heated. Ex. 1004 ¶32; Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1062.

Thus, claim 2 is shown to be rendered obvious by the combination of Quake, Corbett and Lagally.

Dependent Claims 3-5

Claim 3 of the '148 patent recites "providing a detector to detect, analyze, characterize, or monitor the target DNA or RNA molecule in the at least one member of the plurality of plugs

[48] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

during and/or after the reaction has occurred." Claim 4 of the '148 patent further recites "wherein the at least one other molecule includes one or more fluorescent labels and the detector is configured to monitor progress of the reaction by detecting fluorescence emissions from the one or more fluorescent labels." Claim 5 recites "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels."

Quake discloses that "[t]he device of the invention may also comprise a **detection region** which is within or coincident with at least a portion of the main channel at or downstream of the droplet extrusion region. The device may also have a detector, preferably an **optical detector** such as a microscope, associated with the detection region." Ex. 1034 at 6:28-31 [emphasis added]. "A preferred 'forward' sorting algorithm consists of running molecules, cells or virions from the input channel to the waste channel, until a molecule, cell or virion is identified to have an optically detectable signal *(e.g.* **fluorescence**) that is above a pre-set threshold, at which point voltages are temporarily changed to electro-osmotically **divert the molecule [] to the collection channel**." *Id.* at 20:22-26 [emphasis added].

Corbett similarly teaches that "[d]ownstream of temperature zone 34 is in-line analysis means 29 and recovery means 28. Connected to recovery means 28 is **detection means** 30. Detection means 30 determines when a reaction mixture in the carrier fluid reaches recovery means 28. This may be done by measuring the conductivity or **optical density** of the stream of carrier fluid." Ex. 1010 at 8:12-18, [emphasis added].

Lagally teaches "[s]tochastic PCR amplification of single DNA template molecules followed by **capillary electrophoresis** (CE) analysis of the products is demonstrated in an integrated microfluidic device." Ex. 1028 at 1, [emphasis added]. The results of capillary electrophoresis were generally detected optically at the time of filing. Ex. 1011 at 46 (Dolnick).

"The amplified product, labeled with an intercalating **fluorescent** dye, is directly injected into the gel-filled capillary channel for electrophoretic analysis." Ex. 1028 at 1, [emphasis added].

As to claim 5, under the broadest reasonable interpretation the phrase "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels" requires simply that at least some of the droplets or plugs are transmitted to an outlet or other channel. Quake teaches an outlet channel as discussed above in connection with claim 1. See also Ex. 1034 at Fig. 16A, outlet 1602. If claim 5 is more narrowly interpreted as requiring selective diversion of the droplets, is also met by Quake. As noted above, Quake teaches that "[a] preferred 'forward' sorting algorithm consists of running molecules, cells or virions from the input channel to the waste channel, until a molecule, cell or virion is identified to have an optically detectable signal (e.g. fluorescence) that is above a pre-set threshold, at which point voltages are temporarily changed to electro-osmotically divert the molecule [] to the collection channel." *Id.* at 20:22-26. A skilled artisan would have considered it obvious to employ such a technique to sort droplets after they are subjected to the optical detection. Ex. 1004 ¶68,69. For instance, a skilled artisan would have found it advantageous to sort and collect droplets which tested positive or negative for the presence of a certain substance or property, especially in the context of droplet generators which are used to encapsulate on average one template molecule per droplet. *Id.* ¶82.

In the combined method of Quake, Corbett and Lagally, microfluidic droplet reactors are used to perform PCR reactions after which the reaction products are detected optically and sorted by diverting certain droplets into collection channels. Ex. 1004 ¶69.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1062.

Thus, claims 3-5 are shown to be rendered obvious by the combination of Quake, Corbett and Lagally.

Dependent Claims 6-8

Claim 6 of the '148 patent recites "wherein the oil is a fluorinated oil." Claim 7 of the '148 patent recites "wherein the carrier fluid further comprises a surfactant." Claim 8 of the '148 patent recites "wherein the surfactant is a fluorinated surfactant."

Quake discloses that "the first phase or fluid which flows through the main channel can be a non-polar solvent, such as decane (e.g., tetradecane or hexadecane) or another oil (for example, **mineral oil**)." Ex. 1034 at 6:8-10, [emphasis added]. "The fluids used in the invention may contain additives, such as agents which reduce surface tensions (surfactants). **Exemplary surfactants** include Tween, Span, **fluorinated oils**, and other agents that are soluble in oil relative to water. Surfactants may aid in controlling or optimizing droplet size, flow and uniformity, for example by reducing the shear force needed to extrude or inject droplets into an intersecting channel." *Id.* at 35:18-22, [emphasis added].

Additional correspondence between these claim elements and the cited references is shown in the claim chart submitted herewith as Exhibit 1062 and discussed in the Declaration of Dr. Shaqfeh (Ex. 1004).

Thus, claims 6-8 are shown to be rendered obvious by the combination of Quake, Corbett, and Lagally.

B. The Combination of Quake, Corbett and Burns II Renders Obvious Claims 1-8 of the '148 Patent Under (pre-AIA) 35 U.S.C. § 103(a)

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Quake), ii) microfluidic PCR devices were well known (Corbett and Burns II), and iii) there were compelling reasons to use

the microfluidic droplet reactors to conduct microfluidic PCR (Lagally, Kopp, Burns II and Vogelstein).

As to the first feature – forming plugs from continuously flowing immiscible fluids – the '148 patent's teachings in this regard were copied almost verbatim from the Quake PCT (Ex. 1034). The table below compares the Quake PCT publication with the Ismagilov specification, which was filed about two months later.

Quake PCT (published March 21, 2002) (Ex. 1034)	**Ismagilov provisional application (filed May 9, 2002) (Ex. 1003) and '148 patent (Ex. 1001)**
34:32-35:7 The pressure at the droplet extrusion region can also be regulated by adjusting the pressure on the main and sample inlets, for example, with pressurized syringes feeding into those inlets. By controlling the pressure difference between the oil and water sources at the droplet extrusion region, the size and periodicity of the droplets generated may be regulated. Alternatively, a valve may be placed at or coincident to either the droplet extrusion region or the sample inlet connected thereto to control the flow of solution into the droplet extrusion region, thereby controlling the size and periodicity of the droplets.	Provisional at 22:12-19, '148 patent at 21:56-65 The pressure at the plug-forming region can also be regulated by adjusting the pressure on the main and sample inlets, for example with pressurized syringes feeding into those inlets. By controlling the difference between the oil and water flow rates at the plug-forming region, the size and periodicity of the plugs generated may be regulated. Alternatively, a valve may be placed at or coincident to either the plug forming region or the sample inlet connected thereto to control the flow of solution into the plug-forming region, thereby controlling the size and periodicity of the plugs.

Attached as Ex. 1035 is a table showing the numerous passages which Ismagilov copied key disclosures verbatim or nearly verbatim from the Quake PCT. These were not merely background or "state of the art" descriptions; rather, they go to the heart of the claimed subject matter.

The disclosure of Quake was summarized by the Patent Trial and Appeal Board in its November 15, 2015 decision. Therein the Board found that Quake discloses a droplet reactor in which droplets are formed by continuous streams of immiscible fluids:

> Quake relates to microfluidic devices and methods for analyzing and/or sorting biological materials. Ex. 1004, Abstract [which corresponds to Ex. 1033]….
> The devices comprise a main channel, through which a pressurized stream of oil is passed, and at least one sample inlet channel, through which a pressurized stream of aqueous solution is passed. *Id.* A junction joins the main channel with the sample inlet channel. *Id.* By adjusting the pressure of the oil and/or the aqueous solution, a pressure difference can be established such that the stream of aqueous solution is sheared off at a regular frequency as it enters the oil stream, thereby forming droplets. *Id.* Figure 16A of Quake is reproduced below:

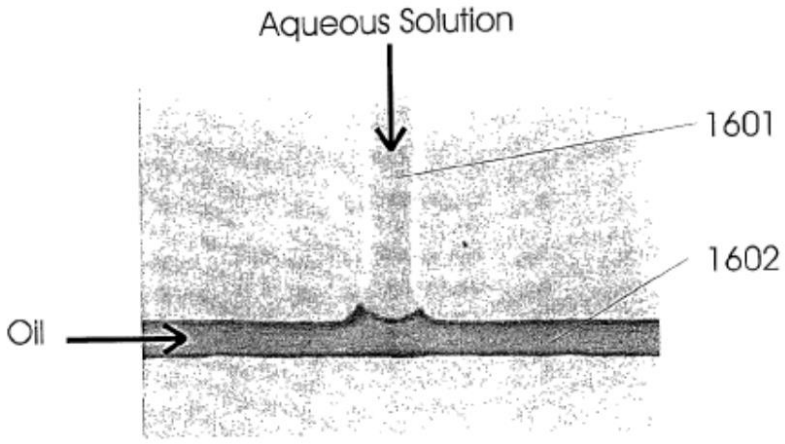


FIG. 16A

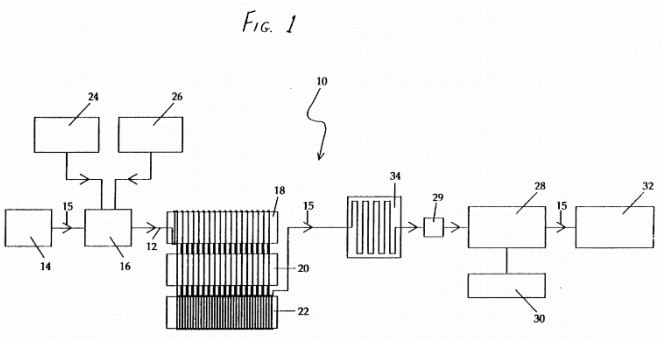
> Figure 16A depicts the channel architecture for the droplet extrusion region. Channel 1601 containing an aqueous solution intersects with the main channel 1602 containing oil. [*Id.*] ¶ 292.
> In preferred embodiments, the droplets have a volume of approximately 0.1 to 100 picoliters. *Id.* According to Quake, microfabrication of the device "permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." *Id.* ¶ 80.

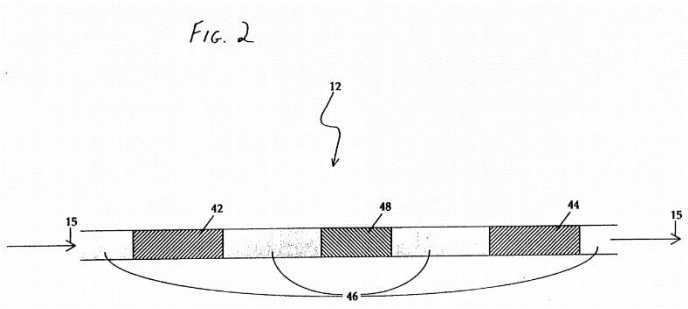
Ex. 1052 at 11-12.

The Patent Owner did not contest in the *inter partes* review proceeding that Quake met the plug-formed-by-continuous-streams limitations of the '148 patent. Ex. 1051 *passim*; Ex. 1052, *passim*. Moreover, Quake himself during prosecution characterized his invention as involving continuous streams.[49] Accordingly, notwithstanding statements to the contrary during *ex parte* prosecution of the '148 patent (discussed in Section IV.C above) it is clear that Quake does in fact teach forming plugs from continuous streams of immiscible fluids.

Turning the second issue, as of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Corbett and Burns II. In 1991, about eleven years prior to the earliest effective filing date, Corbett disclosed using a microchannel or capillary device to conduct DNA amplification reactions such as PCR by injecting plugs of reactants into a carrier stream that is passed through different temperature zones. Corbett explained that as of 1991 "the most critical problem in applying nucleic acid amplification procedures, particularly in repetitive diagnostic assays in a clinical laboratory, is contamination of the reaction mixture by small amounts of DNA which contain sequences capable of being amplified under the conditions of assay." Ex. 1010 at 3:7-12. "Automation of procedures minimize[s] the opportunity for such contamination and allows the implementation of stringent quality control." *Id.* at 3:15-19. *"*As is shown in FIG. 1, the apparatus 10 comprises tube 12 through which a fluid stream passes. As is more clearly shown in FIG. 2, the fluid stream includes pockets of reaction mixtures 42 and 44 and purging solution 48 separated by carrier fluid 46." *Id.* at 7:58-62.

[49] When distinguishing his invention over one of the embodiments of Shaw Stewart in which reagent is periodically injected, Quake noted that his "invention relates, in part, to the discovery that combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid (e.g., decane) it is possible to produce small, discrete, monodisperse droplets of the aqueous solution flowing in the second fluid." Ex. 1058 at September 25, 2006 response, p. 15.





"The pump is actuated and the reaction mixture of 20 μl or less is injected into the mineral oil or silicone oil carrier fluid and specific DNA sequences (whose limits are defined by the oligonucleotide primers) present in the sample is amplified as it passes cyclically through the temperature zones." Ex. 1010 at 9:35-40. Corbett thus demonstrates that it was known at least as of 1991 that reduction of contamination risk in PCR could be achieved by performing the PCR on microliter sized plugs of reactant in capillary tube which passes through various temperature zones. Ex. 1004 ¶77.

In 1996, Burns II reported the use of microfluidic devices to perform PCR. Ex. 1008. Burns II explained that "[n]o new biochemistries or DNA detection methods are required for their use; the components simply reproduce current laboratory procedures in a micron-sized environment." *Id.* at 5560. In other words, the PCR chemistry did not need to be modified – it worked without modification on the microfluidic scale. In the method of Burns II, a sample containing a DNA plasmid was loaded into one of the reaction wells (A) and a mixture of Taq enzyme and buffer was loaded into the other. *Id.* at 5558. Each formed droplets of about 60 nl in

the parallel 500 x 25 µm channels (B) which came together at the Y junction to form a single larger droplet (about 120 nl). *Id.* at 5558-59; Ex. 1004 ¶40.

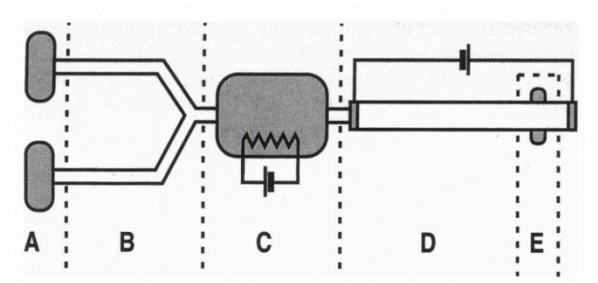


FIG. 1. Example of an integrated DNA analysis system, represented schematically. The individual components of the system are injection entry ports (*A*), liquid pumping channels (*B*), thermally controlled reaction chamber (*C*), electrophoresis channel (*D*), and DNA band migration detector (*E*). Each component would have associated sensors, control circuitry, and external connections.

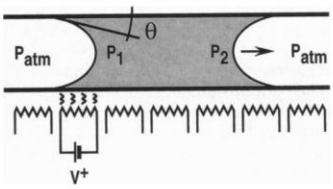


FIG. 2. Schematic drawing showing the principle of thermally induced drop motion in a closed channel. The case of a single aqueous drop in a hydrophilic channel is presented, where V is an applied voltage, P_{atm} is atmospheric pressure, P_1 is the receding-edge internal pressure, P_2 is the advancing-edge internal pressure, and θ is the contact angle of the liquid–gas–solid interface. The contact angle will depend on the surface characteristics of the channel and the constituents of the drop, with a hydrophilic interaction giving θ between 0 and 90 degrees, and a hydrophobic surface giving θ between 90 and 180 degrees. Surface treatments can also reduce contact angle hysteresis and, therefore, reduce the temperature difference necessary for drop motion.

The droplet then passed through the resistive heater portion (C) in which PCR amplification occurred. *Id.* at 5556. Afterwards the amplification results were optically detected in zone D via gel electrophoresis *Id.* at 5559. Burns II noted that "[t]he use of common fabrication methods allows the assembly of increasingly complex, multicomponent, integrated systems from a small, defined set of standardized elements" and that "[f]uture design efforts have a strong impetus to reduce component size and improve efficiency." *Id.* at 5560-61.

In sum, Corbett and Burns II show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Quake is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[50] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing

[50] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

immiscible fluids (as taught in Quake) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake to conduct microfluidic PCR as taught by Corbett and Burns II. Stated a different way, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic droplet reactor of Quake, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing.

Quake **specifically suggests that his microfluidic device could be used to perform PCR**. Quake explained that "[m]icrofabrication permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." Ex. 1034 at 23:26-30. Quake noted that "PCR is [] generally the method of choice to detect viral DNA or RNA directly in clinical specimens. The advantage of PCR for viral diagnostics is its high sensitivity; PCR can detect very low numbers of viruses in a small clinical specimen." *Id.* at 2:31-3:2.

More generally, a skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic droplet reactor of Quake because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed,**

portability, and cost." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Quake to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Quake would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as

the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241;

Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Quake to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Burns II) to incorporate a droplet reactor (Quake). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Burns II,. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the single fluid in microfluidic chip reactor (e.g., Burns II) or droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Corbett). Droplet reactors also **decrease contamination and increase the**

number of reactors that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling

> profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced

> microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic**

droplet (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Quake could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful in miniaturizing and modifying Corbett or modifying Burns II to work in the microfluidic droplet reactor of Quake, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[51] As discussed in Section VI.A.3, above, Higuchi I-III

[51] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail in the following sections, the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake to conduct microfluidic PCR as taught by Corbett and Burns II. Ex. 1004 ¶¶109-112. Alternatively, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic droplet reactor of Quake, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing. *Id*.

As explained in more detail below, in the accompanying claim chart (Ex. 1062) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake to conduct microfluidic PCR as taught by Corbett and Burns II. Ex. 1004 ¶¶109-112. Alternatively, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic droplet reactor of Quake, as evidenced by Burns II's contemporaneous report that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing. *Id*.

Independent Claim 1

Claim 1[a]: A method comprising the steps of: providing a microfluidic system comprising one or more channels

Quake discloses "[a] microfluidic device for analyzing and/or sorting biological materials (e.g., molecules such as polynucleotides and polypeptides, including proteins and enzymes; viruses and cells) and methods for its use are provide[d]. . . . Ex. 1034 Abstract. For instance, **in those embodiments where aqueous droplets are used as microreactors for chemical reactions (including biochemical reactions) or are used to analyze and/or sort biomaterials**, a water soluble surfactant such as SDS may denature or inactivate the contents of the droplet." Ex. 1034 at 28:20-23 [emphasis added].

Quake's device comprises a main channel, through which a pressurized stream of oil is passed, and at least one sample inlet channel, through which a pressurized stream of aqueous solution is passed: "The channel architecture for the droplet extrusion region of the first device is shown in FIG. 16A. In this device, the inlet channel 1601 (inner diameter 30 μm) intersects the main channel 1602 (inner diameter 30 μm) at a T-intersection (i.e., an angle perpendicular to the main channel). Other intersections and angles may be used. The walls of the inlet and main channels were not tapered in this device." Ex. 1034 at 79:23-28.

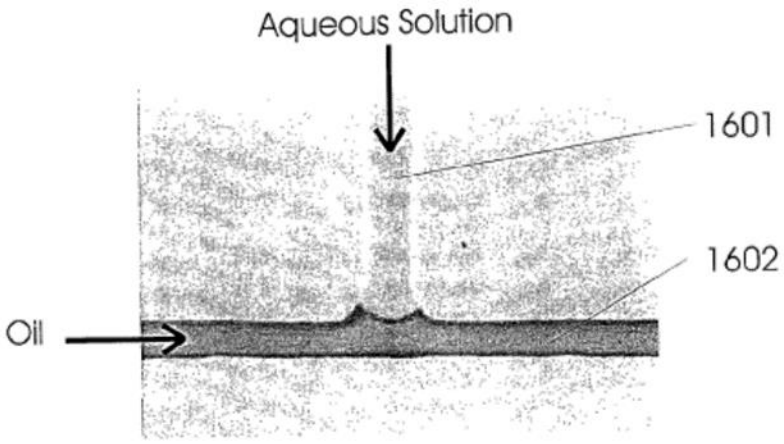


FIG. 16A

During the *inter partes* review proceeding, Patent Owner did not contest that Quake met this limitation. Ex. 1051, *passim*. The PTAB appears to have concluded that this limitation was met by Quake. Ex. 1052, *passim*.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1063.

Claim 1[b]: continuously flowing an aqueous fluid containing at least one biological molecule and at least one reagent for conducting the reaction between the biological molecule and the at least one reagent through a first channel of the at least two channels;

Quake teaches that "[a] first fluid flows through the main channel, and a second fluid, which is incompatible or immiscible with the second fluid, passes through the inlet region so that droplets of the second fluid are sheared into the main channel. … The second phase or fluid which passes through the inlet region can be an **aqueous solution**, for example ultra pure water, TE buffer, phosphate buffer saline and acetate buffer. The second fluid may also contain a **biological sample** (e.g., molecules of an enzyme or a substrate, or one or more cells, or one or more viral particles) for analysis or sorting in the device. In preferred embodiments the second fluid includes a biological sample that comprises one or more **molecules**, cells, virions or particles." Ex. 1034 at 6:6-16, [emphasis added]. Quake further teaches that "[i]n preferred embodiments, a first fluid, which may be referred to as an 'extrusion' or 'barrier' fluid, passes (i.e., flows) through the main channel of the device and a second fluid, referred to as a "sample" or "droplet" fluid, passes or flows through the inlet region. ... Thus, droplets of the sample fluid containing the biological material for analysis, reaction or sorting are sheared at the droplet extrusion region into the flow of the extrusion fluid in the main channel." *Id.* at 7:30-8:5.

Regarding Quake's disclosure of the "continuous" limitation, see the discussion set forth at element 1[c], below.

In the instant combination, the microfluidic droplet reactor of Quake is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[52] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Quake) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake to conduct microfluidic PCR as taught by Corbett and Burns II. Ex. 1004 ¶¶109-112. Stated a different way, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic droplet reactor of Quake, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing. *Id.*

Quake **specifically suggests that his microfluidic device could be used to perform PCR**. Quake explained that "[m]icrofabrication permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." Ex. 1034 at 23:26-30. Quake noted that "PCR is [] generally the method of choice to detect viral DNA or RNA directly in clinical specimens. The advantage of PCR for viral diagnostics is its high sensitivity; PCR can detect very low numbers of viruses in a small clinical specimen." *Id.* at 2:31-3:2.

[52] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

More generally, a skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic droplet reactor of Quake because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565, [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions and the use of two continuous streams of immiscible fluids would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. Likewise, the reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the

droplet reactor of Quake to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Quake would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample

leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Quake to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Burns II) to incorporate a droplet reactor (Quake). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Burns II. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the single fluid in microfluidic chip reactor (e.g., Burns II) or droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Corbett). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA

fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.

Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In

1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Quake could be successfully modified to perform PCR reactions. Ex. 1004 ¶109-112.

As further evidence that a skilled artisan would have expected to be successful in miniaturizing and modifying Corbett or modifying Burns II to work in the microfluidic droplet reactor of Quake, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that

falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[53] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained further in the accompanying claim chart (Ex. 1063) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake to conduct microfluidic PCR as taught by Burns II. Ex. 1004 ¶¶109-112. Alternatively, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic droplet reactor of Quake, as evidenced by contemporaneous reports by Corbett, Kopp, Burns II and Lagally that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing. *Id*.

[53] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

With regard to the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other," this is merely a description of a PCR reaction. Ex. 1004 ¶31,32. As explained in Corbett, Kopp, Burns II, and Lagally, in a PCR reaction the sample DNA or RNA is admixed with various reagents including polymerase under conditions in which no reaction occurs; only when heat is applied does the PCR reaction occur. Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68. In the PCR of the combined method, as in virtually any PCR method, the DNA and polymermase do not react until mixture of reagents is heated. *Id*.; see also Ex. 1004 ¶32. The combined method thus meets the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other" this is merely a description of a PCR reaction. Ex. 1004 ¶31,32.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1063.

Claim 1[c]: controlling flow rates of said aqueous fluid and said carrier fluid to partition the continuously flowing aqueous fluid with the continuously flowing carrier fluid to form a plurality of plugs of the aqueous fluid, each having a substantially uniform size of about 200 µm or less, wherein the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule

As discussed above, the written descriptive support in the '148 patent for this claim element (which was the supposed point of novelty) was copied almost verbatim from Quake. The table below shows the correspondence between the relevant disclosures in the Quake PCT and the Ismagilov provisional application and '148 patent.

Quake PCT (published March 21, 2002) (Ex. 1034)	**Ismagilov provisional application (filed May 9, 2002) (Ex. 1003) and '148 patent (Ex. 1001)**
34:32-35:7 The pressure at the droplet extrusion region can also be regulated by adjusting the pressure on the main and sample inlets, for example, with pressurized syringes feeding into those inlets. By controlling the pressure difference between the oil and water sources at the droplet extrusion region, the size and periodicity of the droplets generated may be regulated. Alternatively, a valve may be placed at or coincident to either the droplet extrusion region or the sample inlet connected thereto to control the flow of solution into the droplet extrusion region, thereby controlling the size and periodicity of the droplets.	Provisional at 22:12-19, '148 patent at 21:56-65 The pressure at the plug-forming region can also be regulated by adjusting the pressure on the main and sample inlets, for example with pressurized syringes feeding into those inlets. By controlling the difference between the oil and water flow rates at the plug-forming region, the size and periodicity of the plugs generated may be regulated. Alternatively, a valve may be placed at or coincident to either the plug forming region or the sample inlet connected thereto to control the flow of solution into the plug-forming region, thereby controlling the size and periodicity of the plugs.

The disclosure of Quake was summarized by the Patent Trial and Appeal Board in its November 15, 2015 decision. Therein the Board found that Quake discloses a droplet reactor in which droplets are formed by continuous streams of immiscible fluids:

> Quake relates to microfluidic devices and methods for analyzing and/or sorting biological materials. Ex. 1004, Abstract [which corresponds to Ex. 1033]….
> The devices comprise a main channel, through which a pressurized stream of oil is passed, and at least one sample inlet channel, through which a pressurized stream of aqueous solution is passed. *Id.* A junction joins the main channel with the sample inlet channel. *Id.* By adjusting the pressure of the oil and/or the aqueous solution, a pressure difference can be established such that the stream of aqueous solution is sheared off at a regular frequency as it enters the oil stream, thereby forming droplets. *Id.* Figure 16A of Quake is reproduced below:

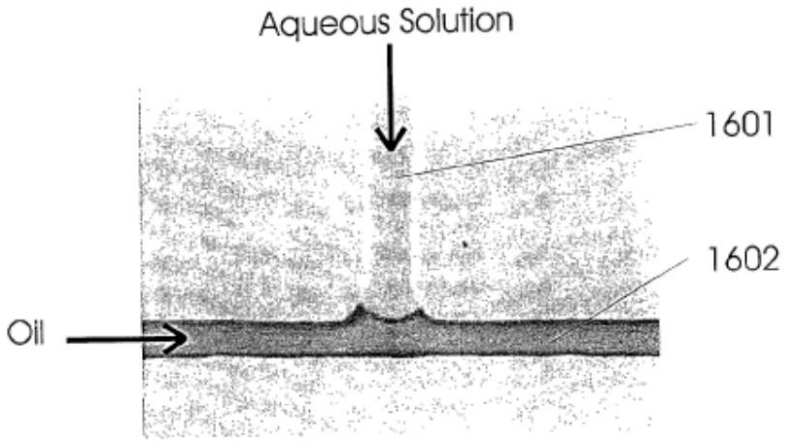


FIG. 16A

> Figure 16A depicts the channel architecture for the droplet extrusion region. Channel 1601 containing an aqueous solution intersects with the main channel 1602 containing oil. [*Id.*] ¶ 292.
>
> In preferred embodiments, the droplets have a volume of approximately 0.1 to 100 picoliters. *Id.* According to Quake, microfabrication of the device "permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." *Id.* ¶ 80.

Ex. 1052 at 11-12.

The Patent Owner did not contest in the *inter partes* review proceeding that Quake met the plug-formed-by-continuous-streams limitations of the '148 patent. Ex. 1051 *passim*; Ex. 1052, *passim*. Moreover, Quake himself during prosecution characterized his invention as involving continuous streams.[54] Accordingly, notwithstanding statements to the contrary during *ex parte* prosecution of the '148 patent (discussed in Section IV.C above) it is clear that Quake does in fact teach forming plugs from continuous streams of immiscible fluids.

[54] When distinguishing his invention over one of the embodiments of Shaw Stewart in which reagent is periodically injected, Quake noted that his "invention relates, in part, to the discovery that combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid (e.g., decane) it is possible to produce small, discrete, monodisperse droplets of the aqueous solution flowing in the second fluid." Ex. 1058 at September 25, 2006 response, p. 15.

Turning to the recitation "each having a substantially uniform size of about 200 μm or less," Quake teaches that "the droplets have a diameter that is smaller than the diameter of the microchannel; i.e., preferably less than 60 μm." Ex. 1034 at 27:29-30. Fig. 21 of Quake shows the droplets sizes generated by the Quake droplet generator were all less than 60 μm.

This leaves the recitation that "the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule." As discussed above, in the combined method of Quake, Corbett and Lagally, the samples are separated into a number of discrete droplets. That has the advantages of i) overcoming the signal-to-noise limitations associated with certain other PCR techniques and ii) enabling the detection of relatively rare mutations, dislocations and allelic imbalances. Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Lagally expressly teaches that "[t]he event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a **Poisson** distribution, demonstrating that we are operating at the single-molecule level." Ex. 1028 at 566 [emphasis added]. Vogelstein likewise teaches that the aforementioned advantages are realized by **reducing the number of sample molecules per droplet to zero or one.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶82**.** That ensures that any amplification that occurs in a given droplet can be readily identified as containing the target sequence. *Id.* Thus, in the combined method the droplets have either one or zero template molecules.

As suggested by Lagally, any process which is generating droplets containing about one template molecule per droplet is expected to conform to a random, or Poisson, distribution. Ex. 1028 at 566; Ex. 1004 ¶48. It was known in the art that "[i]f a solution containing x molecules of

enzyme per ml is dispersed into droplets of v ml volume, the average number of enzyme molecules per droplet n is equal to vx. Because of statistical fluctuations each droplet will not have exactly n enzyme molecules. The probability p(r) of finding 0, 1, 2, 3, . .. , r enzyme molecules in a droplet **is given by the Poisson law**." Ex. 1112 at 1985, [emphasis added]; see also Ex. 1111 at 1667 ("Spectra observed in a time sequence for an average of 0.6 dye molecule in the probed volume exhibited the expected Poisson distribution for actually measuring 0, 1, 2, or 3 molecules.").

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1063.

Claim 1[d]: providing conditions suitable for a polymerase-chain reaction in at least one plug of the plurality of plugs such that the target DNA or RNA is amplified

Quake teaches that "droplets of the sample fluid containing the biological material for analysis, **reaction** or sorting are sheared at the droplet extrusion region into the flow of the extrusion fluid in the main channel." Ex. 1034 at 7:30-8:5 [emphasis added]. "Microfabrication permits other technologies to be integrated or combined with flow cytometry on a single chip, such as **PCR** [i.e., a reaction]." Ex. 1034 at 23:26-30 [emphasis added].

As discussed above in connection with element 1[b], in the instant combination Quake is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[55] These limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in

[55] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

Quake) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1063.

Thus, claim 1 is shown to be rendered obvious by the combination of Quake, Corbett and Burns II.

Dependent Claim 2

Claim 2 of the '148 patent recites "wherein the step of providing conditions includes heating."

As discussed above in connection claim elements 1[b] and 1[c], in the combined method (and in virtually any other PCR reaction) the DNA and polymerase undergo polymerase chain reaction when heated. Ex. 1004 ¶32; Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1063.

Thus, claim 2 is shown to be rendered obvious by the combination of Quake, Corbett and Burns II.

Dependent Claims 3-5

Claim 3 of the '148 patent recites "providing a detector to detect, analyze, characterize, or monitor the target DNA or RNA molecule in the at least one member of the plurality of plugs during and/or after the reaction has occurred." Claim 4 of the '148 patent further recites "wherein the at least one other molecule includes one or more fluorescent labels and the detector is configured to monitor progress of the reaction by detecting fluorescence emissions from the

one or more fluorescent labels." Claim 5 recites "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels."

Quake discloses that "[t]he device of the invention may also comprise a **detection region** which is within or coincident with at least a portion of the main channel at or downstream of the droplet extrusion region. The device may also have a detector, preferably an **optical detector** such as a microscope, associated with the detection region." Ex. 1034 at 6:28-31, [emphasis added]. "A preferred 'forward' sorting algorithm consists of running molecules, cells or virions from the input channel to the waste channel, until a molecule, cell or virion is identified to have an optically detectable signal *(e.g.* **fluorescence**) that is above a pre-set threshold, at which point voltages are temporarily changed to electro-osmotically **divert the molecule [] to the collection channel**." *Id.* at 20:22-26, [emphasis added].

Corbett similarly teaches that "[d]ownstream of temperature zone 34 is in-line analysis means 29 and recovery means 28. Connected to recovery means 28 is **detection means** 30. Detection means 30 determines when a reaction mixture in the carrier fluid reaches recovery means 28. This may be done by measuring the conductivity or **optical density** of the stream of carrier fluid." Ex. 1010 at 8:12-18 [emphasis added].

Burns II teaches that "[c]apillary gel electrophoresis of DNA samples was performed using a Beckman P/ACE instrument with a laser-induced fluorescence detector and 37 cm length, 100 μm diameter, linear polymerase gel capillary according to manufacturer's recommendation." Ex. 1008 at 5558. The results of capillary electrophoresis were generally detected optically at the time of filing. Ex. 1011 at 46 (Dolnick). "The amplified product, labeled with an intercalating **fluorescent** dye, is directly injected into the gel-filled capillary channel for electrophoretic analysis." Ex. 1028 at 1 [emphasis added].

As to claim 5, under the broadest reasonable interpretation the phrase "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels" requires simply that at least some of the droplets or plugs are transmitted to an outlet or other channel. Quake teaches an outlet channel as discussed above in connection with claim 1. See also Ex. 1034 at Fig. 16A, outlet 1602. If claim 5 is more narrowly interpreted as requiring selective diversion of the droplets, is also met by Quake. As noted above, Quake teaches that "[a] preferred 'forward' sorting algorithm consists of running molecules, cells or virions from the input channel to the waste channel, until a molecule, cell or virion is identified to have an optically detectable signal (e.g. fluorescence) that is above a pre-set threshold, at which point voltages are temporarily changed to electro-osmotically divert the molecule [] to the collection channel." *Id.* at 20:22-26. A skilled artisan would have considered it obvious to employ such a technique to sort droplets after they are subjected to the optical detection. Ex. 1004 ¶68,69. For instance, a skilled artisan would have found it advantageous to sort and collect droplets which tested positive or negative for the presence of a certain substance or property, especially in the context of droplet generators which are used to encapsulate on average one template molecule per droplet. *Id.* ¶82.

In the combined method of Quake, Corbett and Lagally, microfluidic droplet reactors are used to perform PCR reactions after which the reaction products are detected optically and sorted by diverting certain droplets into collection channels. Ex. 1004 ¶69.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1063.

Thus, claims 3-5 are shown to be rendered obvious by the combination of Quake, Corbett and Burns II.

Dependent Claims 6-8

Claim 6 of the '148 patent recites "wherein the oil is a fluorinated oil." Claim 7 of the '148 patent recites "wherein the carrier fluid further comprises a surfactant." Claim 8 of the '148 patent recites "wherein the surfactant is a fluorinated surfactant."

Quake discloses that "the first phase or fluid which flows through the main channel can be a non-polar solvent, such as decane (e.g., tetradecane or hexadecane) or another oil (for example, **mineral oil**)." Ex. 1034 at 6:8-10, [emphasis added]. "The fluids used in the invention may contain additives, such as agents which reduce surface tensions (surfactants). **Exemplary surfactants** include Tween, Span, **fluorinated oils**, and other agents that are soluble in oil relative to water. Surfactants may aid in controlling or optimizing droplet size, flow and uniformity, for example by reducing the shear force needed to extrude or inject droplets into an intersecting channel." *Id.* at 35:18-22, [emphasis added].

Additional correspondence between these claim elements and the cited references is shown in the claim chart submitted herewith as Exhibit 1063 and discussed in the Declaration of Dr. Shaqfeh (Ex. 1004).

Thus, claims 6-8 are shown to be rendered obvious by the combination of Quake, Corbett and Burns II.

C. The Combination of Quake, Corbett and Vogelstein Renders Obvious Claims 1-8 of the '148 Patent Under (pre-AIA) 35 U.S.C. § 103(a)

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Quake), ii) microfluidic PCR devices were well known (Corbett and Vogelstein), and iii) there were compelling reasons to use the microfluidic droplet reactors to conduct microfluidic PCR (Lagally, Kopp, Burns II and Vogelstein).

As to the first feature – forming plugs from continuously flowing immiscible fluids – the '148 patent's teachings in this regard were copied almost verbatim from the Quake PCT (Ex. 1034). The table below compares the Quake PCT publication with the Ismagilov specification, which was filed about two months later.

Quake PCT (published March 21, 2002) (Ex. 1034)	**Ismagilov provisional application (filed May 9, 2002) (Ex. 1003) and '148 patent (Ex. 1001)**
34:32-35:7 The pressure at the droplet extrusion region can also be regulated by adjusting the pressure on the main and sample inlets, for example, with pressurized syringes feeding into those inlets. By controlling the pressure difference between the oil and water sources at the droplet extrusion region, the size and periodicity of the droplets generated may be regulated. Alternatively, a valve may be placed at or coincident to either the droplet extrusion region or the sample inlet connected thereto to control the flow of solution into the droplet extrusion region, thereby controlling the size and periodicity of the droplets.	Provisional at 22:12-19, '148 patent at 21:56-65 The pressure at the plug-forming region can also be regulated by adjusting the pressure on the main and sample inlets, for example with pressurized syringes feeding into those inlets. By controlling the difference between the oil and water flow rates at the plug-forming region, the size and periodicity of the plugs generated may be regulated. Alternatively, a valve may be placed at or coincident to either the plug forming region or the sample inlet connected thereto to control the flow of solution into the plug-forming region, thereby controlling the size and periodicity of the plugs.

Attached as Ex. 1035 is a table showing the numerous passages which Ismagilov copied key disclosures verbatim or nearly verbatim from the Quake PCT. These were not merely background or "state of the art" descriptions; rather, they go to the heart of the claimed subject matter.

The disclosure of Quake was summarized by the Patent Trial and Appeal Board in its November 15, 2015 decision. Therein the Board found that Quake discloses a droplet reactor in which droplets are formed by continuous streams of immiscible fluids:

> Quake relates to microfluidic devices and methods for analyzing and/or sorting biological materials. Ex. 1004, Abstract [which corresponds to Ex. 1033]….

> The devices comprise a main channel, through which a pressurized stream of oil is passed, and at least one sample inlet channel, through which a pressurized stream of aqueous solution is passed. *Id.* A junction joins the main channel with the sample inlet channel. *Id.* By adjusting the pressure of the oil and/or the aqueous solution, a pressure difference can be established such that the stream of aqueous solution is sheared off at a regular frequency as it enters the oil stream, thereby forming droplets. *Id.* Figure 16A of Quake is reproduced below:

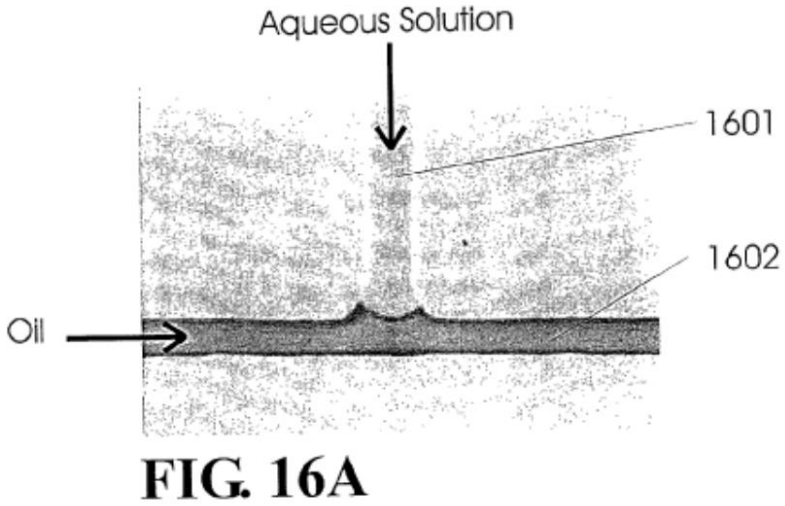


FIG. 16A

> Figure 16A depicts the channel architecture for the droplet extrusion region. Channel 1601 containing an aqueous solution intersects with the main channel 1602 containing oil. [*Id.*] ¶ 292.
>
> In preferred embodiments, the droplets have a volume of approximately 0.1 to 100 picoliters. *Id.* According to Quake, microfabrication of the device "permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." *Id.* ¶ 80.

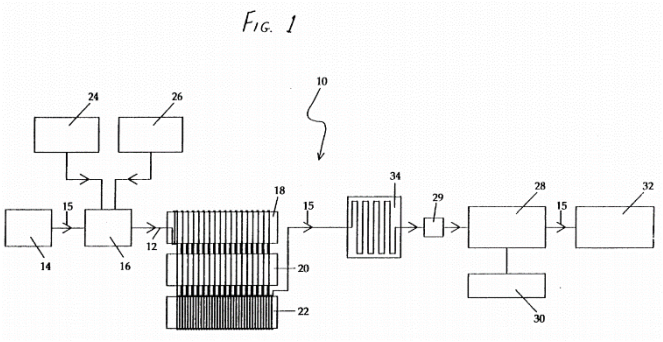
Ex. 1052 at 11-12.

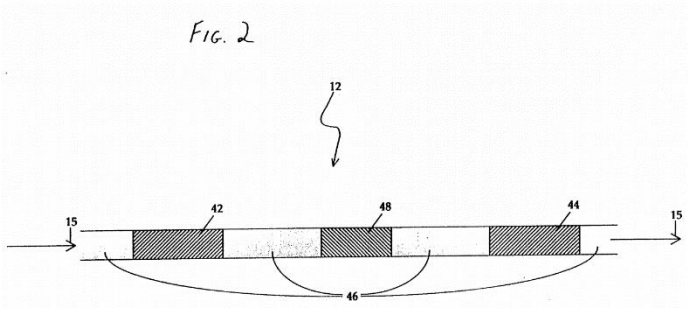
The Patent Owner did not contest in the *inter partes* review proceeding that Quake met the plug-formed-by-continuous-streams limitations of the '148 patent. Ex. 1051 *passim*; Ex. 1052, *passim*. Moreover, Quake himself during prosecution characterized his invention as

involving continuous streams.[56] Accordingly, notwithstanding statements to the contrary during *ex parte* prosecution of the '148 patent (discussed in Section IV.C above) it is clear that Quake does in fact teach forming plugs from continuous streams of immiscible fluids.

Turning the second issue, as of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Corbett and Vogelstein. In 1991, about eleven years prior to the earliest effective filing date, Corbett disclosed using a microchannel or capillary device to conduct DNA amplification reactions such as PCR by injecting plugs of reactants into a carrier stream that is passed through different temperature zones. Corbett explained that as of 1991 "the most critical problem in applying nucleic acid amplification procedures, particularly in repetitive diagnostic assays in a clinical laboratory, is contamination of the reaction mixture by small amounts of DNA which contain sequences capable of being amplified under the conditions of assay." Ex. 1010 at 3:7-12. "Automation of procedures minimize[s] the opportunity for such contamination and allows the implementation of stringent quality control." *Id.* at 3:15-19. "As is shown in FIG. 1, the apparatus 10 comprises tube 12 through which a fluid stream passes. As is more clearly shown in FIG. 2, the fluid stream includes pockets of reaction mixtures 42 and 44 and purging solution 48 separated by carrier fluid 46." *Id.* at 7:58-62.

[56] When distinguishing his invention over one of the embodiments of Shaw Stewart in which reagent is periodically injected, Quake noted that his "invention relates, in part, to the discovery that combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid (e.g., decane) it is possible to produce small, discrete, monodisperse droplets of the aqueous solution flowing in the second fluid." Ex. 1058 at September 25, 2006 response, p. 15.





"The pump is actuated and the reaction mixture of 20 µl or less is injected into the mineral oil or silicone oil carrier fluid and specific DNA sequences (whose limits are defined by the oligonucleotide primers) present in the sample is amplified as it passes cyclically through the temperature zones." Ex. 1010 at 9:35-40. Corbett thus demonstrates that it was known at least as of 1991 that reduction of contamination risk in PCR could be achieved by performing the PCR on microliter sized plugs of reactant in capillary tube which passes through various temperature zones. Ex. 1004 ¶77.

By 1999, Vogelstein had reported **various applications for single-molecule microfluidic PCR**. Ex. 1044 at 9241. In a seminal article which has been cited over by over 1,000 other publications, Vogelstein explained that PCR amplification of small aliquots or droplets containing single molecules was effective for "detection of a small number of mutant-containing cells among a large excess of normal cells." *Id.* at 9236. In Vogelstein's method, the DNA was diluted into 7 µl volumes such that, on average, there was one template molecule for every two wells (i.e., approximately half of the wells contained no DNA template molecule and

approximately half contained a single DNA template molecule). *Id.* at 9236, 9237. This avoided the problem of background noise impairing the determination of whether each volume contained the mutant sequence. *Id.* By counting the number of volumes which indicated wild type or mutant templates, the presence or absence of the mutation (e.g., a sequence associated with colorectal cancer) can be determined. *Id.* at 9238. The accuracy of the test is a function of the number of discrete volumes into which the sample is diluted. *Id.* at 9239. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. *Id.* at 9239. ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude.") *Id.* 9239. The article explained that the same technique would be useful for **detecting a wide array of mutations, dislocations and allelic imbalances**, as summarized in Table 1. *Id.* at 9241. Vogelstein thus taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby overcoming the signal-to-noise limitations associated with certain other PCR techniques and enabling the detection of relatively rare mutations, dislocations and allelic imbalances. Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84.

In sum, Corbett and Vogelstein show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Quake is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules

and at least one other molecule").[57] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Quake) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake to conduct microfluidic PCR as taught by Corbett and Vogelstein. Ex. 1004 ¶109-112. Stated a different way, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic droplet reactor of Quake, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing.

Quake **specifically suggests that his microfluidic device could be used to perform PCR**. Quake explained that "[m]icrofabrication permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." Ex. 1034 at 23:26-27. Quake noted that "PCR is [] generally the method of choice to detect viral DNA or RNA directly in clinical specimens. The advantage of PCR for viral diagnostics is its high sensitivity; PCR can detect very low numbers of viruses in a small clinical specimen." *Id.* at 2:31-3:2.

More generally, skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic droplet reactor of Quake because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting

[57] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Quake to perform PCR advantageously would have allowed PCR reactions to be Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Quake would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the

limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each

contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Quake to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Vogelstein) to incorporate a droplet reactor (Quake). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Vogelstein. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to droplets produced

using injection of an aqueous fluid into an immiscible fluid (e.g., Corbett). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize

> noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson

> distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at

1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Quake could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful in miniaturizing and modifying Corbett or modifying Vogelstein to work in the microfluidic droplet reactor of Quake, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[58] As discussed in Section VI.A.3, above, Higuchi I-III

[58] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the

demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail below, in the accompanying claim chart (Ex. 1064) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake to conduct microfluidic PCR as taught by Corbett and Vogelstein. Ex. 1004 ⁋⁋109-112. Alternatively, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic droplet reactor of Quake, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing. *Id*.

Independent Claim 1

Claim 1[a]: A method comprising the steps of: providing a microfluidic system comprising one or more channels

Quake discloses "[a] microfluidic device for analyzing and/or sorting biological materials (e.g., molecules such as polynucleotides and polypeptides, including proteins and enzymes; viruses and cells) and methods for its use are provide[d]. . . . Ex. 1034 Abstract. For instance, **in those embodiments where aqueous droplets are used as microreactors for chemical reactions (including biochemical reactions) or are used to analyze and/or sort**

references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

biomaterials, a water soluble surfactant such as SDS may denature or inactivate the contents of the droplet." Ex. 1034 at 28:20-23 [emphasis added].

Quake's device comprises a main channel, through which a pressurized stream of oil is passed, and at least one sample inlet channel, through which a pressurized stream of aqueous solution is passed: "The channel architecture for the droplet extrusion region of the first device is shown in FIG. 16A. In this device, the inlet channel 1601 (inner diameter 30 μm) intersects the main channel 1602 (inner diameter 30 μm) at a T-intersection (i.e., an angle perpendicular to the main channel). Other intersections and angles may be used. The walls of the inlet and main channels were not tapered in this device." Ex. 1034 at 79:23-28.

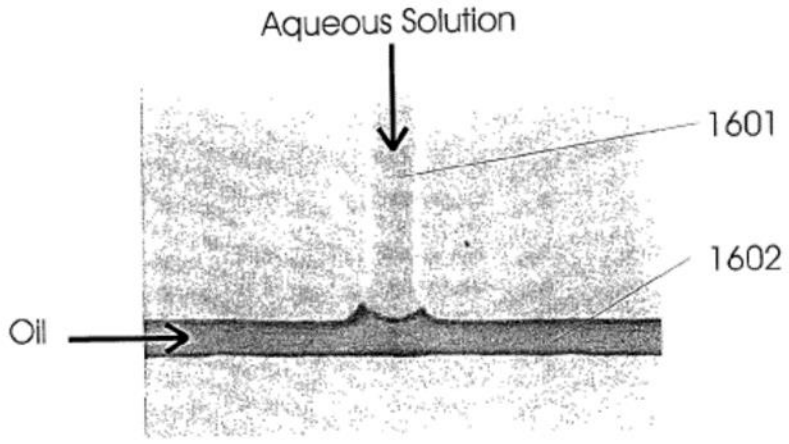


FIG. 16A

During the *inter partes* review proceeding, Patent Owner did not contest that Quake met this limitation. Ex. 1051, *passim*. The PTAB appears to have concluded that this limitation was met by Quake. Ex. 1052, *passim*.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1064.

Claim 1[b]: providing within the one or more channels a continuously flowing carrier fluid comprising an oil and a continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule in the

fluid that can react with the target DNA or RNA molecules under conditions in which the target DNA or RNA molecules and the other molecules in the fluid do not react with each other

Quake teaches that "[a] first fluid flows through the main channel, and a second fluid, which is incompatible or immiscible with the second fluid, passes through the inlet region so that droplets of the second fluid are sheared into the main channel. … The second phase or fluid which passes through the inlet region can be an **aqueous solution**, for example ultra pure water, TE buffer, phosphate buffer saline and acetate buffer. The second fluid may also contain a **biological sample** (e.g., molecules of an enzyme or a substrate, or one or more cells, or one or more viral particles) for analysis or sorting in the device. In preferred embodiments the second fluid includes a biological sample that comprises one or more **molecules**, cells, virions or particles." Ex. 1034 at 6:6-16 [emphasis added]. Quake further teaches that "[i]n preferred embodiments, a first fluid, which may be referred to as an 'extrusion' or 'barrier' fluid, passes (i.e., flows) through the main channel of the device and a second fluid, referred to as a "sample" or "droplet" fluid, passes or flows through the inlet region. ... Thus, droplets of the sample fluid containing the biological material for analysis, reaction or sorting are sheared at the droplet extrusion region into the flow of the extrusion fluid in the main channel." *Id.* at 7:30-8:5.

Regarding Quake's disclosure of the "continuous" limitation, see the discussion set forth at element 1[c], below.

In the instant combination, the microfluidic droplet reactor of Quake is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[59] These limitations are met by using the microfluidic droplet

[59] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

reactors with two continuously flowing immiscible fluids (as taught in Quake) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake to conduct microfluidic PCR in droplets as taught by Corbett, or to modify the reactions of Vogelstein to be performed in microfluidic droplets instead of continuous flow of a single fluid in a microfluidic channel. Ex. 1004 ¶109-112. Stated a different way, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to use two continuous streams of immiscible fluid as used in a microfluidic droplet reactor of Quake, as evidenced by contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing. *Id.*

Quake **specifically suggests that his microfluidic device could be used to perform PCR**. Quake explained that "[m]icrofabrication permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." Ex. 1034 at 23:26-27. Quake noted that "PCR is [] generally the method of choice to detect viral DNA or RNA directly in clinical specimens. The advantage of PCR for viral diagnostics is its high sensitivity; PCR can detect very low numbers of viruses in a small clinical specimen." *Id.* at 2:31-3:2.

More generally, a skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic droplet reactor of Quake because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis

added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions and the use of two continuous streams of immiscible fluids would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. Likewise, the reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Quake to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Quake would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from

the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each

contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239. ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude.") *Id.* 9239. Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Quake to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Vogelstein) to incorporate a droplet reactor (Quake). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Vogelstein. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to droplets produced

using injection of an aqueous fluid into an immiscible fluid (e.g., Corbett). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize

> noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson

> distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at

1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Quake could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful in miniaturizing and modifying Corbett or modifying Lagally to work in the microfluidic droplet reactor of Quake, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[60] As discussed in Section VI.A.3, above, Higuchi I-III

[60] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the

demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained further in the accompanying claim chart (Ex. 1064) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake to conduct microfluidic PCR as taught by Corbett and Vogelstein. Ex. 1004 ¶¶109-112. Alternatively, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic droplet reactor of Quake, as evidenced by contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing. *Id.*

With regard to the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other," this is merely a description of a PCR reaction. Ex. 1004 ¶31,32. As explained in Corbett, Kopp, Burns II, and Lagally, in a PCR reaction the sample DNA or RNA is admixed with various reagents including polymerase under conditions in which no reaction occurs; only when heat is applied does the PCR reaction occur. Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68. In the PCR of the combined method, as in virtually any PCR method, the DNA and polymermase do not react until mixture

references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

of reagents is heated. *Id*.; see also Ex. 1004 ¶32. The combined method thus meets the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other" this is merely a description of a PCR reaction. Ex. 1004 ¶31,32.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1064.

Claim 1[c]: controlling flow rates of said aqueous fluid and said carrier fluid to partition the continuously flowing aqueous fluid with the continuously flowing carrier fluid to form a plurality of plugs of the aqueous fluid, each having a substantially uniform size of about 200 µm or less, wherein the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule

As discussed above, the written descriptive support in the '148 patent for this claim element (which was the supposed point of novelty) was copied almost verbatim from Quake. The table below shows the correspondence between the relevant disclosures in the Quake PCT and the Ismagilov provisional application and '148 patent.

Quake PCT (published March 21, 2002) (Ex. 1034)	**Ismagilov provisional application (filed May 9, 2002) (Ex. 1003) and '148 patent (Ex. 1001)**
34:32-35:7 The pressure at the droplet extrusion region can also be regulated by adjusting the pressure on the main and sample inlets, for example, with pressurized syringes feeding into those inlets. By controlling the pressure difference between the oil and water sources at the droplet extrusion region, the size and periodicity of the droplets generated may be regulated. Alternatively, a valve may be placed at or coincident to either the droplet extrusion region or the sample inlet connected thereto to control the flow of solution into the droplet extrusion region, thereby	Provisional at 22:12-19, '148 patent at 21:56-65 The pressure at the plug-forming region can also be regulated by adjusting the pressure on the main and sample inlets, for example with pressurized syringes feeding into those inlets. By controlling the difference between the oil and water flow rates at the plug-forming region, the size and periodicity of the plugs generated may be regulated. Alternatively, a valve may be placed at or coincident to either the plug forming region or the sample inlet connected thereto to control the flow of solution into the plug-

controlling the size and periodicity of the droplets.	forming region, thereby controlling the size and periodicity of the plugs.

The disclosure of Quake was summarized by the Patent Trial and Appeal Board in its November 15, 2015 decision. Therein the Board found that Quake discloses a droplet reactor in which droplets are formed by continuous streams of immiscible fluids:

> Quake relates to microfluidic devices and methods for analyzing and/or sorting biological materials. Ex. 1004, Abstract [which corresponds to Ex. 1033]….
> The devices comprise a main channel, through which a pressurized stream of oil is passed, and at least one sample inlet channel, through which a pressurized stream of aqueous solution is passed. *Id.* A junction joins the main channel with the sample inlet channel. *Id.* By adjusting the pressure of the oil and/or the aqueous solution, a pressure difference can be established such that the stream of aqueous solution is sheared off at a regular frequency as it enters the oil stream, thereby forming droplets. *Id.* Figure 16A of Quake is reproduced below:

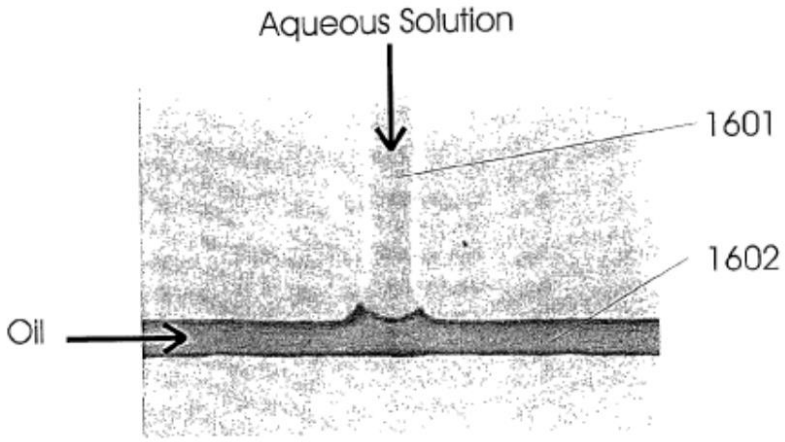


FIG. 16A

> Figure 16A depicts the channel architecture for the droplet extrusion region. Channel 1601 containing an aqueous solution intersects with the main channel 1602 containing oil. [*Id.*] ¶ 292.
> In preferred embodiments, the droplets have a volume of approximately 0.1 to 100 picoliters. *Id.* According to Quake, microfabrication of the device "permits

> other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." *Id.* ¶ 80.

Ex. 1052 at 11-12.

The Patent Owner did not contest in the *inter partes* review proceeding that Quake met the plug-formed-by-continuous-streams limitations of the '148 patent. Ex. 1051 *passim*; Ex. 1052, *passim*. Moreover, Quake himself during prosecution characterized his invention as involving continuous streams.[61] Accordingly, notwithstanding statements to the contrary during *ex parte* prosecution of the '148 patent (discussed in Section IV.C above) it is clear that Quake does in fact teach forming plugs from continuous streams of immiscible fluids.

Turning to the recitation "each having a substantially uniform size of about 200 μm or less," Quake teaches that "the droplets have a diameter that is smaller than the diameter of the microchannel; i.e., preferably less than 60 μm." Ex. 1034 at 27:29-30. Fig. 21 of Quake shows the droplets sizes generated by the Quake droplet generator were all less than 60 μm.

This leaves the recitation that "the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule." As discussed above, in the combined method of Quake, Corbett and Lagally, the samples are separated into a number of discrete droplets. That has the advantages of i) overcoming the signal-to-noise limitations associated with certain other PCR techniques and ii) enabling the detection of relatively rare mutations, dislocations and allelic imbalances. Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Lagally expressly teaches that "[t]he

[61] When distinguishing his invention over one of the embodiments of Shaw Stewart in which reagent is periodically injected, Quake noted that his "invention relates, in part, to the discovery that combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid (e.g., decane) it is possible to produce small, discrete, monodisperse droplets of the aqueous solution flowing in the second fluid." Ex. 1058 at September 25, 2006 response, p. 15.

event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a **Poisson** distribution, demonstrating that we are operating at the single-molecule level." Ex. 1028 at 566 [emphasis added]. Vogelstein likewise teaches that the aforementioned advantages are realized by **reducing the number of sample molecules per droplet to zero or one.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶82**.** That ensures that any amplification that occurs in a given droplet can be readily identified as containing the target sequence. *Id.* Thus, in the combined method the droplets have either one or zero template molecules.

As suggested by Lagally, any process which is generating droplets containing about one template molecule per droplet is expected to conform to a random, or Poisson, distribution. Ex. 1028 at 566; Ex. 1004 ¶48. It was known in the art that "[i]f a solution containing x molecules of enzyme per ml is dispersed into droplets of v ml volume, the average number of enzyme molecules per droplet n is equal to vx. Because of statistical fluctuations each droplet will not have exactly n enzyme molecules. The probability p(r) of finding 0, 1, 2, 3, . .. , r enzyme molecules in a droplet **is given by the Poisson law**." Ex. 1112 at 1985 [emphasis added]; see also Ex. 1111 at 1667 ("Spectra observed in a time sequence for an average of 0.6 dye molecule in the probed volume exhibited the expected Poisson distribution for actually measuring 0, 1, 2, or 3 molecules.").

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1064.

Claim 1[d]: providing conditions suitable for a polymerase-chain reaction in at least one plug of the plurality of plugs such that the target DNA or RNA is amplified

Quake teaches that "droplets of the sample fluid containing the biological material for analysis, **reaction** or sorting are sheared at the droplet extrusion region into the flow of the

extrusion fluid in the main channel." Ex. 1034 at 7:30-8:5 [emphasis added]. "Microfabrication permits other technologies to be integrated or combined with flow cytometry on a single chip, such as **PCR** [i.e., a reaction]." Ex. 1034 at 23:26-30 [emphasis added].

As discussed above in connection with element 1[b], in the instant combination Quake is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[62] These limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Quake) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1064.

Thus, claim 1 is shown to be rendered obvious by the combination of Quake, Corbett and Vogelstein.

Dependent Claim 2

Claim 2 of the '148 patent recites "wherein the step of providing conditions includes heating."

As discussed above in connection claim elements 1[b] and 1[c], in the combined method (and in virtually any other PCR reaction) the DNA and polymerase undergo polymerase chain reaction when heated. Ex. 1004 ¶32; Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68.

[62] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1064.

Thus, claim 2 is shown to be rendered obvious by the combination of Quake, Corbett and Vogelstein.

Dependent Claims 3-5

Claim 3 of the '148 patent recites "providing a detector to detect, analyze, characterize, or monitor the target DNA or RNA molecule in the at least one member of the plurality of plugs during and/or after the reaction has occurred." Claim 4 of the '148 patent recites "wherein the at least one other molecule includes one or more fluorescent labels and the detector is configured to monitor progress of the reaction by detecting fluorescence emissions from the one or more fluorescent labels." Claim 5 recites "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels."

Quake discloses that "[t]he device of the invention may also comprise a **detection region** which is within or coincident with at least a portion of the main channel at or downstream of the droplet extrusion region. The device may also have a detector, preferably an **optical detector** such as a microscope, associated with the detection region." Ex. 1034 at 6:28-31, [emphasis added]. "A preferred 'forward' sorting algorithm consists of running molecules, cells or virions from the input channel to the waste channel, until a molecule, cell or virion is identified to have an optically detectable signal *(e.g.* **fluorescence**) that is above a pre-set threshold, at which point voltages are temporarily changed to electro-osmotically **divert the molecule [] to the collection channel**." *Id.* at 20:22-26, [emphasis added].

Corbett similarly teaches that "[d]ownstream of temperature zone 34 is in-line analysis means 29 and recovery means 28. Connected to recovery means 28 is **detection means** 30. Detection means 30 determines when a reaction mixture in the carrier fluid reaches recovery

means 28. This may be done by measuring the conductivity or **optical density** of the stream of carrier fluid." Ex. 1010 at 8:12-18, [emphasis added].

Vogelstein teaches that the "[r]eactions were analyzed immediately or stored at room temperature for up to 36 h before **fluorescence** analysis." Ex. 1044 at 9236, [emphasis added].

As to claim 5, under the broadest reasonable interpretation the phrase "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels" requires simply that at least some of the droplets or plugs are transmitted to an outlet or other channel. Quake teaches an outlet channel as discussed above in connection with claim 1. See also Ex. 1034 at Fig. 16A, outlet 1602. If claim 5 is more narrowly interpreted as requiring selective diversion of the droplets, is also met by Quake. As noted above, Quake teaches that "[a] preferred 'forward' sorting algorithm consists of running molecules, cells or virions from the input channel to the waste channel, until a molecule, cell or virion is identified to have an optically detectable signal (e.g. fluorescence) that is above a pre-set threshold, at which point voltages are temporarily changed to electro-osmotically divert the molecule [] to the collection channel." *Id.* at 20:22-26. A skilled artisan would have considered it obvious to employ such a technique to sort droplets after they are subjected to the optical detection. Ex. 1004 ¶68,69. For instance, a skilled artisan would have found it advantageous to sort and collect droplets which tested positive or negative for the presence of a certain substance or property, especially in the context of droplet generators which are used to encapsulate on average one template molecule per droplet. *Id.* ¶82.

In the combined method of Quake, Corbett and Lagally, microfluidic droplet reactors are used to perform PCR reactions after which the reaction products are detected optically and sorted by diverting certain droplets into collection channels. Ex. 1004 ¶69.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1064.

Thus, claims 3-5 are shown to be rendered obvious by the combination of Quake, Corbett and Vogelstein.

Dependent Claims 6-8

Claim 6 of the '148 patent recites "wherein the oil is a fluorinated oil." Claim 7 of the '148 patent recites "wherein the carrier fluid further comprises a surfactant." Claim 8 of the '148 patent recites "wherein the surfactant is a fluorinated surfactant."

Quake discloses that "the first phase or fluid which flows through the main channel can be a non-polar solvent, such as decane (e.g., tetradecane or hexadecane) or another oil (for example, **mineral oil**)." Ex. 1034 at 6:8-10, [emphasis added]. "The fluids used in the invention may contain additives, such as agents which reduce surface tensions (surfactants). **Exemplary surfactants** include Tween, Span, **fluorinated oils**, and other agents that are soluble in oil relative to water. Surfactants may aid in controlling or optimizing droplet size, flow and uniformity, for example by reducing the shear force needed to extrude or inject droplets into an intersecting channel." *Id.* at 35:18-22, [emphasis added].

Additional correspondence between these claim elements and the cited references is shown in the claim chart submitted herewith as Exhibit 1064 and discussed in the Declaration of Dr. Shaqfeh (Ex. 1004).

Thus, claims 6-8 are shown to be rendered obvious by the combination of Quake, Corbett and Vogelstein.

D. The Combination of Quake, Kopp and Lagally Renders Obvious Claims 1-8 of the '148 Patent Under (pre-AIA) 35 U.S.C. § 103(a)

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Quake), ii) microfluidic PCR devices were well known (Kopp and Lagally), and iii) there were compelling reasons to use the microfluidic droplet reactors to conduct microfluidic PCR (Kopp, Burns II, Lagally and Vogelstein).

As to the first feature – forming plugs from continuously flowing immiscible fluids – the '148 patent's teachings in this regard were copied almost verbatim from the Quake PCT (Ex. 1034). The table below compares the Quake PCT publication with the Ismagilov specification, which was filed about three months later.

Quake PCT (published March 21, 2002) (Ex. 1034)	**Ismagilov provisional application (filed May 9, 2002) (Ex. 1003) and '148 patent (Ex. 1001)**
34:32-35:7 The pressure at the droplet extrusion region can also be regulated by adjusting the pressure on the main and sample inlets, for example, with pressurized syringes feeding into those inlets. By controlling the pressure difference between the oil and water sources at the droplet extrusion region, the size and periodicity of the droplets generated may be regulated. Alternatively, a valve may be placed at or coincident to either the droplet extrusion region or the sample inlet connected thereto to control the flow of solution into the droplet extrusion region, thereby controlling the size and periodicity of the droplets.	Provisional at 22:12-19, '148 patent at 21:56-65 The pressure at the plug-forming region can also be regulated by adjusting the pressure on the main and sample inlets, for example with pressurized syringes feeding into those inlets. By controlling the difference between the oil and water flow rates at the plug-forming region, the size and periodicity of the plugs generated may be regulated. Alternatively, a valve may be placed at or coincident to either the plug forming region or the sample inlet connected thereto to control the flow of solution into the plug-forming region, thereby controlling the size and periodicity of the plugs.

Attached as Ex. 1035 is a table showing the numerous passages which Ismagilov copied key disclosures verbatim or nearly verbatim from the Quake PCT. These were not merely

background or "state of the art" descriptions; rather, they go to the heart of the claimed subject matter.

The disclosure of Quake was summarized by the Patent Trial and Appeal Board in its November 15, 2015 decision. Therein the Board found that Quake discloses a droplet reactor in which droplets are formed by continuous streams of immiscible fluids:

> Quake relates to microfluidic devices and methods for analyzing and/or sorting biological materials. Ex. 1004, Abstract [which corresponds to Ex. 1033]….
> The devices comprise a main channel, through which a pressurized stream of oil is passed, and at least one sample inlet channel, through which a pressurized stream of aqueous solution is passed. *Id.* A junction joins the main channel with the sample inlet channel. *Id.* By adjusting the pressure of the oil and/or the aqueous solution, a pressure difference can be established such that the stream of aqueous solution is sheared off at a regular frequency as it enters the oil stream, thereby forming droplets. *Id.* Figure 16A of Quake is reproduced below:

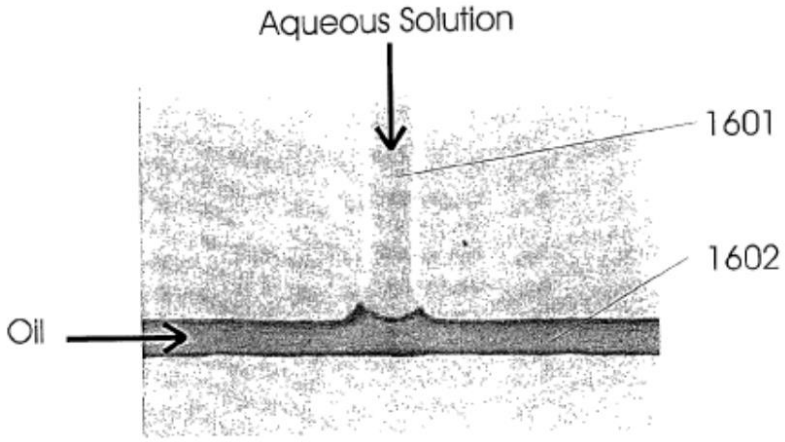


FIG. 16A

> Figure 16A depicts the channel architecture for the droplet extrusion region. Channel 1601 containing an aqueous solution intersects with the main channel 1602 containing oil. [*Id.*] ¶ 292.
> In preferred embodiments, the droplets have a volume of approximately 0.1 to 100 picoliters. *Id.* According to Quake, microfabrication of the device "permits

> other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." *Id.* ¶ 80.

Ex. 1052 at 11-12.

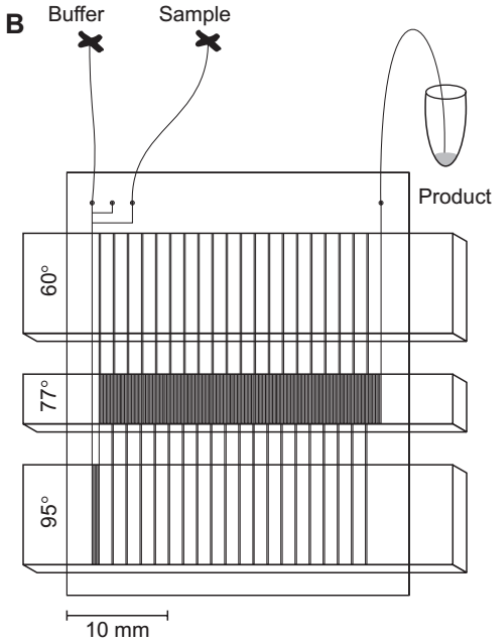
The Patent Owner did not contest in the *inter partes* review proceeding that Quake met the plug-formed-by-continuous-streams limitations of the '148 patent. Ex. 1051 *passim*; Ex. 1052, *passim*. Moreover, Quake himself during prosecution characterized his invention as involving continuous streams.[63] Accordingly, notwithstanding statements to the contrary during *ex parte* prosecution of the '148 patent (discussed in Section IV.C above) it is clear that Quake does in fact teach forming plugs from continuous streams of immiscible fluids.

Turning the second issue, as of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Kopp and Lagally. By 1998 Kopp *et al.* had successfully implemented continuous flow PCR on a chip. In his 1988 article (not previously presented to the USPTO), Kopp explained that "[a] wide range of microreactors, microcapillary electrophoresis devices, and microcell manipulation devices have been described in recent years." Ex. 1027 at 1046. Building on that body of work, Kopp's group developed

> [a] micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in **continuous flow** at high speed. The device is analogous to an electronic amplifier and relies on the movement of sample through thermostated temperature zones on a glass microchip. Input and output of material (DNA) is **continuous**, and amplification is independent of input concentration. . . .

[63] When distinguishing his invention over one of the embodiments of Shaw Stewart in which reagent is periodically injected, Quake noted that his "invention relates, in part, to the discovery that combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid (e.g., decane) it is possible to produce small, discrete, monodisperse droplets of the aqueous solution flowing in the second fluid." Ex. 1058 at September 25, 2006 response, p. 15.

> The results demonstrate that with a very simple device and virtually no optimization, PCR can be performed in **continuous flow**, yielding product quality and quantity comparable to standard thermal cycling methods (Fig. 2B). . . .
>
> We suggest that cross-contamination in a **continuous-flow** format may be much less of a problem than in a stationary-tube PCR format. Thus, a flow-through PCR chip can be reused for extended periods without major cleaning. . . .



Ex. 1027 at 1046-1047 [emphasis added]. Kopp *et al.* also explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications:

> To build a complex system analogous to an electronic integrated circuit, it is necessary to integrate a variety of other continuously working parts. A cursory glance at developments in miniaturized total analysis systems (uTAS) during the past 5 years reveals that most of the relevant components, such as **continuous-flow mixers** (8), **continuous-flow microreactors** (9), **continuous separations** (10), and high-speed capillary gel electrophoresis (as a conventional batch process), have already been

> presented (11). Accordingly, the **continuous-flow microamplifier** should allow the creation of highly elaborate analysis and synthesis systems.
>
> Although chemical amplification reactors will obviously not be applicable to all reactions, neither will they be limited to PCR. Devices of this kind open up whole new areas of application in medical diagnostics. For example, the online amplification and monitoring of a specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy.

Ex. 1027 at 1047 [emphasis added].

In 2001, in another article not previously considered by the Office, Lagally reported using a microfluidic reactor to conduct single-molecule DNA amplification by PCR. Lagally summarized the state of the art as of February 2001, explaining continuous flow PCR microreactors had recently undergone substantial evolution:

> The advent of biological microdevices [6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods16 or **continuous flow** through three differentially heated regions.18 Unfortunately, most of these designs either require bulk heating,16,17 making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample

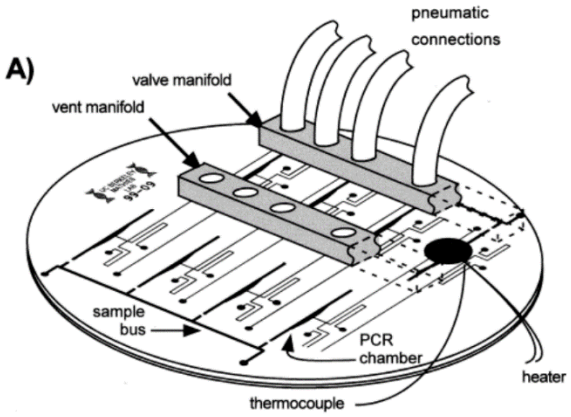
> volumes.18 Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.20,21 The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.21 This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566 [emphasis added].

Lagally *et al.* developed a further improvement – a device and method for achieving single molecule amplification in a microchannel environment.

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced

> microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> Microfluidic PCR-CE device. The PCR chambers are connected to a common sample bus through a set of valves. Hydrophobic vents at the other end of the PCR chambers are used to locate the sample and to eliminate gas. The PCR chambers are directly connected to the cross channel of the CE system for product injection and analysis. Two aluminum manifolds, one each for the vents and valves, are placed onto the respective ports and clamped in place using vacuum. The manifolds connect to external solenoid valves for pressure and vacuum actuation. Thermal cycling is accomplished using a resistive heater and a miniature thermocouple below the 280-nL chamber.



Ex. 1028 at 566 (see figure legend).

In sum, Kopp and Lagally show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Quake is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[64] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Quake) to perform PCR reactions, wherein each droplet or slug

[64] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

Quake **specifically suggests that his microfluidic device could be used to perform PCR**. Quake explained that "[m]icrofabrication permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." Ex. 1034 at 23:26-27. Quake noted that "PCR is [] generally the method of choice to detect viral DNA or RNA directly in clinical specimens. The advantage of PCR for viral diagnostics is its high sensitivity; PCR can detect very low numbers of viruses in a small clinical specimen." *Id.* at 2:31-3:2.

More generally, a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake to conduct microfluidic PCR as taught by Kopp and Lagally. A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic droplet reactor of Quake because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. Ex. 1004 ¶¶109-112. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to

provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Quake to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Quake would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to

achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Quake to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Kopp or Lagally) to incorporate a droplet reactor (Quake). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Kopp or Lagally. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the single fluid in microfluidic chip reactor (e.g., Lagally) or droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Kopp). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high

speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/µL, in volumes down to 1 µL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The

> first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic

> diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent

with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Quake could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful modifying Kopp or Lagally to work in the microfluidic droplet reactor of Quake, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[65] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail below, in the accompanying claim chart (Ex. 1065) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the

[65] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

microfluidic droplet reactor of Quake to conduct microfluidic PCR as taught by Kopp and Lagally.

Independent Claim 1

Claim 1[a]: A method comprising the steps of: providing a microfluidic system comprising one or more channels

Quake discloses "[a] microfluidic device for analyzing and/or sorting biological materials (e.g., molecules such as polynucleotides and polypeptides, including proteins and enzymes; viruses and cells) and methods for its use are provide[d]. . . . Ex. 1034 Abstract. For instance, **in those embodiments where aqueous droplets are used as microreactors for chemical reactions (including biochemical reactions) or are used to analyze and/or sort biomaterials**, a water soluble surfactant such as SDS may denature or inactivate the contents of the droplet." Ex. 1034 at 28:20-23 [emphasis added].

Quake's device comprises a main channel, through which a pressurized stream of oil is passed, and at least one sample inlet channel, through which a pressurized stream of aqueous solution is passed: "The channel architecture for the droplet extrusion region of the first device is shown in FIG. 16A. In this device, the inlet channel 1601 (inner diameter 30 μm) intersects the main channel 1602 (inner diameter 30 μm) at a T-intersection (i.e., an angle perpendicular to the main channel). Other intersections and angles may be used. The walls of the inlet and main channels were not tapered in this device." Ex. 1034 at 79:23-28.

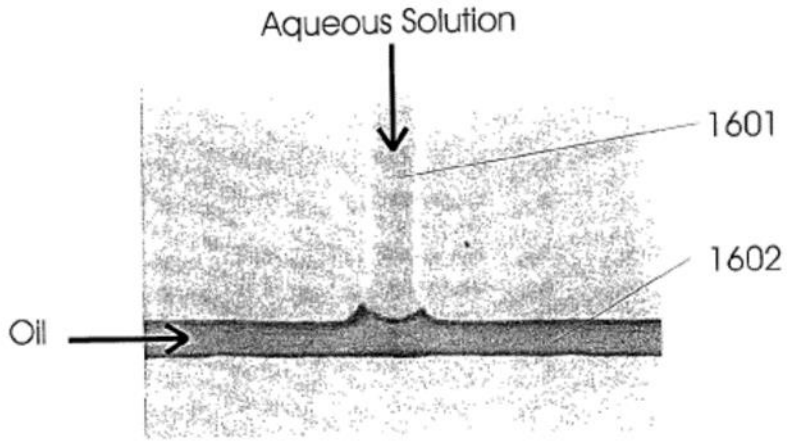


FIG. 16A

During the *inter partes* review proceeding, Patent Owner did not contest that Quake met this limitation. Ex. 1051, *passim*. The PTAB appears to have concluded that this limitation was met by Quake. Ex. 1052, *passim*.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1065.

> **Claim 1[b]: providing within the one or more channels a continuously flowing carrier fluid comprising an oil and a continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule in the fluid that can react with the target DNA or RNA molecules under conditions in which the target DNA or RNA molecules and the other molecules in the fluid do not react with each other**

Quake teaches that "[a] first fluid flows through the main channel, and a second fluid, which is incompatible or immiscible with the second fluid, passes through the inlet region so that droplets of the second fluid are sheared into the main channel. … The second phase or fluid which passes through the inlet region can be an **aqueous solution**, for example ultra pure water, TE buffer, phosphate buffer saline and acetate buffer. The second fluid may also contain a **biological sample** (e.g., molecules of an enzyme or a substrate, or one or more cells, or one or more viral particles) for analysis or sorting in the device. In preferred embodiments the second

fluid includes a biological sample that comprises one or more **molecules**, cells, virions or particles." Ex. 1034 at 6:6-16 [emphasis added]. Quake further teaches that "[i]n preferred embodiments, a first fluid, which may be referred to as an 'extrusion' or 'barrier' fluid, passes (i.e., flows) through the main channel of the device and a second fluid, referred to as a "sample" or "droplet" fluid, passes or flows through the inlet region. ... Thus, droplets of the sample fluid containing the biological material for analysis, reaction or sorting are sheared at the droplet extrusion region into the flow of the extrusion fluid in the main channel." *Id.* at 7:30-8:5.

Regarding Quake's disclosure of the "continuous" limitation, see the discussion set forth at element 1[c], below.

In the instant combination, the microfluidic droplet reactor of Quake is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[66] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Quake) to perform PCR reactions (as taught in Kopp and Lagally), wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

Quake **specifically suggests that his microfluidic device could be used to perform PCR**. Quake explained that "[m]icrofabrication permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." Ex. 1034 at 23:26-27. Quake noted that "PCR is [] generally the method of choice to detect viral DNA or RNA directly in

[66] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

clinical specimens. The advantage of PCR for viral diagnostics is its high sensitivity; PCR can detect very low numbers of viruses in a small clinical specimen." *Id.* at 2:31-3:2.

More generally, a skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic droplet reactor of Quake because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. Likewise, the reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the

droplet reactor of Quake to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Quake would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample

leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Quake to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Kopp or Lagally) to incorporate a droplet reactor (Quake). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Kopp or Lagally. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the single fluid in microfluidic chip reactor (e.g., Bu Lagally) or droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Kopp). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material

and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.

Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In

1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Quake could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful in modifying Lagally or Kopp to work in the microfluidic droplet reactor of Quake, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's

earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[67] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained further in the accompanying claim chart (Ex. 1065) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake to conduct microfluidic PCR as taught by Kopp and Lagally.

With regard to the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other," this is merely a description of a PCR reaction. Ex. 1004 ¶31,32. As explained in Corbett, Kopp, Burns II, and Lagally, in a PCR reaction the sample DNA or RNA is admixed with various reagents including polymerase under conditions in which no reaction occurs; only when heat is applied does the PCR reaction occur. (Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68.) In the PCR of the combined

[67] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

method, as in virtually any PCR method, the DNA and polymermase do not react until mixture of reagents is heated. (*Id.*; see also Ex. 1004 ¶32.) The combined method thus meets the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other" this is merely a description of a PCR reaction. Ex. 1004 ¶31,32.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1065.

Claim 1[c]: controlling flow rates of said aqueous fluid and said carrier fluid to partition the continuously flowing aqueous fluid with the continuously flowing carrier fluid to form a plurality of plugs of the aqueous fluid, each having a substantially uniform size of about 200 µm or less, wherein the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule

As discussed above, the written descriptive support in the '148 patent for this claim element (which was the supposed point of novelty) was copied almost verbatim from Quake. The table below shows the correspondence between the relevant disclosures in the Quake PCT and the Ismagilov provisional application and '148 patent.

Quake PCT (published March 21, 2002) (Ex. 1034)	**Ismagilov provisional application (filed May 9, 2002) (Ex. 1003) and '148 patent (Ex. 1001)**
34:32-35:7 The pressure at the droplet extrusion region can also be regulated by adjusting the pressure on the main and sample inlets, for example, with pressurized syringes feeding into those inlets. By controlling the pressure difference between the oil and water sources at the droplet extrusion region, the size and periodicity of the droplets generated may be regulated. Alternatively, a valve may be placed at or coincident to either the droplet extrusion region or the sample inlet connected thereto	Provisional at 22:12-19, '148 patent at 21:56-65 The pressure at the plug-forming region can also be regulated by adjusting the pressure on the main and sample inlets, for example with pressurized syringes feeding into those inlets. By controlling the difference between the oil and water flow rates at the plug-forming region, the size and periodicity of the plugs generated may be regulated. Alternatively, a valve may be placed at or coincident to either the plug forming region or the sample inlet connected thereto to

to control the flow of solution into the droplet extrusion region, thereby controlling the size and periodicity of the droplets.	control the flow of solution into the plug-forming region, thereby controlling the size and periodicity of the plugs.

The disclosure of Quake was summarized by the Patent Trial and Appeal Board in its November 15, 2015 decision. Therein the Board found that Quake discloses a droplet reactor in which droplets are formed by continuous streams of immiscible fluids:

> Quake relates to microfluidic devices and methods for analyzing and/or sorting biological materials. Ex. 1004, Abstract [which corresponds to Ex. 1033]…. The devices comprise a main channel, through which a pressurized stream of oil is passed, and at least one sample inlet channel, through which a pressurized stream of aqueous solution is passed. *Id.* A junction joins the main channel with the sample inlet channel. *Id.* By adjusting the pressure of the oil and/or the aqueous solution, a pressure difference can be established such that the stream of aqueous solution is sheared off at a regular frequency as it enters the oil stream, thereby forming droplets. *Id.* Figure 16A of Quake is reproduced below:

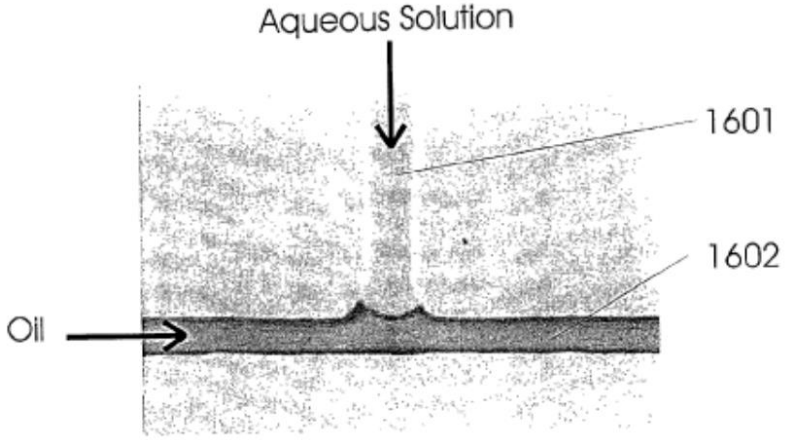


FIG. 16A

> Figure 16A depicts the channel architecture for the droplet extrusion region. Channel 1601 containing an aqueous solution intersects with the main channel 1602 containing oil. [*Id.*] ¶ 292.

> In preferred embodiments, the droplets have a volume of approximately 0.1 to 100 picoliters. *Id.* According to Quake, microfabrication of the device "permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." *Id.* ¶ 80.

Ex. 1052 at 11-12.

The Patent Owner did not contest in the *inter partes* review proceeding that Quake met the plug-formed-by-continuous-streams limitations of the '148 patent. Ex. 1051 *passim*; Ex. 1052, *passim*. Moreover, Quake himself during prosecution characterized his invention as involving continuous streams.[68] Accordingly, notwithstanding statements to the contrary during *ex parte* prosecution of the '148 patent (discussed in Section IV.C above) it is clear that Quake does in fact teach forming plugs from continuous streams of immiscible fluids.

Turning to the recitation "each having a substantially uniform size of about 200 μm or less," Quake teaches that "the droplets have a diameter that is smaller than the diameter of the microchannel; i.e., preferably less than 60 μm." Ex. 1034 at 27:29-30. Fig. 21 of Quake shows the droplets sizes generated by the Quake droplet generator were all less than 60 μm.

This leaves the recitation that "the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule." As discussed above, in the combined method of Quake, Corbett and Lagally, the samples are separated into a number of discrete droplets. That has the advantages of i) overcoming the signal-to-noise limitations associated with certain other PCR

[68] When distinguishing his invention over one of the embodiments of Shaw Stewart in which reagent is periodically injected, Quake noted that his "invention relates, in part, to the discovery that combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid (e.g., decane) it is possible to produce small, discrete, monodisperse droplets of the aqueous solution flowing in the second fluid." Ex. 1058 at September 25, 2006 response, p. 15.

techniques and ii) enabling the detection of relatively rare mutations, dislocations and allelic imbalances. Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Lagally expressly teaches that "[t]he event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a **Poisson** distribution, demonstrating that we are operating at the single-molecule level." Ex. 1028 at 566 [emphasis added]. Vogelstein likewise teaches that the aforementioned advantages are realized by **reducing the number of sample molecules per droplet to zero or one.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶82**.** That ensures that any amplification that occurs in a given droplet can be readily identified as containing the target sequence. *Id.* Thus, in the combined method the droplets have either one or zero template molecules.

As suggested by Lagally, any process which is generating droplets containing about one template molecule per droplet is expected to conform to a random, or Poisson, distribution. Ex. 1028 at 566; Ex. 1004 ¶48. It was known in the art that "[i]f a solution containing x molecules of enzyme per ml is dispersed into droplets of v ml volume, the average number of enzyme molecules per droplet n is equal to vx. Because of statistical fluctuations each droplet will not have exactly n enzyme molecules. The probability p(r) of finding 0, 1, 2, 3, . .. , r enzyme molecules in a droplet **is given by the Poisson law**." Ex. 1112 at 1985 [emphasis added]; see also Ex. 1111 at 1667 ("Spectra observed in a time sequence for an average of 0.6 dye molecule in the probed volume exhibited the expected Poisson distribution for actually measuring 0, 1, 2, or 3 molecules.").

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1065.

Claim 1[d]: providing conditions suitable for a polymerase-chain reaction in at least one plug of the plurality of plugs such that the target DNA or RNA is amplified

Quake teaches that "droplets of the sample fluid containing the biological material for analysis, **reaction** or sorting are sheared at the droplet extrusion region into the flow of the extrusion fluid in the main channel." Ex. 1034 at 7:30-8:5 [emphasis added]. "Microfabrication permits other technologies to be integrated or combined with flow cytometry on a single chip, such as **PCR** [i.e., a reaction]." Ex. 1034 at 23:26-30 [emphasis added].

As discussed above in connection with element 1[b], in the instant combination Quake is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[69] These limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Quake) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1065.

Thus, claim 1 is shown to be rendered obvious by the combination of Quake, Kopp and Lagally.

Dependent Claim 2

Claim 2 of the '148 patent recites "wherein the step of providing conditions includes heating."

As discussed above in connection claim elements 1[b] and 1[c], in the combined method (and in virtually any other PCR reaction) the DNA and polymerase undergo polymerase chain

[69] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

reaction when heated. Ex. 1004 ¶32; Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1065.

Thus, claim 2 is shown to be rendered obvious by the combination of Quake, Kopp and Lagally.

Dependent Claims 3-5

Claim 3 of the '148 patent recites "providing a detector to detect, analyze, characterize, or monitor the target DNA or RNA molecule in the at least one member of the plurality of plugs during and/or after the reaction has occurred." Claim 4 of the '148 patent recites "wherein the at least one other molecule includes one or more fluorescent labels and the detector is configured to monitor progress of the reaction by detecting fluorescence emissions from the one or more fluorescent labels." Claim 5 recites "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels."

Quake discloses that "[t]he device of the invention may also comprise a **detection region** which is within or coincident with at least a portion of the main channel at or downstream of the droplet extrusion region. The device may also have a detector, preferably an **optical detector** such as a microscope, associated with the detection region." Ex. 1034 at 6:28-31, [emphasis added]. "A preferred 'forward' sorting algorithm consists of running molecules, cells or virions from the input channel to the waste channel, until a molecule, cell or virion is identified to have an optically detectable signal *(e.g.* **fluorescence**) that is above a pre-set threshold, at which point voltages are temporarily changed to electro-osmotically **divert the molecule [] to the collection channel**." *Id.* at 20:22-26, [emphasis added].

Lagally teaches "[s]tochastic PCR amplification of single DNA template molecules followed by **capillary electrophoresis** (CE) analysis of the products is demonstrated in an integrated microfluidic device." Ex. 1028 at 1, [emphasis added]. The results of capillary electrophoresis were generally detected optically at the time of filing. Ex. 1011 at 46 (Dolnick). "The amplified product, labeled with an intercalating **fluorescent** dye, is directly injected into the gel-filled capillary channel for electrophoretic analysis." Ex. 1028 at 1, [emphasis added].

As to claim 5, under the broadest reasonable interpretation the phrase "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels" requires simply that at least some of the droplets or plugs are transmitted to an outlet or other channel. Quake teaches an outlet channel as discussed above in connection with claim 1. See also Ex. 1034 at Fig. 16A, outlet 1602. If claim 5 is more narrowly interpreted as requiring selective diversion of the droplets, is also met by Quake. As noted above, Quake teaches that "[a] preferred 'forward' sorting algorithm consists of running molecules, cells or virions from the input channel to the waste channel, until a molecule, cell or virion is identified to have an optically detectable signal (e.g. fluorescence) that is above a pre-set threshold, at which point voltages are temporarily changed to electro-osmotically divert the molecule [] to the collection channel." *Id.* at 20:22-26. A skilled artisan would have considered it obvious to employ such a technique to sort droplets after they are subjected to the optical detection. Ex. 1004 ¶68,69. For instance, a skilled artisan would have found it advantageous to sort and collect droplets which tested positive or negative for the presence of a certain substance or property, especially in the context of droplet generators which are used to encapsulate on average one template molecule per droplet. *Id.* ¶82.

In the combined method of Quake, Kopp and Lagally, microfluidic droplet reactors are used to perform PCR reactions after which the reaction products are detected optically and sorted by diverting certain droplets into collection channels. Ex. 1004 ¶69.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1065.

Thus, claims 3-5 are shown to be rendered obvious by the combination of Quake, Kopp and Lagally.

Dependent Claims 6-8

Claim 6 of the '148 patent recites "wherein the oil is a fluorinated oil." Claim 7 of the '148 patent recites "wherein the carrier fluid further comprises a surfactant." Claim 8 of the '148 patent recites "wherein the surfactant is a fluorinated surfactant."

Quake discloses that "the first phase or fluid which flows through the main channel can be a non-polar solvent, such as decane (e.g., tetradecane or hexadecane) or another oil (for example, **mineral oil**)." Ex. 1034 at 6:8-10, [emphasis added]. "The fluids used in the invention may contain additives, such as agents which reduce surface tensions (surfactants). **Exemplary surfactants** include Tween, Span, **fluorinated oils**, and other agents that are soluble in oil relative to water. Surfactants may aid in controlling or optimizing droplet size, flow and uniformity, for example by reducing the shear force needed to extrude or inject droplets into an intersecting channel." *Id.* at 35:18-22, [emphasis added].

Additional correspondence between these claim elements and the cited references is shown in the claim chart submitted herewith as Exhibit 1065 and discussed in the Declaration of Dr. Shaqfeh (Ex. 1004).

Thus, claims 6-8 are shown to be rendered obvious by the combination of Quake, Kopp and Lagally.

E. The Combination of Quake, Kopp and Burns II Renders Obvious Claims 1-8 of the '148 Patent Under (pre-AIA) 35 U.S.C. § 103(a)

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Quake), ii) microfluidic PCR devices were well known (Kopp and Burns II), and iii) there were compelling reasons to use the microfluidic droplet reactors to conduct microfluidic PCR (Kopp, Burns II, Lagally and Vogelstein).

As to the first feature – forming plugs from continuously flowing immiscible fluids – the '148 patent's teachings in this regard were copied almost verbatim from the Quake PCT (Ex. 1034). The table below compares the Quake PCT publication with the Ismagilov specification, which was filed about three months later.

Quake PCT (published March 21, 2002) (Ex. 1034)	**Ismagilov provisional application (filed May 9, 2002) (Ex. 1003) and '148 patent (Ex. 1001)**
34:32-35:7 The pressure at the droplet extrusion region can also be regulated by adjusting the pressure on the main and sample inlets, for example, with pressurized syringes feeding into those inlets. By controlling the pressure difference between the oil and water sources at the droplet extrusion region, the size and periodicity of the droplets generated may be regulated. Alternatively, a valve may be placed at or coincident to either the droplet extrusion region or the sample inlet connected thereto to control the flow of solution into the droplet extrusion region, thereby controlling the size and periodicity of the droplets.	Provisional at 22:12-19, '148 patent at 21:56-65 The pressure at the plug-forming region can also be regulated by adjusting the pressure on the main and sample inlets, for example with pressurized syringes feeding into those inlets. By controlling the difference between the oil and water flow rates at the plug-forming region, the size and periodicity of the plugs generated may be regulated. Alternatively, a valve may be placed at or coincident to either the plug forming region or the sample inlet connected thereto to control the flow of solution into the plug-forming region, thereby controlling the size and periodicity of the plugs.

Attached as Ex. 1035 is a table showing the numerous passages which Ismagilov copied key disclosures verbatim or nearly verbatim from the Quake PCT. These were not merely background or "state of the art" descriptions; rather, they go to the heart of the claimed subject matter.

The disclosure of Quake was summarized by the Patent Trial and Appeal Board in its November 15, 2015 decision. Therein the Board found that Quake discloses a droplet reactor in which droplets are formed by continuous streams of immiscible fluids:

> Quake relates to microfluidic devices and methods for analyzing and/or sorting biological materials. Ex. 1004, Abstract [which corresponds to Ex. 1033]…. The devices comprise a main channel, through which a pressurized stream of oil is passed, and at least one sample inlet channel, through which a pressurized stream of aqueous solution is passed. *Id.* A junction joins the main channel with the sample inlet channel. *Id.* By adjusting the pressure of the oil and/or the aqueous solution, a pressure difference can be established such that the stream of aqueous solution is sheared off at a regular frequency as it enters the oil stream, thereby forming droplets. *Id.* Figure 16A of Quake is reproduced below:

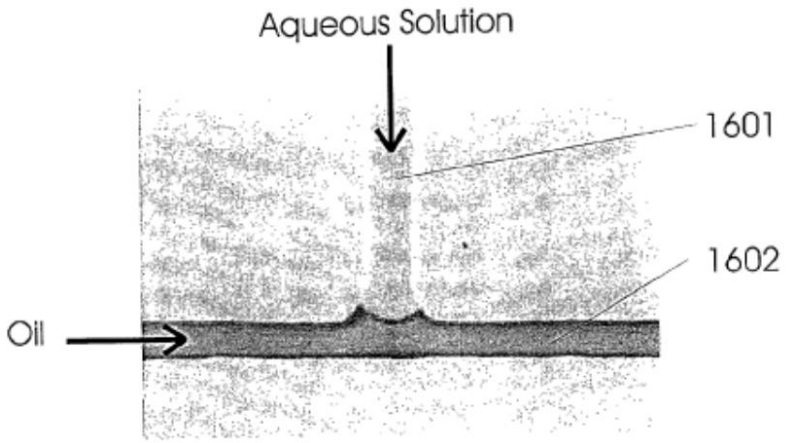


FIG. 16A

> Figure 16A depicts the channel architecture for the droplet extrusion region. Channel 1601 containing an aqueous solution intersects with the main channel 1602 containing oil. [*Id.*] ¶ 292.

> In preferred embodiments, the droplets have a volume of approximately 0.1 to 100 picoliters. *Id.* According to Quake, microfabrication of the device "permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." *Id.* ¶ 80.

Ex. 1052 at 11-12.

The Patent Owner did not contest in the *inter partes* review proceeding that Quake met the plug-formed-by-continuous-streams limitations of the '148 patent. Ex. 1051 *passim*; Ex. 1052, *passim*. Moreover, Quake himself during prosecution characterized his invention as involving continuous streams.[70] Accordingly, notwithstanding statements to the contrary during *ex parte* prosecution of the '148 patent (discussed in Section IV.C above) it is clear that Quake does in fact teach forming plugs from continuous streams of immiscible fluids.

Turning the second issue, as of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Kopp and Burns II. In 1996, Burns II reported the use of microfluidic devices to perform PCR. Ex. 1008. Burns II explained that "[n]o new biochemistries or DNA detection methods are required for their use; the components simply reproduce current laboratory procedures in a micron-sized environment." *Id.* at 5560. In other words, the PCR chemistry did not need to be modified – it worked without modification on the microfluidic scale. In the method of Burns II, a sample containing a DNA plasmid was loaded into one of the reaction wells (A) and a mixture of Taq enzyme and buffer was loaded into the other. *Id.* at 5558. Each formed droplets of about 60 nl in the parallel 500 x

[70] When distinguishing his invention over one of the embodiments of Shaw Stewart in which reagent is periodically injected, Quake noted that his "invention relates, in part, to the discovery that combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid (e.g., decane) it is possible to produce small, discrete, monodisperse droplets of the aqueous solution flowing in the second fluid." Ex. 1058 at September 25, 2006 response, p. 15.

25 µm channels (B) which came together at the Y junction to form a single larger droplet (about 120 nl). *Id.* at 5558-59; Ex. 1004 ¶40.

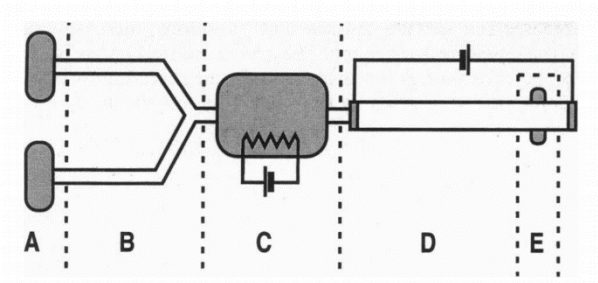


FIG. 1. Example of an integrated DNA analysis system, represented schematically. The individual components of the system are injection entry ports (*A*), liquid pumping channels (*B*), thermally controlled reaction chamber (*C*), electrophoresis channel (*D*), and DNA band migration detector (*E*). Each component would have associated sensors, control circuitry, and external connections.

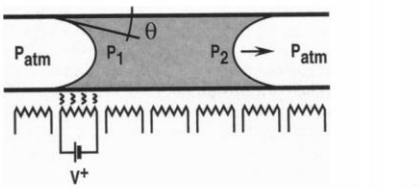


FIG. 2. Schematic drawing showing the principle of thermally induced drop motion in a closed channel. The case of a single aqueous drop in a hydrophilic channel is presented, where V is an applied voltage, P_{atm} is atmospheric pressure, P_1 is the receding-edge internal pressure, P_2 is the advancing-edge internal pressure, and θ is the contact angle of the liquid–gas–solid interface. The contact angle will depend on the surface characteristics of the channel and the constituents of the drop, with a hydrophilic interaction giving θ between 0 and 90 degrees, and a hydrophobic surface giving θ between 90 and 180 degrees. Surface treatments can also reduce contact angle hysteresis and, therefore, reduce the temperature difference necessary for drop motion.

The droplet then passed through the resistive heater portion (C) in which PCR amplification occurred. *Id.* at 5556. Afterwards the amplification results were optically detected in zone D via gel electrophoresis *Id.* at 5559. Burns II noted that "[t]he use of common fabrication methods allows the assembly of increasingly complex, multicomponent, integrated systems from a small, defined set of standardized elements" and that "[f]uture design efforts have a strong impetus to reduce component size and improve efficiency." *Id.* at 5560-61.

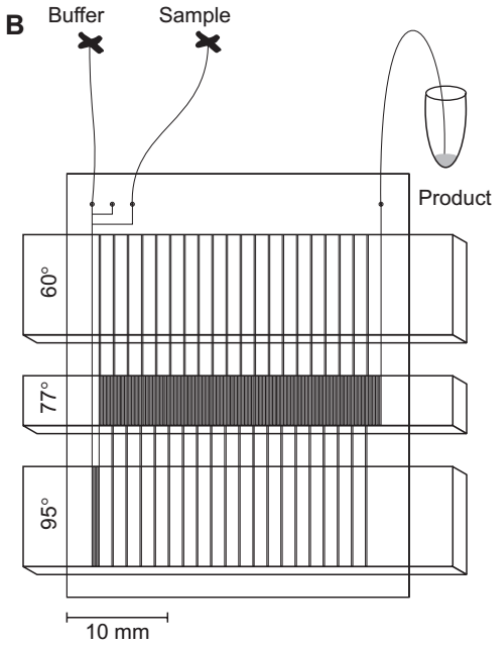
By the late 1990s Kopp *et al*. had successfully implemented continuous flow PCR on a chip. In his 1988 article (not previously presented to the USPTO), Kopp explained that "[a] wide range of microreactors, microcapillary electrophoresis devices, and microcell manipulation devices have been described in recent years." Ex. 1027 at 1046. Building on that body of work, Kopp's group developed

> [a] micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in **continuous flow** at high speed. The device is analogous to an electronic amplifier and relies on the movement of sample through thermostated temperature zones on a glass

microchip. Input and output of material (DNA) is **continuous**, and amplification is independent of input concentration. . . .

The results demonstrate that with a very simple device and virtually no optimization, PCR can be performed in **continuous flow**, yielding product quality and quantity comparable to standard thermal cycling methods (Fig. 2B). . . .

We suggest that cross-contamination in a **continuous-flow** format may be much less of a problem than in a stationary-tube PCR format. Thus, a flow-through PCR chip can be reused for extended periods without major cleaning. . . .



Ex. 1027 at 1046-1047 [emphasis added]. Kopp *et al.* also explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications:

To build a complex system analogous to an electronic integrated circuit, it is necessary to integrate a variety of other continuously working parts. A cursory glance at developments in miniaturized total analysis systems (uTAS) during the past 5 years reveals that most of the relevant components, such as **continuous-flow mixers** (8), **continuous-flow**

> **microreactors** (9), **continuous separations** (10), and high-speed capillary gel electrophoresis (as a conventional batch process), have already been presented (11). Accordingly, the **continuous-flow microamplifier** should allow the creation of highly elaborate analysis and synthesis systems.
>
> Although chemical amplification reactors will obviously not be applicable to all reactions, neither will they be limited to PCR. Devices of this kind open up whole new areas of application in medical diagnostics. For example, the online amplification and monitoring of a specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy.

Ex. 1027 at 1047 [emphasis added].

In sum, Kopp and Burns II show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Quake is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[71] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Quake) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

Quake **specifically suggests that his microfluidic device could be used to perform PCR**. Quake explained that "[m]icrofabrication permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." Ex. 1034 at 23:26-27. Quake

[71] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

noted that "PCR is [] generally the method of choice to detect viral DNA or RNA directly in clinical specimens. The advantage of PCR for viral diagnostics is its high sensitivity; PCR can detect very low numbers of viruses in a small clinical specimen." *Id.* at 2:31-3:2.

More generally, a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake, to conduct microfluidic PCR as taught by Kopp and Burns II. A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic droplet reactor of Quake because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing**).** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance,

identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Quake to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Quake would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a

sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Quake to dilute the samples and thereby enable the detection of

mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Kopp or Burns II) to incorporate a droplet reactor (Quake). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Kopp or Burns II. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the single fluid in microfluidic chip reactor (e.g., Burns II) or droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Kopp). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.

Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with

> sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Quake could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful modifying Kopp or Burns II to work in the microfluidic droplet reactor of Quake, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention

was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[72] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail below, in the accompanying claim chart (Ex. 1066) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake to conduct microfluidic PCR as taught by Kopp and Burns II.

Independent Claim 1

Claim 1[a]: A method comprising the steps of: providing a microfluidic system comprising one or more channels

[72] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

Quake discloses "[a] microfluidic device for analyzing and/or sorting biological materials (e.g., molecules such as polynucleotides and polypeptides, including proteins and enzymes; viruses and cells) and methods for its use are provide[d]. . . . Ex. 1034 Abstract. For instance, **in those embodiments where aqueous droplets are used as microreactors for chemical reactions (including biochemical reactions) or are used to analyze and/or sort biomaterials**, a water soluble surfactant such as SDS may denature or inactivate the contents of the droplet." Ex. 1034 at 28:20-23 [emphasis added].

Quake's device comprises a main channel, through which a pressurized stream of oil is passed, and at least one sample inlet channel, through which a pressurized stream of aqueous solution is passed: "The channel architecture for the droplet extrusion region of the first device is shown in FIG. 16A. In this device, the inlet channel 1601 (inner diameter 30 μm) intersects the main channel 1602 (inner diameter 30 μm) at a T-intersection (i.e., an angle perpendicular to the main channel). Other intersections and angles may be used. The walls of the inlet and main channels were not tapered in this device." Ex. 1034 at 79:23-28.

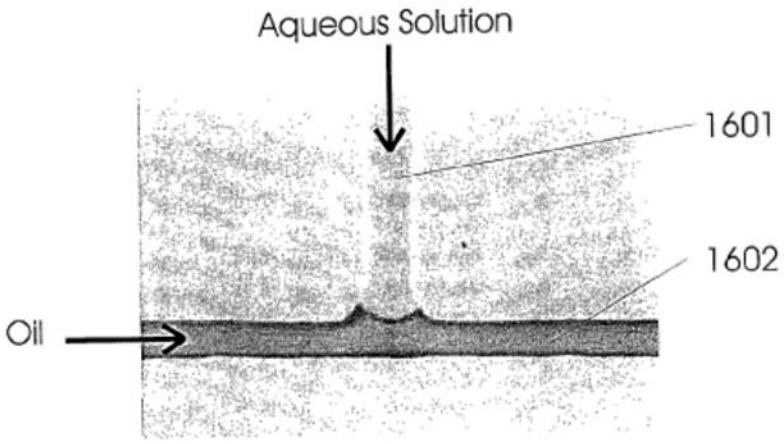


FIG. 16A

During the *inter partes* review proceeding, Patent Owner did not contest that Quake met this limitation. Ex. 1051, *passim*. The PTAB appears to have concluded that this limitation was met by Quake. Ex. 1052, *passim*.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1066.

Claim 1[b]: providing within the one or more channels a continuously flowing carrier fluid comprising an oil and a continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule in the fluid that can react with the target DNA or RNA molecules under conditions in which the target DNA or RNA molecules and the other molecules in the fluid do not react with each other

Quake teaches that "[a] first fluid flows through the main channel, and a second fluid, which is incompatible or immiscible with the second fluid, passes through the inlet region so that droplets of the second fluid are sheared into the main channel. … The second phase or fluid which passes through the inlet region can be an **aqueous solution**, for example ultra pure water, TE buffer, phosphate buffer saline and acetate buffer. The second fluid may also contain a **biological sample** (e.g., molecules of an enzyme or a substrate, or one or more cells, or one or more viral particles) for analysis or sorting in the device. In preferred embodiments the second fluid includes a biological sample that comprises one or more **molecules**, cells, virions or particles." Ex. 1034 at 6:6-16 [emphasis added]. Quake further teaches that "[i]n preferred embodiments, a first fluid, which may be referred to as an 'extrusion' or 'barrier' fluid, passes (i.e., flows) through the main channel of the device and a second fluid, referred to as a "sample" or "droplet" fluid, passes or flows through the inlet region. ... Thus, droplets of the sample fluid containing the biological material for analysis, reaction or sorting are sheared at the droplet extrusion region into the flow of the extrusion fluid in the main channel." *Id.* at 7:30-8:5.

Regarding Quake's disclosure of the "continuous" limitation, see the discussion set forth at element 1[c], below.

In the instant combination, the microfluidic droplet reactor of Quake is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[73] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Quake) to perform PCR reactions (as taught in Kopp and Burns II), wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

Quake **specifically suggests that his microfluidic device could be used to perform PCR**. Quake explained that "[m]icrofabrication permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." Ex. 1034 at 23:26-27. Quake noted that "PCR is [] generally the method of choice to detect viral DNA or RNA directly in clinical specimens. The advantage of PCR for viral diagnostics is its high sensitivity; PCR can detect very low numbers of viruses in a small clinical specimen." *Id.* at 2:31-3:2.

More generally, a skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic droplet reactor of Quake because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis

[73] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. Likewise, the reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Quake to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Quake would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07.

In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of**

relatively rare mutations, dislocations and allelic imbalances. Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Quake to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Kopp or Burns II,) to incorporate a droplet reactor (Quake). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Kopp or Burns II. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the single fluid in microfluidic chip reactor (e.g., Burns II) or droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Kopp). Droplet reactors also **decrease**

contamination and increase the number of reactors that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk

> heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with

> microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching**

whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Burns II, a skilled artisan would have expected that the microfluidic droplet reactor of Quake could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful in modifying Kopp or Lagally to work in the microfluidic droplet reactor of Quake, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[74] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of

[74] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations

microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained further in the accompanying claim chart (Ex. 1066) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake to conduct microfluidic PCR as taught by Kopp and Burns II.

With regard to the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other," this is merely a description of a PCR reaction. Ex. 1004 ¶31,32. As explained in Corbett, Kopp, Burns II, and Lagally, in a PCR reaction the sample DNA or RNA is admixed with various reagents including polymerase under conditions in which no reaction occurs; only when heat is applied does the PCR reaction occur. Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68. In the PCR of the combined method, as in virtually any PCR method, the DNA and polymermase do not react until mixture of reagents is heated. *Id*.; see also Ex. 1004 ¶32. The combined method thus meets the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other" this is merely a description of a PCR reaction. Ex. 1004 ¶31,32.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1066.

Claim 1[c]: controlling flow rates of said aqueous fluid and said carrier fluid to partition the continuously flowing aqueous fluid with the continuously flowing carrier fluid to form a plurality of plugs of the aqueous fluid, each having a

omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

substantially uniform size of about 200 µm or less, wherein the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule

As discussed above, the written descriptive support in the '148 patent for this claim element (which was the supposed point of novelty) was copied almost verbatim from Quake. The table below shows the correspondence between the relevant disclosures in the Quake PCT and the Ismagilov provisional application and '148 patent.

Quake PCT (published March 21, 2002) (Ex. 1034)	**Ismagilov provisional application (filed May 9, 2002) (Ex. 1003) and '148 patent (Ex. 1001)**
34:32-35:7 The pressure at the droplet extrusion region can also be regulated by adjusting the pressure on the main and sample inlets, for example, with pressurized syringes feeding into those inlets. By controlling the pressure difference between the oil and water sources at the droplet extrusion region, the size and periodicity of the droplets generated may be regulated. Alternatively, a valve may be placed at or coincident to either the droplet extrusion region or the sample inlet connected thereto to control the flow of solution into the droplet extrusion region, thereby controlling the size and periodicity of the droplets.	Provisional at 22:12-19, '148 patent at 21:56-65 The pressure at the plug-forming region can also be regulated by adjusting the pressure on the main and sample inlets, for example with pressurized syringes feeding into those inlets. By controlling the difference between the oil and water flow rates at the plug-forming region, the size and periodicity of the plugs generated may be regulated. Alternatively, a valve may be placed at or coincident to either the plug forming region or the sample inlet connected thereto to control the flow of solution into the plug-forming region, thereby controlling the size and periodicity of the plugs.

The disclosure of Quake was summarized by the Patent Trial and Appeal Board in its November 15, 2015 decision. Therein the Board found that Quake discloses a droplet reactor in which droplets are formed by continuous streams of immiscible fluids:

> Quake relates to microfluidic devices and methods for analyzing and/or sorting biological materials. Ex. 1004, Abstract [which corresponds to Ex. 1033]….
> The devices comprise a main channel, through which a pressurized stream of oil is passed, and at least one sample inlet channel, through which a pressurized

stream of aqueous solution is passed. *Id.* A junction joins the main channel with the sample inlet channel. *Id.* By adjusting the pressure of the oil and/or the aqueous solution, a pressure difference can be established such that the stream of aqueous solution is sheared off at a regular frequency as it enters the oil stream, thereby forming droplets. *Id.* Figure 16A of Quake is reproduced below:

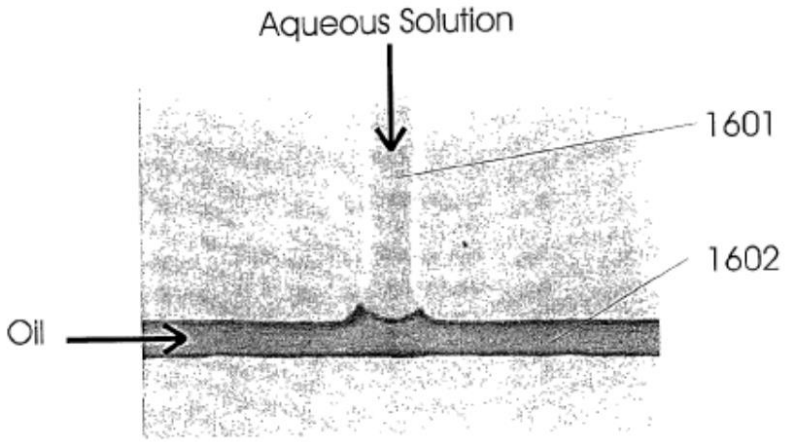


FIG. 16A

Figure 16A depicts the channel architecture for the droplet extrusion region. Channel 1601 containing an aqueous solution intersects with the main channel 1602 containing oil. [*Id.*] ¶ 292.

In preferred embodiments, the droplets have a volume of approximately 0.1 to 100 picoliters. *Id.* According to Quake, microfabrication of the device "permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." *Id.* ¶ 80.

Ex. 1052 at 11-12.

The Patent Owner did not contest in the *inter partes* review proceeding that Quake met the plug-formed-by-continuous-streams limitations of the '148 patent. Ex. 1051 *passim*; Ex. 1052, *passim*. Moreover, Quake himself during prosecution characterized his invention as involving continuous streams.[75] Accordingly, notwithstanding statements to the contrary during

[75] When distinguishing his invention over one of the embodiments of Shaw Stewart in which reagent is periodically injected, Quake noted that his "invention relates, in part, to the discovery that combining a flowing stream of an

ex parte prosecution of the '148 patent (discussed in Section IV.C above) it is clear that Quake does in fact teach forming plugs from continuous streams of immiscible fluids.

Turning to the recitation "each having a substantially uniform size of about 200 μm or less," Quake teaches that "the droplets have a diameter that is smaller than the diameter of the microchannel; i.e., preferably less than 60 μm." Ex. 1034 at 27:29-30. Fig. 21 of Quake shows the droplets sizes generated by the Quake droplet generator were all less than 60 μm.

This leaves the recitation that "the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule." As discussed above, in the combined method of Quake, Corbett and Lagally, the samples are separated into a number of discrete droplets. That has the advantages of i) overcoming the signal-to-noise limitations associated with certain other PCR techniques and ii) enabling the detection of relatively rare mutations, dislocations and allelic imbalances. Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Lagally expressly teaches that "[t]he event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a **Poisson** distribution, demonstrating that we are operating at the single-molecule level." Ex. 1028 at 566. Vogelstein likewise teaches that the aforementioned advantages are realized by **reducing the number of sample molecules per droplet to zero or one.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶82**.** That ensures that any amplification that occurs in a given droplet can be readily identified as containing the target sequence. *Id.* Thus, in the combined method the droplets have either one or zero template molecules.

aqueous solution and a flowing stream of an immiscible fluid (e.g., decane) it is possible to produce small, discrete, monodisperse droplets of the aqueous solution flowing in the second fluid." Ex. 1058 at September 25, 2006 response, p. 15.

As suggested by Lagally, any process which is generating droplets containing about one template molecule per droplet is expected to conform to a random, or Poisson, distribution. Ex. 1028 at 566; Ex. 1004 ¶48. It was known in the art that "[i]f a solution containing x molecules of enzyme per ml is dispersed into droplets of v ml volume, the average number of enzyme molecules per droplet n is equal to vx. Because of statistical fluctuations each droplet will not have exactly n enzyme molecules. The probability p(r) of finding 0, 1, 2, 3, . .. , r enzyme molecules in a droplet **is given by the Poisson law**." Ex. 1112 at 1985 [emphasis added]; see also Ex. 1111 at 1667 ("Spectra observed in a time sequence for an average of 0.6 dye molecule in the probed volume exhibited the expected Poisson distribution for actually measuring 0, 1, 2, or 3 molecules.").

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1066.

Claim 1[d]: providing conditions suitable for the reaction in the at least one plug involving the at least one biological molecule and the at least one reagent to form a reaction product.

Quake teaches that "droplets of the sample fluid containing the biological material for analysis, **reaction** or sorting are sheared at the droplet extrusion region into the flow of the extrusion fluid in the main channel." Ex. 1034 at 7:30-8:5 [emphasis added]. "Microfabrication permits other technologies to be integrated or combined with flow cytometry on a single chip, such as **PCR** [i.e., a reaction]." Ex. 1034 at 23:26-30 [emphasis added].

As discussed above in connection with element 1[b], in the instant combination Quake is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or

RNA molecules and at least one other molecule").[76] These limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Quake) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1066.

Thus, claim 1 is shown to be rendered obvious by the combination of Quake, Kopp and Burns II.

Dependent Claim 2

Claim 2 of the '148 patent recites "wherein the step of providing conditions includes heating."

As discussed above in connection claim elements 1[b] and 1[c], in the combined method (and in virtually any other PCR reaction) the DNA and polymerase undergo polymerase chain reaction when heated. Ex. 1004 ¶32; Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68.)

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1066.

Thus, claim 2 is shown to be rendered obvious by the combination of Quake, Kopp and Burns II.

Dependent Claims 3-5

Claim 3 of the '148 patent recites "providing a detector to detect, analyze, characterize, or monitor the target DNA or RNA molecule in the at least one member of the plurality of plugs

[76] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

during and/or after the reaction has occurred." Claim 4 of the '148 patent further recites "wherein the at least one other molecule includes one or more fluorescent labels and the detector is configured to monitor progress of the reaction by detecting fluorescence emissions from the one or more fluorescent labels." Claim 5 recites "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels."

Quake discloses that "[t]he device of the invention may also comprise a **detection region** which is within or coincident with at least a portion of the main channel at or downstream of the droplet extrusion region. The device may also have a detector, preferably an **optical detector** such as a microscope, associated with the detection region." Ex. 1034 at 6:28-31, [emphasis added]. "A preferred 'forward' sorting algorithm consists of running molecules, cells or virions from the input channel to the waste channel, until a molecule, cell or virion is identified to have an optically detectable signal *(e.g.* **fluorescence**) that is above a pre-set threshold, at which point voltages are temporarily changed to electro-osmotically **divert the molecule [] to the collection channel**." *Id.* at 20:22-26, [emphasis added].

Burns II teaches that "[c]apillary gel electrophoresis of DNA samples was performed using a Beckman P/ACE instrument with a laser-induced fluorescence detector and 37 cm length, 100 μm diameter, linear polymerase gel capillary according to manufacturer's recommendation." Ex. 1008 at 5558. The results of capillary electrophoresis were generally detected optically at the time of filing. Ex. 1011 at 46 (Dolnick). "The amplified product, labeled with an intercalating **fluorescent** dye, is directly injected into the gel-filled capillary channel for electrophoretic analysis." Ex. 1028 at 1, [emphasis added].

As to claim 5, under the broadest reasonable interpretation the phrase "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels"

requires simply that at least some of the droplets or plugs are transmitted to an outlet or other channel. Quake teaches an outlet channel as discussed above in connection with claim 1. See also Ex. 1034 at Fig. 16A, outlet 1602. If claim 5 is more narrowly interpreted as requiring selective diversion of the droplets, is also met by Quake. As noted above, Quake teaches that "[a] preferred 'forward' sorting algorithm consists of running molecules, cells or virions from the input channel to the waste channel, until a molecule, cell or virion is identified to have an optically detectable signal (e.g. fluorescence) that is above a pre-set threshold, at which point voltages are temporarily changed to electro-osmotically divert the molecule [] to the collection channel." *Id.* at 20:22-26. A skilled artisan would have considered it obvious to employ such a technique to sort droplets after they are subjected to the optical detection. Ex. 1004 ¶68,69. For instance, a skilled artisan would have found it advantageous to sort and collect droplets which tested positive or negative for the presence of a certain substance or property, especially in the context of droplet generators which are used to encapsulate on average one template molecule per droplet. *Id.* ¶82.

In the combined method of Quake, Kopp and Burns II, microfluidic droplet reactors are used to perform PCR reactions after which the reaction products are detected optically and sorted by diverting certain droplets into collection channels. Ex. 1004 ¶69.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1066.

Thus, claims 3-5 are shown to be rendered obvious by the combination of Quake, Kopp and Burns II.

Dependent Claims 6-8

Claim 6 of the '148 patent recites "wherein the oil is fluorinated oil." Claim 7 of the '148 patent recites "wherein the carrier fluid further comprises a surfactant." Claim 8 of the '148 patent recites "wherein the surfactant is a fluorinated surfactant."

Quake discloses that "the first phase or fluid which flows through the main channel can be a non-polar solvent, such as decane (e.g., tetradecane or hexadecane) or another oil (for example, **mineral oil**)." Ex. 1034 at 6:8-10, [emphasis added]. "The fluids used in the invention may contain additives, such as agents which reduce surface tensions (surfactants). **Exemplary surfactants** include Tween, Span, **fluorinated oils**, and other agents that are soluble in oil relative to water. Surfactants may aid in controlling or optimizing droplet size, flow and uniformity, for example by reducing the shear force needed to extrude or inject droplets into an intersecting channel." *Id.* at 35:18-22, [emphasis added].

Additional correspondence between these claim elements and the cited references is shown in the claim chart submitted herewith as Exhibit 1066 and discussed in the Declaration of Dr. Shaqfeh (Ex. 1004).

Thus, claims 6-8 are shown to be rendered obvious by the combination of Quake, Kopp and Burns II.

F. The Combination of Quake, Kopp and Vogelstein Renders Obvious Claims 1-8 of the '148 Patent Under (pre-AIA) 35 U.S.C. § 103(a)

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Quake), ii) microfluidic PCR devices were well known (Kopp and Vogelstein), and iii) there were compelling reasons to use the microfluidic droplet reactors to conduct microfluidic PCR (Kopp, Burns II, Lagally and Vogelstein).

As to the first feature – forming plugs from continuously flowing immiscible fluids – the '148 patent's teachings in this regard were copied almost verbatim from the Quake PCT (Ex. 1034). The table below compares the Quake PCT publication with the Ismagilov specification, which was filed about three months later.

Quake PCT (published March 21, 2002) (Ex. 1034)	**Ismagilov provisional application (filed May 9, 2002) (Ex. 1003) and '148 patent (Ex. 1001)**
34:32-35:7 The pressure at the droplet extrusion region can also be regulated by adjusting the pressure on the main and sample inlets, for example, with pressurized syringes feeding into those inlets. By controlling the pressure difference between the oil and water sources at the droplet extrusion region, the size and periodicity of the droplets generated may be regulated. Alternatively, a valve may be placed at or coincident to either the droplet extrusion region or the sample inlet connected thereto to control the flow of solution into the droplet extrusion region, thereby controlling the size and periodicity of the droplets.	Provisional at 22:12-19, '148 patent at 21:56-65 The pressure at the plug-forming region can also be regulated by adjusting the pressure on the main and sample inlets, for example with pressurized syringes feeding into those inlets. By controlling the difference between the oil and water flow rates at the plug-forming region, the size and periodicity of the plugs generated may be regulated. Alternatively, a valve may be placed at or coincident to either the plug forming region or the sample inlet connected thereto to control the flow of solution into the plug-forming region, thereby controlling the size and periodicity of the plugs.

Attached as Ex. 1035 is a table showing the numerous passages which Ismagilov copied key disclosures verbatim or nearly verbatim from the Quake PCT. These were not merely background or "state of the art" descriptions; rather, they go to the heart of the claimed subject matter.

The disclosure of Quake was summarized by the Patent Trial and Appeal Board in its November 15, 2015 decision. Therein the Board found that Quake discloses a droplet reactor in which droplets are formed by continuous streams of immiscible fluids:

> Quake relates to microfluidic devices and methods for analyzing and/or sorting biological materials. Ex. 1004, Abstract [which corresponds to Ex. 1033]….

> The devices comprise a main channel, through which a pressurized stream of oil is passed, and at least one sample inlet channel, through which a pressurized stream of aqueous solution is passed. *Id.* A junction joins the main channel with the sample inlet channel. *Id.* By adjusting the pressure of the oil and/or the aqueous solution, a pressure difference can be established such that the stream of aqueous solution is sheared off at a regular frequency as it enters the oil stream, thereby forming droplets. *Id.* Figure 16A of Quake is reproduced below:

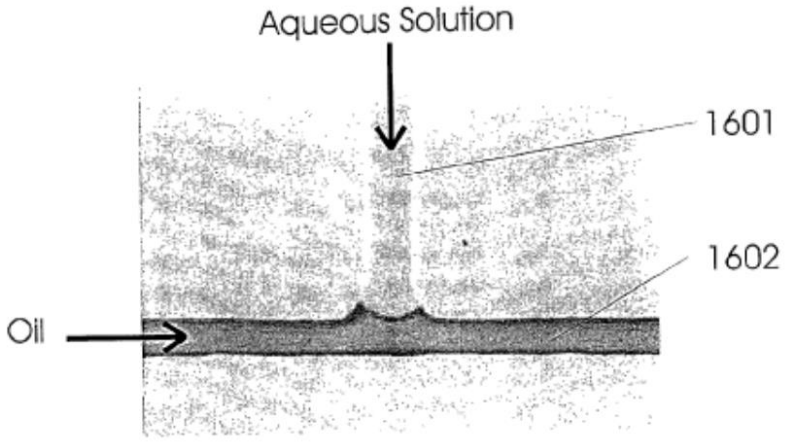


FIG. 16A

> Figure 16A depicts the channel architecture for the droplet extrusion region. Channel 1601 containing an aqueous solution intersects with the main channel 1602 containing oil. [*Id.*] ¶ 292.
>
> In preferred embodiments, the droplets have a volume of approximately 0.1 to 100 picoliters. *Id.* According to Quake, microfabrication of the device "permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." *Id.* ¶ 80.

Ex. 1052 at 11-12.

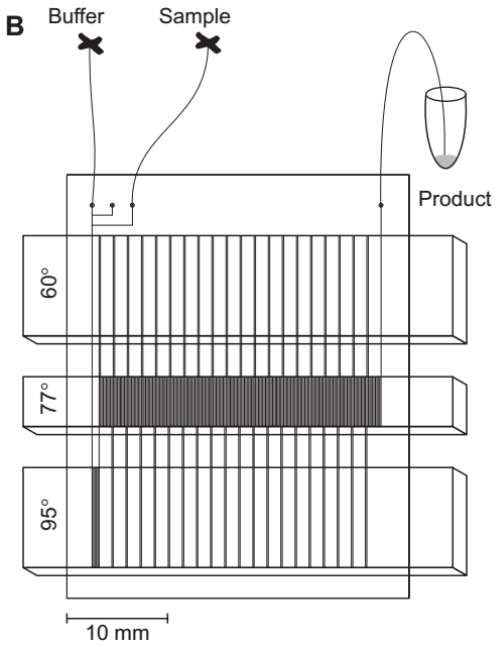
The Patent Owner did not contest in the *inter partes* review proceeding that Quake met the plug-formed-by-continuous-streams limitations of the '148 patent. Ex. 1051 *passim*; Ex. 1052, *passim*. Moreover, Quake himself during prosecution characterized his invention as

involving continuous streams.[77] Accordingly, notwithstanding statements to the contrary during *ex parte* prosecution of the '148 patent (discussed in Section IV.C above) it is clear that Quake does in fact teach forming plugs from continuous streams of immiscible fluids.

Turning the second issue, as of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Kopp and Vogelstein. By the late 1990s Kopp et al. had successfully implemented continuous flow PCR on a chip. In his 1988 article (not previously presented to the USPTO), Kopp explained that "[a] wide range of microreactors, microcapillary electrophoresis devices, and microcell manipulation devices have been described in recent years." Ex. 1027 at 1046. Building on that body of work, Kopp's group developed

> [a] micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in **continuous flow** at high speed. The device is analogous to an electronic amplifier and relies on the movement of sample through thermostated temperature zones on a glass microchip. Input and output of material (DNA) is **continuous**, and amplification is independent of input concentration. . . .
>
> The results demonstrate that with a very simple device and virtually no optimization, PCR can be performed in **continuous flow**, yielding product quality and quantity comparable to standard thermal cycling methods (Fig. 2B). . . .
>
> We suggest that cross-contamination in a **continuous-flow** format may be much less of a problem than in a stationary-tube PCR format. Thus, a flow-through PCR chip can be reused for extended periods without major cleaning. . . .

[77] When distinguishing his invention over one of the embodiments of Shaw Stewart in which reagent is periodically injected, Quake noted that his "invention relates, in part, to the discovery that combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid (e.g., decane) it is possible to produce small, discrete, monodisperse droplets of the aqueous solution flowing in the second fluid." Ex. 1058 at September 25, 2006 response, p. 15.



Ex. 1027 at 1046-1047 [emphasis added]. Kopp *et al.* also explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications:

> To build a complex system analogous to an electronic integrated circuit, it is necessary to integrate a variety of other continuously working parts. A cursory glance at developments in miniaturized total analysis systems (uTAS) during the past 5 years reveals that most of the relevant components, such as **continuous-flow mixers** (8), **continuous-flow microreactors** (9), **continuous separations** (10), and high-speed capillary gel electrophoresis (as a conventional batch process), have already been presented (11). Accordingly, the **continuous-flow microamplifier** should allow the creation of highly elaborate analysis and synthesis systems.
>
> Although chemical amplification reactors will obviously not be applicable to all reactions, neither will they be limited to PCR. Devices of this kind open up whole new areas of application in medical diagnostics. For example, the online amplification and monitoring of a specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy.

Ex. 1027 at 1047 [emphasis added].

By 1999, Vogelstein had reported **various applications for single-molecule microfluidic PCR**. Ex. 1044 at 9241. In a seminal article which has been cited over by over 1,000 other publications, Vogelstein explained that PCR amplification of small aliquots or droplets containing single molecules was effective for "detection of a small number of mutant-containing cells among a large excess of normal cells." *Id.* at 9236. In Vogelstein's method, the DNA was diluted into 7 µl volumes such that, on average, there was one template molecule for every two wells (i.e., approximately half of the wells contained no DNA template molecule and approximately half contained a single DNA template molecule). *Id.* at 9236, 9237. This avoided the problem of background noise impairing the determination of whether each volume contained the mutant sequence. *Id.* By counting the number of volumes which indicated wild type or mutant templates, the presence or absence of the mutation (e.g., a sequence associated with colorectal cancer) can be determined. *Id.* at 9238. The accuracy of the test is a function of the number of discrete volumes into which the sample is diluted. *Id.* at 9239. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. *Id.* at 9239. ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude.") *Id.* 9239. The article explained that the same technique would be useful for **detecting a wide array of mutations, dislocations and allelic imbalances**, as summarized in Table 1. *Id.* at 9241. Vogelstein thus taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby overcoming the signal-to-noise limitations associated with certain other PCR

techniques and enabling the detection of relatively rare mutations, dislocations and allelic imbalances. Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84.

In sum, Kopp and Vogelstein show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Quake is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[78] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Quake) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

Quake **specifically suggests that his microfluidic device could be used to perform PCR**. Quake explained that "[m]icrofabrication permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." Ex. 1034 at 23:26-27. Quake noted that "PCR is [] generally the method of choice to detect viral DNA or RNA directly in clinical specimens. The advantage of PCR for viral diagnostics is its high sensitivity; PCR can detect very low numbers of viruses in a small clinical specimen." *Id.* at 2:31-3:2.

More generally, a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake, to conduct microfluidic PCR as taught by Kopp and Vogelstein. A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic droplet reactor of Quake because doing so have provided the substantial

[78] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Quake to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Quake would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026

(Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Quake to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Kopp or Vogelstein) to

incorporate a droplet reactor (Quake). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Kopp or Vogelstein. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Kopp). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors

> were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of

> single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority

date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Quake could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful modifying Kopp or Lagally to work in the microfluidic droplet reactor of Quake, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e).

Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[79] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail below, in the accompanying claim chart (Ex. 1067) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake to conduct microfluidic PCR as taught by Kopp and Vogelstein.

Independent Claim 1

Claim 1[a]: A method comprising the steps of: providing a microfluidic system comprising one or more channels

Quake discloses "[a] microfluidic device for analyzing and/or sorting biological materials (e.g., molecules such as polynucleotides and polypeptides, including proteins and enzymes; viruses and cells) and methods for its use are provide[d]. . . . Ex. 1034 at Abstract. For instance, **in those embodiments where aqueous droplets are used as microreactors for chemical reactions (including biochemical reactions) or are used to analyze and/or sort biomaterials**, a water soluble surfactant such as SDS may denature or inactivate the contents of the droplet." Ex. 1034 at 28:20-23 [emphasis added].

[79] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

Quake's device comprises a main channel, through which a pressurized stream of oil is passed, and at least one sample inlet channel, through which a pressurized stream of aqueous solution is passed: "The channel architecture for the droplet extrusion region of the first device is shown in FIG. 16A. In this device, the inlet channel 1601 (inner diameter 30 μm) intersects the main channel 1602 (inner diameter 30 μm) at a T-intersection (i.e., an angle perpendicular to the main channel). Other intersections and angles may be used. The walls of the inlet and main channels were not tapered in this device." Ex. 1034 at 79:23-28.

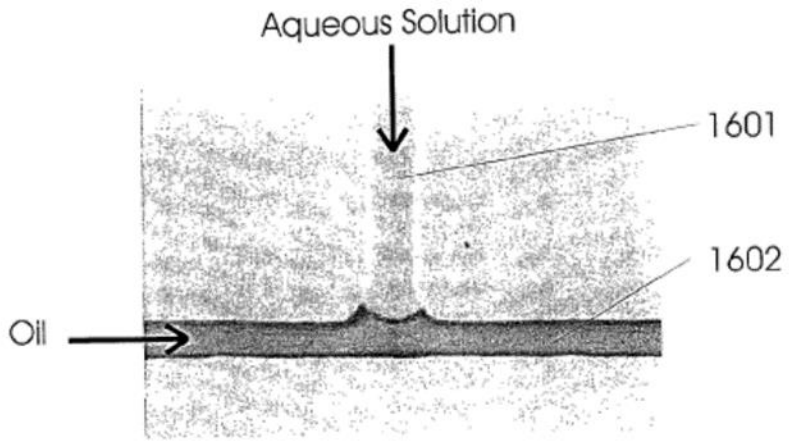


FIG. 16A

During the *inter partes* review proceeding, Patent Owner did not contest that Quake met this limitation. Ex. 1051, *passim*. The PTAB appears to have concluded that this limitation was met by Quake. Ex. 1052, *passim*.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1067.

Claim 1[b]: providing within the one or more channels a continuously flowing carrier fluid comprising an oil and a continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule in the fluid that can react with the target DNA or RNA molecules under conditions in which the target DNA or RNA molecules and the other molecules in the fluid do not react with each other

Quake teaches that "[a] first fluid flows through the main channel, and a second fluid, which is incompatible or immiscible with the second fluid, passes through the inlet region so that droplets of the second fluid are sheared into the main channel. … The second phase or fluid which passes through the inlet region can be an **aqueous solution**, for example ultra pure water, TE buffer, phosphate buffer saline and acetate buffer. The second fluid may also contain a **biological sample** (e.g., molecules of an enzyme or a substrate, or one or more cells, or one or more viral particles) for analysis or sorting in the device. In preferred embodiments the second fluid includes a biological sample that comprises one or more **molecules**, cells, virions or particles." Ex. 1034 at 6:6-16 [emphasis added]. Quake further teaches that "[i]n preferred embodiments, a first fluid, which may be referred to as an 'extrusion' or 'barrier' fluid, passes (i.e., flows) through the main channel of the device and a second fluid, referred to as a "sample" or "droplet" fluid, passes or flows through the inlet region. ... Thus, droplets of the sample fluid containing the biological material for analysis, reaction or sorting are sheared at the droplet extrusion region into the flow of the extrusion fluid in the main channel." *Id.* at 7:30-8:5.

Regarding Quake's disclosure of the "continuous" limitation, see the discussion set forth at element 1[c], below.

In the instant combination, the microfluidic droplet reactor of Quake is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[80] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Quake) to perform PCR reactions (as taught in Kopp and

[80] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

Vogelstein), wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

Quake **specifically suggests that his microfluidic device could be used to perform PCR**. Quake explained that "[m]icrofabrication permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." Ex. 1034 at 23:26-27. Quake noted that "PCR is [] generally the method of choice to detect viral DNA or RNA directly in clinical specimens. The advantage of PCR for viral diagnostics is its high sensitivity; PCR can detect very low numbers of viruses in a small clinical specimen." *Id.* at 2:31-3:2.

More generally, a skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic droplet reactor of Quake because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. Likewise, the reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such

portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Quake to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Quake would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004

¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
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Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Quake to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Kopp or Vogelstein) to incorporate a droplet reactor (Quake). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Kopp or Vogelstein. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Kopp). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers,

continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with

> polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ∼1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic

> diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent

with the observations of Kopp and Burns II, a skilled artisan would have expected that the microfluidic droplet reactor of Quake could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful in modifying Kopp or Lagally to work in the microfluidic droplet reactor of Quake, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[81] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained further in the accompanying claim chart (Ex. 1067) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled

[81] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Quake to conduct microfluidic PCR as taught by Kopp and Vogelstein.

With regard to the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other," this is merely a description of a PCR reaction. Ex. 1004 ¶31,32. As explained in Corbett, Kopp, Burns II, and Lagally, in a PCR reaction the sample DNA or RNA is admixed with various reagents including polymerase under conditions in which no reaction occurs; only when heat is applied does the PCR reaction occur. Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68. In the PCR of the combined method, as in virtually any PCR method, the DNA and polymermase do not react until mixture of reagents is heated. *Id*.; see also Ex. 1004 ¶32. The combined method thus meets the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other" this is merely a description of a PCR reaction. Ex. 1004 ¶31,32.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1067.

Claim 1[c]: controlling flow rates of said aqueous fluid and said carrier fluid to partition the continuously flowing aqueous fluid with the continuously flowing carrier fluid to form a plurality of plugs of the aqueous fluid, each having a substantially uniform size of about 200 µm or less, wherein the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule

As discussed above, the written descriptive support in the '148 patent for this claim element (which was the supposed point of novelty) was copied almost verbatim from Quake. The table below shows the correspondence between the relevant disclosures in the Quake PCT and the Ismagilov provisional application and '148 patent.

Quake PCT (published March 21, 2002) (Ex. 1034)	**Ismagilov provisional application (filed May 9, 2002) (Ex. 1003) and '148 patent (Ex. 1001)**
34:32-35:7 The pressure at the droplet extrusion region can also be regulated by adjusting the pressure on the main and sample inlets, for example, with pressurized syringes feeding into those inlets. By controlling the pressure difference between the oil and water sources at the droplet extrusion region, the size and periodicity of the droplets generated may be regulated. Alternatively, a valve may be placed at or coincident to either the droplet extrusion region or the sample inlet connected thereto to control the flow of solution into the droplet extrusion region, thereby controlling the size and periodicity of the droplets.	Provisional at 22:12-19, '148 patent at 21:56-65 The pressure at the plug-forming region can also be regulated by adjusting the pressure on the main and sample inlets, for example with pressurized syringes feeding into those inlets. By controlling the difference between the oil and water flow rates at the plug-forming region, the size and periodicity of the plugs generated may be regulated. Alternatively, a valve may be placed at or coincident to either the plug forming region or the sample inlet connected thereto to control the flow of solution into the plug-forming region, thereby controlling the size and periodicity of the plugs.

The disclosure of Quake was summarized by the Patent Trial and Appeal Board in its November 15, 2015 decision. Therein the Board found that Quake discloses a droplet reactor in which droplets are formed by continuous streams of immiscible fluids:

> Quake relates to microfluidic devices and methods for analyzing and/or sorting biological materials. Ex. 1004, Abstract [which corresponds to Ex. 1033]….
> The devices comprise a main channel, through which a pressurized stream of oil is passed, and at least one sample inlet channel, through which a pressurized stream of aqueous solution is passed. *Id.* A junction joins the main channel with the sample inlet channel. *Id.* By adjusting the pressure of the oil and/or the aqueous solution, a pressure difference can be established such that the stream of aqueous solution is sheared off at a regular frequency as it enters the oil stream, thereby forming droplets. *Id.* Figure 16A of Quake is reproduced below:

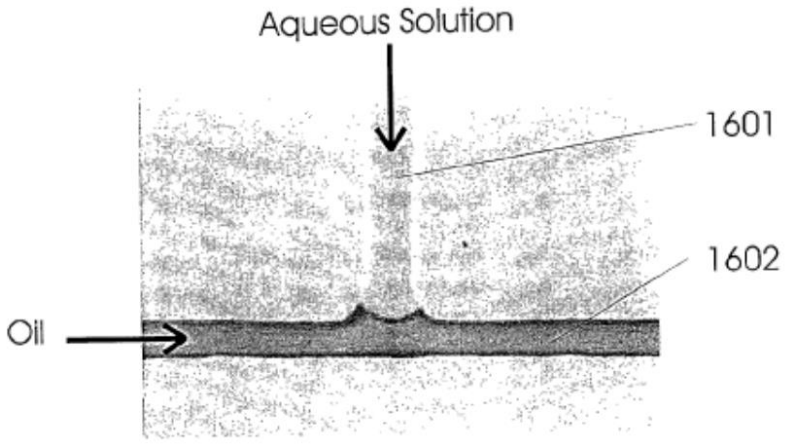


FIG. 16A

> Figure 16A depicts the channel architecture for the droplet extrusion region. Channel 1601 containing an aqueous solution intersects with the main channel 1602 containing oil. [*Id.*] ¶ 292.
>
> In preferred embodiments, the droplets have a volume of approximately 0.1 to 100 picoliters. *Id.* According to Quake, microfabrication of the device "permits other technologies to be integrated or combined with flow cytometry on a single chip, such as PCR." *Id.* ¶ 80.

Ex. 1052 at 11-12.

The Patent Owner did not contest in the *inter partes* review proceeding that Quake met the plug-formed-by-continuous-streams limitations of the '148 patent. Ex. 1051 *passim*; Ex. 1052, *passim*. Moreover, Quake himself during prosecution characterized his invention as involving continuous streams.[82] Accordingly, notwithstanding statements to the contrary during *ex parte* prosecution of the '148 patent (discussed in Section IV.C above) it is clear that Quake does in fact teach forming plugs from continuous streams of immiscible fluids.

[82] When distinguishing his invention over one of the embodiments of Shaw Stewart in which reagent is periodically injected, Quake noted that his "invention relates, in part, to the discovery that combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid (e.g., decane) it is possible to produce small, discrete, monodisperse droplets of the aqueous solution flowing in the second fluid." Ex. 1058 at September 25, 2006 response, p. 15.

Turning to the recitation "each having a substantially uniform size of about 200 μm or less," Quake teaches that "the droplets have a diameter that is smaller than the diameter of the microchannel; i.e., preferably less than 60 μm." Ex. 1034 at 27:29-30. Fig. 21 of Quake shows the droplets sizes generated by the Quake droplet generator were all less than 60 μm.

This leaves the recitation that "the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule." As discussed above, in the combined method of Quake, Corbett and Lagally, the samples are separated into a number of discrete droplets. That has the advantages of i) overcoming the signal-to-noise limitations associated with certain other PCR techniques and ii) enabling the detection of relatively rare mutations, dislocations and allelic imbalances. Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Lagally expressly teaches that "[t]he event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a **Poisson** distribution, demonstrating that we are operating at the single-molecule level." Ex. 1028 at 566 [emphasis added]. Vogelstein likewise teaches that the aforementioned advantages are realized by **reducing the number of sample molecules per droplet to zero or one.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶82**.** That ensures that any amplification that occurs in a given droplet can be readily identified as containing the target sequence. *Id.* Thus, in the combined method the droplets have either one or zero template molecules.

As suggested by Lagally, any process which is generating droplets containing about one template molecule per droplet is expected to conform to a random, or Poisson, distribution. Ex. 1028 at 566; Ex. 1004 ¶48. It was known in the art that "[i]f a solution containing x molecules of

enzyme per ml is dispersed into droplets of v ml volume, the average number of enzyme molecules per droplet n is equal to vx. Because of statistical fluctuations each droplet will not have exactly n enzyme molecules. The probability p(r) of finding 0, 1, 2, 3, . .. , r enzyme molecules in a droplet **is given by the Poisson law**." Ex. 1112 at 1985 [emphasis added]; see also Ex. 1111 at 1667 ("Spectra observed in a time sequence for an average of 0.6 dye molecule in the probed volume exhibited the expected Poisson distribution for actually measuring 0, 1, 2, or 3 molecules.").

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1067.

Claim 1[d]: providing conditions suitable for a polymerase-chain reaction in at least one plug of the plurality of plugs such that the target DNA or RNA is amplified

Quake teaches that "droplets of the sample fluid containing the biological material for analysis, **reaction** or sorting are sheared at the droplet extrusion region into the flow of the extrusion fluid in the main channel." Ex. 1034 at 7:30-8:5 [emphasis added]. "Microfabrication permits other technologies to be integrated or combined with flow cytometry on a single chip, such as **PCR** [i.e., a reaction]." Ex. 1034 at 23:26-30 [emphasis added].

As discussed above in connection with element 1[b], in the instant combination Quake is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[83] These limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in

[83] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

Quake) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1067.

Thus, claim 1 is shown to be rendered obvious by the combination of Quake, Kopp and Vogelstein.

Dependent Claim 2

Claim 2 of the '148 patent recites "wherein the step of providing conditions includes heating."

As discussed above in connection claim elements 1[b] and 1[c], in the combined method (and in virtually any other PCR reaction) the DNA and polymerase undergo polymerase chain reaction when heated. Ex. 1004 ¶32; Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1067.

Thus, claim 2 is shown to be rendered obvious by the combination of Quake, Kopp and Vogelstein.

Dependent Claims 3-5

Claim 3 of the '148 patent recites "providing a detector to detect, analyze, characterize, or monitor the target DNA or RNA molecule in the at least one member of the plurality of plugs during and/or after the reaction has occurred." Claim 4 of the '148 patent further recites "wherein the at least one other molecule includes one or more fluorescent labels and the detector is configured to monitor progress of the reaction by detecting fluorescence emissions from the

one or more fluorescent labels." Claim 5 recites "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels."

Quake discloses that "[t]he device of the invention may also comprise a **detection region** which is within or coincident with at least a portion of the main channel at or downstream of the droplet extrusion region. The device may also have a detector, preferably an **optical detector** such as a microscope, associated with the detection region." Ex. 1034 at 6:28-31, [emphasis added]. "A preferred 'forward' sorting algorithm consists of running molecules, cells or virions from the input channel to the waste channel, until a molecule, cell or virion is identified to have an optically detectable signal *(e.g.* **fluorescence**) that is above a pre-set threshold, at which point voltages are temporarily changed to electro-osmotically **divert the molecule [] to the collection channel**." *Id.* at 20:22-26, [emphasis added].

Vogelstein teaches that the "[r]eactions were analyzed immediately or stored at room temperature for up to 36 h before fluorescence analysis." Ex. 1044 at 9236.

As to claim 5, under the broadest reasonable interpretation the phrase "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels" requires simply that at least some of the droplets or plugs are transmitted to an outlet or other channel. Quake teaches an outlet channel as discussed above in connection with claim 1. See also Ex. 1034 at Fig. 16A, outlet 1602. If claim 5 is more narrowly interpreted as requiring selective diversion of the droplets, is also met by Quake. As noted above, Quake teaches that "[a] preferred 'forward' sorting algorithm consists of running molecules, cells or virions from the input channel to the waste channel, until a molecule, cell or virion is identified to have an optically detectable signal (e.g. fluorescence) that is above a pre-set threshold, at which point voltages are temporarily changed to electro-osmotically divert the molecule [] to the collection

channel." *Id.* at 20:22-26. A skilled artisan would have considered it obvious to employ such a technique to sort droplets after they are subjected to the optical detection. Ex. 1004 ¶68,69. For instance, a skilled artisan would have found it advantageous to sort and collect droplets which tested positive or negative for the presence of a certain substance or property, especially in the context of droplet generators which are used to encapsulate on average one template molecule per droplet. *Id.* ¶82.

In the combined method of Quake, Kopp and Vogelstein, microfluidic droplet reactors are used to perform PCR reactions after which the reaction products are detected optically and sorted by diverting certain droplets into collection channels. Ex. 1004 ¶69.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1067.

Thus, claims 3-5 are shown to be rendered obvious by the combination of Quake, Kopp and Vogelstein.

Dependent Claims 6-8

Claim 6 of the '148 patent recites "wherein the oil is a fluorinated oil." Claim 7 of the '148 patent recites "wherein the carrier fluid further comprises a surfactant." Claim 8 of the '148 patent recites "wherein the surfactant is a fluorinated surfactant."

Quake discloses that "the first phase or fluid which flows through the main channel can be a non-polar solvent, such as decane (e.g., tetradecane or hexadecane) or another oil (for example, **mineral oil**)." Ex. 1034 at 6:8-10, [emphasis added]. "The fluids used in the invention may contain additives, such as agents which reduce surface tensions (surfactants). **Exemplary surfactants** include Tween, Span, **fluorinated oils**, and other agents that are soluble in oil relative to water. Surfactants may aid in controlling or optimizing droplet size, flow and

uniformity, for example by reducing the shear force needed to extrude or inject droplets into an intersecting channel." *Id.* at 35:18-22, [emphasis added].

Additional correspondence between these claim elements and the cited references is shown in the claim chart submitted herewith as Exhibit 1067 and discussed in the Declaration of Dr. Shaqfeh (Ex. 1004).

Thus, claims 6-8 are shown to be rendered obvious by the combination of Quake, Kopp and Vogelstein.

G. The Combination of Shaw Stewart, Corbett and Lagally Renders Obvious Claims 1-8 of the '148 Patent Under (pre-AIA) 35 U.S.C. § 103(a)

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Shaw Stewart), ii) microfluidic PCR devices were well known (Corbett and Lagally), and iii) there were compelling reasons to use the microfluidic droplet reactors to conduct microfluidic PCR (Kopp, Burns II, Lagally and Vogelstein).

The Shaw Stewart British Application teaches conducting reactions in microdroplets formed from two continuously flowing immiscible fluids. The Shaw Stewart British Application discloses a "droplet reactor" and illustrates at Figure 1 a carrier phase 1 flowing along a conduit into which reagent 2 is injected, thereby forming droplet 4. Ex. 1040 at 1:1-20, 70-90. "[I]f large numbers of droplets are required, a continuous flow of reagent through the opening will be produced, while a continuous current of carrier phase flows down the tube. When each droplet

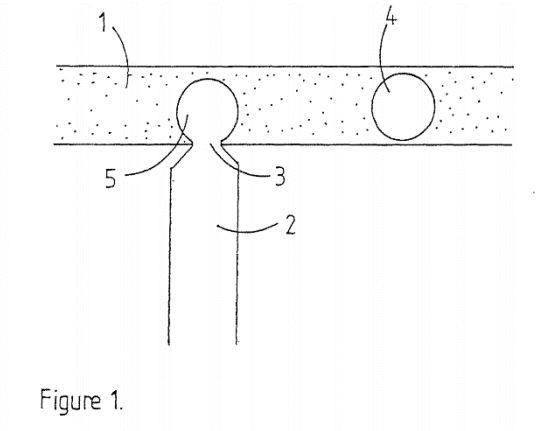


Figure 1.

almost spans the tube, it will be broken off. The exact size of the droplets produced will depend on the relative and absolute magnitude of the two currents." *Id.* at 1:83-90.

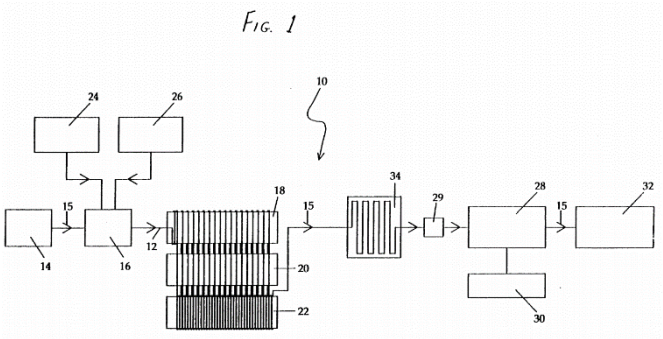
Significantly, the exclusive licensee of the '148 patent affirmatively (and correctly) took the position that Shaw Stewart teaches plug or droplet formation combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid. More particularly, Bio-Rad (who recently acquired RainDance, the original exclusive licensee of the '148 patent, Ex. 1061, Ex. 1053 ¶71) explained that another party had mischaracterized the Shaw Stewart reference by failing to point out the above-quoted portion of Shaw Stewart, in which Shaw Stewart specifically teaches that in certain embodiments droplets are formed from intersecting and continuously flowing streams.

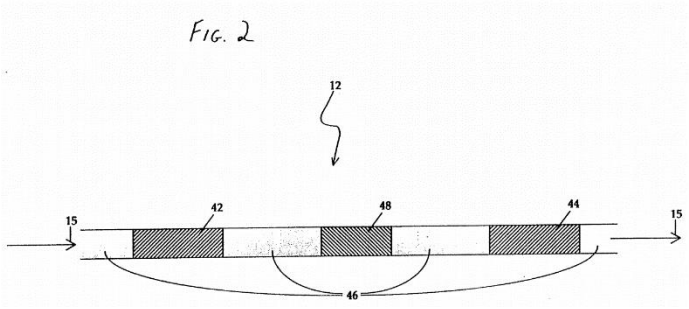
> To overcome Stewart I and secure allowance of their patent, the Patent Owner ("PO") mischaracterized the device disclosed in Stewart I (and hence Stewart II), misdirecting the Examiner to irrelevant portions of Stewart I while ignoring other portions that directly contradicted PO's arguments. *See, e.g.*, Ex. 1003 at 174-180. For example, one of PO's primary arguments was that Stewart I only disclosed introducing fluid in a "stepwise fashion," whereas in the '503 Patent droplets are formed by "combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid." *Id.* at 174. In truth, however, Stewart I also discloses combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid:
>
> > If large numbers of droplets are required, a continuous flow of reagent through the opening will be produced, while a continuous current of carrier phase flows down the tube. When each droplet almost spans the tube, it will be broken off.
>
> Ex. 1008, pg. 1, col. 2, ll. 83-88.

Ex. 1059 at 2-3.

Thus the exclusive licensee (Bio-Rad) expressly agreed that Shaw Stewart discloses forming plugs from intersecting streams of continuously flowing immiscible fluids.

As of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Corbett and Lagally. In 1991, about eleven years prior to the earliest effective filing date, Corbett disclosed using a microchannel or capillary device to conduct DNA amplification reactions such as PCR by injecting plugs of reactants into a carrier stream that is passed through different temperature zones. Corbett explained that as of 1991 "the most critical problem in applying nucleic acid amplification procedures, particularly in repetitive diagnostic assays in a clinical laboratory, is contamination of the reaction mixture by small amounts of DNA which contain sequences capable of being amplified under the conditions of assay." Ex. 1010 at 3:7-12."Automation of procedures minimize[s] the opportunity for such contamination and allows the implementation of stringent quality control." *Id.* at 3:15-19. *"*As is shown in FIG. 1, the apparatus 10 comprises tube 12 through which a fluid stream passes. As is more clearly shown in FIG. 2, the fluid stream includes pockets of reaction mixtures 42 and 44 and purging solution 48 separated by carrier fluid 46." *Id.* at 7:58-62.





"The pump is actuated and the reaction mixture of 20 µl or less is injected into the mineral oil or silicone oil carrier fluid and specific DNA sequences (whose limits are defined by the oligonucleotide primers) present in the sample is amplified as it passes cyclically through the temperature zones." Ex. 1010 at 9:35-40. Corbett thus demonstrates that it was known at least as of 1991 that reduction of contamination risk in PCR could be achieved by performing the PCR on microliter sized plugs of reactant in capillary tube which passes through various temperature zones. Ex. 1004 ¶77.

In 2001, in another article not previously considered by the Office, Lagally reported using a microfluidic droplet reactor to conduct single-molecule DNA amplification by PCR. Lagally summarized the state of the art as of February 2001, explaining continuous flow PCR microreactors had recently undergone substantial evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/µL, in volumes down to 1 µL.[11] Since then, stand-alone PCR reactors have been constructed in

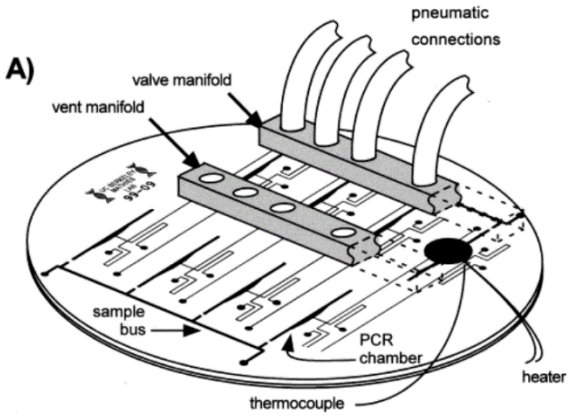
> silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

Lagally *et al.* developed a further improvement – a device and method for achieving single molecule amplification in a microchannel environment.

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control.

> The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> Microfluidic PCR-CE device. The PCR chambers are connected to a common sample bus through a set of valves. Hydrophobic vents at the other end of the PCR chambers are used to locate the sample and to eliminate gas. The PCR chambers are directly connected to the cross channel of the CE system for product injection and analysis. Two aluminum manifolds, one each for the vents and valves, are placed onto the respective ports and clamped in place using vacuum. The manifolds connect to external solenoid valves for pressure and vacuum actuation. Thermal cycling is accomplished using a resistive heater and a miniature thermocouple below the 280-nL chamber.



Ex. 1028 at 566 (see figure legend).

In sum, Corbett and Lagally show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Shaw Stewart are relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA

molecules and at least one other molecule").[84] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Shaw Stewart) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Shaw Stewart to conduct microfluidic PCR as taught by Corbett and Lagally. Stated a different way, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic droplet reactor of Shaw Stewart, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing.

A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic droplet reactor of Shaw Stewart because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004

[84] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

¶105, 107. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Shaw Stewart to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Shaw Stewart would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for

nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the

assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

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Application	Example	Probe 1 detects	Probe 2 detects
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Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Shaw Stewart to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Lagally) to incorporate a droplet reactor (Shaw Stewart). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Lagally. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the single fluid in microfluidic chip reactor (e.g., Lagally) or droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Corbett). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually

> transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .

> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an

autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactors Shaw Stewart could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful in miniaturizing and modifying Corbett or modifying Lagally to work in the microfluidic droplet reactor of Shaw Stewart, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[85] As discussed in Section VI.A.3, above, Higuchi I-III

[85] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail below, in the accompanying claim chart (Ex. 1068) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Shaw Stewart to conduct microfluidic PCR as taught by Corbett and Lagally. Ex. 1004 ¶¶109-112. Alternatively, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic droplet reactor of Shaw Stewart, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing. *Id.*

Independent Claim 1

Claim 1[a]: A method comprising the steps of: providing a microfluidic system comprising one or more channels

Shaw Stewart discloses that "[t]he method of the invention may be used for initiating and controlling a chemical reaction or preparing mixtures of reagents.…" Ex. 1040 at 1:7-9. "The system is particularly suited **to the manipulation of microscopic quantities of reagents, with volumes of less than one microlit[er]**,…" *Id.* at 1:20-22 [emphasis added].

As shown in Shaw Stewart's Figure 1, "[t]he reagent is introduced into a tube containing carrier phase (1) from a side arm (2) by being sucked or pushed through a small opening (3). When the correct volume has passed into the tube, it is separated and carried away by a current of carrier phase, down the tube." Ex. 1040 at 1:70-75.

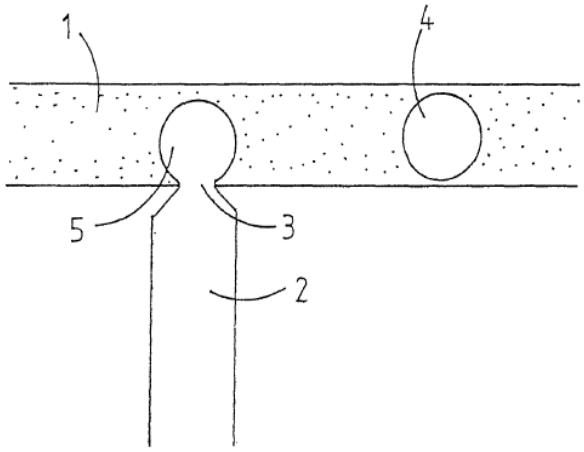

Figure 1.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1068 and in the Shaqfeh Declaration (Ex. 1004).

Claim 1[b]: providing within the one or more channels a continuously flowing carrier fluid comprising an oil and a continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule in the fluid that can react with the target DNA or RNA molecules under conditions in which the target DNA or RNA molecules and the other molecules in the fluid do not react with each other

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In the instant combination, the microfluidic droplet reactor of Shaw Stewart are relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[86] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Shaw Stewart) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

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provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Shaw Stewart to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

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Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

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increase the number of reactors that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

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> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching**

whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactors Shaw Stewart could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful in miniaturizing and modifying Corbett or modifying Lagally to work in the microfluidic droplet reactor of Shaw Stewart, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[87] As discussed in Section VI.A.3, above, Higuchi I-III

[87] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the

demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained further in the accompanying claim chart (Ex. 1068) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Shaw Stewart to conduct microfluidic PCR as taught by Corbett and Lagally. Ex. 1004 ¶¶109-112. Alternatively, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modifying it to incorporate the microfluidic droplet reactor of Shaw Stewart, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing. *Id*.

With regard to the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other," this is merely a description of a PCR reaction. Ex. 1004 ¶31,32. As explained in Corbett, Kopp, Burns II, and Lagally, in a PCR reaction the sample DNA or RNA is admixed with various reagents including polymerase under conditions in which no reaction occurs; only when heat is applied does the PCR reaction occur. Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68. In the PCR of the combined method, as in virtually any PCR method, the DNA and polymermase do not react until mixture

level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

of reagents is heated. *Id*.; see also Ex. 1004 ¶32. The combined method thus meets the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other" this is merely a description of a PCR reaction. Ex. 1004 ¶31,32.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1068.

Claim 1[c]: controlling flow rates of said aqueous fluid and said carrier fluid to partition the continuously flowing aqueous fluid with the continuously flowing carrier fluid to form a plurality of plugs of the aqueous fluid, each having a substantially uniform size of about 200 µm or less, wherein the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule

As discussed above, the Shaw Stewart British Application teaches conducting reactions in microdroplets formed from two continuously flowing immiscible fluids. Significantly, the exclusive licensee of the '148 patent affirmatively (and correctly) took the position that Shaw Stewart teaches plug or droplet formation combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid. More particularly, Bio-Rad (who recently acquired RainDance, the original exclusive licensee of the '148 patent, Ex. 1061, Ex. 1053 ¶71) explained that another party had mischaracterized the Shaw Stewart reference by failing to point out the above-quoted portion of Shaw Stewart, in which Shaw Stewart specifically teaches that in certain embodiments droplets are formed from intersecting and continuously flowing streams.

> To overcome Stewart I and secure allowance of their patent, the Patent Owner ("PO") mischaracterized the device disclosed in Stewart I (and hence Stewart II), misdirecting the Examiner to irrelevant portions of Stewart I while ignoring other portions that directly contradicted PO's arguments. *See, e.g.*, Ex. 1003 at 174-180. For example, one of PO's primary arguments was that Stewart I

> only disclosed introducing fluid in a "stepwise fashion," whereas in the '503 Patent droplets are formed by "combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid." *Id.* at 174. In truth, however, Stewart I also discloses combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid:
>
> > If large numbers of droplets are required, a continuous flow of reagent through the opening will be produced, while a continuous current of carrier phase flows down the tube. When each droplet almost spans the tube, it will be broken off.
>
> Ex. 1008, pg. 1, col. 2, ll. 83-88.

Ex. 1059 at 2-3.

Thus the exclusive licensee (Bio-Rad) expressly agreed that Shaw Stewart discloses forming plugs from intersecting streams of continuously flowing immiscible fluids.

Turning to the recitation "each having a substantially uniform size of about 200 µm or less," Shaw Stewart teaches that the volumes of the droplets are 1 microliter or less. Ex. 1040 at 1:20-23. 1,000,000 microliters are equal to one liter. One liter is equal to 1×10^{-3} cubic meters. Assuming the droplets are spheres, this equates to a droplet diameter of 1,200 µm. Shaw Stewart did not put a lower limit on the size of the droplets and contemplated droplet sizes below 200 µm. *Id.* Moreover, it must be noted that Shaw Stewart dates back to 1984, when microfabrication techniques were not as refined and channels could not readily be made as small as they could circa the year 2000. Quake (filed in the year 2000) teaches that "the droplets have a diameter that is smaller than the diameter of the microchannel; i.e., preferably less than 60 µm." Ex. 1034 at 27:29-30. Fig. 21 of Quake shows the droplets sizes generated by the Quake droplet generator were all less than 60 µm. Burns II (1996) similarly discloses uses of 60 nl droplets (which equates to a droplet diameter of about 62 µm). Ex. 1008 at 5558. Burns I (2001) discloses that the droplets are 100 µm to 400 µm in diameter. Ex. 1007 at 10. Accordingly, it would have been

obvious to a skilled artisan that as of the earliest claimed priority date (2002) droplets below 200 µm in diameter could, and should, be used in order to reduce reagent usage and reaction time and achieve the various other benefits described above with the combination. Ex. 1004 ¶36, 66.

This leaves the recitation that "the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule." As discussed above, in the combined method of Quake, Corbett and Lagally, the samples are separated into a number of discrete droplets. That has the advantages of i) overcoming the signal-to-noise limitations associated with certain other PCR techniques and ii) enabling the detection of relatively rare mutations, dislocations and allelic imbalances. Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Lagally expressly teaches that "[t]he event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a **Poisson** distribution, demonstrating that we are operating at the single-molecule level." Ex. 1028 at 566 [emphasis added]. Vogelstein likewise teaches that the aforementioned advantages are realized by **reducing the number of sample molecules per droplet to zero or one.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶82**.** That ensures that any amplification that occurs in a given droplet can be readily identified as containing the target sequence. *Id.* Thus, in the combined method the droplets have either one or zero template molecules.

As suggested by Lagally, any process which is generating droplets containing about one template molecule per droplet is expected to conform to a random, or Poisson, distribution. Ex. 1028 at 566; Ex. 1004 ¶48. It was known in the art that "[i]f a solution containing x molecules of enzyme per ml is dispersed into droplets of v ml volume, the average number of enzyme

molecules per droplet n is equal to vx. Because of statistical fluctuations each droplet will not have exactly n enzyme molecules. The probability p(r) of finding 0, 1, 2, 3, . .. , r enzyme molecules in a droplet **is given by the Poisson law**." Ex. 1112 at 1985 [emphasis added]; see also Ex. 1111 at 1667 ("Spectra observed in a time sequence for an average of 0.6 dye molecule in the probed volume exhibited the expected Poisson distribution for actually measuring 0, 1, 2, or 3 molecules.").

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1068.

Claim 1[d]: providing conditions suitable for a polymerase-chain reaction in at least one plug of the plurality of plugs such that the target DNA or RNA is amplified

Shaw Stewart discloses that "[r]egions of the device can be heated by electric circuits or coils or by electromagnetic radiation to allow the merged droplets to be incubated at the required temperature." Ex. 1040 at 2:44-47. "If a colour change reaction is involved, such a colour change can be recorded immediately or after incubation, using thermostatically controlled heating coil (27)." *Id.* at 3:58-60.

As discussed above in connection with element 1[b], in the instant combination Shaw Stewart is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[88] These limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Shaw Stewart) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

[88] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1068.

Thus, claim 1 is shown to be rendered obvious by the combination of Shaw Stewart, Corbett and Lagally.

Dependent Claim 2

Claim 2 of the '148 patent recites "wherein the step of providing conditions includes heating."

As discussed above in connection claim elements 1[b] and 1[c], in the combined method (and in virtually any other PCR reaction) the DNA and polymerase undergo polymerase chain reaction when heated. Ex. 1004 ¶32; Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1068.

Thus, claim 2 is shown to be rendered obvious by the combination of Shaw Stewart, Corbett and Lagally.

Dependent Claims 3-5

Claim 3 of the '148 patent recites "providing a detector to detect, analyze, characterize, or monitor the target DNA or RNA molecule in the at least one member of the plurality of plugs during and/or after the reaction has occurred." Claim 4 of the '148 patent further recites "wherein the at least one other molecule includes one or more fluorescent labels and the detector is configured to monitor progress of the reaction by detecting fluorescence emissions from the one or more fluorescent labels." Claim 5 recites "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels."

Shaw Stewart discloses that "parts of the device itself can be adapted to form the sample chambers of the standard instruments of chemical or biochemical analysis." Ex. 1040 at 2:61-64. "For example ducts can be formed with two plain transparent walls to form the sample chambers of spectrophotometers." Ex. 1040 at 2:64-66.

Corbett similarly teaches that "[d]ownstream of temperature zone 34 is in-line analysis means 29 and recovery means 28. Connected to recovery means 28 is **detection means** 30. Detection means 30 determines when a reaction mixture in the carrier fluid reaches recovery means 28. This may be done by measuring the conductivity or **optical density** of the stream of carrier fluid." Ex. 1010 at 8:12-18, [emphasis added].

Lagally teaches "[s]tochastic PCR amplification of single DNA template molecules followed by **capillary electrophoresis** (CE) analysis of the products is demonstrated in an integrated microfluidic device." Ex. 1028 at 1, [emphasis added]. The results of capillary electrophoresis were generally detected optically at the time of filing. Ex. 1011 at 46 (Dolnick). "The amplified product, labeled with an intercalating **fluorescent** dye, is directly injected into the gel-filled capillary channel for electrophoretic analysis." Ex. 1028 at 1, [emphasis added].

Turning to claim 5, under the broadest reasonable interpretation the phrase "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels" requires simply that at least some of the droplets or plugs are transmitted to an outlet or other channel. Shaw Stewart teaches an outlet channel leading to reservoir 19 and thus meets claim 5 under the broadest reasonable interpretation. Ex. 1041 at 3:57-62. If claim 5 is more narrowly interpreted as requiring selective diversion of the droplets, that was well known. For instance, Quake teaches that "[a] preferred 'forward' sorting algorithm consists of running molecules, cells or virions from the input channel to the waste channel, until a molecule, cell or

virion is identified to have an optically detectable signal (e.g. fluorescence) that is above a pre-set threshold, at which point voltages are temporarily changed to electro-osmotically divert the molecule [] to the collection channel." Ex. 1034 at 20:22-26. Fu likewise discloses a microfabricated fluorescence-activiated cell sorter for use in connection with emulsion or droplet based systems. Ex. 1113 at 1109. A skilled artisan would have considered it obvious to employ such a technique to sort droplets after they are subjected to the optical detection. Ex. 1004 ¶68,69. For instance, a skilled artisan would have found it advantageous to sort and collect droplets which tested positive or negative for the presence of a certain substance or property, especially in the context of droplet generators which are used to encapsulate on average one template molecule per droplet. *Id.* ¶82.

In the combined method of Shaw Stewart, Corbett and Lagally, microfluidic droplet reactors are used to perform PCR reactions after which the reaction products are detected optically and sorted by diverting certain droplets into collection channels. Ex. 1004 ¶69.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1068.

Thus, claims 3-5 are shown to be rendered obvious by the combination of Shaw Stewart, Corbett and Lagally.

Dependent Claims 6-8

Claim 6 of the '148 patent recites "wherein the oil is a fluorinated oil." Claim 7 of the '148 patent recites "wherein the carrier fluid further comprises a surfactant." Claim 8 of the '148 patent recites "wherein the surfactant is a fluorinated surfactant."

Shaw Stewart discloses that "[s]uitable carrier phases include **mineral oils**, water, light silicons, or **Freons**." Ex. 1040 at 1:39-41, [emphasis added]; (Freons are comprised of chorofluorocarbons. Ex. 1108). "**Surface acting agents** may also be included in the carrier

and/or reagents phases to produce suitable surface properties, for example to allow efficient merging." Ex. 1040 at 1:43-47, [emphasis added].

Additional correspondence between these claim elements and the cited references is shown in the claim chart submitted herewith as Exhibit 1068 and discussed in the Declaration of Dr. Shaqfeh (Ex. 1004).

To the extent the Office determines that any element of claims 6-8 is not disclosed by Shaw Stewart, it would have been obvious to use the recited fluorinated surfactants and oils in light of Quake's disclosure that fluourinated oils serve as surfactants which "aid in controlling or optimizing droplet size, flow and uniformity, for example by reducing the shear force needed to extrude or inject droplets into an intersecting channel." Ex. 1034 at 35:18-22.

Thus, claims 6-8 are shown to be rendered obvious by the combination of Shaw Stewart, Corbett and Lagally and further in view of Quake.

H. The Combination of Shaw Stewart, Corbett and Burns II Renders Obvious Claims 1-8 of the '148 Patent Under (pre-AIA) 35 U.S.C. § 103(a)

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Shaw Stewart), ii) microfluidic PCR devices were well known (Corbett and Burns II), and iii) there were compelling reasons to use the microfluidic droplet reactors to conduct microfluidic PCR (Lagally, Kopp, Burns II and Vogelstein).

The Shaw Stewart British Application teaches conducting reactions in microdroplets formed from two continuously flowing immiscible fluids. The Shaw Stewart British Application discloses a "droplet reactor" and illustrates at Figure 1 a carrier phase 1 flowing along a conduit into which reagent 2 is injected, thereby forming droplet 4. Ex. 1040 at 1:1-20, 70-90. "[I]f large numbers of droplets are required, a continuous flow of reagent through the opening will be produced, while a continuous current of carrier phase flows down the tube. When each droplet almost spans the tube, it will be broken off. The exact size of the droplets produced will depend on the relative and absolute magnitude of the two currents." *Id.* at 1:83-90.

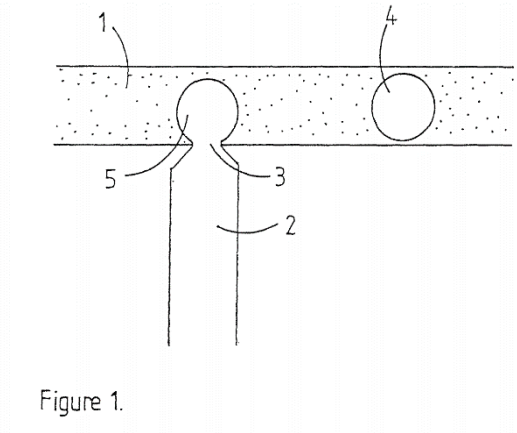


Figure 1.

Significantly, the exclusive licensee of the '148 patent affirmatively (and correctly) took the position that Shaw Stewart teaches plug or droplet formation combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid. More particularly, Bio-Rad (who recently acquired RainDance, the original exclusive licensee of the '148 patent, Ex. 1061, Ex. 1053 ¶71) explained that another party had mischaracterized the Shaw Stewart reference by failing to point out the above-quoted portion of Shaw Stewart, in which Shaw Stewart specifically teaches that in certain embodiments droplets are formed from intersecting and continuously flowing streams.

> To overcome Stewart I and secure allowance of their patent, the Patent Owner ("PO") mischaracterized the device disclosed in Stewart I (and hence Stewart II), misdirecting the Examiner to irrelevant portions of Stewart I while ignoring other portions that directly contradicted PO's arguments. *See, e.g.*, Ex. 1003 at 174-180. For example, one of PO's primary arguments was that Stewart I

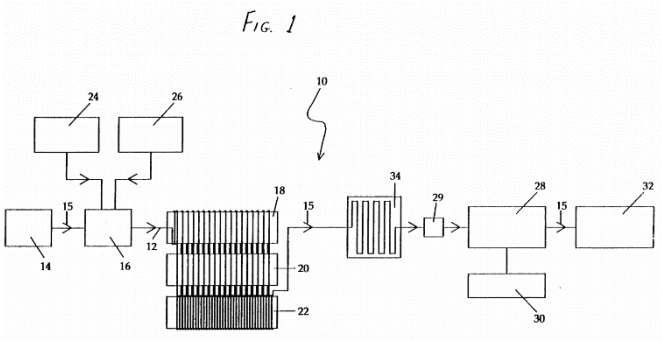
> only disclosed introducing fluid in a "stepwise fashion," whereas in the '503 Patent droplets are formed by "combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid." *Id.* at 174. In truth, however, Stewart I also discloses combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid:
>
> > If large numbers of droplets are required, a continuous flow of reagent through the opening will be produced, while a continuous current of carrier phase flows down the tube. When each droplet almost spans the tube, it will be broken off.
>
> Ex. 1008, pg. 1, col. 2, ll. 83-88.

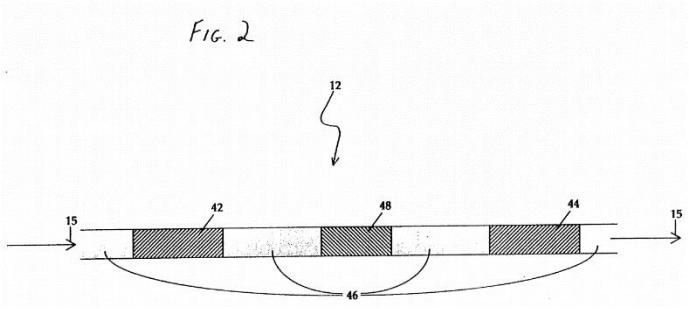
Ex. 1059 at 2-3.

Thus the exclusive licensee (Bio-Rad) expressly agreed that Shaw Stewart discloses forming plugs from intersecting streams of continuously flowing immiscible fluids.

As of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Corbett and Burns II. In 1991, about eleven years prior to the earliest effective filing date, Corbett disclosed using a microchannel or capillary device to conduct DNA amplification reactions such as PCR by injecting plugs of reactants into a carrier stream that is passed through different temperature zones. Corbett explained that as of 1991 "the most critical problem in applying nucleic acid amplification procedures, particularly in repetitive diagnostic assays in a clinical laboratory, is contamination of the reaction mixture by small amounts of DNA which contain sequences capable of being amplified under the conditions of assay." Ex. 1010 at 3:7-12. "Automation of procedures minimize[s] the opportunity for such contamination and allows the implementation of stringent quality control." *Id.* at 3:15-19. *"*As is shown in FIG. 1, the apparatus 10 comprises tube 12 through which a fluid stream passes. As is more clearly shown in FIG. 2, the fluid stream

includes pockets of reaction mixtures 42 and 44 and purging solution 48 separated by carrier fluid 46." *Id.* at 7:58-62.





"The pump is actuated and the reaction mixture of 20 μl or less is injected into the mineral oil or silicone oil carrier fluid and specific DNA sequences (whose limits are defined by the oligonucleotide primers) present in the sample is amplified as it passes cyclically through the temperature zones." Ex. 1010 at 9:35-40. Corbett thus demonstrates that it was known at least as of 1991 that reduction of contamination risk in PCR could be achieved by performing the PCR on microliter sized plugs of reactant in capillary tube which passes through various temperature zones. Ex. 1004 ¶77.

In 1996, Burns II reported the use of microfluidic devices to perform PCR. Ex. 1008. Burns II explained that "[n]o new biochemistries or DNA detection methods are required for their use; the components simply reproduce current laboratory procedures in a micron-sized environment." *Id.* at 5560. In other words, the PCR chemistry did not need to be modified – it worked without modification on the microfluidic scale. In the method of Burns II, a sample

containing a DNA plasmid was loaded into one of the reaction wells (A) and a mixture of Taq enzyme and buffer was loaded into the other. *Id.* at 5558. Each formed droplets of about 60 nl in the parallel 500 x 25 µm channels (B) which came together at the Y junction to form a single larger droplet (about 120 nl). *Id.* at 5558-59; Ex. 1004 ¶40.

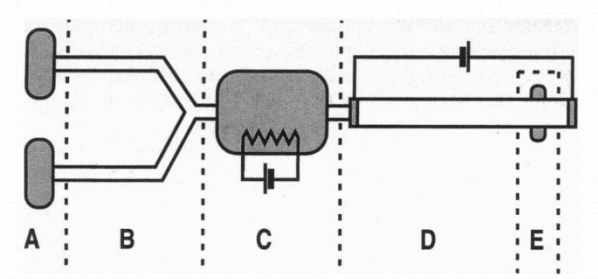


FIG. 1. Example of an integrated DNA analysis system, represented schematically. The individual components of the system are injection entry ports (*A*), liquid pumping channels (*B*), thermally controlled reaction chamber (*C*), electrophoresis channel (*D*), and DNA band migration detector (*E*). Each component would have associated sensors, control circuitry, and external connections.

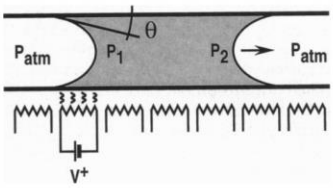


FIG. 2. Schematic drawing showing the principle of thermally induced drop motion in a closed channel. The case of a single aqueous drop in a hydrophilic channel is presented, where V is an applied voltage, P_{atm} is atmospheric pressure, P_1 is the receding-edge internal pressure, P_2 is the advancing-edge internal pressure, and θ is the contact angle of the liquid–gas–solid interface. The contact angle will depend on the surface characteristics of the channel and the constituents of the drop, with a hydrophilic interaction giving θ between 0 and 90 degrees, and a hydrophobic surface giving θ between 90 and 180 degrees. Surface treatments can also reduce contact angle hysteresis and, therefore, reduce the temperature difference necessary for drop motion.

The droplet then passed through the resistive heater portion (C) in which PCR amplification occurred. *Id.* at 5556. Afterwards the amplification results were optically detected in zone D via gel electrophoresis *Id.* at 5559. Burns II noted that "[t]he use of common fabrication methods allows the assembly of increasingly complex, multicomponent, integrated systems from a small, defined set of standardized elements" and that "[f]uture design efforts have a strong impetus to reduce component size and improve efficiency." *Id.* at 5560-61.

In sum, Corbett and Burns II show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Shaw Stewart is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA

molecules and at least one other molecule").[89] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Shaw Stewart) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Shaw Stewart to conduct PCR as taught by Corbett and Burns II. Stated a different way, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic droplet reactor of Shaw Stewart, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing.

A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic droplet reactor of Shaw Stewart because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the

[89] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Shaw Stewart to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Shaw Stewart would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target

effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 (“It is also possible that Dig-PCR can be performed in

microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

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Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
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Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Shaw Stewart to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to

increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.

Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In

1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Shaw Stewart could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful in miniaturizing and modifying Corbett or modifying Burns II to work in the microfluidic droplet reactor of Shaw Stewart, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet

reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[90] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail below, in the accompanying claim chart (Ex. 1069) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Shaw Stewart to conduct microfluidic PCR as taught by Corbett and Burns II. Ex. 1004 ¶¶109-112. Alternatively, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic droplet reactor of Shaw Stewart, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing. *Id*.

[90] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

Independent Claim 1

Claim 1[a]: A method comprising the steps of: providing a microfluidic system comprising one or more channels

Shaw Stewart discloses that "[t]he method of the invention may be used for initiating and controlling a chemical reaction or preparing mixtures of reagents.…" Ex. 1040 at 1:7-9. "The system is particularly suited **to the manipulation of microscopic quantities of reagents, with volumes of less than one microlit[er]**,…" *Id.* at 1:20-22 [emphasis added].

As shown in Shaw Stewart's Figure 1, "[t]he reagent is introduced into a tube containing carrier phase (1) from a side arm (2) by being sucked or pushed through a small opening (3). When the correct volume has passed into the tube, it is separated and carried away by a current of carrier phase, down the tube." Ex. 1040 at 1:70-75.

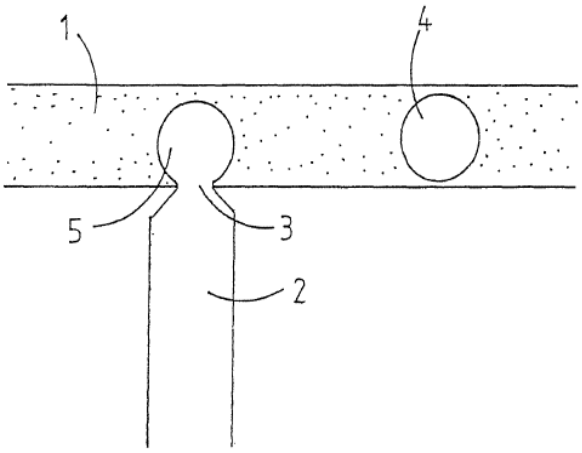

Figure 1.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1069.

Claim 1[b]: providing within the one or more channels a continuously flowing carrier fluid comprising an oil and a continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule in the fluid that can react with the target DNA or RNA molecules under conditions in which the target DNA or RNA molecules and the other molecules in the fluid do not react with each other

As shown in Shaw Stewart's Figure 1, "[t]he reagent is introduced into a tube containing carrier phase (1) from a side arm (2) by being sucked or pushed through a small opening (3). When the correct volume has passed into the tube, it is separated and carried away by a current of carrier phase, down the tube." Ex. 1040 at 1:70-75. Shaw Stewart teaches that "[f]or **aqueous reagents**, glass tubing and a light silicon carrier phase would be particularly suitable, as this combination would prevent wetting of the glass, due to a strong interaction at the glass-silicon interface." Ex. 1040 at 1:62-66 [emphasis added]. "[I]f large numbers of droplets are required, **a continuous flow of reagent** through the opening will be produced, while a **continuous current of carrier phase flows** down the tube." Ex. 1040 at 1:83-86 [emphasis added].

In the instant combination, the microfluidic droplet reactor of Shaw Stewart are relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[91] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Shaw Stewart) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Shaw Stewart to conduct microfluidic PCR as taught by Corbett and Burns II. Stated a different way, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modifying it to incorporate the microfluidic droplet reactor of Shaw Stewart, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that

[91] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

PCR had been widely and successfully implemented at the microfluidic scale at the time of filing.

A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic droplet reactor of Shaw Stewart because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as

to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Shaw Stewart to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Shaw Stewart would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely

reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
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Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Shaw Stewart to dilute the samples and thereby enable the detection of

mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Burns II) to incorporate a droplet reactor (Shaw Stewart). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Burns II. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the single fluid in microfluidic chip reactor (e.g., Burns II) or droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Corbett). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

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Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactors Shaw Stewart could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful in miniaturizing and modifying Corbett or modifying Burns II to work in the microfluidic droplet

reactor of Shaw Stewart, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[92] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained further in the accompanying claim chart (Ex. 1069) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Shaw Stewart to conduct microfluidic PCR as taught by Corbett and Burns II. Ex. 1004 ¶¶109-112. Alternatively, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modifying it to incorporate the microfluidic droplet reactor of Shaw

[92] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

Stewart, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing. *Id*.

With regard to the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other," this is merely a description of a PCR reaction. Ex. 1004 ¶31,32. As explained in Corbett, Kopp, Burns II, and Lagally, in a PCR reaction the sample DNA or RNA is admixed with various reagents including polymerase under conditions in which no reaction occurs; only when heat is applied does the PCR reaction occur. Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68. In the PCR of the combined method, as in virtually any PCR method, the DNA and polymermase do not react until mixture of reagents is heated. *Id*.; see also Ex. 1004 ¶32. The combined method thus meets the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other" this is merely a description of a PCR reaction. Ex. 1004 ¶31,32.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1069.

Claim 1[c]: controlling flow rates of said aqueous fluid and said carrier fluid to partition the continuously flowing aqueous fluid with the continuously flowing carrier fluid to form a plurality of plugs of the aqueous fluid, each having a substantially uniform size of about 200 µm or less, wherein the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule

As discussed above, the Shaw Stewart British Application teaches conducting reactions in microdroplets formed from two continuously flowing immiscible fluids. Significantly, the exclusive licensee of the '148 patent affirmatively (and correctly) took the position that Shaw

Stewart teaches plug or droplet formation combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid. More particularly, Bio-Rad (who recently acquired RainDance, the original exclusive licensee of the '148 patent, Ex. 1061, Ex. 1053 ¶71) explained that another party had mischaracterized the Shaw Stewart reference by failing to point out the above-quoted portion of Shaw Stewart, in which Shaw Stewart specifically teaches that in certain embodiments droplets are formed from intersecting and continuously flowing streams.

> To overcome Stewart I and secure allowance of their patent, the Patent Owner ("PO") mischaracterized the device disclosed in Stewart I (and hence Stewart II), misdirecting the Examiner to irrelevant portions of Stewart I while ignoring other portions that directly contradicted PO's arguments. *See, e.g.*, Ex. 1003 at 174-180. For example, one of PO's primary arguments was that Stewart I only disclosed introducing fluid in a "stepwise fashion," whereas in the '503 Patent droplets are formed by "combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid." *Id.* at 174. In truth, however, Stewart I also discloses combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid:
>
> > If large numbers of droplets are required, a continuous flow of reagent through the opening will be produced, while a continuous current of carrier phase flows down the tube. When each droplet almost spans the tube, it will be broken off.
>
> Ex. 1008, pg. 1, col. 2, ll. 83-88.

Ex. 1059 at 2-3.

Thus the exclusive licensee (Bio-Rad) expressly agreed that Shaw Stewart discloses forming plugs from intersecting streams of continuously flowing immiscible fluids.

Turning to the recitation "each having a substantially uniform size of about 200 µm or less," Shaw Stewart teaches that the volumes of the droplets are 1 microliter or less. Ex. 1040 at

1:20-23. 1,000,000 microliters are equal to one liter. One liter is equal to 1 x 10^{-3} cubic meters. Assuming the droplets are spheres, this equates to a droplet diameter of 1,200 µm. Shaw Stewart did not put a lower limit on the size of the droplets and contemplated droplet sizes below 200 µm. *Id.* Moreover, it must be noted that Shaw Stewart dates back to 1984, when microfabrication techniques were not as refined and channels could not readily be made as small as they could circa the year 2000. Quake (filed in the year 2000) teaches that "the droplets have a diameter that is smaller than the diameter of the microchannel; i.e., preferably less than 60 µm." Ex. 1034 at 27:29-30. Fig. 21 of Quake shows the droplets sizes generated by the Quake droplet generator were all less than 60 µm. Burns II (1996) similarly discloses uses of 60 nl droplets (which equates to a droplet diameter of about 62 µm). Ex. 1008 at 5558. Burns I (2001) discloses that the droplets are 100 µm to 400 µm in diameter. Ex. 1007 at 10. Accordingly, it would have been obvious to a skilled artisan that as of the earliest claimed priority date (2002) droplets below 200 µm in diameter could, and should, be used in order to reduce reagent usage and reaction time and achieve the various other benefits described above with the combination. Ex. 1004 ¶36, 66.

This leaves the recitation that "the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule." As discussed above, in the combined method of Quake, Corbett and Lagally, the samples are separated into a number of discrete droplets. That has the advantages of i) overcoming the signal-to-noise limitations associated with certain other PCR techniques and ii) enabling the detection of relatively rare mutations, dislocations and allelic imbalances. Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Lagally expressly teaches that "[t]he event amplitudes and frequencies at a mean occupancy of ∼1 template molecule in the reactor

are found to conform to a **Poisson** distribution, demonstrating that we are operating at the single-molecule level." Ex. 1028 at 566 [emphasis added]. Vogelstein likewise teaches that the aforementioned advantages are realized by **reducing the number of sample molecules per droplet to zero or one.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶82**.** That ensures that any amplification that occurs in a given droplet can be readily identified as containing the target sequence. *Id.* Thus, in the combined method the droplets have either one or zero template molecules.

As suggested by Lagally, any process which is generating droplets containing about one template molecule per droplet is expected to conform to a random, or Poisson, distribution. Ex. 1028 at 566; Ex. 1004 ¶48. It was known in the art that "[i]f a solution containing x molecules of enzyme per ml is dispersed into droplets of v ml volume, the average number of enzyme molecules per droplet n is equal to vx. Because of statistical fluctuations each droplet will not have exactly n enzyme molecules. The probability p(r) of finding 0, 1, 2, 3, . .. , r enzyme molecules in a droplet **is given by the Poisson law**." Ex. 1112 at 1985 [emphasis added]; see also Ex. 1111 at 1667 ("Spectra observed in a time sequence for an average of 0.6 dye molecule in the probed volume exhibited the expected Poisson distribution for actually measuring 0, 1, 2, or 3 molecules.").

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1069.

Claim 1[d]: providing conditions suitable for a polymerase-chain reaction in at least one plug of the plurality of plugs such that the target DNA or RNA is amplified

Shaw Stewart discloses that "[r]egions of the device can be heated by electric circuits or coils or by electromagnetic radiation to allow the merged droplets to be incubated at the required temperature." Ex. 1040 at 2:44-47. "If a colour change reaction is involved, such a colour

change can be recorded immediately or after incubation, using thermostatically controlled heating coil (27)." *Id.* at 3:58-60.

As discussed above in connection with element 1[b], in the instant combination Shaw Stewart is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[93] These limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Shaw Stewart) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1069.

Thus, claim 1 is shown to be rendered obvious by the combination of Shaw Stewart, Corbett and Burns II.

Dependent Claim 2

Claim 2 of the '148 patent recites "wherein the step of providing conditions includes heating."

As discussed above in connection claim elements 1[b] and 1[c], in the combined method (and in virtually any other PCR reaction) the DNA and polymerase undergo polymerase chain reaction when heated. Ex. 1004 ¶32; Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68.)

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1069.

[93] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

Thus, claim 2 is shown to be rendered obvious by the combination of Shaw Stewart, Corbett and Burns II.

Dependent Claims 3-5

Claim 3 of the '148 patent recites "providing a detector to detect, analyze, characterize, or monitor the target DNA or RNA molecule in the at least one member of the plurality of plugs during and/or after the reaction has occurred." Claim 4 of the '148 patent further recites "wherein the at least one other molecule includes one or more fluorescent labels and the detector is configured to monitor progress of the reaction by detecting fluorescence emissions from the one or more fluorescent labels." Claim 5 recites "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels."

Shaw Stewart discloses that "parts of the device itself can be adapted to form the sample chambers of the standard instruments of chemical or biochemical analysis." Ex. 1040 at 2:61-64. "For example ducts can be formed with two plain transparent walls to form the sample chambers of spectrophotometers." Ex. 1040 at 2:64-66.

Corbett similarly teaches that "[d]ownstream of temperature zone 34 is in-line analysis means 29 and recovery means 28. Connected to recovery means 28 is **detection means** 30. Detection means 30 determines when a reaction mixture in the carrier fluid reaches recovery means 28. This may be done by measuring the conductivity or **optical density** of the stream of carrier fluid." Ex. 1010 at 8:12-18, [emphasis added].

Burns II teaches that "[c]apillary gel electrophoresis of DNA samples was performed using a Beckman P/ACE instrument with a laser-induced fluorescence detector and 37 cm length, 100 μm diameter, linear polymerase gel capillary according to manufacturer's recommendation." Ex. 1008 at 5558. The results of capillary electrophoresis were generally detected optically at the time of filing. Ex. 1011 at 46 (Dolnick). "The amplified product, labeled

with an intercalating **fluorescent** dye, is directly injected into the gel-filled capillary channel for electrophoretic analysis." Ex. 1028 at 1, [emphasis added].

Turning to claim 5, under the broadest reasonable interpretation the phrase "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels" requires simply that at least some of the droplets or plugs are transmitted to an outlet or other channel. Shaw Stewart teaches an outlet channel leading to reservoir 19 and thus meets claim 5 under the broadest reasonable interpretation. Ex. 1041 at 3:57-62. If claim 5 is more narrowly interpreted as requiring selective diversion of the droplets, that was well known. For instance, Quake teaches that "[a] preferred 'forward' sorting algorithm consists of running molecules, cells or virions from the input channel to the waste channel, until a molecule, cell or virion is identified to have an optically detectable signal (e.g. fluorescence) that is above a pre-set threshold, at which point voltages are temporarily changed to electro-osmotically divert the molecule [] to the collection channel." Ex. 1034 at 20:22-26. Fu likewise discloses a microfabricated fluorescence-activiated cell sorter for use in connection with emulsion or droplet based systems. Ex. 1113 at 1109. A skilled artisan would have considered it obvious to employ such a technique to sort droplets after they are subjected to the optical detection. Ex. 1004 ¶68,69. For instance, a skilled artisan would have found it advantageous to sort and collect droplets which tested positive or negative for the presence of a certain substance or property, especially in the context of droplet generators which are used to encapsulate on average one template molecule per droplet. *Id.* ¶82.

In the combined method of Shaw Stewart, Corbett and Burns II, microfluidic droplet reactors are used to perform PCR reactions after which the reaction products are detected optically and sorted by diverting certain droplets into collection channels. Ex. 1004 ¶69.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1069.

Thus, claims 3-5 are shown to be rendered obvious by the combination of Shaw Stewart, Corbett and Burns II.

Dependent Claims 6-8

Claim 6 of the '148 patent recites "wherein the oil is a fluorinated oil." Claim 7 of the '148 patent recites "wherein the carrier fluid further comprises a surfactant." Claim 8 of the '148 patent recites "wherein the surfactant is a fluorinated surfactant."

Shaw Stewart discloses that "[s]uitable carrier phases include **mineral oils**, water, light silicons, or **Freons**." Ex. 1040 at 1:39-41, [emphasis added]; (Freons are comprised of chorofluorocarbons. Ex. 1108). "**Surface acting agents** may also be included in the carrier and/or reagents phases to produce suitable surface properties, for example to allow efficient merging." Ex. 1040 at 1:43-47, [emphasis added].

Additional correspondence between these claim elements and the cited references is shown in the claim chart submitted herewith as Exhibit 1069 and discussed in the Declaration of Dr. Shaqfeh (Ex. 1004).

To the extent the Office determines that any element of claims 6-8 is not disclosed by Shaw Stewart, it would have been obvious to use the recited fluorinated surfactants and oils in light of Quake's disclosure that fluourinated oils serve as surfactants which "aid in controlling or optimizing droplet size, flow and uniformity, for example by reducing the shear force needed to extrude or inject droplets into an intersecting channel." Ex. 1034 at 35:18-22.

Thus, claims 6-8 are shown to be rendered obvious by the combination of Shaw Stewart, Corbett and Burns II and further in view of Quake.

I. The Combination of Shaw Stewart, Corbett and Vogelstein Renders Obvious Claims 1-8 of the '148 Patent Under (pre-AIA) 35 U.S.C. § 103(a)

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Shaw Stewart), ii) microfluidic PCR devices were well known (Corbett and Vogelstein), and iii) there were compelling reasons to use the microfluidic droplet reactors to conduct microfluidic PCR (Lagally, Kopp, Burns II and Vogelstein).

The Shaw Stewart British Application teaches conducting reactions in microdroplets formed from two continuously flowing immiscible fluids. The Shaw Stewart British Application discloses a "droplet reactor" and illustrates at Figure 1 a carrier phase 1 flowing along a conduit into which reagent 2 is injected, thereby forming droplet 4. Ex. 1040 at 1:1-20, 70-90. "[I]f large numbers of droplets are required, a continuous flow of reagent through the opening will be produced, while a continuous current of carrier phase flows down the tube. When each droplet almost spans the tube, it will be broken off. The exact size of the droplets produced will depend on the relative and absolute magnitude of the two currents." *Id.* at 1:83-90.

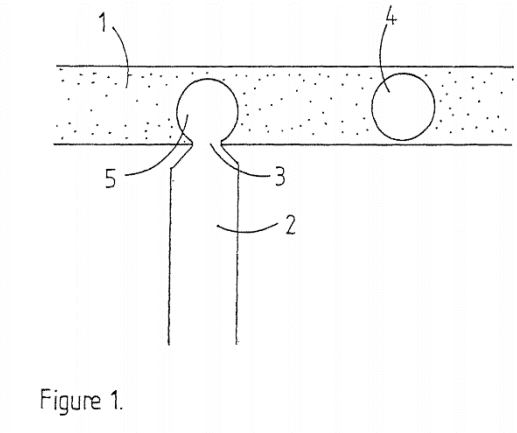


Figure 1.

Significantly, the exclusive licensee of the '148 patent affirmatively (and correctly) took the position that Shaw Stewart teaches plug or droplet formation combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid. More particularly, Bio-Rad (who recently acquired RainDance, the original exclusive licensee of the '148 patent, Ex. 1061, Ex. 1053 ¶71) explained that another party had mischaracterized the Shaw Stewart reference by failing to point out the above-quoted portion of Shaw Stewart, in which Shaw Stewart

specifically teaches that in certain embodiments droplets are formed from intersecting and continuously flowing streams.

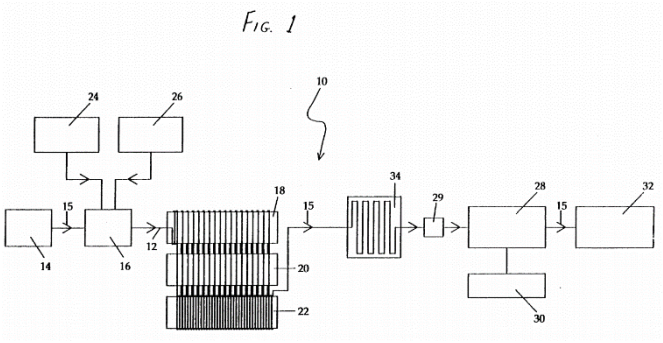
> To overcome Stewart I and secure allowance of their patent, the Patent Owner ("PO") mischaracterized the device disclosed in Stewart I (and hence Stewart II), misdirecting the Examiner to irrelevant portions of Stewart I while ignoring other portions that directly contradicted PO's arguments. *See, e.g.*, Ex. 1003 at 174-180. For example, one of PO's primary arguments was that Stewart I only disclosed introducing fluid in a "stepwise fashion," whereas in the '503 Patent droplets are formed by "combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid." *Id.* at 174. In truth, however, Stewart I also discloses combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid:
>
> > If large numbers of droplets are required, a continuous flow of reagent through the opening will be produced, while a continuous current of carrier phase flows down the tube. When each droplet almost spans the tube, it will be broken off.
>
> Ex. 1008, pg. 1, col. 2, ll. 83-88.

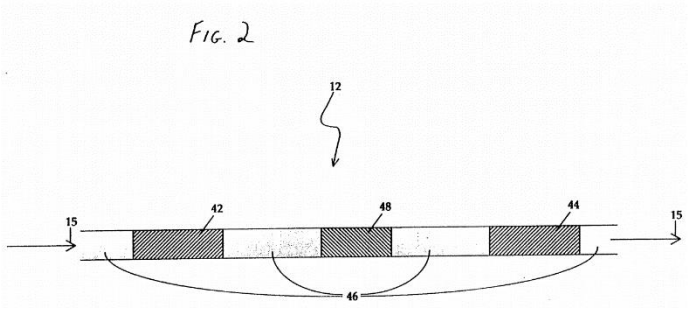
Ex. 1059 at 2-3.

Thus the exclusive licensee (Bio-Rad) expressly agreed that Shaw Stewart discloses forming plugs from intersecting streams of continuously flowing immiscible fluids.

As of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Corbett and Vogelstein. In 1991, about eleven years prior to the earliest effective filing date, Corbett disclosed using a microchannel or capillary device to conduct DNA amplification reactions such as PCR by injecting plugs of reactants into a carrier stream that is passed through different temperature zones. Corbett explained that as of 1991 "the most critical problem in applying nucleic acid amplification

procedures, particularly in repetitive diagnostic assays in a clinical laboratory, is contamination of the reaction mixture by small amounts of DNA which contain sequences capable of being amplified under the conditions of assay." Ex. 1010 at 3:7-12. "Automation of procedures minimize[s] the opportunity for such contamination and allows the implementation of stringent quality control." *Id.* at 3:15-19. "As is shown in FIG. 1, the apparatus 10 comprises tube 12 through which a fluid stream passes. As is more clearly shown in FIG. 2, the fluid stream includes pockets of reaction mixtures 42 and 44 and purging solution 48 separated by carrier fluid 46." *Id.* at 7:58-62.





"The pump is actuated and the reaction mixture of 20 µl or less is injected into the mineral oil or silicone oil carrier fluid and specific DNA sequences (whose limits are defined by the oligonucleotide primers) present in the sample is amplified as it passes cyclically through the temperature zones." Ex. 1010 at 9:35-40. Corbett thus demonstrates that it was known at least as of 1991 that reduction of contamination risk in PCR could be achieved by performing the PCR

on microliter sized plugs of reactant in capillary tube which passes through various temperature zones. Ex. 1004 ¶77.

By 1999, Vogelstein had reported **various applications for single-molecule microfluidic PCR**. Ex. 1044 at 9241. In a seminal article which has been cited over by over 1,000 other publications, Vogelstein explained that PCR amplification of small aliquots or droplets containing single molecules was effective for "detection of a small number of mutant-containing cells among a large excess of normal cells." *Id.* at 9236. In Vogelstein's method, the DNA was diluted into 7 µl volumes such that, on average, there was one template molecule for every two wells (i.e., approximately half of the wells contained no DNA template molecule and approximately half contained a single DNA template molecule). *Id.* at 9236, 9237. This avoided the problem of background noise impairing the determination of whether each volume contained the mutant sequence. *Id.* By counting the number of volumes which indicated wild type or mutant templates, the presence or absence of the mutation (e.g., a sequence associated with colorectal cancer) can be determined. *Id.* at 9238. The accuracy of the test is a function of the number of discrete volumes into which the sample is diluted. *Id.* at 9239. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. *Id.* at 9239. ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude.") *Id.* 9239. The article explained that the same technique would be useful for **detecting a wide array of mutations, dislocations and allelic imbalances**, as summarized in Table 1. *Id.* at 9241. Vogelstein thus taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template

molecule, thereby overcoming the signal-to-noise limitations associated with certain other PCR techniques and enabling the detection of relatively rare mutations, dislocations and allelic imbalances. Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84.

In sum, Corbett and Vogelstein show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Shaw Stewart is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[94] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Shaw Stewart) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Shaw Stewart to conduct microfluidic PCR as taught by Corbett and Vogelstein. Ex. 1004 ⁋⁋109-112. Stated a different way, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic droplet reactor of Shaw Stewart, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing.

A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic droplet reactor of Shaw Stewart because doing so have provided the substantial

[94] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Shaw Stewart to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Shaw Stewart would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026

(Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Shaw Stewart to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Vogelstein) to

incorporate a droplet reactor (Shaw Stewart). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Vogelstein. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Corbett). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors

> were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of

> single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority

date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Shaw Stewart could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful in miniaturizing and modifying Corbett or modifying Vogelstein to work in the microfluidic droplet reactor of Shaw Stewart, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese

rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[95] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail below, in the accompanying claim chart (Ex. 1070) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Shaw Stewart to conduct microfluidic PCR as taught by Corbett and Vogelstein. Ex. 1004 ⁋⁋109-112. Alternatively, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic droplet reactor of Shaw Stewart, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing. *Id*.

Independent Claim 1

Claim 1[a]: A method comprising the steps of: providing a microfluidic system comprising one or more channels

Shaw Stewart discloses that "[t]he method of the invention may be used for initiating and controlling a chemical reaction or preparing mixtures of reagents…." Ex. 1040 at 1:7-9.

[95] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

"The system is particularly suited **to the manipulation of microscopic quantities of reagents, with volumes of less than one microlit[er]**,…" *Id.* at 1:20-22 [emphasis added].

As shown in Shaw Stewart's Figure 1, "[t]he reagent is introduced into a tube containing carrier phase (1) from a side arm (2) by being sucked or pushed through a small opening (3). When the correct volume has passed into the tube, it is separated and carried away by a current of carrier phase, down the tube." Ex. 1040 at 1:70-75.

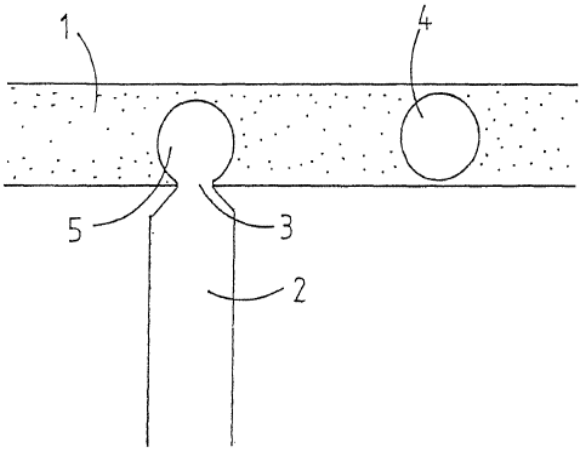


Figure 1.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1070.

Claim 1[b]: providing within the one or more channels a continuously flowing carrier fluid comprising an oil and a continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule in the fluid that can react with the target DNA or RNA molecules under conditions in which the target DNA or RNA molecules and the other molecules in the fluid do not react with each other

As shown in Shaw Stewart's Figure 1, "[t]he reagent is introduced into a tube containing carrier phase (1) from a side arm (2) by being sucked or pushed through a small opening (3). When the correct volume has passed into the tube, it is separated and carried away by a current of carrier phase, down the tube." Ex. 1040 at 1:70-75. Shaw Stewart teaches that "[f]or **aqueous**

reagents, glass tubing and a light silicon carrier phase would be particularly suitable, as this combination would prevent wetting of the glass, due to a strong interaction at the glass-silicon interface." Ex. 1040 at 1:62-66 [emphasis added]. "[I]f large numbers of droplets are required, **a continuous flow of reagent** through the opening will be produced, while a **continuous current of carrier phase flows** down the tube." Ex. 1040 at 1:83-86 [emphasis added].

In the instant combination, the microfluidic droplet reactor of Shaw Stewart are relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[96] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Shaw Stewart) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Shaw Stewart to conduct microfluidic PCR as taught by Corbett and Vogelstein. Ex. 1004 ⁋⁋109-112. Stated a different way, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modifying it to incorporate the microfluidic droplet reactor of Shaw Stewart, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing.

A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic droplet reactor of Shaw Stewart because doing so have provided the substantial

[96] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Shaw Stewart to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Shaw Stewart would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026

(Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Shaw Stewart to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Vogelstein) to

incorporate a droplet reactor (Shaw Stewart). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Vogelstein. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Corbett). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors

> were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of

> single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority

date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactors Shaw Stewart could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful in miniaturizing and modifying Corbett or modifying Vogelstein to work in the microfluidic droplet reactor of Shaw Stewart, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese

rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[97] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained further in the accompanying claim chart (Ex. 1070) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Shaw Stewart to conduct microfluidic PCR as taught by Corbett and Vogelstein. Ex. 1004 ⁋⁋109-112. Alternatively, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modifying it to incorporate the microfluidic droplet reactor of Shaw Stewart, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing. *Id*.

With regard to the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other," this is merely a description of a PCR reaction. Ex. 1004 ¶31,32. As explained in Corbett, Kopp, Burns II, and Lagally, in a PCR reaction the sample DNA or RNA is admixed with various

[97] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

reagents including polymerase under conditions in which no reaction occurs; only when heat is applied does the PCR reaction occur. Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68. In the PCR of the combined method, as in virtually any PCR method, the DNA and polymermase do not react until mixture of reagents is heated. *Id*.; see also Ex. 1004 ¶32. The combined method thus meets the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other" this is merely a description of a PCR reaction. Ex. 1004 ¶31,32.)

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1070.

Claim 1[c]: controlling flow rates of said aqueous fluid and said carrier fluid to partition the continuously flowing aqueous fluid with the continuously flowing carrier fluid to form a plurality of plugs of the aqueous fluid, each having a substantially uniform size of about 200 µm or less, wherein the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule

As discussed above, the Shaw Stewart British Application teaches conducting reactions in microdroplets formed from two continuously flowing immiscible fluids. Significantly, the exclusive licensee of the '148 patent affirmatively (and correctly) took the position that Shaw Stewart teaches plug or droplet formation combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid. More particularly, Bio-Rad (who recently acquired RainDance, the original exclusive licensee of the '148 patent, Ex. 1061, Ex. 1053 ¶71) explained that another party had mischaracterized the Shaw Stewart reference by failing to point out the above-quoted portion of Shaw Stewart, in which Shaw Stewart specifically teaches that in certain embodiments droplets are formed from intersecting and continuously flowing streams.

> To overcome Stewart I and secure allowance of their patent, the Patent Owner ("PO") mischaracterized the device disclosed in Stewart I (and hence Stewart II), misdirecting the Examiner to irrelevant portions of Stewart I while ignoring other portions that directly contradicted PO's arguments. *See, e.g.*, Ex. 1003 at 174-180. For example, one of PO's primary arguments was that Stewart I only disclosed introducing fluid in a "stepwise fashion," whereas in the '503 Patent droplets are formed by "combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid." *Id.* at 174. In truth, however, Stewart I also discloses combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid:
>
> > If large numbers of droplets are required, a continuous flow of reagent through the opening will be produced, while a continuous current of carrier phase flows down the tube. When each droplet almost spans the tube, it will be broken off.
>
> Ex. 1008, pg. 1, col. 2, ll. 83-88.

Ex. 1059 at 2-3.

Thus the exclusive licensee (Bio-Rad) expressly agreed that Shaw Stewart discloses forming plugs from intersecting streams of continuously flowing immiscible fluids.

Turning to the recitation "each having a substantially uniform size of about 200 µm or less," Shaw Stewart teaches that the volumes of the droplets are 1 microliter or less. Ex. 1040 at 1:20-23. 1,000,000 microliters are equal to one liter. One liter is equal to 1×10^{-3} cubic meters. Assuming the droplets are spheres, this equates to a droplet diameter of 1,200 µm. Shaw Stewart did not put a lower limit on the size of the droplets and contemplated droplet sizes below 200 µm. *Id.* Moreover, it must be noted that Shaw Stewart dates back to 1984, when microfabrication techniques were not as refined and channels could not readily be made as small as they could circa the year 2000. Quake (filed in the year 2000) teaches that "the droplets have a diameter that

is smaller than the diameter of the microchannel; i.e., preferably less than 60 μm." Ex. 1034 at 27:29-30. Fig. 21 of Quake shows the droplets sizes generated by the Quake droplet generator were all less than 60 μm. Burns II (1996) similarly discloses uses of 60 nl droplets (which equates to a droplet diameter of about 62 μm). Ex. 1008 at 5558. Burns I (2001) discloses that the droplets are 100 μm to 400 μm in diameter. Ex. 1007 at 10. Accordingly, it would have been obvious to a skilled artisan that as of the earliest claimed priority date (2002) droplets below 200 μm in diameter could, and should, be used in order to reduce reagent usage and reaction time and achieve the various other benefits described above with the combination. Ex. 1004 ¶36, 66.

This leaves the recitation that "the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule." As discussed above, in the combined method of Quake, Corbett and Lagally, the samples are separated into a number of discrete droplets. That has the advantages of i) overcoming the signal-to-noise limitations associated with certain other PCR techniques and ii) enabling the detection of relatively rare mutations, dislocations and allelic imbalances. Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Lagally expressly teaches that "[t]he event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a **Poisson** distribution, demonstrating that we are operating at the single-molecule level." Ex. 1028 at 566 [emphasis added]. Vogelstein likewise teaches that the aforementioned advantages are realized by **reducing the number of sample molecules per droplet to zero or one.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶82**.** That ensures that any amplification that occurs in a given droplet can be readily identified as containing the target

sequence. *Id.* Thus, in the combined method the droplets have either one or zero template molecules.

As suggested by Lagally, any process which is generating droplets containing about one template molecule per droplet is expected to conform to a random, or Poisson, distribution. Ex. 1028 at 566; Ex. 1004 ¶48. It was known in the art that "[i]f a solution containing x molecules of enzyme per ml is dispersed into droplets of v ml volume, the average number of enzyme molecules per droplet n is equal to vx. Because of statistical fluctuations each droplet will not have exactly n enzyme molecules. The probability p(r) of finding 0, 1, 2, 3, . .. , r enzyme molecules in a droplet **is given by the Poisson law**." Ex. 1112 at 1985 [emphasis added]; see also Ex. 1111 at 1667 ("Spectra observed in a time sequence for an average of 0.6 dye molecule in the probed volume exhibited the expected Poisson distribution for actually measuring 0, 1, 2, or 3 molecules.").

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1070.

Claim 1[d]: providing conditions suitable for a polymerase-chain reaction in at least one plug of the plurality of plugs such that the target DNA or RNA is amplified

Shaw Stewart discloses that "[r]egions of the device can be heated by electric circuits or coils or by electromagnetic radiation to allow the merged droplets to be incubated at the required temperature." Ex. 1040 at 2:44-47. "If a colour change reaction is involved, such a colour change can be recorded immediately or after incubation, using thermostatically controlled heating coil (27)." *Id.* at 3:58-60.

As discussed above in connection with element 1[b], in the instant combination Shaw Stewart is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target

DNA or RNA molecules and at least one other molecule").[98] These limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Shaw Stewart) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1070.

Thus, claim 1 is shown to be rendered obvious by the combination of Shaw Stewart, Corbett and Vogelstein.

Dependent Claim 2

Claim 2 of the '148 patent recites "wherein the step of providing conditions includes heating."

As discussed above in connection claim elements 1[b] and 1[c], in the combined method (and in virtually any other PCR reaction) the DNA and polymerase undergo polymerase chain reaction when heated. Ex. 1004 ¶32; Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1070.

Thus, claim 2 is shown to be rendered obvious by the combination of Shaw Stewart, Corbett and Vogelstein.

Dependent Claims 3-5

Claim 3 of the '148 patent recites "providing a detector to detect, analyze, characterize, or monitor the target DNA or RNA molecule in the at least one member of the plurality of plugs

[98] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

during and/or after the reaction has occurred." Claim 4 of the '148 patent further recites "wherein the at least one other molecule includes one or more fluorescent labels and the detector is configured to monitor progress of the reaction by detecting fluorescence emissions from the one or more fluorescent labels." Claim 5 recites "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels."

Shaw Stewart discloses that "parts of the device itself can be adapted to form the sample chambers of the standard instruments of chemical or biochemical analysis." Ex. 1040 at 2:61-64. "For example ducts can be formed with two plain transparent walls to form the sample chambers of spectrophotometers." Ex. 1040 at 2:64-66.

Corbett similarly teaches that "[d]ownstream of temperature zone 34 is in-line analysis means 29 and recovery means 28. Connected to recovery means 28 is **detection means** 30. Detection means 30 determines when a reaction mixture in the carrier fluid reaches recovery means 28. This may be done by measuring the conductivity or **optical density** of the stream of carrier fluid." Ex. 1010 at 8:12-18, [emphasis added].

Vogelstein teaches that the "[r]eactions were analyzed immediately or stored at room temperature for up to 36 h before **fluorescence analysis**." Ex. 1044 at 9236, [emphasis added].

In the combined method of Shaw Stewart, Corbett and Vogelstein, microfluidic droplet reactors are used to perform PCR reactions after which the reaction products are detected optically and sorted by diverting certain droplets into collection channels. Ex. 1004 ¶69.

Turning to claim 5, under the broadest reasonable interpretation the phrase "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels" requires simply that at least some of the droplets or plugs are transmitted to an outlet or other channel. Shaw Stewart teaches an outlet channel leading to reservoir 19 and thus meets

claim 5 under the broadest reasonable interpretation. Ex. 1041 at 3:57-62. If claim 5 is more narrowly interpreted as requiring selective diversion of the droplets, that was well known. For instance, Quake teaches that "[a] preferred 'forward' sorting algorithm consists of running molecules, cells or virions from the input channel to the waste channel, until a molecule, cell or virion is identified to have an optically detectable signal (e.g. fluorescence) that is above a pre-set threshold, at which point voltages are temporarily changed to electro-osmotically divert the molecule [] to the collection channel." Ex. 1034 at 20:22-26. Fu likewise discloses a microfabricated fluorescence-activiated cell sorter for use in connection with emulsion or droplet based systems. Ex. 1113 at 1109. A skilled artisan would have considered it obvious to employ such a technique to sort droplets after they are subjected to the optical detection. Ex. 1004 ¶68,69. For instance, a skilled artisan would have found it advantageous to sort and collect droplets which tested positive or negative for the presence of a certain substance or property, especially in the context of droplet generators which are used to encapsulate on average one template molecule per droplet. *Id.* ¶82.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1070.

Thus, claims 3-5 are shown to be rendered obvious by the combination of Shaw Stewart, Corbett and Vogelstein.

Dependent Claims 6-8

Claim 6 of the '148 patent recites "wherein the oil is fluorinated oil." Claim 7 of the '148 patent recites "wherein the carrier fluid further comprises a surfactant." Claim 8 of the '148 patent recites "wherein the surfactant is a fluorinated surfactant."

Shaw Stewart discloses that "[s]uitable carrier phases include **mineral oils**, water, light silicons, or **Freons**." Ex. 1040 at 1:39-41, [emphasis added]; (Freons are comprised of

chorofluorocarbons. Ex. 1108). "**Surface acting agents** may also be included in the carrier and/or reagents phases to produce suitable surface properties, for example to allow efficient merging." *Id.* at 1:43-47, [emphasis added].

Additional correspondence between these claim elements and the cited references is shown in the claim chart submitted herewith as Exhibit 1070 and discussed in the Declaration of Dr. Shaqfeh (Ex. 1004).

To the extent the Office determines that any element of claims 6-8 is not disclosed by Shaw Stewart, it would have been obvious to use the recited fluorinated surfactants and oils in light of Quake's disclosure that fluourinated oils serve as surfactants which "aid in controlling or optimizing droplet size, flow and uniformity, for example by reducing the shear force needed to extrude or inject droplets into an intersecting channel." Ex. 1034 at 35:18-22.

Thus, claims 6-8 are shown to be rendered obvious by the combination of Shaw Stewart, Corbett and Vogelstein and further in view of Quake.

J. The Combination of Shaw Stewart, Kopp and Lagally Renders Obvious Claims 1-8 of the '148 Patent Under (pre-AIA) 35 U.S.C. § 103(a)

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Shaw Stewart), ii) microfluidic PCR devices were well known (Kopp and Lagally), and iii) there were compelling reasons to use the microfluidic droplet reactors to conduct microfluidic PCR (Kopp, Burns II, Lagally and Vogelstein).

The Shaw Stewart British Application teaches conducting reactions in microdroplets formed from two continuously flowing immiscible fluids. The Shaw Stewart British Application discloses a "droplet reactor" and illustrates at Figure 1 a carrier phase 1 flowing along a conduit into which reagent 2 is injected, thereby forming droplet 4. Ex. 1040 at 1:1-20, 70-90. "[I]f large numbers of droplets are required, a continuous flow of reagent through the opening will be produced, while a continuous current of carrier phase flows down the tube. When each droplet almost spans the tube, it will be broken off. The exact size of the droplets produced will depend on the relative and absolute magnitude of the two currents." *Id.* at 1:83-90.

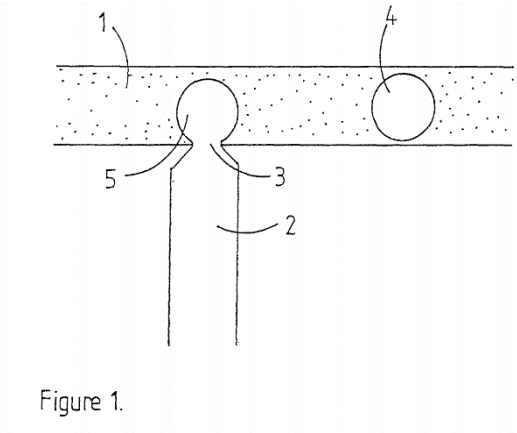


Figure 1.

Significantly, the exclusive licensee of the '148 patent affirmatively (and correctly) took the position that Shaw Stewart teaches plug or droplet formation combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid. More particularly, Bio-Rad (who recently acquired RainDance, the original exclusive licensee of the '148 patent, Ex. 1061, Ex. 1053 ¶71) explained that another party had mischaracterized the Shaw Stewart reference by failing to point out the above-quoted portion of Shaw Stewart, in which Shaw Stewart specifically teaches that in certain embodiments droplets are formed from intersecting and continuously flowing streams.

> To overcome Stewart I and secure allowance of their patent, the Patent Owner ("PO") mischaracterized the device disclosed in Stewart I (and hence Stewart II), misdirecting the Examiner to irrelevant portions of Stewart I while ignoring other portions that directly contradicted PO's arguments. *See, e.g.*, Ex. 1003 at 174-180. For example, one of PO's primary arguments was that Stewart I

> only disclosed introducing fluid in a "stepwise fashion," whereas in the '503 Patent droplets are formed by "combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid." *Id.* at 174. In truth, however, Stewart I also discloses combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid:
>
> > If large numbers of droplets are required, a continuous flow of reagent through the opening will be produced, while a continuous current of carrier phase flows down the tube. When each droplet almost spans the tube, it will be broken off.
>
> Ex. 1008, pg. 1, col. 2, ll. 83-88.

Ex. 1059 at 2-3.

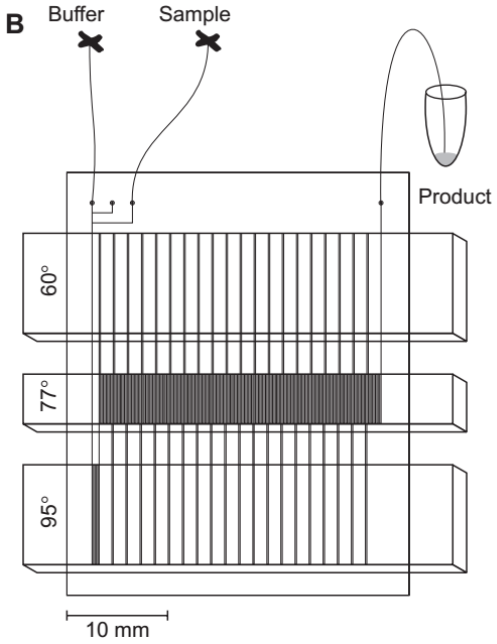
Thus the exclusive licensee (Bio-Rad) expressly agreed that Shaw Stewart discloses forming plugs from intersecting streams of continuously flowing immiscible fluids.

As of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Kopp and Lagally. By 1998 Kopp *et al.* had successfully implemented continuous flow PCR on a chip. In his 1988 article (not previously presented to the USPTO), Kopp explained that "[a] wide range of microreactors, microcapillary electrophoresis devices, and microcell manipulation devices have been described in recent years." Ex. 1027 at 1046. Building on that body of work, Kopp's group developed

> [a] micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in **continuous flow** at high speed. The device is analogous to an electronic amplifier and relies on the movement of sample through thermostated temperature zones on a glass microchip. Input and output of material (DNA) is **continuous**, and amplification is independent of input concentration. . . .
>
> The results demonstrate that with a very simple device and virtually no optimization, PCR can be performed in **continuous flow**, yielding product

quality and quantity comparable to standard thermal cycling methods (Fig. 2B). . . .

We suggest that cross-contamination in a **continuous-flow** format may be much less of a problem than in a stationary-tube PCR format. Thus, a flow-through PCR chip can be reused for extended periods without major cleaning. . . .



Ex. 1027 at 1046-1047 [emphasis added]. Kopp *et al.* also explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications:

To build a complex system analogous to an electronic integrated circuit, it is necessary to integrate a variety of other continuously working parts. A cursory glance at developments in miniaturized total analysis systems (uTAS) during the past 5 years reveals that most of the relevant components, such as **continuous-flow mixers** (8), **continuous-flow microreactors** (9), **continuous separations** (10), and high-speed capillary gel electrophoresis (as a conventional batch process), have already been presented (11). Accordingly, the **continuous-flow microamplifier** should allow the creation of highly elaborate analysis and synthesis systems.

> Although chemical amplification reactors will obviously not be applicable to all reactions, neither will they be limited to PCR. Devices of this kind open up whole new areas of application in medical diagnostics. For example, the online amplification and monitoring of a specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy.

Ex. 1027 at 1047 [emphasis added].

In 2001, in another article not previously considered by the Office, Lagally reported using a microfluidic reactor to conduct single-molecule DNA amplification by PCR. Lagally summarized the state of the art as of February 2001, explaining continuous flow PCR microreactors had recently undergone substantial evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually

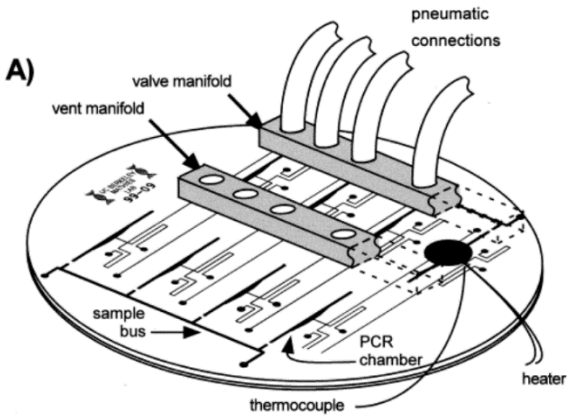
> transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

Lagally *et al.* developed a further improvement – a device and method for achieving single molecule amplification in a microchannel environment.

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ∼1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .

Microfluidic PCR-CE device. The PCR chambers are connected to a common sample bus through a set of valves. Hydrophobic vents at the other end of the PCR chambers are used to locate the sample and to eliminate gas. The PCR chambers are directly connected to the cross channel of the CE system for product injection and analysis. Two aluminum manifolds, one each for the vents and valves, are placed onto the respective ports and clamped in place using vacuum. The manifolds connect to external solenoid valves for pressure and vacuum actuation. Thermal cycling is accomplished using a resistive heater and a miniature thermocouple below the 280-nL chamber.



Ex. 1028 at 566 (see figure legend).

In sum, Kopp and Lagally show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Shaw Stewart is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[99] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Shaw Stewart) to perform PCR reactions, wherein each

[99] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Shaw Stewart to conduct microfluidic PCR as taught by Kopp and Lagally. A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic reactors of Shaw Stewart because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as

to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Shaw Stewart to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Shaw Stewart would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely

reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Shaw Stewart to dilute the samples and thereby enable the detection of

mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Kopp or Lagally) to incorporate a droplet reactor (Shaw Stewart). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Kopp, Burns II, Lagally or Vogelstein. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the single fluid in microfluidic chip reactor (e.g., Burns II or Lagally) or droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Corbett or Kopp). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.

Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with

> sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Shaw Stewart could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful modifying Kopp or Lagally to work in the microfluidic reactor of Shaw Stewart, the new teachings set forth

above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[100] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail below, in the accompanying claim chart (Ex. 1071) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Shaw Stewart to conduct microfluidic PCR as taught by Kopp and Lagally.

Independent Claim 1

Claim 1[a]: A method comprising the steps of: providing a microfluidic system comprising one or more channels

[100] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

Shaw Stewart discloses that "[t]he method of the invention may be used for initiating and controlling a chemical reaction or preparing mixtures of reagents.…" Ex. 1040 at 1:7-9. "The system is particularly suited **to the manipulation of microscopic quantities of reagents, with volumes of less than one microlit[er]**,…" *Id.* at 1:20-22 [emphasis added].

As shown in Shaw Stewart's Figure 1, "[t]he reagent is introduced into a tube containing carrier phase (1) from a side arm (2) by being sucked or pushed through a small opening (3). When the correct volume has passed into the tube, it is separated and carried away by a current of carrier phase, down the tube." Ex. 1040 at 1:70-75.

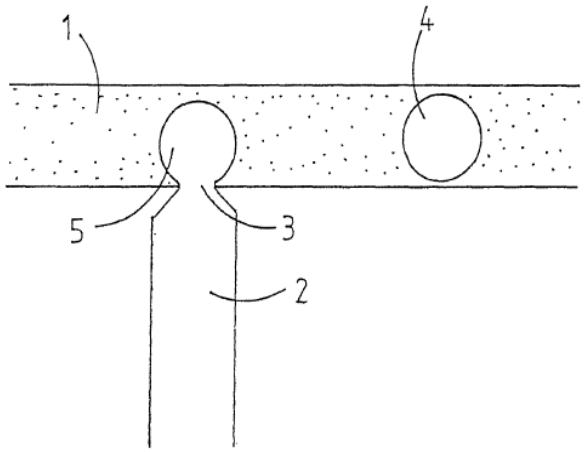


Figure 1.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1071 and in the Shaqfeh Declaration (Ex. 1004).

Claim 1[b]: providing within the one or more channels a continuously flowing carrier fluid comprising an oil and a continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule in the fluid that can react with the target DNA or RNA molecules under conditions in which the target DNA or RNA molecules and the other molecules in the fluid do not react with each other

As shown in Shaw Stewart's Figure 1, "[t]he reagent is introduced into a tube containing carrier phase (1) from a side arm (2) by being sucked or pushed through a small opening (3).

When the correct volume has passed into the tube, it is separated and carried away by a current of carrier phase, down the tube." Ex. 1040 at 1:70-75. Shaw Stewart teaches that "[f]or **aqueous reagents**, glass tubing and a light silicon carrier phase would be particularly suitable, as this combination would prevent wetting of the glass, due to a strong interaction at the glass-silicon interface." Ex. 1040 at 1:62-66 [emphasis added]. "[I]f large numbers of droplets are required, **a continuous flow of reagent** through the opening will be produced, while a **continuous current of carrier phase flows** down the tube." Ex. 1040 at 1:83-86 [emphasis added].

In the instant combination, the microfluidic droplet reactor of Shaw Stewart are relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule") Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Shaw Stewart) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Shaw Stewart to conduct microfluidic PCR as taught by Kopp and Lagally. A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic reactors of Shaw Stewart because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he

combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Shaw Stewart to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Shaw Stewart would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as

the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241;

Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Shaw Stewart to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Kopp or Lagally) to incorporate a droplet reactor (Shaw Stewart). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Kopp or Lagally. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the single fluid in microfluidic chip reactor (e.g., Lagally) or droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Kopp). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip.

Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling

> profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced

> microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic**

droplet (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactors Shaw Stewart could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful in modifying Kopp or Lagally to work in the microfluidic droplet reactor of Shaw Stewart, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[101] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of

[101] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary

microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained further in the accompanying claim chart (Ex. 1071) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Shaw Stewart to conduct microfluidic PCR as taught by Kopp and Lagally.

With regard to the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other," this is merely a description of a PCR reaction. Ex. 1004 ¶31,32. As explained in Corbett, Kopp, Burns II, and Lagally, in a PCR reaction the sample DNA or RNA is admixed with various reagents including polymerase under conditions in which no reaction occurs; only when heat is applied does the PCR reaction occur. Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68. In the PCR of the combined method, as in virtually any PCR method, the DNA and polymermase do not react until mixture of reagents is heated. *Id*.; see also Ex. 1004 ¶32. The combined method thus meets the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other" this is merely a description of a PCR reaction. Ex. 1004 ¶31,32.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1071.

Claim 1[c]: controlling flow rates of said aqueous fluid and said carrier fluid to partition the continuously flowing aqueous fluid with the continuously flowing carrier fluid to form a plurality of plugs of the aqueous fluid, each having a

consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

substantially uniform size of about 200 µm or less, wherein the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule

As discussed above, the Shaw Stewart British Application teaches conducting reactions in microdroplets formed from two continuously flowing immiscible fluids. Significantly, the exclusive licensee of the '148 patent affirmatively (and correctly) took the position that Shaw Stewart teaches plug or droplet formation combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid. More particularly, Bio-Rad (who recently acquired RainDance, the original exclusive licensee of the '148 patent, Ex. 1061, Ex. 1053 ¶71) explained that another party had mischaracterized the Shaw Stewart reference by failing to point out the above-quoted portion of Shaw Stewart, in which Shaw Stewart specifically teaches that in certain embodiments droplets are formed from intersecting and continuously flowing streams.

> To overcome Stewart I and secure allowance of their patent, the Patent Owner ("PO") mischaracterized the device disclosed in Stewart I (and hence Stewart II), misdirecting the Examiner to irrelevant portions of Stewart I while ignoring other portions that directly contradicted PO's arguments. *See, e.g.*, Ex. 1003 at 174-180. For example, one of PO's primary arguments was that Stewart I
>
> only disclosed introducing fluid in a "stepwise fashion," whereas in the '503 Patent droplets are formed by "combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid." *Id.* at 174. In truth, however, Stewart I also discloses combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid:
>
> > If large numbers of droplets are required, a continuous flow of reagent through the opening will be produced, while a continuous current of carrier phase flows down the tube. When each droplet almost spans the tube, it will be broken off.
>
> Ex. 1008, pg. 1, col. 2, ll. 83-88.

Ex. 1059 at 2-3.

Thus the exclusive licensee (Bio-Rad) expressly agreed that Shaw Stewart discloses forming plugs from intersecting streams of continuously flowing immiscible fluids.

Turning to the recitation "each having a substantially uniform size of about 200 μm or less," Shaw Stewart teaches that the volumes of the droplets are 1 microliter or less. Ex. 1040 at 1:20-23. 1,000,000 microliters are equal to one liter. One liter is equal to 1×10^{-3} cubic meters. Assuming the droplets are spheres, this equates to a droplet diameter of 1,200 μm. Shaw Stewart did not put a lower limit on the size of the droplets and contemplated droplet sizes below 200 μm. *Id.* Moreover, it must be noted that Shaw Stewart dates back to 1984, when microfabrication techniques were not as refined and channels could not readily be made as small as they could circa the year 2000. Quake (filed in the year 2000) teaches that "the droplets have a diameter that is smaller than the diameter of the microchannel; i.e., preferably less than 60 μm." Ex. 1034 at 27:29-30. Fig. 21 of Quake shows the droplets sizes generated by the Quake droplet generator were all less than 60 μm. Burns II (1996) similarly discloses uses of 60 nl droplets (which equates to a droplet diameter of about 62 μm). Ex. 1008 at 5558. Burns I (2001) discloses that the droplets are 100 μm to 400 μm in diameter. Ex. 1007 at 10. Accordingly, it would have been obvious to a skilled artisan that as of the earliest claimed priority date (2002) droplets below 200 μm in diameter could, and should, be used in order to reduce reagent usage and reaction time and achieve the various other benefits described above with the combination. Ex. 1004 ¶36, 66.

This leaves the recitation that "the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule." As discussed above, in the combined method of Quake, Corbett

and Lagally, the samples are separated into a number of discrete droplets. That has the advantages of i) overcoming the signal-to-noise limitations associated with certain other PCR techniques and ii) enabling the detection of relatively rare mutations, dislocations and allelic imbalances. Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Lagally expressly teaches that "[t]he event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a **Poisson** distribution, demonstrating that we are operating at the single-molecule level." Ex. 1028 at 566 [emphasis added]. Vogelstein likewise teaches that the aforementioned advantages are realized by **reducing the number of sample molecules per droplet to zero or one.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶82**.** That ensures that any amplification that occurs in a given droplet can be readily identified as containing the target sequence. *Id.* Thus, in the combined method the droplets have either one or zero template molecules.

As suggested by Lagally, any process which is generating droplets containing about one template molecule per droplet is expected to conform to a random, or Poisson, distribution. Ex. 1028 at 566; Ex. 1004 ¶48. It was known in the art that "[i]f a solution containing x molecules of enzyme per ml is dispersed into droplets of v ml volume, the average number of enzyme molecules per droplet n is equal to vx. Because of statistical fluctuations each droplet will not have exactly n enzyme molecules. The probability p(r) of finding 0, 1, 2, 3, . .. , r enzyme molecules in a droplet **is given by the Poisson law**." Ex. 1112 at 1985 [emphasis added]; see also Ex. 1111 at 1667 ("Spectra observed in a time sequence for an average of 0.6 dye molecule in the probed volume exhibited the expected Poisson distribution for actually measuring 0, 1, 2, or 3 molecules.").

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1071.

Claim 1[d]: providing conditions suitable for a polymerase-chain reaction in at least one plug of the plurality of plugs such that the target DNA or RNA is amplified

Shaw Stewart discloses that "[r]egions of the device can be heated by electric circuits or coils or by electromagnetic radiation to allow the merged droplets to be incubated at the required temperature." Ex. 1040 at 2:44-47. "If a colour change reaction is involved, such a colour change can be recorded immediately or after incubation, using thermostatically controlled heating coil (27)." *Id.* at 3:58-60.

As discussed above in connection with element 1[b], in the instant combination Shaw Stewart is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[102] These limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Shaw Stewart) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1071.

Thus, claim 1 is shown to be rendered obvious by the combination of Shaw Stewart, Kopp and Lagally.

[102] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

Dependent Claim 2

Claim 2 of the '148 patent recites "wherein the step of providing conditions includes heating."

As discussed above in connection claim elements 1[b] and 1[c], in the combined method (and in virtually any other PCR reaction) the DNA and polymerase undergo polymerase chain reaction when heated. Ex. 1004 ¶32; Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1071.

Thus, claim 2 is shown to be rendered obvious by the combination of Shaw Stewart, Kopp and Lagally.

Dependent Claims 3-5

Claim 3 of the '148 patent recites "providing a detector to detect, analyze, characterize, or monitor the target DNA or RNA molecule in the at least one member of the plurality of plugs during and/or after the reaction has occurred." Claim 4 of the '148 patent further recites "wherein the at least one other molecule includes one or more fluorescent labels and the detector is configured to monitor progress of the reaction by detecting fluorescence emissions from the one or more fluorescent labels." Claim 5 recites "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels."

Shaw Stewart discloses that "parts of the device itself can be adapted to form the sample chambers of the standard instruments of chemical or biochemical analysis." Ex. 1040 at 2:61-64. "For example ducts can be formed with two plain transparent walls to form the sample chambers of spectrophotometers." Ex. 1040 at 2:64-66.

Lagally teaches "[s]tochastic PCR amplification of single DNA template molecules followed by **capillary electrophoresis** (CE) analysis of the products is demonstrated in an integrated microfluidic device." Ex. 1028 at 1, [emphasis added]. The results of capillary electrophoresis were generally detected optically at the time of filing. Ex. 1011 at 46 (Dolnick). "The amplified product, labeled with an intercalating **fluorescent** dye, is directly injected into the gel-filled capillary channel for electrophoretic analysis." Ex. 1028 at 1, [emphasis added].

In the combined method of Shaw Stewart, Kopp and Lagally, microfluidic droplet reactors are used to perform PCR reactions after which the reaction products are detected optically and sorted by diverting certain droplets into collection channels. Ex. 1004 ¶69.

Turning to claim 5, under the broadest reasonable interpretation the phrase "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels" requires simply that at least some of the droplets or plugs are transmitted to an outlet or other channel. Shaw Stewart teaches an outlet channel leading to reservoir 19 and thus meets claim 5 under the broadest reasonable interpretation. Ex. 1041 at 3:57-62. If claim 5 is more narrowly interpreted as requiring selective diversion of the droplets, that was well known. For instance, Quake teaches that "[a] preferred 'forward' sorting algorithm consists of running molecules, cells or virions from the input channel to the waste channel, until a molecule, cell or virion is identified to have an optically detectable signal (e.g. fluorescence) that is above a pre-set threshold, at which point voltages are temporarily changed to electro-osmotically divert the molecule [] to the collection channel." Ex. 1034 at 20:22-26. Fu likewise discloses a microfabricated fluorescence-activiated cell sorter for use in connection with emulsion or droplet based systems. Ex. 1113 at 1109. A skilled artisan would have considered it obvious to employ such a technique to sort droplets after they are subjected to the optical detection. Ex. 1004

¶68,69. For instance, a skilled artisan would have found it advantageous to sort and collect droplets which tested positive or negative for the presence of a certain substance or property, especially in the context of droplet generators which are used to encapsulate on average one template molecule per droplet. *Id.* ¶82.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1071.

Thus, claims 3-5 are shown to be rendered obvious by the combination of Shaw Stewart, Kopp and Lagally.

Dependent Claims 6-8

Claim 6 of the '148 patent recites "wherein the oil is a fluorinated oil." Claim 7 of the '148 patent recites "wherein the carrier fluid further comprises a surfactant." Claim 8 of the '148 patent recites "wherein the surfactant is a fluorinated surfactant."

Shaw Stewart discloses that "[s]uitable carrier phases include **mineral oils**, water, light silicons, or **Freons**." Ex. 1040 at 1:39-41, [emphasis added]; (Freons are comprised of chorofluorocarbons. Ex. 1108). "**Surface acting agents** may also be included in the carrier and/or reagents phases to produce suitable surface properties, for example to allow efficient merging." *Id.* at 1:43-47, [emphasis added].

Additional correspondence between these claim elements and the cited references is shown in the claim chart submitted herewith as Exhibit 1071 and discussed in the Declaration of Dr. Shaqfeh (Ex. 1004).

To the extent the Office determines that any element of claims 6-8 is not disclosed by Shaw Stewart, it would have been obvious to use the recited fluorinated surfactants and oils in light of Quake's disclosure that fluourinated oils serve as surfactants which "aid in controlling or

optimizing droplet size, flow and uniformity, for example by reducing the shear force needed to extrude or inject droplets into an intersecting channel." Ex. 1034 at 35:18-22.

Thus, claims 6-8 are shown to be rendered obvious by the combination of Shaw Stewart, Kopp and Lagally and further in view of Quake.

K. The Combination of Shaw Stewart, Kopp and Burns II Renders Obvious Claims 1-8 of the '148 Patent Under (pre-AIA) 35 U.S.C. § 103(a)

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Shaw Stewart), ii) microfluidic PCR devices were well known (Kopp and Burns II), and iii) there were compelling reasons to use the microfluidic droplet reactors to conduct microfluidic PCR (Kopp, Burns II, Lagally and Vogelstein).

The Shaw Stewart British Application teaches conducting reactions in microdroplets formed from two continuously flowing immiscible fluids. The Shaw Stewart British Application discloses a "droplet reactor" and illustrates at Figure 1 a carrier phase 1 flowing along a conduit into which reagent 2 is injected, thereby forming droplet 4. Ex. 1040 at 1:1-20, 70-90. "[I]f large numbers of droplets are required, a continuous flow of reagent through the opening will be produced, while a continuous current of carrier phase flows down the tube. When each droplet almost spans the tube, it will be broken off. The exact size of the droplets produced will depend on the relative and absolute magnitude of the two currents." *Id.* at 1:83-90.

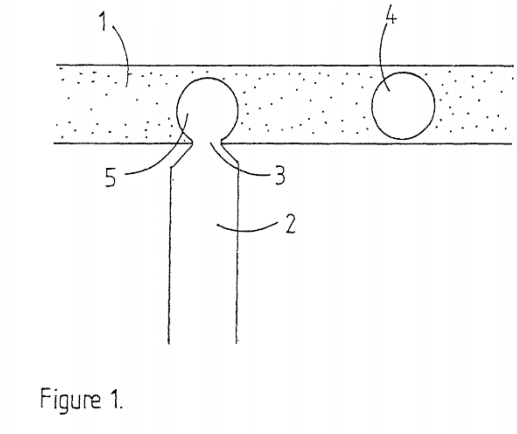


Figure 1.

Significantly, the exclusive licensee of the '148 patent affirmatively (and correctly) took the position that Shaw Stewart teaches plug or droplet formation combining a flowing stream of

an aqueous solution and a flowing stream of an immiscible fluid. More particularly, Bio-Rad (who recently acquired RainDance, the original exclusive licensee of the '148 patent, Ex. 1061, Ex. 1053 ¶71) explained that another party had mischaracterized the Shaw Stewart reference by failing to point out the above-quoted portion of Shaw Stewart, in which Shaw Stewart specifically teaches that in certain embodiments droplets are formed from intersecting and continuously flowing streams.

> To overcome Stewart I and secure allowance of their patent, the Patent Owner ("PO") mischaracterized the device disclosed in Stewart I (and hence Stewart II), misdirecting the Examiner to irrelevant portions of Stewart I while ignoring other portions that directly contradicted PO's arguments. *See, e.g.*, Ex. 1003 at 174-180. For example, one of PO's primary arguments was that Stewart I only disclosed introducing fluid in a "stepwise fashion," whereas in the '503 Patent droplets are formed by "combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid." *Id.* at 174. In truth, however, Stewart I also discloses combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid:
>
> > If large numbers of droplets are required, a continuous flow of reagent through the opening will be produced, while a continuous current of carrier phase flows down the tube. When each droplet almost spans the tube, it will be broken off.
>
> Ex. 1008, pg. 1, col. 2, ll. 83-88.

Ex. 1059 at 2-3.

Thus the exclusive licensee (Bio-Rad) expressly agreed that Shaw Stewart discloses forming plugs from intersecting streams of continuously flowing immiscible fluids.

As of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Kopp and Burns II. In 1996, Burns II

reported the use of microfluidic devices to perform PCR. Ex. 1008. Burns II explained that "[n]o new biochemistries or DNA detection methods are required for their use; the components simply reproduce current laboratory procedures in a micron-sized environment." *Id.* at 5560. In other words, the PCR chemistry did not need to be modified – it worked without modification on the microfluidic scale. In the method of Burns II, a sample containing a DNA plasmid was loaded into one of the reaction wells (A) and a mixture of *Taq* enzyme and buffer was loaded into the other. *Id.* at 5558. Each formed droplets of about 60 nl in the parallel 500 x 25 µm channels (B) which came together at the Y junction to form a single larger droplet (about 120 nl). *Id.* at 5558-59; Ex. 1004 ¶40.

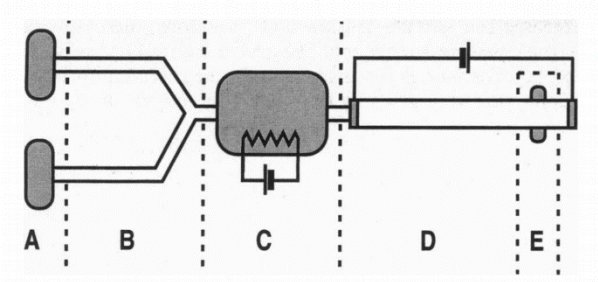


FIG. 1. Example of an integrated DNA analysis system, represented schematically. The individual components of the system are injection entry ports (*A*), liquid pumping channels (*B*), thermally controlled reaction chamber (*C*), electrophoresis channel (*D*), and DNA band migration detector (*E*). Each component would have associated sensors, control circuitry, and external connections.

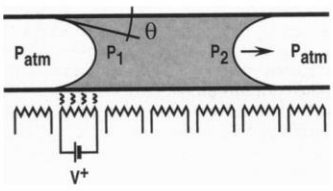


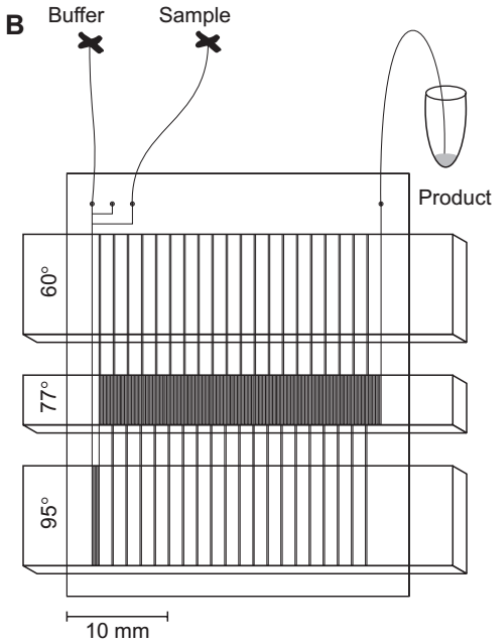
FIG. 2. Schematic drawing showing the principle of thermally induced drop motion in a closed channel. The case of a single aqueous drop in a hydrophilic channel is presented, where V is an applied voltage, P_{atm} is atmospheric pressure, P_1 is the receding-edge internal pressure, P_2 is the advancing-edge internal pressure, and θ is the contact angle of the liquid–gas–solid interface. The contact angle will depend on the surface characteristics of the channel and the constituents of the drop, with a hydrophilic interaction giving θ between 0 and 90 degrees, and a hydrophobic surface giving θ between 90 and 180 degrees. Surface treatments can also reduce contact angle hysteresis and, therefore, reduce the temperature difference necessary for drop motion.

The droplet then passed through the resistive heater portion (C) in which PCR amplification occurred. *Id.* at 5556. Afterwards the amplification results were optically detected in zone D via gel electrophoresis *Id.* at 5559. Burns II noted that "[t]he use of common fabrication methods allows the assembly of increasingly complex, multicomponent, integrated systems from a small, defined set of standardized elements" and that "[f]uture design efforts have a strong impetus to reduce component size and improve efficiency." *Id.* at 5560-61.

By the late 1990s Kopp *et al.* had successfully implemented continuous flow PCR on a chip. In his 1988 article (not previously presented to the USPTO), Kopp explained that "[a] wide

range of microreactors, microcapillary electrophoresis devices, and microcell manipulation devices have been described in recent years." Ex. 1027 at 1046. Building on that body of work, Kopp's group developed

> [a] micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in **continuous flow** at high speed. The device is analogous to an electronic amplifier and relies on the movement of sample through thermostated temperature zones on a glass microchip. Input and output of material (DNA) is **continuous**, and amplification is independent of input concentration. . . .
>
> The results demonstrate that with a very simple device and virtually no optimization, PCR can be performed in **continuous flow**, yielding product quality and quantity comparable to standard thermal cycling methods (Fig. 2B). . . .
>
> We suggest that cross-contamination in a **continuous-flow** format may be much less of a problem than in a stationary-tube PCR format. Thus, a flow-through PCR chip can be reused for extended periods without major cleaning. . . .



Ex. 1027 at 1046-1047 [emphasis added]. Kopp *et al.* also explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications:

> To build a complex system analogous to an electronic integrated circuit, it is necessary to integrate a variety of other continuously working parts. A cursory glance at developments in miniaturized total analysis systems (uTAS) during the past 5 years reveals that most of the relevant components, such as **continuous-flow mixers** (8), **continuous-flow microreactors** (9), **continuous separations** (10), and high-speed capillary gel electrophoresis (as a conventional batch process), have already been presented (11). Accordingly, the **continuous-flow microamplifier** should allow the creation of highly elaborate analysis and synthesis systems.
>
> Although chemical amplification reactors will obviously not be applicable to all reactions, neither will they be limited to PCR. Devices of this kind open up whole new areas of application in medical diagnostics. For example, the online amplification and monitoring of a specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy.

Ex. 1027 at 1047 [emphasis added].

In sum, Kopp and Burns II show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Shaw Stewart is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[103] Ex 1001 at 78:22-24. In the instant combination,

[103] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Shaw Stewart) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Shaw Stewart, to conduct microfluidic PCR as taught by Kopp and Burns II. A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic droplet reactor of Shaw Stewart because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could

demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Shaw Stewart to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Shaw Stewart would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004

¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Shaw Stewart to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Kopp or Burns II) to incorporate a droplet reactor (Shaw Stewart). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Kopp, Burns II, Lagally or Vogelstein. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the single fluid in microfluidic chip reactor (e.g., Burns II or Lagally) or droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Corbett or Kopp). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high

speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The

> first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic

> diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the

microfluidic droplet reactor of Shaw Stewart could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful modifying Kopp or Burns II to work in the microfluidic droplet reactor of Shaw Stewart, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[104] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail below, in the accompanying claim chart (Ex. 1072) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the

[104] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

microfluidic droplet reactor of Shaw Stewart to conduct microfluidic PCR as taught by Kopp and Burns II.

Independent Claim 1

Claim 1[a]: A method comprising the steps of: providing a microfluidic system comprising one or more channels

Shaw Stewart discloses that "[t]he method of the invention may be used for initiating and controlling a chemical reaction or preparing mixtures of reagents.…" Ex. 1040 at 1:7-9. "The system is particularly suited **to the manipulation of microscopic quantities of reagents, with volumes of less than one microlit[er]**,…" *Id.* at 1:20-22 [emphasis added].

As shown in Shaw Stewart's Figure 1, "[t]he reagent is introduced into a tube containing carrier phase (1) from a side arm (2) by being sucked or pushed through a small opening (3). When the correct volume has passed into the tube, it is separated and carried away by a current of carrier phase, down the tube." Ex. 1040 at 1:70-75.

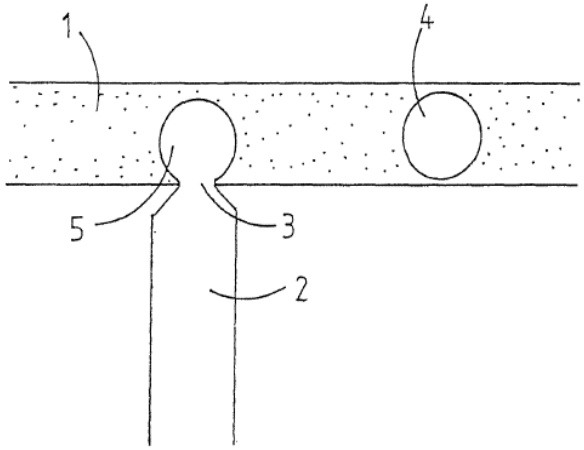


Figure 1.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1072 and in the Shaqfeh Declaration (Ex. 1004).

Claim 1[b]: providing within the one or more channels a continuously flowing carrier fluid comprising an oil and a continuously flowing aqueous fluid

comprising target DNA or RNA molecules and at least one other molecule in the fluid that can react with the target DNA or RNA molecules under conditions in which the target DNA or RNA molecules and the other molecules in the fluid do not react with each other

As shown in Shaw Stewart's Figure 1, "[t]he reagent is introduced into a tube containing carrier phase (1) from a side arm (2) by being sucked or pushed through a small opening (3). When the correct volume has passed into the tube, it is separated and carried away by a current of carrier phase, down the tube." Ex. 1040 at 1:70-75. Shaw Stewart teaches that "[f]or **aqueous reagents**, glass tubing and a light silicon carrier phase would be particularly suitable, as this combination would prevent wetting of the glass, due to a strong interaction at the glass-silicon interface." Ex. 1040 at 1:62-66 [emphasis added]. "[I]f large numbers of droplets are required, **a continuous flow of reagent** through the opening will be produced, while a **continuous current of carrier phase flows** down the tube." Ex. 1040 at 1:83-86 [emphasis added].

In the instant combination, the microfluidic droplet reactor of Shaw Stewart are relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[105] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Shaw Stewart) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Shaw Stewart to conduct microfluidic PCR as taught by Kopp and Burns II. A skilled

[105] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

artisan would have been strongly motivated to perform such PCR reactions in the microfluidic reactors of Shaw Stewart because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Shaw Stewart to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Shaw Stewart would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026

(Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
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Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Shaw Stewart to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Kopp or Burns II) to

incorporate a droplet reactor (Shaw Stewart). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Kopp or Burns II. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the single fluid in microfluidic chip reactor (e.g., Burns II) or droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Kopp). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and

> amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that

"the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactors Shaw Stewart could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful in modifying Kopp or Burns II to work in the microfluidic droplet reactor of Shaw Stewart, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was

published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[106] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained further in the accompanying claim chart (Ex. 1072) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Shaw Stewart to conduct microfluidic PCR as taught by Kopp and Burns II.

With regard to the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other," this is merely a description of a PCR reaction. Ex. 1004 ¶31,32. As explained in Corbett, Kopp, Burns II, and Lagally, in a PCR reaction the sample DNA or RNA is admixed with various reagents including polymerase under conditions in which no reaction occurs; only when heat is applied does the PCR reaction occur. Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68. In the PCR of the combined method, as in virtually any PCR method, the DNA and polymermase do not react until mixture

[106] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

of reagents is heated. *Id*.; see also Ex. 1004 ¶32.) The combined method thus meets the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other" this is merely a description of a PCR reaction. Ex. 1004 ¶31,32.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1072.

Claim 1[c]: controlling flow rates of said aqueous fluid and said carrier fluid to partition the continuously flowing aqueous fluid with the continuously flowing carrier fluid to form a plurality of plugs of the aqueous fluid, each having a substantially uniform size of about 200 µm or less, wherein the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule

As discussed above, the Shaw Stewart British Application teaches conducting reactions in microdroplets formed from two continuously flowing immiscible fluids. Significantly, the exclusive licensee of the '148 patent affirmatively (and correctly) took the position that Shaw Stewart teaches plug or droplet formation combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid. More particularly, Bio-Rad (who recently acquired RainDance, the original exclusive licensee of the '148 patent, Ex. 1061, Ex. 1053 ¶71) explained that another party had mischaracterized the Shaw Stewart reference by failing to point out the above-quoted portion of Shaw Stewart, in which Shaw Stewart specifically teaches that in certain embodiments droplets are formed from intersecting and continuously flowing streams.

> To overcome Stewart I and secure allowance of their patent, the Patent Owner ("PO") mischaracterized the device disclosed in Stewart I (and hence Stewart II), misdirecting the Examiner to irrelevant portions of Stewart I while ignoring other portions that directly contradicted PO's arguments. *See, e.g.*, Ex. 1003 at 174-180. For example, one of PO's primary arguments was that Stewart I

> only disclosed introducing fluid in a "stepwise fashion," whereas in the '503 Patent droplets are formed by "combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid." *Id.* at 174. In truth, however, Stewart I also discloses combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid:
>
> > If large numbers of droplets are required, a continuous flow of reagent through the opening will be produced, while a continuous current of carrier phase flows down the tube. When each droplet almost spans the tube, it will be broken off.
>
> Ex. 1008, pg. 1, col. 2, ll. 83-88.

Ex. 1059 at 2-3.

Thus the exclusive licensee (Bio-Rad) expressly agreed that Shaw Stewart discloses forming plugs from intersecting streams of continuously flowing immiscible fluids.

Turning to the recitation "each having a substantially uniform size of about 200 µm or less," Shaw Stewart teaches that the volumes of the droplets are 1 microliter or less. Ex. 1040 at 1:20-23. 1,000,000 microliters are equal to one liter. One liter is equal to 1×10^{-3} cubic meters. Assuming the droplets are spheres, this equates to a droplet diameter of 1,200 µm. Shaw Stewart did not put a lower limit on the size of the droplets and contemplated droplet sizes below 200 µm. *Id.* Moreover, it must be noted that Shaw Stewart dates back to 1984, when microfabrication techniques were not as refined and channels could not readily be made as small as they could circa the year 2000. Quake (filed in the year 2000) teaches that "the droplets have a diameter that is smaller than the diameter of the microchannel; i.e., preferably less than 60 µm." Ex. 1034 at 27:29-30. Fig. 21 of Quake shows the droplets sizes generated by the Quake droplet generator were all less than 60 µm. Burns II (1996) similarly discloses uses of 60 nl droplets (which equates to a droplet diameter of about 62 µm). Ex. 1008 at 5558. Burns I (2001) discloses that the droplets are 100 µm to 400 µm in diameter. Ex. 1007 at 10. Accordingly, it would have been

obvious to a skilled artisan that as of the earliest claimed priority date (2002) droplets below 200 µm in diameter could, and should, be used in order to reduce reagent usage and reaction time and achieve the various other benefits described above with the combination. Ex. 1004 ¶36, 66. This leaves the recitation that "the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule." As discussed above, in the combined method of Quake, Corbett and Lagally, the samples are separated into a number of discrete droplets. That has the advantages of i) overcoming the signal-to-noise limitations associated with certain other PCR techniques and ii) enabling the detection of relatively rare mutations, dislocations and allelic imbalances. Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Lagally expressly teaches that "[t]he event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a **Poisson** distribution, demonstrating that we are operating at the single-molecule level." Ex. 1028 at 566 [emphasis added]. Vogelstein likewise teaches that the aforementioned advantages are realized by **reducing the number of sample molecules per droplet to zero or one.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶82**.** That ensures that any amplification that occurs in a given droplet can be readily identified as containing the target sequence. *Id.* Thus, in the combined method the droplets have either one or zero template molecules.

As suggested by Lagally, any process which is generating droplets containing about one template molecule per droplet is expected to conform to a random, or Poisson, distribution. Ex. 1028 at 566; Ex. 1004 ¶48. It was known in the art that "[i]f a solution containing x molecules of enzyme per ml is dispersed into droplets of v ml volume, the average number of enzyme molecules per droplet n is equal to vx. Because of statistical fluctuations each droplet will not

have exactly n enzyme molecules. The probability p(r) of finding 0, 1, 2, 3, . .. , r enzyme molecules in a droplet **is given by the Poisson law**." Ex. 1112 at 1985 [emphasis added]; see also Ex. 1111 at 1667 ("Spectra observed in a time sequence for an average of 0.6 dye molecule in the probed volume exhibited the expected Poisson distribution for actually measuring 0, 1, 2, or 3 molecules.").

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1072.

Claim 1[d]: providing conditions suitable for a polymerase-chain reaction in at least one plug of the plurality of plugs such that the target DNA or RNA is amplified

Shaw Stewart discloses that "[r]egions of the device can be heated by electric circuits or coils or by electromagnetic radiation to allow the merged droplets to be incubated at the required temperature." Ex. 1040 at 2:44-47. "If a colour change reaction is involved, such a colour change can be recorded immediately or after incubation, using thermostatically controlled heating coil (27)." *Id.* at 3:58-60.

As discussed above in connection with element 1[b], in the instant combination Shaw Stewart is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[107] These limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Shaw Stewart) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

[107] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1072.

Thus, claim 1 is shown to be rendered obvious by the combination of Shaw Stewart, Kopp and Burns II.

Dependent Claim 2

Claim 2 of the '148 patent recites "wherein the step of providing conditions includes heating."

As discussed above in connection claim elements 1[b] and 1[c], in the combined method (and in virtually any other PCR reaction) the DNA and polymerase undergo polymerase chain reaction when heated. Ex. 1004 ¶32; Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1072.

Thus, claim 2 is shown to be rendered obvious by the combination of Shaw Stewart, Kopp and Burns II.

Dependent Claims 3-5

Claim 3 of the '148 patent recites "providing a detector to detect, analyze, characterize, or monitor the target DNA or RNA molecule in the at least one member of the plurality of plugs during and/or after the reaction has occurred." Claim 4 of the '148 patent further recites "wherein the at least one other molecule includes one or more fluorescent labels and the detector is configured to monitor progress of the reaction by detecting fluorescence emissions from the one or more fluorescent labels." Claim 5 recites "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels."

Shaw Stewart discloses that "parts of the device itself can be adapted to form the sample chambers of the standard instruments of chemical or biochemical analysis." Ex. 1040 at 2:61-64. "For example ducts can be formed with two plain transparent walls to form the sample chambers of spectrophotometers." Ex. 1040 at 2:64-66.

Burns II teaches that "[c]apillary gel electrophoresis of DNA samples was performed using a Beckman P/ACE instrument with a laser-induced fluorescence detector and 37 cm length, 100 μm diameter, linear polymerase gel capillary according to manufacturer's recommendation." Ex. 1008 at 5558. The results of capillary electrophoresis were generally detected optically at the time of filing. Ex. 1011 at 46 (Dolnick). "The amplified product, labeled with an intercalating **fluorescent** dye, is directly injected into the gel-filled capillary channel for electrophoretic analysis." Ex. 1028 at 1, [emphasis added].

In the combined method of Shaw Stewart, Kopp and Burns II, microfluidic droplet reactors are used to perform PCR reactions after which the reaction products are detected optically and sorted by diverting certain droplets into collection channels. Ex. 1004 ¶69.

Turning to claim 5, under the broadest reasonable interpretation the phrase "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels" requires simply that at least some of the droplets or plugs are transmitted to an outlet or other channel. Shaw Stewart teaches an outlet channel leading to reservoir 19 and thus meets claim 5 under the broadest reasonable interpretation. Ex. 1041 at 3:57-62. If claim 5 is more narrowly interpreted as requiring selective diversion of the droplets, that was well known. For instance, Quake teaches that "[a] preferred 'forward' sorting algorithm consists of running molecules, cells or virions from the input channel to the waste channel, until a molecule, cell or virion is identified to have an optically detectable signal (e.g. fluorescence) that is above a pre-

set threshold, at which point voltages are temporarily changed to electro-osmotically divert the molecule [] to the collection channel." Ex. 1034 at 20:22-26. Fu likewise discloses a microfabricated fluorescence-activiated cell sorter for use in connection with emulsion or droplet based systems. Ex. 1113 at 1109. A skilled artisan would have considered it obvious to employ such a technique to sort droplets after they are subjected to the optical detection. Ex. 1004 ¶68,69. For instance, a skilled artisan would have found it advantageous to sort and collect droplets which tested positive or negative for the presence of a certain substance or property, especially in the context of droplet generators which are used to encapsulate on average one template molecule per droplet. *Id.* ¶82.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1072.

Thus, claims 3-5 are shown to be rendered obvious by the combination of Shaw Stewart, Kopp and Burns II.

Dependent Claims 6-8

Claim 6 of the '148 patent recites "wherein the oil is a fluorinated oil." Claim 7 of the '148 patent recites "wherein the carrier fluid further comprises a surfactant." Claim 8 of the '148 patent recites "wherein the surfactant is a fluorinated surfactant."

Shaw Stewart discloses that "[s]uitable carrier phases include **mineral oils**, water, light silicons, or **Freons**." Ex. 1040 at 1:39-41, [emphasis added]; (Freons are comprised of chorofluorocarbons. Ex. 1108). "**Surface acting agents** may also be included in the carrier and/or reagents phases to produce suitable surface properties, for example to allow efficient merging." *Id.* at 1:43-47, [emphasis added].

Additional correspondence between these claim elements and the cited references is shown in the claim chart submitted herewith as Exhibit 1072 and discussed in the Declaration of Dr. Shaqfeh (Ex. 1004).

To the extent the Office determines that any element of claims 6-8 is not disclosed by Shaw Stewart, it would have been obvious to use the recited fluorinated surfactants and oils in light of Quake's disclosure that fluourinated oils serve as surfactants which "aid in controlling or optimizing droplet size, flow and uniformity, for example by reducing the shear force needed to extrude or inject droplets into an intersecting channel." Ex. 1034 at 35:18-22.

Thus, claims 6-8 are shown to be rendered obvious by the combination of Shaw Stewart, Kopp and Burns II and further in view of Quake.

L. The Combination of Shaw Stewart, Kopp and Vogelstein Renders Obvious Claims 1-8 of the '148 Patent Under (pre-AIA) 35 U.S.C. § 103(a)

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Shaw Stewart), ii) microfluidic PCR devices were well known (Kopp and Vogelstein), and iii) there were compelling reasons to use the microfluidic droplet reactors to conduct microfluidic PCR (Kopp, Burns II, Lagally and Vogelstein).

The Shaw Stewart British Application teaches conducting reactions in microdroplets formed from two continuously flowing immiscible fluids. The Shaw Stewart British Application discloses a "droplet reactor" and illustrates at Figure 1 a carrier phase 1 flowing along a conduit into which reagent 2 is injected, thereby forming droplet 4. Ex. 1040 at 1:1-20, 70-90. "[I]f large numbers of droplets are required, a

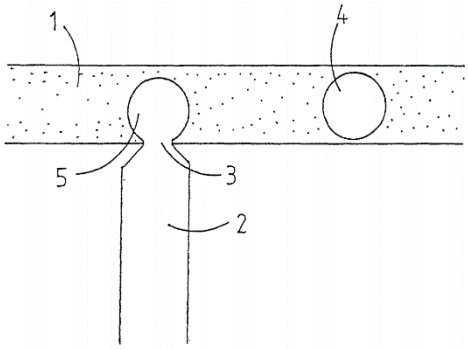


Figure 1.

continuous flow of reagent through the opening will be produced, while a continuous current of carrier phase flows down the tube. When each droplet almost spans the tube, it will be broken off. The exact size of the droplets produced will depend on the relative and absolute magnitude of the two currents." *Id.* at 1:83-90.

Significantly, the exclusive licensee of the '148 patent affirmatively (and correctly) took the position that Shaw Stewart teaches plug or droplet formation combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid. More particularly, Bio-Rad (who recently acquired RainDance, the original exclusive licensee of the '148 patent, Ex. 1061, Ex. 1053 ¶71) explained that another party had mischaracterized the Shaw Stewart reference by failing to point out the above-quoted portion of Shaw Stewart, in which Shaw Stewart specifically teaches that in certain embodiments droplets are formed from intersecting and continuously flowing streams.

> To overcome Stewart I and secure allowance of their patent, the Patent Owner ("PO") mischaracterized the device disclosed in Stewart I (and hence Stewart II), misdirecting the Examiner to irrelevant portions of Stewart I while ignoring other portions that directly contradicted PO's arguments. *See, e.g.*, Ex. 1003 at 174-180. For example, one of PO's primary arguments was that Stewart I

> only disclosed introducing fluid in a "stepwise fashion," whereas in the '503 Patent droplets are formed by "combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid." *Id.* at 174. In truth, however, Stewart I also discloses combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid:
>
> > If large numbers of droplets are required, a continuous flow of reagent through the opening will be produced, while a continuous current of carrier phase flows down the tube. When each droplet almost spans the tube, it will be broken off.
>
> Ex. 1008, pg. 1, col. 2, ll. 83-88.

Ex. 1059 at 2-3.

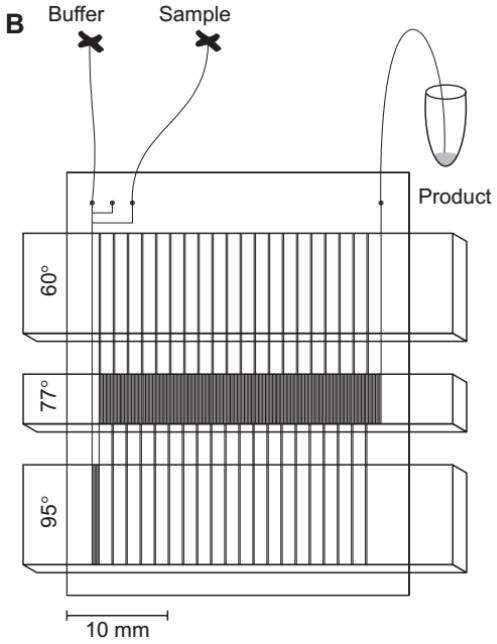
Thus the exclusive licensee (Bio-Rad) expressly agreed that Shaw Stewart discloses forming plugs from intersecting streams of continuously flowing immiscible fluids.

As of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Kopp and Vogelstein. By the late 1990s Kopp et al. had successfully implemented continuous flow PCR on a chip. In his 1988 article (not previously presented to the USPTO), Kopp explained that "[a] wide range of microreactors, microcapillary electrophoresis devices, and microcell manipulation devices have been described in recent years." Ex. 1027 at 1046. Building on that body of work, Kopp's group developed

> [a] micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in **continuous flow** at high speed. The device is analogous to an electronic amplifier and relies on the movement of sample through thermostated temperature zones on a glass microchip. Input and output of material (DNA) is **continuous**, and amplification is independent of input concentration. . . .
>
> The results demonstrate that with a very simple device and virtually no optimization, PCR can be performed in **continuous flow**, yielding product

quality and quantity comparable to standard thermal cycling methods (Fig. 2B). . . .

We suggest that cross-contamination in a **continuous-flow** format may be much less of a problem than in a stationary-tube PCR format. Thus, a flow-through PCR chip can be reused for extended periods without major cleaning. . . .



Ex. 1027 at 1046-1047 [emphasis added]. Kopp *et al.* also explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications:

To build a complex system analogous to an electronic integrated circuit, it is necessary to integrate a variety of other continuously working parts. A cursory glance at developments in miniaturized total analysis systems (uTAS) during the past 5 years reveals that most of the relevant components, such as **continuous-flow mixers** (8), **continuous-flow microreactors** (9), **continuous separations** (10), and high-speed capillary gel electrophoresis (as a conventional batch process), have already been presented (11). Accordingly, the **continuous-flow microamplifier** should allow the creation of highly elaborate analysis and synthesis systems.

> Although chemical amplification reactors will obviously not be applicable to all reactions, neither will they be limited to PCR. Devices of this kind open up whole new areas of application in medical diagnostics. For example, the online amplification and monitoring of a specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy.

Ex. 1027 at 1047 [emphasis added].

By 1999, Vogelstein had reported **various applications for single-molecule microfluidic PCR**. Ex. 1044 at 9241. In a seminal article which has been cited over by over 1,000 other publications, Vogelstein explained that PCR amplification of small aliquots or droplets containing single molecules was effective for "detection of a small number of mutant-containing cells among a large excess of normal cells." *Id.* at 9236. In Vogelstein's method, the DNA was diluted into 7 µl volumes such that, on average, there was one template molecule for every two wells (i.e., approximately half of the wells contained no DNA template molecule and approximately half contained a single DNA template molecule). *Id.* at 9236, 9237. This avoided the problem of background noise impairing the determination of whether each volume contained the mutant sequence. *Id.* By counting the number of volumes which indicated wild type or mutant templates, the presence or absence of the mutation (e.g., a sequence associated with colorectal cancer) can be determined. *Id.* at 9238. The accuracy of the test is a function of the number of discrete volumes into which the sample is diluted. *Id.* at 9239. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. *Id.* at 9239. ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude.") *Id.* 9239.The article explained that the same technique would be useful for **detecting a wide array of mutations, dislocations and allelic**

imbalances, as summarized in Table 1. *Id.* at 9241. Vogelstein thus taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby overcoming the signal-to-noise limitations associated with certain other PCR techniques and enabling the detection of relatively rare mutations, dislocations and allelic imbalances. Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84.

In sum, Kopp and Vogelstein show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Shaw Stewart is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[108] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Shaw Stewart) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Shaw Stewart, to conduct microfluidic PCR as taught by Kopp and Vogelstein. A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic droplet reactor of Shaw Stewart because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as

[108] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing**).** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Shaw Stewart to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Shaw Stewart would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to

then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
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Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
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Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Shaw Stewart to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Kopp or Vogelstein) to incorporate a droplet reactor (Shaw Stewart). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic

PCR devices taught in Kopp or Vogelstein. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Kopp). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in

> silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
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> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control.

> The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-

chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Shaw Stewart could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful modifying Kopp or Vogelstein to work in the microfluidic droplet reactor of Shaw Stewart, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill

in the art.[109] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail below, in the accompanying claim chart (Ex. 1073) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Shaw Stewart to conduct microfluidic PCR as taught by Kopp and Vogelstein.

Independent Claim 1

Claim 1[a]: A method comprising the steps of: providing a microfluidic system comprising one or more channels

Shaw Stewart discloses that "[t]he method of the invention may be used for initiating and controlling a chemical reaction or preparing mixtures of reagents.…" Ex. 1040 at 1:7-9. "The system is particularly suited **to the manipulation of microscopic quantities of reagents, with volumes of less than one microlit[er]**,…" *Id.* at 1:20-22 [emphasis added].

As shown in Shaw Stewart's Figure 1, "[t]he reagent is introduced into a tube containing carrier phase (1) from a side arm (2) by being sucked or pushed through a small opening (3). When the correct volume has passed into the tube, it is separated and carried away by a current of carrier phase, down the tube." Ex. 1040 at 1:70-75.

[109] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

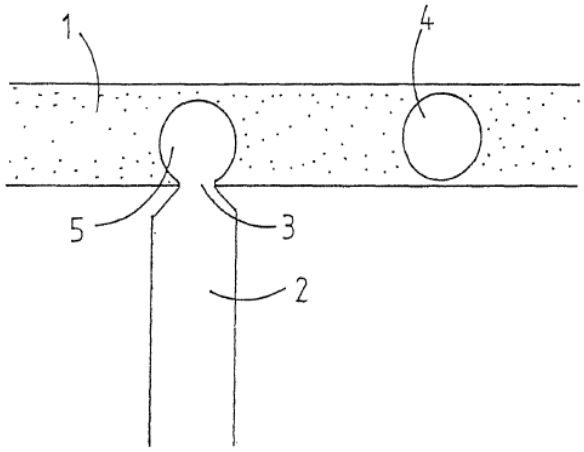

Figure 1.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1073 and in the Shaqfeh Declaration (Ex. 1004).

Claim 1[b]: providing within the one or more channels a continuously flowing carrier fluid comprising an oil and a continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule in the fluid that can react with the target DNA or RNA molecules under conditions in which the target DNA or RNA molecules and the other molecules in the fluid do not react with each other

As shown in Shaw Stewart's Figure 1, "[t]he reagent is introduced into a tube containing carrier phase (1) from a side arm (2) by being sucked or pushed through a small opening (3). When the correct volume has passed into the tube, it is separated and carried away by a current of carrier phase, down the tube." Ex. 1040 at 1:70-75. Shaw Stewart teaches that "[f]or **aqueous reagents**, glass tubing and a light silicon carrier phase would be particularly suitable, as this combination would prevent wetting of the glass, due to a strong interaction at the glass-silicon interface." Ex. 1040 at 1:62-66 [emphasis added]. "[I]f large numbers of droplets are required, **a continuous flow of reagent** through the opening will be produced, while a **continuous current of carrier phase flows** down the tube." Ex. 1040 at 1:83-86 [emphasis added].

In the instant combination, the microfluidic droplet reactor of Shaw Stewart is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[110] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Shaw Stewart) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Shaw Stewart to conduct microfluidic PCR as taught by Kopp and Vogelstein. A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic reactors of Shaw Stewart because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

[110] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Shaw Stewart to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Shaw Stewart would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product

specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

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Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

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Application	Example	Probe 1 detects	Probe 2 detects
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Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Shaw Stewart to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

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In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

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Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

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As explained further in the accompanying claim chart (Ex. 1073) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled

[111] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Shaw Stewart to conduct microfluidic PCR as taught by Kopp and Vogelstein.

With regard to the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other," this is merely a description of a PCR reaction. Ex. 1004 ¶31,32. As explained in Corbett, Kopp, Burns II, and Lagally, in a PCR reaction the sample DNA or RNA is admixed with various reagents including polymerase under conditions in which no reaction occurs; only when heat is applied does the PCR reaction occur. Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68. In the PCR of the combined method, as in virtually any PCR method, the DNA and polymermase do not react until mixture of reagents is heated. *Id*.; see also Ex. 1004 ¶32. The combined method thus meets the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other" this is merely a description of a PCR reaction. Ex. 1004 ¶31,32.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1073.

Claim 1[c]: controlling flow rates of said aqueous fluid and said carrier fluid to partition the continuously flowing aqueous fluid with the continuously flowing carrier fluid to form a plurality of plugs of the aqueous fluid, each having a substantially uniform size of about 200 µm or less, wherein the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule

As discussed above, the Shaw Stewart British Application teaches conducting reactions in microdroplets formed from two continuously flowing immiscible fluids. Significantly, the exclusive licensee of the '148 patent affirmatively (and correctly) took the position that Shaw Stewart teaches plug or droplet formation combining a flowing stream of an aqueous solution

and a flowing stream of an immiscible fluid. More particularly, Bio-Rad (who recently acquired RainDance, the original exclusive licensee of the '148 patent, Ex. 1061, Ex. 1053 ¶71) explained that another party had mischaracterized the Shaw Stewart reference by failing to point out the above-quoted portion of Shaw Stewart, in which Shaw Stewart specifically teaches that in certain embodiments droplets are formed from intersecting and continuously flowing streams.

> To overcome Stewart I and secure allowance of their patent, the Patent Owner ("PO") mischaracterized the device disclosed in Stewart I (and hence Stewart II), misdirecting the Examiner to irrelevant portions of Stewart I while ignoring other portions that directly contradicted PO's arguments. *See, e.g.*, Ex. 1003 at 174-180. For example, one of PO's primary arguments was that Stewart I only disclosed introducing fluid in a "stepwise fashion," whereas in the '503 Patent droplets are formed by "combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid." *Id.* at 174. In truth, however, Stewart I also discloses combining a flowing stream of an aqueous solution and a flowing stream of an immiscible fluid:
>
> > If large numbers of droplets are required, a continuous flow of reagent through the opening will be produced, while a continuous current of carrier phase flows down the tube. When each droplet almost spans the tube, it will be broken off.
>
> Ex. 1008, pg. 1, col. 2, ll. 83-88.

Ex. 1059 at 2-3.

Thus the exclusive licensee (Bio-Rad) expressly agreed that Shaw Stewart discloses forming plugs from intersecting streams of continuously flowing immiscible fluids.

Turning to the recitation "each having a substantially uniform size of about 200 µm or less," Shaw Stewart teaches that the volumes of the droplets are 1 microliter or less. Ex. 1040 at 1:20-23. 1,000,000 microliters are equal to one liter. One liter is equal to 1×10^{-3} cubic meters.

Assuming the droplets are spheres, this equates to a droplet diameter of 1,200 µm. Shaw Stewart did not put a lower limit on the size of the droplets and contemplated droplet sizes below 200 µm. *Id.* Moreover, it must be noted that Shaw Stewart dates back to 1984, when microfabrication techniques were not as refined and channels could not readily be made as small as they could circa the year 2000. Quake (filed in the year 2000) teaches that "the droplets have a diameter that is smaller than the diameter of the microchannel; i.e., preferably less than 60 µm." Ex. 1034 at 27:29-30. Fig. 21 of Quake shows the droplets sizes generated by the Quake droplet generator were all less than 60 µm. Burns II (1996) similarly discloses uses of 60 nl droplets (which equates to a droplet diameter of about 62 µm). Ex. 1008 at 5558. Burns I (2001) discloses that the droplets are 100 µm to 400 µm in diameter. Ex. 1007 at 10. Accordingly, it would have been obvious to a skilled artisan that as of the earliest claimed priority date (2002) droplets below 200 µm in diameter could, and should, be used in order to reduce reagent usage and reaction time and achieve the various other benefits described above with the combination. Ex. 1004 ¶36, 66.

This leaves the recitation that "the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule." As discussed above, in the combined method of Quake, Corbett and Lagally, the samples are separated into a number of discrete droplets. That has the advantages of i) overcoming the signal-to-noise limitations associated with certain other PCR techniques and ii) enabling the detection of relatively rare mutations, dislocations and allelic imbalances. Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Lagally expressly teaches that "[t]he event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a **Poisson** distribution, demonstrating that we are operating at the single-

molecule level." Ex. 1028 at 566 [emphasis added]. Vogelstein likewise teaches that the aforementioned advantages are realized by **reducing the number of sample molecules per droplet to zero or one.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶82**.** That ensures that any amplification that occurs in a given droplet can be readily identified as containing the target sequence. *Id.* Thus, in the combined method the droplets have either one or zero template molecules.

As suggested by Lagally, any process which is generating droplets containing about one template molecule per droplet is expected to conform to a random, or Poisson, distribution. Ex. 1028 at 566; Ex. 1004 ¶48. It was known in the art that "[i]f a solution containing x molecules of enzyme per ml is dispersed into droplets of v ml volume, the average number of enzyme molecules per droplet n is equal to vx. Because of statistical fluctuations each droplet will not have exactly n enzyme molecules. The probability p(r) of finding 0, 1, 2, 3, . .. , r enzyme molecules in a droplet **is given by the Poisson law**." Ex. 1112 at 1985 [emphasis added]; see also Ex. 1111 at 1667 ("Spectra observed in a time sequence for an average of 0.6 dye molecule in the probed volume exhibited the expected Poisson distribution for actually measuring 0, 1, 2, or 3 molecules.").

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1073.

Claim 1[d]: providing conditions suitable for a polymerase-chain reaction in at least one plug of the plurality of plugs such that the target DNA or RNA is amplified

Shaw Stewart discloses that "[r]egions of the device can be heated by electric circuits or coils or by electromagnetic radiation to allow the merged droplets to be incubated at the required temperature." Ex. 1040 at 2:44-47. "If a colour change reaction is involved, such a colour

change can be recorded immediately or after incubation, using thermostatically controlled heating coil (27)." *Id.* at 3:58-60.

As discussed above in connection with element 1[b], in the instant combination Shaw Stewart is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[112] These limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Shaw Stewart) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1073.

Thus, claim 1 is shown to be rendered obvious by the combination of Shaw Stewart, Kopp and Vogelstein.

Dependent Claim 2

Claim 2 of the '148 patent recites "wherein the step of providing conditions includes heating."

As discussed above in connection claim elements 1[b] and 1[c], in the combined method (and in virtually any other PCR reaction) the DNA and polymerase undergo polymerase chain reaction when heated. Ex. 1004 ¶32; Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1073.

[112] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

Thus, claim 2 is shown to be rendered obvious by the combination of Shaw Stewart, Kopp and Vogelstein.

Dependent Claims 3-5

Claim 3 of the '148 patent recites "providing a detector to detect, analyze, characterize, or monitor the target DNA or RNA molecule in the at least one member of the plurality of plugs during and/or after the reaction has occurred." Claim 4 of the '148 patent further recites "wherein the at least one other molecule includes one or more fluorescent labels and the detector is configured to monitor progress of the reaction by detecting fluorescence emissions from the one or more fluorescent labels." Claim 5 recites "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels."

Shaw Stewart discloses that "parts of the device itself can be adapted to form the sample chambers of the standard instruments of chemical or biochemical analysis." Ex. 1040 at 2:61-64. "For example ducts can be formed with two plain transparent walls to form the sample chambers of spectrophotometers." Ex. 1040 at 2:64-66.

Vogelstein teaches that the "[r]eactions were analyzed immediately or stored at room temperature for up to 36 h before **fluorescence analysis**." Ex. 1044 at 9236, [emphasis added].

In the combined method of Shaw Stewart, Kopp and Vogelstein, microfluidic droplet reactors are used to perform PCR reactions after which the reaction products are detected optically and sorted by diverting certain droplets into collection channels. Ex. 1004 ¶69.

Turning to claim 5, under the broadest reasonable interpretation the phrase "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels" requires simply that at least some of the droplets or plugs are transmitted to an outlet or other channel. Shaw Stewart teaches an outlet channel leading to reservoir 19 and thus meets claim 5 under the broadest reasonable interpretation. Ex. 1041 at 3:57-62. If claim 5 is more

narrowly interpreted as requiring selective diversion of the droplets, that was well known. For instance, Quake teaches that "[a] preferred 'forward' sorting algorithm consists of running molecules, cells or virions from the input channel to the waste channel, until a molecule, cell or virion is identified to have an optically detectable signal (e.g. fluorescence) that is above a pre-set threshold, at which point voltages are temporarily changed to electro-osmotically divert the molecule [] to the collection channel." Ex. 1034 at 20:22-26. Fu likewise discloses a microfabricated fluorescence-activiated cell sorter for use in connection with emulsion or droplet based systems. Ex. 1113 at 1109. A skilled artisan would have considered it obvious to employ such a technique to sort droplets after they are subjected to the optical detection. Ex. 1004 ¶68,69. For instance, a skilled artisan would have found it advantageous to sort and collect droplets which tested positive or negative for the presence of a certain substance or property, especially in the context of droplet generators which are used to encapsulate on average one template molecule per droplet. *Id.* ¶82.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1073.

Thus, claims 3-5 are shown to be rendered obvious by the combination of Shaw Stewart, Kopp and Vogelstein.

Dependent Claims 6-8

Claim 6 of the '148 patent recites "wherein the oil is a fluorinated oil." Claim 7 of the '148 patent recites "wherein the carrier fluid further comprises a surfactant." Claim 8 of the '148 patent recites "wherein the surfactant is a fluorinated surfactant."

Shaw Stewart discloses that "[s]uitable carrier phases include **mineral oils**, water, light silicons, or **Freons**." Ex. 1040 at 1:39-41, [emphasis added]; (Freons are comprised of chorofluorocarbons. Ex. 1108). "**Surface acting agents** may also be included in the carrier

and/or reagents phases to produce suitable surface properties, for example to allow efficient merging." *Id.* at 1:43-47, [emphasis added].

Additional correspondence between these claim elements and the cited references is shown in the claim chart submitted herewith as Exhibit 1073 and discussed in the Declaration of Dr. Shaqfeh (Ex. 1004).

To the extent the Office determines that any element of claims 6-8 is not disclosed by Shaw Stewart, it would have been obvious to use the recited fluorinated surfactants and oils in light of Quake's disclosure that fluourinated oils serve as surfactants which "aid in controlling or optimizing droplet size, flow and uniformity, for example by reducing the shear force needed to extrude or inject droplets into an intersecting channel." Ex. 1034 at 35:18-22.

Thus, claims 6-8 are shown to be rendered obvious by the combination of Shaw Stewart, Kopp and Vogelstein and further in view of Quake.

M. The Combination of Burns I, Corbett and Lagally Renders Obvious Claims 1-5 of the '148 Patent Under (pre-AIA) 35 U.S.C. § 103(a)

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Burns I), ii) microfluidic PCR devices were well known (Corbett and Lagally), and iii) there were compelling reasons to use the microfluidic droplet reactors to conduct microfluidic PCR (Kopp, Burns II, Lagally and Vogelstein).

Burns I teaches that plugs in the channels of a microreactor by the continuous flow of fluids at an intersecting channel. Burns I explained that "various methods may be used to generate slugs of liquid within a microreactor." Ex. 1007 at 10. Burns I chose a method in which "the continuous flow of both phases through T or cross-shaped intersections" and "[s]lugs are generated by the action of one phase flowing into the channel whilst the other phase moves into the intersection, eventually cutting off the flow of the first phase into the channel and reversing the process." Ex. 1007 at 11. Fig. 3 shows oil-in-water slug generation and Fig. 4 shows water-in-oil slug generation. *Id.* at 11. In Burns' Fig. 4 embodiment, an aqueous plug of reagents is formed in a continuous oil phase. The continuous flow plug formation used by Burns was a well-known alternative to periodically injecting a measured slug of reactant into the main channel. See Ex. 1010; 1027; Ex. 1025 at 23:2-8. Burns I demonstrates that making slugs or plugs with continuous flows of immiscible fluids was a mere design choice as of 2001.

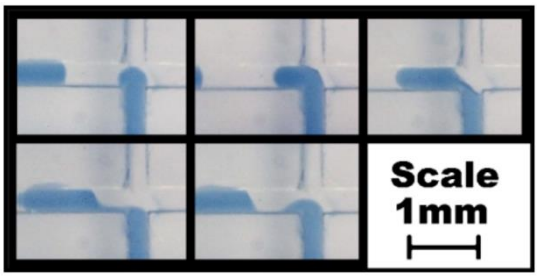
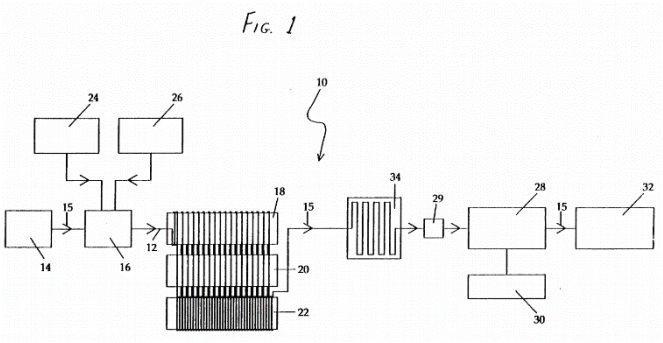
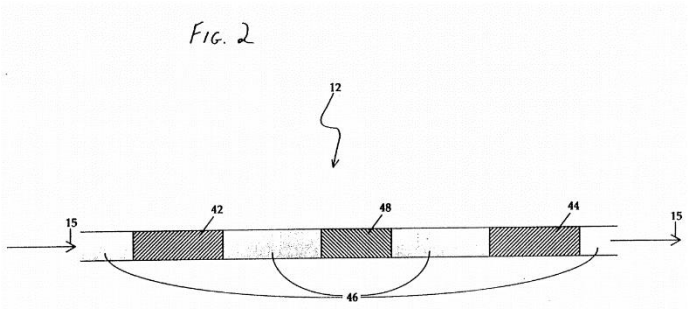


Fig. 3 Typical slug generation in glass device with 380 µm wide channels.

As of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Corbett and Lagally. In 1991, about eleven years prior to the earliest effective filing date, Corbett disclosed using a microchannel or capillary device to conduct DNA amplification reactions such as PCR by injecting plugs of reactants into a carrier stream that is passed through different temperature zones. Corbett explained that as of 1991 "the most critical problem in applying nucleic acid amplification procedures, particularly in repetitive diagnostic assays in a clinical laboratory, is contamination of the reaction mixture by small amounts of DNA which contain sequences capable of being

amplified under the conditions of assay." Ex. 1010 at 3:7-12. "Automation of procedures minimize[s] the opportunity for such contamination and allows the implementation of stringent quality control." *Id.* at 3:15-19. "As is shown in FIG. 1, the apparatus 10 comprises tube 12 through which a fluid stream passes. As is more clearly shown in FIG. 2, the fluid stream includes pockets of reaction mixtures 42 and 44 and purging solution 48 separated by carrier fluid 46." *Id.* at 7:58-62.





"The pump is actuated and the reaction mixture of 20 μl or less is injected into the mineral oil or silicone oil carrier fluid and specific DNA sequences (whose limits are defined by the oligonucleotide primers) present in the sample is amplified as it passes cyclically through the temperature zones." Ex. 1010 at 9:35-40. Corbett thus demonstrates that it was known at least as of 1991 that reduction of contamination risk in PCR could be achieved by performing the PCR on microliter sized plugs of reactant in capillary tube which passes through various temperature zones. Ex. 1004 ¶77.

In 2001, in another article not previously considered by the Office, Lagally reported using a microfluidic reactor to conduct single-molecule DNA amplification by PCR. Lagally summarized the state of the art as of February 2001, explaining continuous flow PCR microreactors had recently undergone substantial evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of

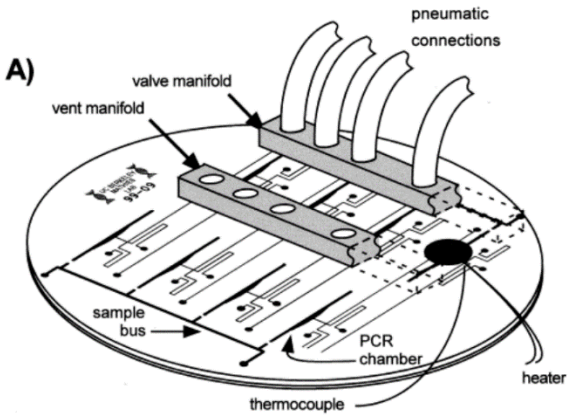
> 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

Lagally *et al.* developed a further improvement – a device and method for achieving single molecule amplification in a microchannel environment.

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ∼1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> Microfluidic PCR-CE device. The PCR chambers are connected to a common sample bus through a set of valves. Hydrophobic

> vents at the other end of the PCR chambers are used to locate the sample and to eliminate gas. The PCR chambers are directly connected to the cross channel of the CE system for product injection and analysis. Two aluminum manifolds, one each for the vents and valves, are placed onto the respective ports and clamped in place using vacuum. The manifolds connect to external solenoid valves for pressure and vacuum actuation. Thermal cycling is accomplished using a resistive heater and a miniature thermocouple below the 280-nL chamber.



Ex. 1028 at 566 (see figure legend).

In sum, Corbett and Lagally show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Burns I is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[113] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Burns I) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

[113] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I to conduct microfluidic PCR as taught by Corbett and Lagally. Stated a different way, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic reactor of Burns I, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing.

A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic reactors of Burns I because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could

demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Burns I to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the droplet reactors of Burns I would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96.

Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Burns I to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Lagally) to incorporate a droplet reactor (Burns I). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Lagally. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the single fluid in microfluidic chip reactor (e.g., Lagally) or droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Corbett). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers,

continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with

> polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ∼1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic

> diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent

with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Burns I could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful in miniaturizing and modifying Corbett or modifying Lagally to work in the microfluidic reactor of Burns I, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[114] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

[114] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

As explained in more detail below, in the accompanying claim chart (Ex. 1074) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I to conduct microfluidic PCR as taught by Corbett and Lagally. Ex. 1004 ¶¶109-112. Alternatively, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic reactor of Burns I, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing. *Id*.

Independent Claim 1

Claim 1[a]: A method comprising the steps of: providing a microfluidic system comprising one or more channels

Burns I discloses "[a] multiphase microreactor based upon the use of slug flow through a narrow channel.…" Ex. 1007 at 10. "The experimental work discussed here is based on the use of alternating slugs of two liquid phases to provide the environment for mass transfer and reaction within the microreactor." Ex. 1007 at 10.

Burns I explains that "[t]he method discussed in this work **uses the continuous flow of both phases through T or cross-shaped intersections**. Slugs are generated by the action of **one phase flowing into the channel whilst the other phase moves into the intersection,** eventually cutting off the flow of the first phase into the channel and reversing the process." Ex. 1007 at 11 [emphasis added].

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1074 and in the Shaqfeh Declaration (Ex. 1004).

Claim 1[b]: providing within the one or more channels a continuously flowing carrier fluid comprising an oil and a continuously flowing aqueous fluid

comprising target DNA or RNA molecules and at least one other molecule in the fluid that can react with the target DNA or RNA molecules under conditions in which the target DNA or RNA molecules and the other molecules in the fluid do not react with each other

As noted above, Burns I teaches that "[t]he method discussed in this work uses the continuous flow of both phases through T or cross-shaped intersections. Slugs are generated by the action of one phase flowing into the channel whilst the other phase moves into the intersection, eventually cutting off the flow of the first phase into the channel and reversing the process." Ex. 1007 at 11. Figure 1 shows that the reactant slug is composed of an "**aqueous phase**." *Id.* at Figure 1 [emphasis added]. Fig. 3 shows oil-in-water slug generation and Fig. 4 shows water-in-oil slug generation. *Id.* at 11. In Burns' Fig. 4 embodiment, an aqueous plug of reagents is formed in a continuous oil phase. The continuous flow plug formation used by Burns was a well-known alternative to periodically injecting a measured slug of reactant into the main channel. See Ex. 1010; 1027; Ex. 1025 at 23:2-8. Burns I demonstrates that making slugs or plugs with continuous flows of immiscible fluids was a mere design choice as of 2001.

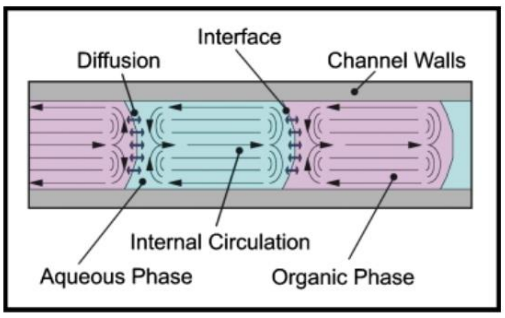


Fig. 1 Illustration of internal circulation generated within immiscible slug flow.

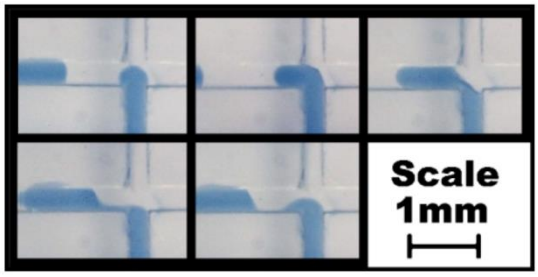


Fig. 3 Typical slug generation in glass device with 380 μm wide channels.

In the instant combination, the microfluidic droplet reactor of Burns I are relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules

and at least one other molecule").[115] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Burns I) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I to conduct microfluidic PCR as taught by Corbett and Lagally. Stated a different way, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modifying it to incorporate the microfluidic droplet reactor of Burns I, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing.

A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic droplet reactor of Burns I because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the

[115] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Burns I to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Burns I would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target

effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 (“It is also possible that Dig-PCR can be performed in

microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Burns I to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Lagally) to incorporate a droplet reactor (Burns I). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Lagally. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the single fluid in microfluidic chip reactor (e.g., Lagally) or droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Corbett). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow

PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.

> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
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> The ability of our device to amplify single DNA molecules provides

> an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an

autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactors Burns I could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful in miniaturizing and modifying Corbett or modifying Lagally to work in the microfluidic droplet reactor of Burns I, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[116] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of microdroplet reactors to create droplets from continuously flowing

[116] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained further in the accompanying claim chart (Ex. 1074) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I to conduct microfluidic PCR as taught by Corbett and Lagally. Ex. 1004 ¶¶109-112. Alternatively, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modifying it to incorporate the microfluidic droplet reactor of Burns I, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing. *Id*.

With regard to the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other," this is merely a description of a PCR reaction. Ex. 1004 ¶31,32. As explained in Corbett, Kopp, Burns II, and Lagally, in a PCR reaction the sample DNA or RNA is admixed with various reagents including polymerase under conditions in which no reaction occurs; only when heat is applied does the PCR reaction occur. Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68. In the PCR of the combined method, as in virtually any PCR method, the DNA and polymermase do not react until mixture of reagents is heated. *Id*.; see also Ex. 1004 ¶32. The combined method thus meets the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other" this is merely a description of a PCR reaction. Ex. 1004 ¶31,32.

Additional correspondence between this claim element and the cited references is shown

in the claim chart submitted herewith as Exhibit 1074.

Claim 1[c]: controlling flow rates of said aqueous fluid and said carrier fluid to partition the continuously flowing aqueous fluid with the continuously flowing carrier fluid to form a plurality of plugs of the aqueous fluid, each having a substantially uniform size of about 200 µm or less, wherein the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule

Burns I teaches that plugs in the channels of a microreactor by the continuous flow of fluids at an intersecting channel. Burns I explained that "various methods may be used to generate slugs of liquid within a microreactor." Ex. 1007 at 10. Burns I chose a method in which "the continuous flow of both phases through T or cross-shaped intersections" and "[s]lugs are generated by the action of one phase flowing into the channel whilst the other phase moves into the intersection, eventually cutting off the flow of the first phase into the channel and reversing the process." Ex. 1007 at 11 [emphasis added]. Fig. 3 shows oil-in-water slug generation and Fig. 4 shows water-in-oil slug generation. *Id.* at 11. In Burns' Fig. 4 embodiment, an aqueous plug of reagents is formed in a continuous oil phase. The continuous flow plug formation used by Burns was a well-known alternative to periodically injecting a measured slug of reactant into the main channel. See Ex. 1010; 1027; Ex. 1025 at 23:2-8. Burns I demonstrates that making slugs or plugs with continuous flows of immiscible fluids was a mere design choice as of 2001.

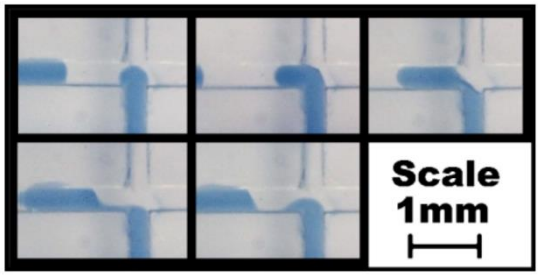


Fig. 3 Typical slug generation in glass device with 380 µm wide channels.

Turning to the recitation "each having a substantially uniform size of about 200 µm or less," Burns I discloses that the droplets are 100 µm to 400 µm in diameter. Ex. 1007 at 10. Moreover, it was well known that droplets smaller than 200 µm could be advantageously used in

droplet generators. Quake teaches that "the droplets have a diameter that is smaller than the diameter of the microchannel; i.e., preferably less than 60 μm." Ex. 1034 at 27:29-30. Fig. 21 of Quake shows the droplets sizes generated by the Quake droplet generator were all less than 60 μm. Burns II (1996) similarly discloses uses of 60 nl droplets (which equates to a droplet diameter of about 62 μm). Ex. 1008 at 5558. Accordingly, it would have been obvious to a skilled artisan that as of the earliest claimed priority date (2002) droplets below 200 μm in diameter could, and should, be used in order to reduce reagent usage and reaction time and achieve the various other benefits described above with the combination. Ex. 1004 ¶36, 66.

This leaves the recitation that "the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule." As discussed above, in the combined method of Quake, Corbett and Lagally, the samples are separated into a number of discrete droplets. That has the advantages of i) overcoming the signal-to-noise limitations associated with certain other PCR techniques and ii) enabling the detection of relatively rare mutations, dislocations and allelic imbalances. Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Lagally expressly teaches that "[t]he event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a **Poisson** distribution, demonstrating that we are operating at the single-molecule level." Ex. 1028 at 566 [emphasis added]. Vogelstein likewise teaches that the aforementioned advantages are realized by **reducing the number of sample molecules per droplet to zero or one.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶82**.** That ensures that any amplification that occurs in a given droplet can be readily identified as containing the target

sequence. *Id.* Thus, in the combined method the droplets have either one or zero template molecules.

As suggested by Lagally, any process which is generating droplets containing about one template molecule per droplet is expected to conform to a random, or Poisson, distribution. Ex. 1028 at 566; Ex. 1004 ¶48. It was known in the art that "[i]f a solution containing x molecules of enzyme per ml is dispersed into droplets of v ml volume, the average number of enzyme molecules per droplet n is equal to vx. Because of statistical fluctuations each droplet will not have exactly n enzyme molecules. The probability p(r) of finding 0, 1, 2, 3, . .. , r enzyme molecules in a droplet **is given by the Poisson law**." Ex. 1112 at 1985 [emphasis added]; see also Ex. 1111 at 1667 ("Spectra observed in a time sequence for an average of 0.6 dye molecule in the probed volume exhibited the expected Poisson distribution for actually measuring 0, 1, 2, or 3 molecules.").

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1074.

Claim 1[d]: providing conditions suitable for a polymerase-chain reaction in at least one plug of the plurality of plugs such that the target DNA or RNA is amplified

Burns I discloses that "[t]he high heat and mass transfer capability offered by capillary microreactors allows high intensity reactions to be precisely controlled for optimum yield and selectivity." Ex. 1007 at 10. "The internal circulation, which is stimulated within the slugs by their passage along the channel, is responsible for a **large enhancement** in the interfacial mass transfer and the **reaction rate**." Ex. 1007 at 10.

As discussed above in connection with element 1[b], in the instant combination Burns I is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or

RNA molecules and at least one other molecule").[117] These limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Burns I) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1074.

Thus, claim 1 is shown to be rendered obvious by the combination of Burns I, Corbett and Lagally.

Dependent Claim 2

Claim 2 of the '148 patent recites "wherein the step of providing conditions includes heating."

As discussed above in connection claim elements 1[b] and 1[c], in the combined method (and in virtually any other PCR reaction) the DNA and polymerase undergo polymerase chain reaction when heated. Ex. 1004 ¶32; Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68.

Thus, claim 2 is shown to be rendered obvious by the combination of Burns I, Corbett and Lagally.

Dependent Claims 3-5

Claim 3 of the '148 patent recites "providing a detector to detect, analyze, characterize, or monitor the target DNA or RNA molecule in the at least one member of the plurality of plugs during and/or after the reaction has occurred." Claim 4 of the '148 patent further recites "wherein the at least one other molecule includes one or more fluorescent labels and the detector

[117] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

is configured to monitor progress of the reaction by detecting fluorescence emissions from the one or more fluorescent labels." Claim 5 recites "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels."

Corbett similarly teaches that "[d]ownstream of temperature zone 34 is in-line analysis means 29 and recovery means 28. Connected to recovery means 28 is **detection means** 30. Detection means 30 determines when a reaction mixture in the carrier fluid reaches recovery means 28. This may be done by measuring the conductivity or **optical density** of the stream of carrier fluid." Ex. 1010 at 8:12-18, [emphasis added].

Lagally teaches "[s]tochastic PCR amplification of single DNA template molecules followed by **capillary electrophoresis** (CE) analysis of the products is demonstrated in an integrated microfluidic device." Ex. 1028 at 1, [emphasis added]. The results of capillary electrophoresis were generally detected optically at the time of filing. Ex. 1011 at 46 (Dolnick). "The amplified product, labeled with an intercalating **fluorescent** dye, is directly injected into the gel-filled capillary channel for electrophoretic analysis." Ex. 1028 at 1, [emphasis added].

Turning to claim 5, under the broadest reasonable interpretation the phrase "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels" requires simply that at least some of the droplets or plugs are transmitted to an outlet or other channel. Burns I teaches an outlet channel downstream of the droplet generating junction and thus meets claim 5 under the broadest reasonable interpretation. Ex. 1007 at 11. If claim 5 is more narrowly interpreted as requiring selective diversion of the droplets, that was well known. For instance, Quake teaches that "[a] preferred 'forward' sorting algorithm consists of running molecules, cells or virions from the input channel to the waste channel, until a molecule, cell or virion is identified to have an optically detectable signal (e.g. fluorescence) that

is above a pre-set threshold, at which point voltages are temporarily changed to electro-osmotically divert the molecule [] to the collection channel." Ex. 1034 at 20:22-26. Fu likewise discloses a microfabricated fluorescence-activiated cell sorter for use in connection with emulsion or droplet based systems. Ex. 1113 at 1109. A skilled artisan would have considered it obvious to employ such a technique to sort droplets after they are subjected to the optical detection. Ex. 1004 ¶68,69. For instance, a skilled artisan would have found it advantageous to sort and collect droplets which tested positive or negative for the presence of a certain substance or property, especially in the context of droplet generators which are used to encapsulate on average one template molecule per droplet. *Id.* ¶82.

In the combined method of Burns I, Corbett and Lagally, microfluidic droplet reactors are used to perform PCR reactions after which the reaction products are detected optically and sorted by diverting certain droplets into collection channels. Ex. 1004 ¶69.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1074.

Thus, claims 3-5 are shown to be rendered obvious by the combination of Burns I, Corbett and Lagally.

N. The Combination of Burns I, Corbett and Burns II Renders Obvious Claims 1-5 of the '148 Patent Under (pre-AIA) 35 U.S.C. § 103(a)

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Burns I), ii) microfluidic PCR devices were well known (Corbett and Burns II), and iii) there were compelling reasons to use the microfluidic droplet reactors to conduct microfluidic PCR (Lagally, Kopp, Burns II and Vogelstein).

Burns I teaches that plugs in the channels of a microreactor by the continuous flow of fluids at an intersecting channel. Burns I explained that "various methods may be used to generate slugs of liquid within a microreactor." Ex. 1007 at 10. Burns I chose a method in which "the continuous flow of both phases through T or cross-shaped intersections" and "[s]lugs are generated by the action of one phase flowing into the channel whilst the other phase moves into the intersection, eventually cutting off the flow of the first phase into the channel and reversing the process." Ex. 1007 at 11. Fig. 3 shows oil-in-water slug generation and Fig. 4 shows water-in-oil slug generation. *Id.* at 11. In Burns' Fig. 4 embodiment, an aqueous plug of reagents is formed in a continuous oil phase. The continuous flow plug formation used by Burns was a well-known alternative to periodically injecting a measured slug of reactant into the main channel. See Ex. 1010; 1027; Ex. 1025 at 23:2-8. Burns I demonstrates that making slugs or plugs with continuous flows of immiscible fluids was a mere design choice as of 2001.

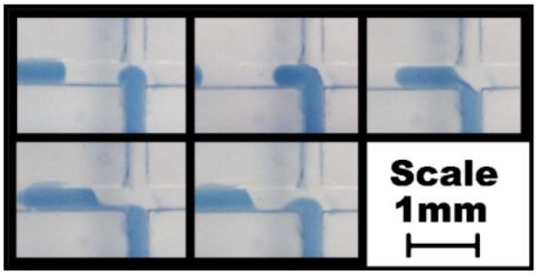
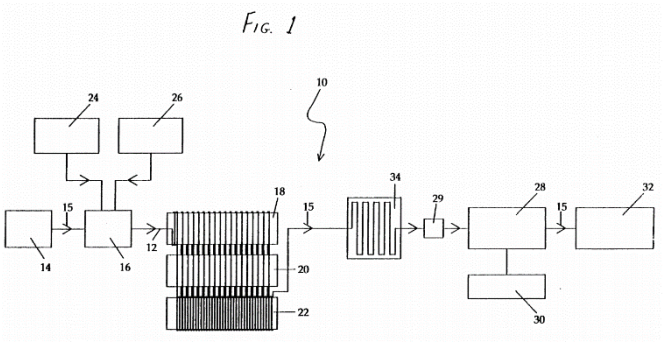
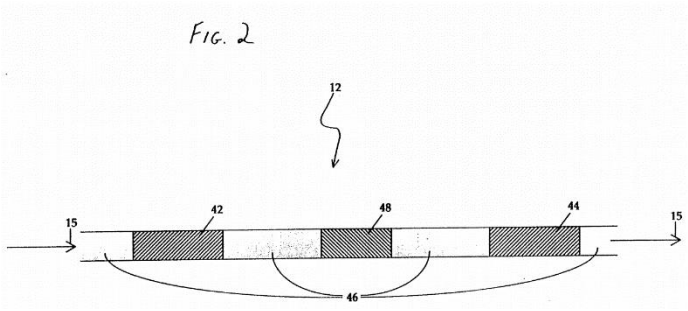


Fig. 3 Typical slug generation in glass device with 380 μm wide channels.

As of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Corbett and Burns II. In 1991, about eleven years prior to the earliest effective filing date, Corbett disclosed using a microchannel or capillary device to conduct DNA amplification reactions such as PCR by injecting plugs of reactants into a carrier stream that is passed through different temperature zones. Corbett explained that as of 1991 "the most critical problem in applying nucleic acid amplification procedures, particularly in repetitive diagnostic assays in a clinical laboratory, is contamination of the reaction mixture by small amounts of DNA which contain sequences capable of being

amplified under the conditions of assay." Ex. 1010 at 3:7-12. "Automation of procedures minimize[s] the opportunity for such contamination and allows the implementation of stringent quality control." *Id.* at 3:15-19. "As is shown in FIG. 1, the apparatus 10 comprises tube 12 through which a fluid stream passes. As is more clearly shown in FIG. 2, the fluid stream includes pockets of reaction mixtures 42 and 44 and purging solution 48 separated by carrier fluid 46." *Id.* at 7:58-62.





"The pump is actuated and the reaction mixture of 20 μl or less is injected into the mineral oil or silicone oil carrier fluid and specific DNA sequences (whose limits are defined by the oligonucleotide primers) present in the sample is amplified as it passes cyclically through the temperature zones." Ex. 1010 at 9:35-40. Corbett thus demonstrates that it was known at least as of 1991 that reduction of contamination risk in PCR could be achieved by performing the PCR on microliter sized plugs of reactant in capillary tube which passes through various temperature zones. Ex. 1004 ¶77.

In 1996, Burns II reported the use of microfluidic devices to perform PCR. Ex. 1008. Burns II explained that "[n]o new biochemistries or DNA detection methods are required for their use; the components simply reproduce current laboratory procedures in a micron-sized environment." *Id.* at 5560. In other words, the PCR chemistry did not need to be modified – it worked without modification on the microfluidic scale. In the method of Burns II, a sample containing a DNA plasmid was loaded into one of the reaction wells (A) and a mixture of Taq enzyme and buffer was loaded into the other. *Id.* at 5558. Each formed droplets of about 60 nl in the parallel 500 x 25 µm channels (B) which came together at the Y junction to form a single larger droplet (about 120 nl). *Id.* at 5558-59; Ex. 1004 ¶40.

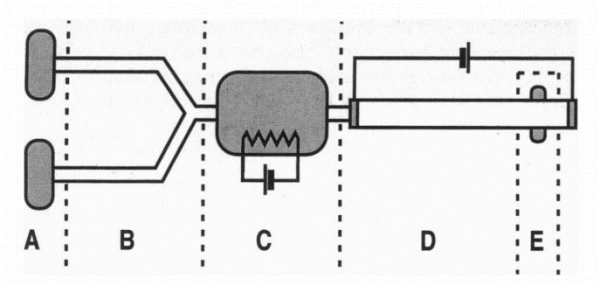


FIG. 1. Example of an integrated DNA analysis system, represented schematically. The individual components of the system are injection entry ports (*A*), liquid pumping channels (*B*), thermally controlled reaction chamber (*C*), electrophoresis channel (*D*), and DNA band migration detector (*E*). Each component would have associated sensors, control circuitry, and external connections.

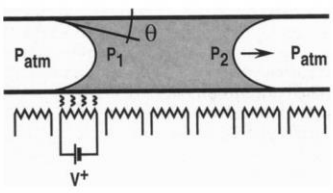


FIG. 2. Schematic drawing showing the principle of thermally induced drop motion in a closed channel. The case of a single aqueous drop in a hydrophilic channel is presented, where V is an applied voltage, P_{atm} is atmospheric pressure, P_1 is the receding-edge internal pressure, P_2 is the advancing-edge internal pressure, and θ is the contact angle of the liquid–gas–solid interface. The contact angle will depend on the surface characteristics of the channel and the constituents of the drop, with a hydrophilic interaction giving θ between 0 and 90 degrees, and a hydrophobic surface giving θ between 90 and 180 degrees. Surface treatments can also reduce contact angle hysteresis and, therefore, reduce the temperature difference necessary for drop motion.

The droplet then passed through the resistive heater portion (C) in which PCR amplification occurred. *Id.* at 5556. Afterwards the amplification results were optically detected in zone D via gel electrophoresis *Id.* at 5559. Burns II noted that "[t]he use of common fabrication methods allows the assembly of increasingly complex, multicomponent, integrated systems from a small, defined set of standardized elements" and that "[f]uture design efforts have a strong impetus to reduce component size and improve efficiency." *Id.* at 5560-61.

In sum, Corbett and Burns II show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Burns I is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[118] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Burns I) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I to conduct microfluidic PCR as taught by Corbett and Burns II. Stated a different way, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic reactor of Burns I, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing.

A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic reactor of Burns I because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at

[118] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Burns I to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the droplet reactors of Burns I would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the

reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241;

Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Burns I to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Burns II) to incorporate a droplet reactor (Burns I). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Burns II. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the single fluid in microfluidic chip reactor (e.g., Burns II) or droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Corbett). Droplet reactors also **decrease contamination and increase the**

number of reactors that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling

> profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced

> microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic**

droplet (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Burns I could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful in miniaturizing and modifying Corbett or modifying Burns II to work in the microfluidic reactor of Burns I, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[119] As discussed in Section VI.A.3, above, Higuchi I-III

[119] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

thus demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail below, in the accompanying claim chart (Ex. 1075) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I to conduct microfluidic PCR as taught by Corbett and Burns II. Ex. 1004 ¶¶109-112. Alternatively, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic reactor of Burns I, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing. *Id*.

Independent Claim 1

Claim 1[a]: A method comprising the steps of: providing a microfluidic system comprising one or more channels

Burns I discloses "[a] multiphase microreactor based upon the use of slug flow through a narrow channel.…" Ex. 1007 at 10. "The experimental work discussed here is based on the use of alternating slugs of two liquid phases to provide the environment for mass transfer and reaction within the microreactor." Ex. 1007 at 10.

Burns I explains that "[t]he method discussed in this work **uses the continuous flow of both phases through T or cross-shaped intersections**. Slugs are generated by the action of **one phase flowing into the channel whilst the other phase moves into the intersection,** eventually cutting off the flow of the first phase into the channel and reversing the process." Ex. 1007 at 11 [emphasis added].

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1075.

Claim 1[b]: providing within the one or more channels a continuously flowing carrier fluid comprising an oil and a continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule in the fluid that can react with the target DNA or RNA molecules under conditions in which the target DNA or RNA molecules and the other molecules in the fluid do not react with each other

As noted above, Burns I teaches that "[t]he method discussed in this work uses the continuous flow of both phases through T or cross-shaped intersections. Slugs are generated by the action of one phase flowing into the channel whilst the other phase moves into the intersection, eventually cutting off the flow of the first phase into the channel and reversing the process." Ex. 1007 at 11. Figure 1 shows that the reactant slug is composed of an "**aqueous phase**." *Id.* at Figure 1 [emphasis added]. Fig. 3 shows oil-in-water slug generation and Fig. 4 shows water-in-oil slug generation. *Id.* at 11. In Burns' Fig. 4 embodiment, an aqueous plug of reagents is formed in a continuous oil phase. The continuous flow plug formation used by Burns was a well-known alternative to periodically injecting a measured slug of reactant into the main channel. See Ex. 1010; 1027; Ex. 1025 at 23:2-8. Burns I demonstrates that making slugs or plugs with continuous flows of immiscible fluids was a mere design choice as of 2001.

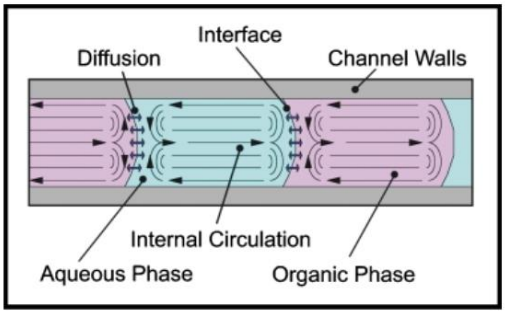


Fig. 1 Illustration of internal circulation generated within immiscible slug flow.

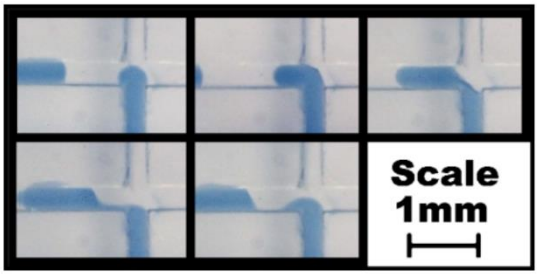


Fig. 3 Typical slug generation in glass device with 380 μm wide channels.

In the instant combination, the microfluidic droplet reactor of Burns I is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[120] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Burns I) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I to conduct microfluidic PCR as taught by Corbett and Burns II. Stated a different way, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modifying it to incorporate the microfluidic droplet reactor of Burns I, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing.

A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic droplet reactor of Burns I because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at

[120] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Burns I to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the droplet reactors of Burns I would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the

reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241;

Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Burns I to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Burns II) to incorporate a droplet reactor (Burns I). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Burns II. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the single fluid in microfluidic chip reactor (e.g., Burns II) or droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Corbett). Droplet reactors also **decrease contamination and increase the**

number of reactors that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling

> profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced

> microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic**

droplet (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactors Burns I could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful in miniaturizing and modifying Corbett or modifying Burns II to work in the microfluidic droplet reactor of Burns I, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[121] As discussed in Section VI.A.3, above, Higuchi I-

[121] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

III demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained further in the accompanying claim chart (Ex. 1075) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I to conduct microfluidic PCR as taught by Corbett and Burns II. Ex. 1004 ¶¶109-112. Alternatively, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modifying it to incorporate the microfluidic droplet reactor of Burns I, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing. *Id*.

With regard to the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other," this is merely a description of a PCR reaction. Ex. 1004 ¶31,32. As explained in Corbett, Kopp, Burns II, and Lagally, in a PCR reaction the sample DNA or RNA is admixed with various reagents including polymerase under conditions in which no reaction occurs; only when heat is applied does the PCR reaction occur. Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68. In the PCR of the combined method, as in virtually any PCR method, the DNA and polymermase do not react until mixture of reagents is heated. *Id*.; see also Ex. 1004 ¶32. The combined method thus meets the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other" this is merely a description of a PCR reaction. Ex. 1004 ¶31,32.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1075.

Claim 1[c]: controlling flow rates of said aqueous fluid and said carrier fluid to partition the continuously flowing aqueous fluid with the continuously flowing carrier fluid to form a plurality of plugs of the aqueous fluid, each having a substantially uniform size of about 200 µm or less, wherein the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule

Burns I teaches that plugs in the channels of a microreactor by the continuous flow of fluids at an intersecting channel. Burns I explained that "various methods may be used to generate slugs of liquid within a microreactor." Ex. 1007 at 10. Burns I chose a method in which "the continuous flow of both phases through T or cross-shaped intersections" and "[s]lugs are generated by the action of one phase flowing into the channel whilst the other phase moves into the intersection, eventually cutting off the flow of the first phase into the channel and reversing the process." Ex. 1007 at 11. Fig. 3 shows oil-in-water slug generation and Fig. 4 shows water-in-oil slug generation. *Id.* at 11. In Burns' Fig. 4 embodiment, an aqueous plug of reagents is formed in a continuous oil phase. The continuous flow plug formation used by Burns was a well-known alternative to periodically injecting a measured slug of reactant into the main channel. See Ex. 1010; 1027; Ex. 1025 at 23:2-8. Burns I demonstrates that making slugs or plugs with continuous flows of immiscible fluids was a mere design choice as of 2001.

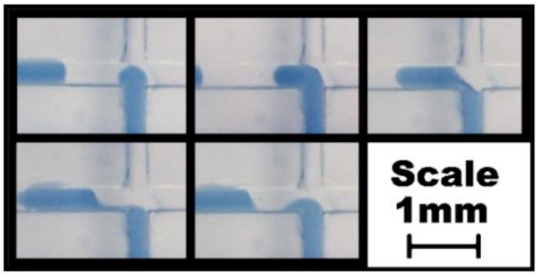


Fig. 3 Typical slug generation in glass device with 380 µm wide channels.

Turning to the recitation "each having a substantially uniform size of about 200 µm or less," Burns I discloses that the droplets are 100 µm to 400 µm in diameter. Ex. 1007 at 10. Moreover, it was well known that droplets smaller than 200 µm could be advantageously used in

droplet generators. Quake teaches that "the droplets have a diameter that is smaller than the diameter of the microchannel; i.e., preferably less than 60 μm." Ex. 1034 at 27:29-30. Fig. 21 of Quake shows the droplets sizes generated by the Quake droplet generator were all less than 60 μm. Burns II (1996) similarly discloses uses of 60 nl droplets (which equates to a droplet diameter of about 62 μm). Ex. 1008 at 5558. Accordingly, it would have been obvious to a skilled artisan that as of the earliest claimed priority date (2002) droplets below 200 μm in diameter could, and should, be used in order to reduce reagent usage and reaction time and achieve the various other benefits described above with the combination. Ex. 1004 ¶36, 66.

This leaves the recitation that "the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule." As discussed above, in the combined method of Quake, Corbett and Lagally, the samples are separated into a number of discrete droplets. That has the advantages of i) overcoming the signal-to-noise limitations associated with certain other PCR techniques and ii) enabling the detection of relatively rare mutations, dislocations and allelic imbalances. Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Lagally expressly teaches that "[t]he event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a **Poisson** distribution, demonstrating that we are operating at the single-molecule level." Ex. 1028 at 566 [emphasis added]. Vogelstein likewise teaches that the aforementioned advantages are realized by **reducing the number of sample molecules per droplet to zero or one.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶82**.** That ensures that any amplification that occurs in a given droplet can be readily identified as containing the target

sequence. *Id.* Thus, in the combined method the droplets have either one or zero template molecules.

As suggested by Lagally, any process which is generating droplets containing about one template molecule per droplet is expected to conform to a random, or Poisson, distribution. Ex. 1028 at 566; Ex. 1004 ¶48. It was known in the art that "[i]f a solution containing x molecules of enzyme per ml is dispersed into droplets of v ml volume, the average number of enzyme molecules per droplet n is equal to vx. Because of statistical fluctuations each droplet will not have exactly n enzyme molecules. The probability p(r) of finding 0, 1, 2, 3, . .. , r enzyme molecules in a droplet **is given by the Poisson law**." Ex. 1112 at 1985 [emphasis added]; see also Ex. 1111 at 1667 ("Spectra observed in a time sequence for an average of 0.6 dye molecule in the probed volume exhibited the expected Poisson distribution for actually measuring 0, 1, 2, or 3 molecules.").

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1075.

Claim 1[d]: providing conditions suitable for a polymerase-chain reaction in at least one plug of the plurality of plugs such that the target DNA or RNA is amplified

Burns I discloses that "[t]he high heat and mass transfer capability offered by capillary microreactors allows high intensity reactions to be precisely controlled for optimum yield and selectivity." Ex. 1007 at 10. "The internal circulation, which is stimulated within the slugs by their passage along the channel, is responsible for a **large enhancement** in the interfacial mass transfer and the **reaction rate**." Ex. 1007 at 10, emphasis added.

As discussed above in connection with element 1[b], in the instant combination Burns I is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or

RNA molecules and at least one other molecule").[122] These limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Burns I) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1075.

Thus, claim 1 is shown to be rendered obvious by the combination of Burns I, Corbett and Burns II.

Dependent Claim 2

Claim 2 of the '148 patent recites "wherein the step of providing conditions includes heating."

As discussed above in connection claim elements 1[b] and 1[c], in the combined method (and in virtually any other PCR reaction) the DNA and polymerase undergo polymerase chain reaction when heated. Ex. 1004 ¶32; Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1075.

Thus, claim 2 is shown to be rendered obvious by the combination of Burns I, Corbett and Burns II.

Dependent Claims 3-5

Claim 3 of the '148 patent recites "providing a detector to detect, analyze, characterize, or monitor the target DNA or RNA molecule in the at least one member of the plurality of plugs

[122] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

during and/or after the reaction has occurred." Claim 4 of the '148 patent further recites "wherein the at least one other molecule includes one or more fluorescent labels and the detector is configured to monitor progress of the reaction by detecting fluorescence emissions from the one or more fluorescent labels." Claim 5 recites "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels."

Corbett similarly teaches that "[d]ownstream of temperature zone 34 is in-line analysis means 29 and recovery means 28. Connected to recovery means 28 is **detection means** 30. Detection means 30 determines when a reaction mixture in the carrier fluid reaches recovery means 28. This may be done by measuring the conductivity or **optical density** of the stream of carrier fluid." Ex. 1010 at 8:12-18, [emphasis added].

Burns II teaches that "[c]apillary gel electrophoresis of DNA samples was performed using a Beckman P/ACE instrument with a laser-induced fluorescence detector and 37 cm length, 100 μm diameter, linear polymerase gel capillary according to manufacturer's recommendation." Ex. 1008 at 5558. The results of capillary electrophoresis were generally detected optically at the time of filing. Ex. 1011 at 46 (Dolnick). "The amplified product, labeled with an intercalating **fluorescent** dye, is directly injected into the gel-filled capillary channel for electrophoretic analysis." Ex. 1028 at 1, [emphasis added].

Turning to claim 5, under the broadest reasonable interpretation the phrase "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels" requires simply that at least some of the droplets or plugs are transmitted to an outlet or other channel. Burns I teaches an outlet channel downstream of the droplet generating junction and thus meets claim 5 under the broadest reasonable interpretation. Ex. 1007 at 11. If claim 5 is more narrowly interpreted as requiring selective diversion of the droplets, that was

well known. For instance, Quake teaches that "[a] preferred 'forward' sorting algorithm consists of running molecules, cells or virions from the input channel to the waste channel, until a molecule, cell or virion is identified to have an optically detectable signal (e.g. fluorescence) that is above a pre-set threshold, at which point voltages are temporarily changed to electro-osmotically divert the molecule [] to the collection channel." Ex. 1034 at 20:22-26. Fu likewise discloses a microfabricated fluorescence-activiated cell sorter for use in connection with emulsion or droplet based systems. Ex. 1113 at 1109. A skilled artisan would have considered it obvious to employ such a technique to sort droplets after they are subjected to the optical detection. Ex. 1004 ¶68,69. For instance, a skilled artisan would have found it advantageous to sort and collect droplets which tested positive or negative for the presence of a certain substance or property, especially in the context of droplet generators which are used to encapsulate on average one template molecule per droplet. *Id.* ¶82.

In the combined method of Burns I, Corbett and Burns II, microfluidic droplet reactors are used to perform PCR reactions after which the reaction products are detected optically and sorted by diverting certain droplets into collection channels. Ex. 1004 ¶69.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1075.

Thus, claims 3-5 are shown to be rendered obvious by the combination of Burns I, Corbett and Burns II.

O. The Combination of Burns I, Corbett and Vogelstein Renders Obvious Claims 1-5 of the '148 Patent Under (pre-AIA) 35 U.S.C. § 103(a)

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Burns I), ii) microfluidic PCR devices were well known (Corbett and Vogelstein), and iii) there were compelling reasons to use

the microfluidic droplet reactors to conduct microfluidic PCR (Lagally, Kopp, Burns II and Vogelstein).

Burns I teaches that plugs in the channels of a microreactor by the continuous flow of fluids at an intersecting channel. Burns I explained that "various methods may be used to generate slugs of liquid within a microreactor." Ex. 1007 at 10. Burns I chose a method in which "the continuous flow of both phases through T or cross-shaped intersections" and "[s]lugs are generated by the action of one phase flowing into the channel whilst the other phase moves into the intersection, eventually cutting off the flow of the first phase into the channel and reversing the process." Ex. 1007 at 11. Fig. 3 shows oil-in-water slug generation and Fig. 4 shows water-in-oil slug generation. *Id.* at 11. In Burns' Fig. 4 embodiment, an aqueous plug of reagents is formed in a continuous oil phase. The continuous flow plug formation used by Burns was a well-known alternative to periodically injecting a measured slug of reactant into the main channel. See Ex. 1010; 1027; Ex. 1025 at 23:2-8. Burns I demonstrates that making slugs or plugs with continuous flows of immiscible fluids was a mere design choice as of 2001.

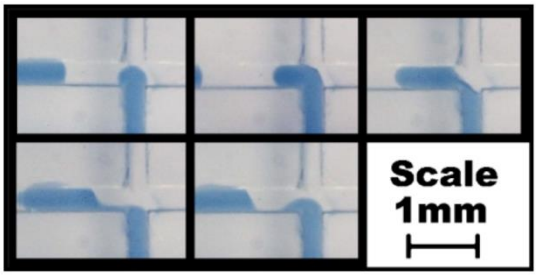


Fig. 3 Typical slug generation in glass device with 380 μm wide channels.

As of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Corbett and Vogelstein. In 1991, about eleven years prior to the earliest effective filing date, Corbett disclosed using a microchannel or capillary device to conduct DNA amplification reactions such as PCR by injecting plugs of reactants into a carrier stream that is passed through different temperature zones. Corbett explained that as of 1991 "the most critical problem in applying nucleic acid

amplification procedures, particularly in repetitive diagnostic assays in a clinical laboratory, is contamination of the reaction mixture by small amounts of DNA which contain sequences capable of being amplified under the conditions of assay." Ex. 1010 at 3:7-12. "Automation of procedures minimize[s] the opportunity for such contamination and allows the implementation of stringent quality control." *Id.* 3:15-19. "As is shown in FIG. 1, the apparatus 10 comprises tube 12 through which a fluid stream passes. As is more clearly shown in FIG. 2, the fluid stream includes pockets of reaction mixtures 42 and 44 and purging solution 48 separated by carrier fluid 46." *Id.* at 7:58-62.

FIG. 1

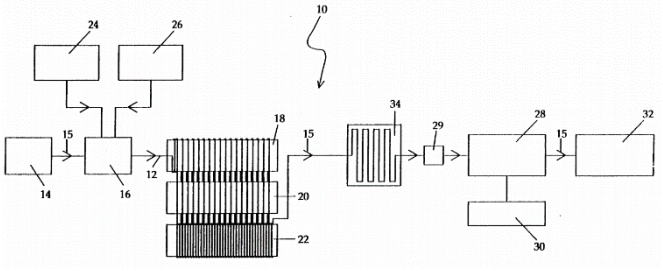
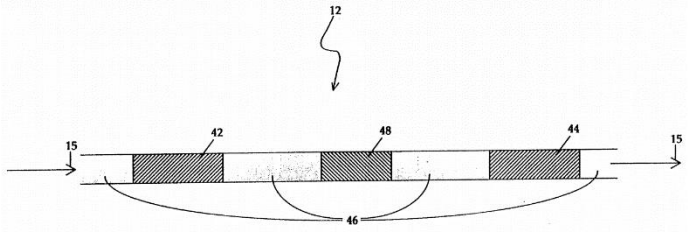


FIG. 2



"The pump is actuated and the reaction mixture of 20 µl or less is injected into the mineral oil or silicone oil carrier fluid and specific DNA sequences (whose limits are defined by the oligonucleotide primers) present in the sample is amplified as it passes cyclically through the temperature zones." Ex. 1010 at 9:35-40. Corbett thus demonstrates that it was known at least as of 1991 that reduction of contamination risk in PCR could be achieved by performing the PCR

on microliter sized plugs of reactant in capillary tube which passes through various temperature zones. Ex. 1004 ¶77.

By 1999, Vogelstein had reported **various applications for single-molecule microfluidic PCR**. Ex. 1044 at 9241. In a seminal article which has been cited over by over 1,000 other publications, Vogelstein explained that PCR amplification of small aliquots or droplets containing single molecules was effective for "detection of a small number of mutant-containing cells among a large excess of normal cells." *Id.* at 9236. In Vogelstein's method, the DNA was diluted into 7 µl volumes such that, on average, there was one template molecule for every two wells (i.e., approximately half of the wells contained no DNA template molecule and approximately half contained a single DNA template molecule). *Id.* at 9236, 9237. This avoided the problem of background noise impairing the determination of whether each volume contained the mutant sequence. *Id.* By counting the number of volumes which indicated wild type or mutant templates, the presence or absence of the mutation (e.g., a sequence associated with colorectal cancer) can be determined. *Id.* at 9238. The accuracy of the test is a function of the number of discrete volumes into which the sample is diluted. *Id.* at 9239. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. *Id.* at 9239. ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude.") *Id.* 9239. The article explained that the same technique would be useful for **detecting a wide array of mutations, dislocations and allelic imbalances**, as summarized in Table 1. *Id.* at 9241. Vogelstein thus taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template

molecule, thereby overcoming the signal-to-noise limitations associated with certain other PCR techniques and enabling the detection of relatively rare mutations, dislocations and allelic imbalances. Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84.

In sum, Corbett and Vogelstein show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Burns I is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[123] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Burns I) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I to conduct microfluidic PCR as taught by Corbett and Vogelstein. Ex. 1004 ⁋⁋109-112. Stated a different way, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic reactor of Burns I, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing.

A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic reactor of Burns I because doing so have provided the substantial benefits

[123] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Burns I to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Burns I would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026

(Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Burns I to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Vogelstein) to

incorporate a droplet reactor (Burns I). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Vogelstein. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Corbett). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors

> were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of

> single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority

date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Burns I could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful in miniaturizing and modifying Corbett or modifying Vogelstein to work in the microfluidic reactor of Burns I, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as

evidence of the level of skill in the art.[124] As discussed in Section VI.A.3, above, Higuchi I-III thus demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail below, in the accompanying claim chart (Ex. 1076) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I to conduct microfluidic PCR as taught by Corbett and Vogelstein. Ex. 1004 ⁋⁋109-112. Alternatively, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modify it to incorporate the microfluidic reactor of Burns I, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing. *Id*.

Independent Claim 1

Claim 1[a]: A method comprising the steps of: providing a microfluidic system comprising one or more channels

Burns I discloses "[a] multiphase microreactor based upon the use of slug flow through a narrow channel.…" Ex. 1007 at 10. "The experimental work discussed here is based on the use

[124] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

of alternating slugs of two liquid phases to provide the environment for mass transfer and reaction within the microreactor." Ex. 1007 at 10.

Burns I explains that "[t]he method discussed in this work **uses the continuous flow of both phases through T or cross-shaped intersections**. Slugs are generated by the action of **one phase flowing into the channel whilst the other phase moves into the intersection,** eventually cutting off the flow of the first phase into the channel and reversing the process." Ex. 1007 at 11 [emphasis added].

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1076.

Claim 1[b]: providing within the one or more channels a continuously flowing carrier fluid comprising an oil and a continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule in the fluid that can react with the target DNA or RNA molecules under conditions in which the target DNA or RNA molecules and the other molecules in the fluid do not react with each other

As noted above, Burns I teaches that "[t]he method discussed in this work uses the continuous flow of both phases through T or cross-shaped intersections. Slugs are generated by the action of one phase flowing into the channel whilst the other phase moves into the intersection, eventually cutting off the flow of the first phase into the channel and reversing the process." Ex. 1007 at 11 [emphasis added]. Figure 1 shows that the reactant slug is composed of an "**aqueous**

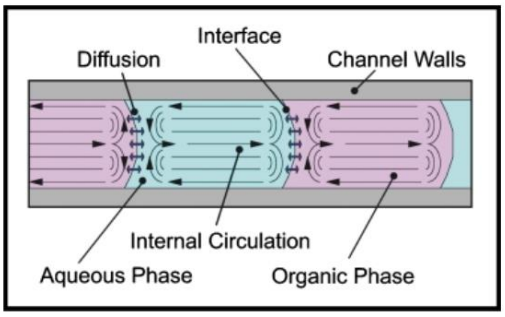


Fig. 1 Illustration of internal circulation generated within immiscible slug flow.

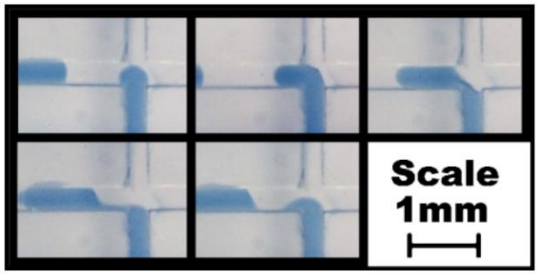


Fig. 3 Typical slug generation in glass device with 380 μm wide channels.

phase." *Id.* at Figure 1 [emphasis added]. Fig. 3 shows oil-in-water slug generation and Fig. 4 shows water-in-oil slug generation. *Id.* at 11. In Burns' Fig. 4 embodiment, an aqueous plug of reagents is formed in a continuous oil phase. The continuous flow plug formation used by Burns was a well-known alternative to periodically injecting a measured slug of reactant into the main channel. See Ex. 1010; 1027; Ex. 1025 at 23:2-8. Burns I demonstrates that making slugs or plugs with continuous flows of immiscible fluids was a mere design choice as of 2001.

In the instant combination, the microfluidic droplet reactor of Burns I is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[125] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Burns I) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I to conduct microfluidic PCR as taught by Corbett and Vogelstein. Ex. 1004 ¶¶109-112. Stated a different way, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modifying it to incorporate the microfluidic droplet reactor of Burns I, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing.

[125] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic droplet reactor of Burns I because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing**).** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the

droplet reactor of Burns I to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the droplet reactors of Burns I would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample

leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

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Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Burns I to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Vogelstein) to incorporate a droplet reactor (Burns I). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Vogelstein. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Corbett). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA

fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.

Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In

1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactors Burns I could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful in miniaturizing and modifying Corbett or modifying Vogelstein to work in the microfluidic droplet reactor of Burns I, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more

than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[126] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained further in the accompanying claim chart (Ex. 1076) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I to conduct microfluidic PCR as taught by Corbett and Vogelstein. Ex. 1004 ⁋⁋109-112. Alternatively, a skilled artisan would have considered it obvious to miniaturize the PCR apparatus of Corbett and modifying it to incorporate the microfluidic droplet reactor of Burns I, as evidenced by Kopp, Burns II, Lagally and Vogelstein's contemporaneous reports that PCR had been widely and successfully implemented at the microfluidic scale at the time of filing. *Id*.

With regard to the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other,"

[126] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

this is merely a description of a PCR reaction. Ex. 1004 ¶31,32. As explained in Corbett, Kopp, Burns II, and Lagally, in a PCR reaction the sample DNA or RNA is admixed with various reagents including polymerase under conditions in which no reaction occurs; only when heat is applied does the PCR reaction occur. Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68. In the PCR of the combined method, as in virtually any PCR method, the DNA and polymermase do not react until mixture of reagents is heated. *Id*.; see also Ex. 1004 ¶32. The combined method thus meets the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other" this is merely a description of a PCR reaction. Ex. 1004 ¶31,32.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1076.

Claim 1[c]: controlling flow rates of said aqueous fluid and said carrier fluid to partition the continuously flowing aqueous fluid with the continuously flowing carrier fluid to form a plurality of plugs of the aqueous fluid, each having a substantially uniform size of about 200 µm or less, wherein the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule

Burns I teaches that plugs in the channels of a microreactor by the continuous flow of fluids at an intersecting channel. Burns I explained that "various methods may be used to generate slugs of liquid within a microreactor." Ex. 1007 at 10. Burns I chose a method in which "the continuous flow of both phases through T or cross-shaped intersections" and "[s]lugs are generated by the action of one phase flowing into the channel whilst the other phase moves into the intersection, eventually cutting off the flow of the first phase into

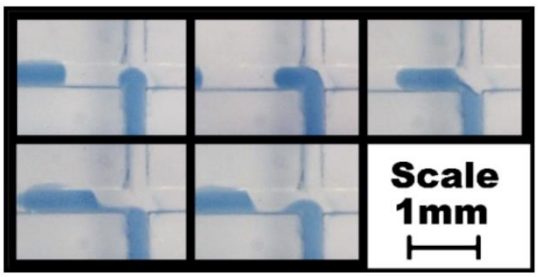


Fig. 3 Typical slug generation in glass device with 380 µm wide channels.

the channel and reversing the process." Ex. 1007 at 11. Fig. 3 shows oil-in-water slug generation and Fig. 4 shows water-in-oil slug generation. *Id.* at 11. In Burns' Fig. 4 embodiment, an aqueous plug of reagents is formed in a continuous oil phase. The continuous flow plug formation used by Burns was a well-known alternative to periodically injecting a measured slug of reactant into the main channel. See Ex. 1010; 1027; Ex. 1025 at 23:2-8. Burns I demonstrates that making slugs or plugs with continuous flows of immiscible fluids was a mere design choice as of 2001.

Turning to the recitation "each having a substantially uniform size of about 200 μm or less," Burns I discloses that the droplets are 100 μm to 400 μm in diameter. (Ex. 1007 at 10.) Moreover, it was well known that droplets smaller than 200 μm could be advantageously used in droplet generators. Quake teaches that "the droplets have a diameter that is smaller than the diameter of the microchannel; i.e., preferably less than 60 μm." Ex. 1034 at 27:29-30. Fig. 21 of Quake shows the droplets sizes generated by the Quake droplet generator were all less than 60 μm. Burns II (1996) similarly discloses uses of 60 nl droplets (which equates to a droplet diameter of about 62 μm). Ex. 1008 at 5558. Accordingly, it would have been obvious to a skilled artisan that as of the earliest claimed priority date (2002) droplets below 200 μm in diameter could, and should, be used in order to reduce reagent usage and reaction time and achieve the various other benefits described above with the combination. Ex. 1004 ¶36, 66.

This leaves the recitation that "the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule." As discussed above, in the combined method of Quake, Corbett and Lagally, the samples are separated into a number of discrete droplets. That has the

advantages of i) overcoming the signal-to-noise limitations associated with certain other PCR techniques and ii) enabling the detection of relatively rare mutations, dislocations and allelic imbalances. Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Lagally expressly teaches that "[t]he event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a **Poisson** distribution, demonstrating that we are operating at the single-molecule level." Ex. 1028 at 566 [emphasis added]. Vogelstein likewise teaches that the aforementioned advantages are realized by **reducing the number of sample molecules per droplet to zero or one.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶82**.** That ensures that any amplification that occurs in a given droplet can be readily identified as containing the target sequence. *Id.* Thus, in the combined method the droplets have either one or zero template molecules.

As suggested by Lagally, any process which is generating droplets containing about one template molecule per droplet is expected to conform to a random, or Poisson, distribution. Ex. 1028 at 566; Ex. 1004 ¶48. It was known in the art that "[i]f a solution containing x molecules of enzyme per ml is dispersed into droplets of v ml volume, the average number of enzyme molecules per droplet n is equal to vx. Because of statistical fluctuations each droplet will not have exactly n enzyme molecules. The probability p(r) of finding 0, 1, 2, 3, . .. , r enzyme molecules in a droplet **is given by the Poisson law**." Ex. 1112 at 1985 [emphasis added]; see also Ex. 1111 at 1667 ("Spectra observed in a time sequence for an average of 0.6 dye molecule in the probed volume exhibited the expected Poisson distribution for actually measuring 0, 1, 2, or 3 molecules.").

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1076.

Claim 1[d]: providing conditions suitable for a polymerase-chain reaction in at least one plug of the plurality of plugs such that the target DNA or RNA is amplified

Burns I discloses that "[t]he high heat and mass transfer capability offered by capillary microreactors allows high intensity reactions to be precisely controlled for optimum yield and selectivity." Ex. 1007 at 10. "The internal circulation, which is stimulated within the slugs by their passage along the channel, is responsible for a **large enhancement** in the interfacial mass transfer and the **reaction rate**." *Id.*

As discussed above in connection with element 1[b], in the instant combination Burns I is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[127] These limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Burns I) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1076.

Thus, claim 1 is shown to be rendered obvious by the combination of Burns I, Corbett and Vogelstein.

Dependent Claim 2

Claim 2 of the '148 patent recites "wherein the step of providing conditions includes heating."

[127] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

As discussed above in connection claim elements 1[b] and 1[c], in the combined method (and in virtually any other PCR reaction) the DNA and polymerase undergo polymerase chain reaction when heated. Ex. 1004 ¶32; Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1076.

Thus, claim 2 is shown to be rendered obvious by the combination of Burns I, Corbett and Vogelstein.

Dependent Claims 3-5

Claim 3 of the '148 patent recites "providing a detector to detect, analyze, characterize, or monitor the target DNA or RNA molecule in the at least one member of the plurality of plugs during and/or after the reaction has occurred." Claim 4 of the '148 patent further recites "wherein the at least one other molecule includes one or more fluorescent labels and the detector is configured to monitor progress of the reaction by detecting fluorescence emissions from the one or more fluorescent labels." Claim 5 recites "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels."

Corbett similarly teaches that "[d]ownstream of temperature zone 34 is in-line analysis means 29 and recovery means 28. Connected to recovery means 28 is **detection means** 30. Detection means 30 determines when a reaction mixture in the carrier fluid reaches recovery means 28. This may be done by measuring the conductivity or **optical density** of the stream of carrier fluid." Ex. 1010 at 8:12-18, [emphasis added].

Vogelstein teaches that the "[r]eactions were analyzed immediately or stored at room temperature for up to 36 h before **fluorescence analysis**." Ex. 1044 at 9236, [emphasis added].

Turning to claim 5, under the broadest reasonable interpretation the phrase "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels" requires simply that at least some of the droplets or plugs are transmitted to an outlet or other channel. Burns I teaches an outlet channel downstream of the droplet generating junction and thus meets claim 5 under the broadest reasonable interpretation. Ex. 1007 at 11. If claim 5 is more narrowly interpreted as requiring selective diversion of the droplets, that was well known. For instance, Quake teaches that "[a] preferred 'forward' sorting algorithm consists of running molecules, cells or virions from the input channel to the waste channel, until a molecule, cell or virion is identified to have an optically detectable signal (e.g. fluorescence) that is above a pre-set threshold, at which point voltages are temporarily changed to electro-osmotically divert the molecule [] to the collection channel." Ex. 1034 at 20:22-26. Fu likewise discloses a microfabricated fluorescence-activiated cell sorter for use in connection with emulsion or droplet based systems. Ex. 1113 at 1109. A skilled artisan would have considered it obvious to employ such a technique to sort droplets after they are subjected to the optical detection. Ex. 1004 ¶68,69. For instance, a skilled artisan would have found it advantageous to sort and collect droplets which tested positive or negative for the presence of a certain substance or property, especially in the context of droplet generators which are used to encapsulate on average one template molecule per droplet. *Id.* ¶82.

In the combined method of Burns I, Corbett and Vogelstein, microfluidic droplet reactors are used to perform PCR reactions after which the reaction products are detected optically and sorted by diverting certain droplets into collection channels. Ex. 1004 ¶69.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1076.

Thus, claims 3-5 are shown to be rendered obvious by the combination of Burns I, Corbett and Vogelstein.

P. The Combination of Burns I, Kopp and Lagally Renders Obvious Claims 1-5 of the '148 Patent Under (pre-AIA) 35 U.S.C. § 103(a)

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Burns I), ii) microfluidic PCR devices were well known (Kopp and Lagally), and iii) there were compelling reasons to use the microfluidic droplet reactors to conduct microfluidic PCR (Kopp, Burns II, Lagally and Vogelstein).

Burns I teaches that plugs in the channels of a microreactor by the continuous flow of fluids at an intersecting channel. Burns I explained that "various methods may be used to generate slugs of liquid within a microreactor." Ex. 1007 at 10. Burns I chose a method in which "the continuous flow of both phases through T or cross-shaped intersections" and "[s]lugs are generated by the action of one phase flowing into the channel whilst the other phase moves into the intersection, eventually cutting off the flow of the first phase into the channel and reversing the process." Ex. 1007 at 11. Fig. 3 shows oil-in-water slug generation and Fig. 4 shows water-in-oil slug generation. *Id.* at 11. In Burns' Fig. 4 embodiment, an aqueous plug of reagents is formed in a continuous oil phase. The continuous flow plug formation used by Burns was a well-known alternative to periodically injecting a measured slug of reactant into the main channel. See Ex. 1010; 1027; Ex. 1025 at 23:2-8. Burns I demonstrates that making slugs or plugs with continuous flows of immiscible fluids was a mere design choice as of 2001.

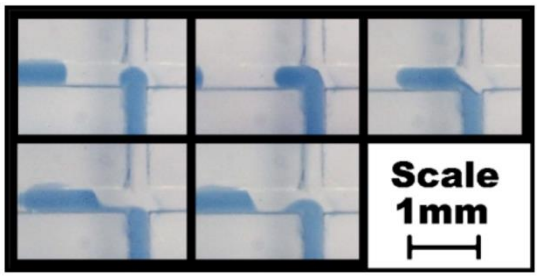
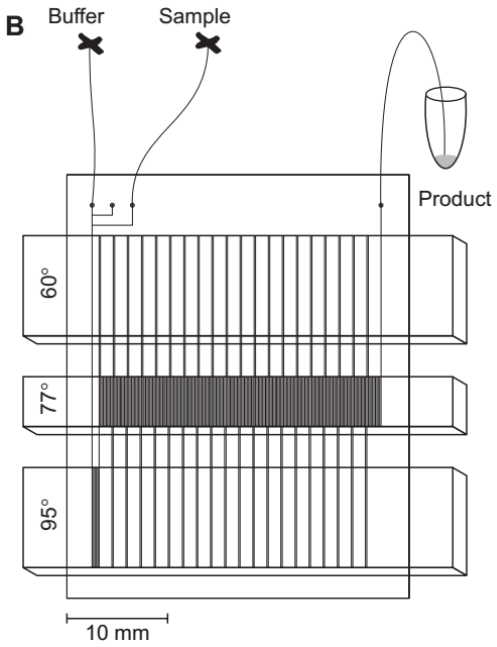


Fig. 3 Typical slug generation in glass device with 380 μm wide channels.

As of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Kopp and Lagally. By 1998 Kopp *et al.* had successfully implemented continuous flow PCR on a chip. In his 1988 article (not previously presented to the USPTO), Kopp explained that "[a] wide range of microreactors, microcapillary electrophoresis devices, and microcell manipulation devices have been described in recent years." Ex. 1027 at 1046. Building on that body of work, Kopp's group developed

> [a] micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in **continuous flow** at high speed. The device is analogous to an electronic amplifier and relies on the movement of sample through thermostated temperature zones on a glass microchip. Input and output of material (DNA) is **continuous**, and amplification is independent of input concentration. . . .
>
> The results demonstrate that with a very simple device and virtually no optimization, PCR can be performed in **continuous flow**, yielding product quality and quantity comparable to standard thermal cycling methods (Fig. 2B). . . .
>
> We suggest that cross-contamination in a **continuous-flow** format may be much less of a problem than in a stationary-tube PCR format. Thus, a flow-through PCR chip can be reused for extended periods without major cleaning. . . .



Ex. 1027 at 1046-1047 [emphasis added]. Kopp *et al.* also explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications:

> To build a complex system analogous to an electronic integrated circuit, it is necessary to integrate a variety of other continuously working parts. A cursory glance at developments in miniaturized total analysis systems (uTAS) during the past 5 years reveals that most of the relevant components, such as **continuous-flow mixers** (8), **continuous-flow microreactors** (9), **continuous separations** (10), and high-speed capillary gel electrophoresis (as a conventional batch process), have already been presented (11). Accordingly, the **continuous-flow microamplifier** should allow the creation of highly elaborate analysis and synthesis systems.
>
> Although chemical amplification reactors will obviously not be applicable to all reactions, neither will they be limited to PCR. Devices of this kind open up whole new areas of application in medical diagnostics. For example, the online amplification and monitoring of a specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy.

Ex. 1027 at 1047 [emphasis added].

In 2001, in another article not previously considered by the Office, Lagally reported using a microfluidic reactor to conduct single-molecule DNA amplification by PCR. Lagally summarized the state of the art as of February 2001, explaining continuous flow PCR microreactors had recently undergone substantial evolution:

> The advent of biological microdevices [6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis Microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods16 or **continuous flow** through three differentially heated regions.18 Unfortunately, most of these designs either require bulk heating,16,17 making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.18 Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.20,21 The first integrated PCRCE device used 10-μL silicon chambers with

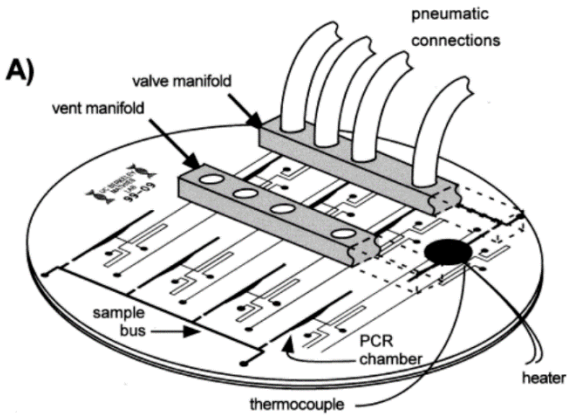
> polysilicon heaters that were attached to a glass CE microchannel.21 This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566 [emphasis added].

Lagally *et al.* developed a further improvement – a device and method for achieving single molecule amplification in a microchannel environment.

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ∼1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> Microfluidic PCR-CE device. The PCR chambers are connected to a common sample bus through a set of valves. Hydrophobic

> vents at the other end of the PCR chambers are used to locate the sample and to eliminate gas. The PCR chambers are directly connected to the cross channel of the CE system for product injection and analysis. Two aluminum manifolds, one each for the vents and valves, are placed onto the respective ports and clamped in place using vacuum. The manifolds connect to external solenoid valves for pressure and vacuum actuation. Thermal cycling is accomplished using a resistive heater and a miniature thermocouple below the 280-nL chamber.



Ex. 1028 at 566 (see figure legend).

In sum, Kopp and Lagally show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Burns I is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[128] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Burns I) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

[128] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I to conduct microfluidic PCR as taught by Kopp and Lagally. A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic reactors of Burns I because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as

to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Burns I to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the droplet reactors of Burns I would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely

reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Burns I to dilute the samples and thereby enable the detection of

mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Kopp or Lagally) to incorporate a droplet reactor (Burns I). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Kopp or Lagally. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the single fluid in microfluidic chip reactor (e.g., Lagally) or droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Kopp). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al*. provided an overview of that evolution:

The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.

Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with

> sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Burns I could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful modifying Kopp or Lagally to work in the microfluidic reactor of Quake, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was

contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[129] As discussed in Section VI.A.3, above, Higuchi I-III thus demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail below, in the accompanying claim chart (Ex. 1077) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I to conduct microfluidic PCR as taught by Kopp and Lagally.

Independent Claim 1

Claim 1[a]: A method comprising the steps of: providing a microfluidic system comprising one or more channels

[129] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

Burns I discloses "[a] multiphase microreactor based upon the use of slug flow through a narrow channel.…" Ex. 1007 at 10. "The experimental work discussed here is based on the use of alternating slugs of two liquid phases to provide the environment for mass transfer and reaction within the microreactor." Ex. 1007 at 10.

Burns I explains that "[t]he method discussed in this work **uses the continuous flow of both phases through T or cross-shaped intersections**. Slugs are generated by the action of **one phase flowing into the channel whilst the other phase moves into the intersection,** eventually cutting off the flow of the first phase into the channel and reversing the process." Ex. 1007 at 11 [emphasis added].

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1077.

Claim 1[b]: providing within the one or more channels a continuously flowing carrier fluid comprising an oil and a continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule in the fluid that can react with the target DNA or RNA molecules under conditions in which the target DNA or RNA molecules and the other molecules in the fluid do not react with each other

As noted above, Burns I teaches that "[t]he method discussed in this work uses the continuous flow of both phases through T or cross-shaped intersections. Slugs are generated by the action of one phase flowing into the channel whilst the other phase moves into the intersection, eventually cutting off the flow of the first phase into the channel and reversing the process." Ex. 1007 at 11. Figure 1 shows that the reactant slug is composed of an "**aqueous phase**." *Id.* at Figure 1 [emphasis

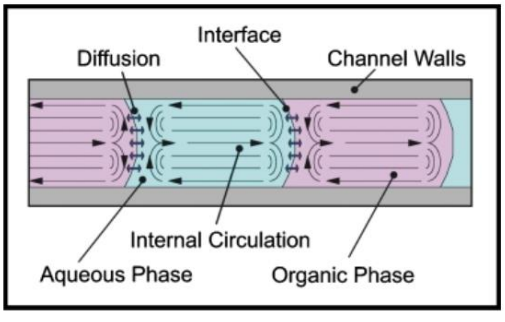


Fig. 1 Illustration of internal circulation generated within immiscible slug flow.

added]. Fig. 3 shows oil-in-water slug generation and Fig. 4 shows water-in-oil slug generation. *Id.* at 11. In Burns' Fig. 4 embodiment, an aqueous plug of reagents is formed in a continuous oil phase. The continuous flow plug formation used by Burns was a well-known alternative to periodically injecting a measured slug of reactant into the main channel. See Ex. 1010; 1027; Ex. 1025 at 23:2-8. Burns I demonstrates that making slugs or plugs with continuous flows of immiscible fluids was a mere design choice as of 2001.

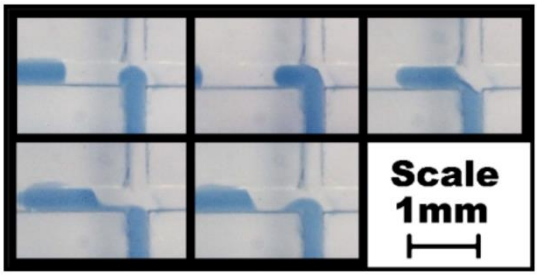


Fig. 3 Typical slug generation in glass device with 380 μm wide channels.

In the instant combination, the microfluidic droplet reactor of Burns I is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[130] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Burns I) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I to conduct microfluidic PCR as taught by Kopp and Lagally. A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic droplet reactor of Burns I because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological

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microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Burns I to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the microfluidic droplet reactor of Burns I would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from

the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

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Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Burns I to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

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Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-

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in the art.[131] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained further in the accompanying claim chart (Ex. 1077) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I to conduct microfluidic PCR as taught by Kopp and Lagally.

With regard to the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other," this is merely a description of a PCR reaction. Ex. 1004 ¶31,32. As explained in Corbett, Kopp, Burns II, and Lagally, in a PCR reaction the sample DNA or RNA is admixed with various reagents including polymerase under conditions in which no reaction occurs; only when heat is applied does the PCR reaction occur. Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68. In the PCR of the combined method, as in virtually any PCR method, the DNA and polymermase do not react until mixture of reagents is heated. *Id*.; see also Ex. 1004 ¶32. The combined method thus meets the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and

[131] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

the other molecules in the fluid do not react with each other" this is merely a description of a PCR reaction. Ex. 1004 ¶31,32.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1077.

Claim 1[c]: controlling flow rates of said aqueous fluid and said carrier fluid to partition the continuously flowing aqueous fluid with the continuously flowing carrier fluid to form a plurality of plugs of the aqueous fluid, each having a substantially uniform size of about 200 µm or less, wherein the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule

Burns I teaches that plugs in the channels of a microreactor by the continuous flow of fluids at an intersecting channel. Burns I explained that "various methods may be used to generate slugs of liquid within a microreactor." Ex. 1007 at 10. Burns I chose a method in which "the continuous flow of both phases through T or cross-shaped intersections" and "[s]lugs are generated by the action of one phase flowing into the channel whilst the other phase moves into the intersection, eventually cutting off the flow of the first phase into the channel and reversing the process." Ex. 1007 at 11. Fig. 3 shows oil-in-water slug generation and Fig. 4 shows water-in-oil slug generation. *Id.* at 11. In Burns' Fig. 4 embodiment, an aqueous plug of reagents is formed in a continuous oil phase. The continuous flow plug formation used by Burns was a well-known alternative to periodically injecting a measured slug of reactant into the main channel. See Ex. 1010; 1027; Ex. 1025 at 23:2-8. Burns I demonstrates that making slugs or plugs with continuous flows of immiscible fluids was a mere design choice as of 2001.

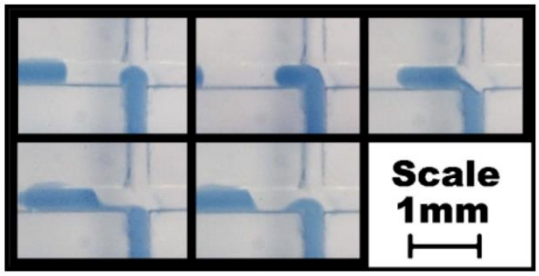


Fig. 3 Typical slug generation in glass device with 380 µm wide channels.

Turning to the recitation "each having a substantially uniform size of about 200 µm or less," Burns I discloses that the droplets are 100 µm to 400 µm in diameter. Ex. 1007 at 10. Moreover, it was well known that droplets smaller than 200 µm could be advantageously used in droplet generators. Quake teaches that "the droplets have a diameter that is smaller than the diameter of the microchannel; i.e., preferably less than 60 µm." Ex. 1034 at 27:29-30. Fig. 21 of Quake shows the droplets sizes generated by the Quake droplet generator were all less than 60 µm. Burns II (1996) similarly discloses uses of 60 nl droplets (which equates to a droplet diameter of about 62 µm). Ex. 1008 at 5558. Accordingly, it would have been obvious to a skilled artisan that as of the earliest claimed priority date (2002) droplets below 200 µm in diameter could, and should, be used in order to reduce reagent usage and reaction time and achieve the various other benefits described above with the combination. Ex. 1004 ¶36, 66.

This leaves the recitation that "the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule." As discussed above, in the combined method of Quake, Corbett and Lagally, the samples are separated into a number of discrete droplets. That has the advantages of i) overcoming the signal-to-noise limitations associated with certain other PCR techniques and ii) enabling the detection of relatively rare mutations, dislocations and allelic imbalances. Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Lagally expressly teaches that "[t]he event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a **Poisson** distribution, demonstrating that we are operating at the single-molecule level." Ex. 1028 at 566 [emphasis added]. Vogelstein likewise teaches that the aforementioned advantages are realized by **reducing the number of sample molecules per**

droplet to zero or one. Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶82**.** That ensures that any amplification that occurs in a given droplet can be readily identified as containing the target sequence. *Id.* Thus, in the combined method the droplets have either one or zero template molecules.

As suggested by Lagally, any process which is generating droplets containing about one template molecule per droplet is expected to conform to a random, or Poisson, distribution. Ex. 1028 at 566; Ex. 1004 ¶48. It was known in the art that "[i]f a solution containing x molecules of enzyme per ml is dispersed into droplets of v ml volume, the average number of enzyme molecules per droplet n is equal to vx. Because of statistical fluctuations each droplet will not have exactly n enzyme molecules. The probability p(r) of finding 0, 1, 2, 3, . .. , r enzyme molecules in a droplet **is given by the Poisson law**." Ex. 1112 at 1985 [emphasis added]; see also Ex. 1111 at 1667 ("Spectra observed in a time sequence for an average of 0.6 dye molecule in the probed volume exhibited the expected Poisson distribution for actually measuring 0, 1, 2, or 3 molecules.").

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1077.

Claim 1[d]: providing conditions suitable for a polymerase-chain reaction in at least one plug of the plurality of plugs such that the target DNA or RNA is amplified

Burns I discloses that "[t]he high heat and mass transfer capability offered by capillary microreactors allows high intensity reactions to be precisely controlled for optimum yield and selectivity." Ex. 1007 at 10. "The internal circulation, which is stimulated within the slugs by their passage along the channel, is responsible for a **large enhancement** in the interfacial mass transfer and the **reaction rate**." *Id.* [emphasis added].

As discussed above in connection with element 1[b], in the instant combination Burns I is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[132] These limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Burns I) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1077.

Thus, claim 1 is shown to be rendered obvious by the combination of Burns I, Kopp and Lagally.

Dependent Claim 2

Claim 2 of the '148 patent recites "wherein the step of providing conditions includes heating."

As discussed above in connection claim elements 1[b] and 1[c], in the combined method (and in virtually any other PCR reaction) the DNA and polymerase undergo polymerase chain reaction when heated. Ex. 1004 ¶32; Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1077.

Thus, claim 2 is shown to be rendered obvious by the combination of Burns I, Kopp and Lagally.

[132] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

Dependent Claims 3-5

Claim 3 of the '148 patent recites "providing a detector to detect, analyze, characterize, or monitor the target DNA or RNA molecule in the at least one member of the plurality of plugs during and/or after the reaction has occurred." Claim 4 of the '148 patent further recites "wherein the at least one other molecule includes one or more fluorescent labels and the detector is configured to monitor progress of the reaction by detecting fluorescence emissions from the one or more fluorescent labels." Claim 5 recites "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels."

Lagally teaches "[s]tochastic PCR amplification of single DNA template molecules followed by **capillary electrophoresis** (CE) analysis of the products is demonstrated in an integrated microfluidic device." Ex. 1028 at 1, [emphasis added]. The results of capillary electrophoresis were generally detected optically at the time of filing. Ex. 1011 at 46 (Dolnick). "The amplified product, labeled with an intercalating **fluorescent** dye, is directly injected into the gel-filled capillary channel for electrophoretic analysis." Ex. 1028 at 1, [emphasis added].

In the combined method of Burns I, Kopp and Lagally, microfluidic droplet reactors are used to perform PCR reactions after which the reaction products are detected optically and sorted by diverting certain droplets into collection channels. Ex. 1004 ¶69.

Turning to claim 5, under the broadest reasonable interpretation the phrase "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels" requires simply that at least some of the droplets or plugs are transmitted to an outlet or other channel. Burns I teaches an outlet channel downstream of the droplet generating junction and thus meets claim 5 under the broadest reasonable interpretation. Ex. 1007 at 11. If claim 5 is more narrowly interpreted as requiring selective diversion of the droplets, that was well known. For instance, Quake teaches that "[a] preferred 'forward' sorting algorithm consists

of running molecules, cells or virions from the input channel to the waste channel, until a molecule, cell or virion is identified to have an optically detectable signal (e.g. fluorescence) that is above a pre-set threshold, at which point voltages are temporarily changed to electro-osmotically divert the molecule [] to the collection channel." Ex. 1034 at 20:22-26. Fu likewise discloses a microfabricated fluorescence-activiated cell sorter for use in connection with emulsion or droplet based systems. Ex. 1113 at 1109. A skilled artisan would have considered it obvious to employ such a technique to sort droplets after they are subjected to the optical detection. Ex. 1004 ¶68,69. For instance, a skilled artisan would have found it advantageous to sort and collect droplets which tested positive or negative for the presence of a certain substance or property, especially in the context of droplet generators which are used to encapsulate on average one template molecule per droplet. *Id.* ¶82.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1077.

Thus, claims 3-5 are shown to be rendered obvious by the combination of Burns I, Kopp and Lagally.

Q. The Combination of Burns I, Kopp and Burns II Renders Obvious Claims 1-5 of the '148 Patent Under (pre-AIA) 35 U.S.C. § 103(a)

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Burns I), ii) microfluidic PCR devices were well known (Kopp and Burns II), and iii) there were compelling reasons to use the microfluidic droplet reactors to conduct microfluidic PCR (Kopp, Burns II, Lagally and Vogelstein).

Burns I teaches that plugs in the channels of a microreactor by the continuous flow of fluids at an intersecting channel. Burns I explained that "various methods may be used to

generate slugs of liquid within a microreactor." Ex. 1007 at 10. Burns I chose a method in which "the continuous flow of both phases through T or cross-shaped intersections" and "[s]lugs are generated by the action of one phase flowing into the channel whilst the other phase moves into the intersection, eventually cutting off the flow of the first phase into the channel and reversing the process." Ex. 1007 at 11. Fig. 3 shows oil-in-water slug generation and Fig. 4 shows water-in-oil slug generation. *Id.* at 11. In Burns' Fig. 4 embodiment, an aqueous plug of reagents is formed in a continuous oil phase. The continuous flow plug formation used by Burns was a well-known alternative to periodically injecting a measured slug of reactant into the main channel. See Ex. 1010; 1027; Ex. 1025 at 23:2-8. Burns I demonstrates that making slugs or plugs with continuous flows of immiscible fluids was a mere design choice as of 2001.

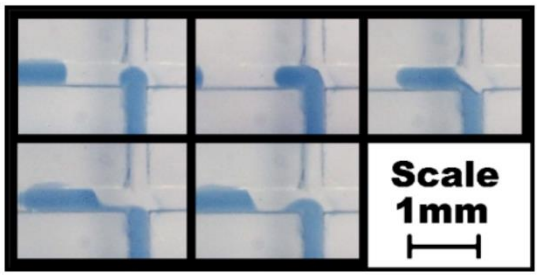


Fig. 3 Typical slug generation in glass device with 380 µm wide channels.

As of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Kopp and Burns II. In 1996, Burns II reported the use of microfluidic devices to perform PCR. Ex. 1008. Burns II explained that "[n]o new biochemistries or DNA detection methods are required for their use; the components simply reproduce current laboratory procedures in a micron-sized environment." *Id.* at 5560. In other words, the PCR chemistry did not need to be modified – it worked without modification on the microfluidic scale. In the method of Burns II, a sample containing a DNA plasmid was loaded into one of the reaction wells (A) and a mixture of Taq enzyme and buffer was loaded into the other. *Id.* at 5558. Each formed droplets of about 60 nl in the parallel 500 x 25 µm channels (B)

which came together at the Y junction to form a single larger droplet (about 120 nl). *Id.* at 5558-59; Ex. 1004 ¶40.

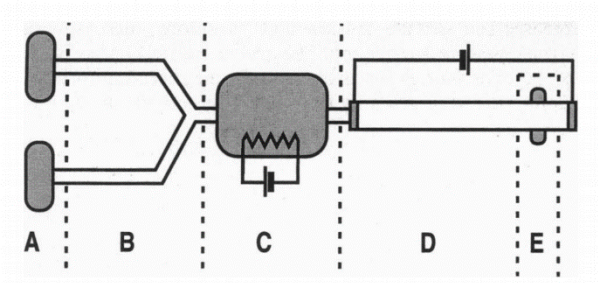


FIG. 1. Example of an integrated DNA analysis system, represented schematically. The individual components of the system are injection entry ports (*A*), liquid pumping channels (*B*), thermally controlled reaction chamber (*C*), electrophoresis channel (*D*), and DNA band migration detector (*E*). Each component would have associated sensors, control circuitry, and external connections.

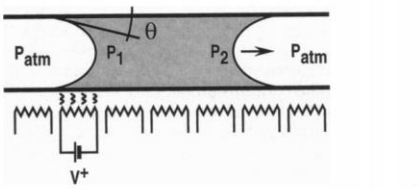


FIG. 2. Schematic drawing showing the principle of thermally induced drop motion in a closed channel. The case of a single aqueous drop in a hydrophilic channel is presented, where V is an applied voltage, P_{atm} is atmospheric pressure, P_1 is the receding-edge internal pressure, P_2 is the advancing-edge internal pressure, and θ is the contact angle of the liquid–gas–solid interface. The contact angle will depend on the surface characteristics of the channel and the constituents of the drop, with a hydrophilic interaction giving θ between 0 and 90 degrees, and a hydrophobic surface giving θ between 90 and 180 degrees. Surface treatments can also reduce contact angle hysteresis and, therefore, reduce the temperature difference necessary for drop motion.

The droplet then passed through the resistive heater portion (C) in which PCR amplification occurred. *Id.* at 5556. Afterwards the amplification results were optically detected in zone D via gel electrophoresis *Id.* at 5559. Burns II noted that "[t]he use of common fabrication methods allows the assembly of increasingly complex, multicomponent, integrated systems from a small, defined set of standardized elements" and that "[f]uture design efforts have a strong impetus to reduce component size and improve efficiency." *Id.* at 5560-61.

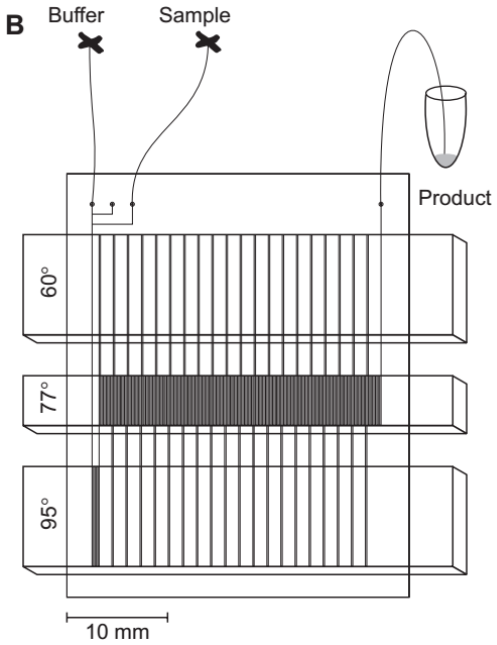
By the late 1990s Kopp *et al.* had successfully implemented continuous flow PCR on a chip. In his 1988 article (not previously presented to the USPTO), Kopp explained that "[a] wide range of microreactors, microcapillary electrophoresis devices, and microcell manipulation devices have been described in recent years." Ex. 1027 at 1046. Building on that body of work, Kopp's group developed

> [a] micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in **continuous flow** at high speed. The device is analogous to an electronic amplifier and relies on the movement of sample through thermostated temperature zones on a glass

microchip. Input and output of material (DNA) is **continuous**, and amplification is independent of input concentration. . . .

The results demonstrate that with a very simple device and virtually no optimization, PCR can be performed in **continuous flow**, yielding product quality and quantity comparable to standard thermal cycling methods (Fig. 2B). . . .

We suggest that cross-contamination in a **continuous-flow** format may be much less of a problem than in a stationary-tube PCR format. Thus, a flow-through PCR chip can be reused for extended periods without major cleaning. . . .



Ex. 1027 at 1046-1047 [emphasis added]. Kopp *et al.* also explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications:

To build a complex system analogous to an electronic integrated circuit, it is necessary to integrate a variety of other continuously working parts. A cursory glance at developments in miniaturized total analysis systems (uTAS) during the past 5 years reveals that most of the relevant components, such as **continuous-flow mixers** (8), **continuous-flow**

> **microreactors** (9), **continuous separations** (10), and high-speed capillary gel electrophoresis (as a conventional batch process), have already been presented (11). Accordingly, the **continuous-flow microamplifier** should allow the creation of highly elaborate analysis and synthesis systems.
>
> Although chemical amplification reactors will obviously not be applicable to all reactions, neither will they be limited to PCR. Devices of this kind open up whole new areas of application in medical diagnostics. For example, the online amplification and monitoring of a specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy.

Ex. 1027 at 1047 [emphasis added].

In sum, Kopp and Burns II show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Burns I is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[133] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Burns I) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I, to conduct microfluidic PCR as taught by Kopp and Burns II. A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic reactor of

[133] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

Burns I because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Burns I to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the droplet reactors of Burns I would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026

(Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Burns I to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Kopp or Burns II) to

incorporate a droplet reactor (Burns I). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Kopp or Burns II. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the single fluid in microfluidic chip reactor (e.g., Burns II) or droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Kopp). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and

> amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that

"the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Burns I could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful modifying Kopp or Burns II to work in the microfluidic reactor of Burns I, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact

that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[134] As discussed in Section VI.A.3, above, Higuchi I-III thus demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail below, in the accompanying claim chart (Ex. 1078) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I to conduct microfluidic PCR as taught by Kopp and Burns II.

Independent Claim 1

Claim 1[a]: A method comprising the steps of: providing a microfluidic system comprising one or more channels

Burns I discloses "[a] multiphase microreactor based upon the use of slug flow through a narrow channel…." Ex. 1007 at 10. "The experimental work discussed here is based on the use of alternating slugs of two liquid phases to provide the environment for mass transfer and reaction within the microreactor." *Id.*

Burns I explains that "[t]he method discussed in this work **uses the continuous flow of both phases through T or cross-shaped intersections**. Slugs are generated by the action of **one**

[134] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

phase flowing into the channel whilst the other phase moves into the intersection, eventually cutting off the flow of the first phase into the channel and reversing the process." Ex. 1007 at 11 [emphasis added].

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1078.

Claim 1[b]: providing within the one or more channels a continuously flowing carrier fluid comprising an oil and a continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule in the fluid that can react with the target DNA or RNA molecules under conditions in which the target DNA or RNA molecules and the other molecules in the fluid do not react with each other

As noted above, Burns I teaches that "[t]he method discussed in this work uses the continuous flow of both phases through T or cross-shaped intersections. Slugs are generated by the action of one phase flowing into the channel whilst the other phase moves into the intersection, eventually cutting off the flow of the first phase into the channel and reversing the process." Ex. 1007 at 11. Figure 1 shows that the reactant slug is composed of an "**aqueous phase**." *Id.* at Figure 1 [emphasis added]. Fig. 3 shows oil-in-water slug generation and Fig. 4 shows water-in-oil slug generation. *Id.* at 11. In Burns' Fig. 4 embodiment, an aqueous plug of reagents is formed in a continuous oil phase. The continuous flow plug formation used by Burns was a well-known alternative to periodically

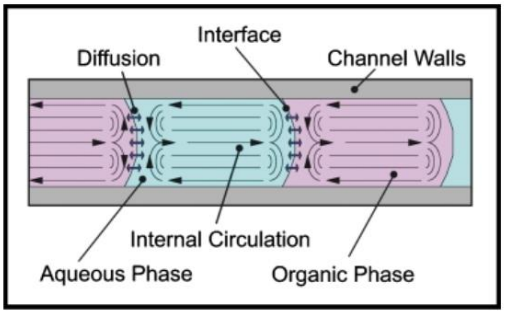


Fig. 1 Illustration of internal circulation generated within immiscible slug flow.

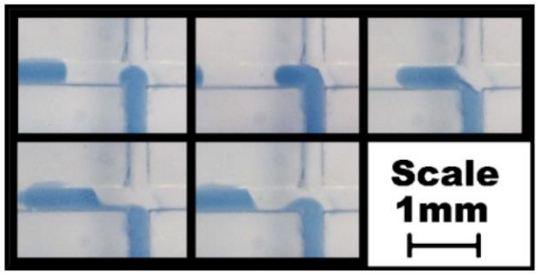


Fig. 3 Typical slug generation in glass device with 380 µm wide channels.

injecting a measured slug of reactant into the main channel. See Ex. 1010; 1027; Ex. 1025 at 23:2-8. Burns I demonstrates that making slugs or plugs with continuous flows of immiscible fluids was a mere design choice as of 2001.

In the instant combination, the microfluidic droplet reactor of Burns I is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[135] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Burns I) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I to conduct microfluidic PCR as taught by Kopp and Burns II. A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic droplet reactor of Burns I because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The reduction of fluid plug size would also have increased the rate at which

[135] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Burns I to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the droplet reactors of Burns I would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such

binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 (“It is also possible that Dig-PCR can be performed in

microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Burns I to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Kopp or Burns II) to incorporate a droplet reactor (Burns I). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Kopp or Burns II. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to the single fluid in microfluidic chip reactor (e.g., Burns II) or droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Kopp). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually

> transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ∼1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .

> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an

autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactors Burns I could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful in modifying Burns II or Kopp to work in the microfluidic droplet reactor of Burns I, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[136] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of

[136] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained further in the accompanying claim chart (Ex. 1078) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I to conduct microfluidic PCR as taught by Kopp and Burns II.

With regard to the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other," this is merely a description of a PCR reaction. Ex. 1004 ¶31,32. As explained in Corbett, Kopp, Burns II, and Lagally, in a PCR reaction the sample DNA or RNA is admixed with various reagents including polymerase under conditions in which no reaction occurs; only when heat is applied does the PCR reaction occur. Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68. In the PCR of the combined method, as in virtually any PCR method, the DNA and polymermase do not react until mixture of reagents is heated. *Id*.; see also Ex. 1004 ¶32. The combined method thus meets the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other" this is merely a description of a PCR reaction. Ex. 1004 ¶31,32.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1078.

Claim 1[c]: controlling flow rates of said aqueous fluid and said carrier fluid to partition the continuously flowing aqueous fluid with the continuously flowing carrier fluid to form a plurality of plugs of the aqueous fluid, each having a substantially uniform size of about 200 µm or less, wherein the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member

of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule

Burns I teaches that plugs in the channels of a microreactor by the continuous flow of fluids at an intersecting channel. Burns I explained that "various methods may be used to generate slugs of liquid within a microreactor." Ex. 1007 at 10. Burns I chose a method in which "the continuous flow of both phases through T or cross-shaped intersections" and "[s]lugs are generated by the action of one phase flowing into the channel whilst the other phase moves into the intersection, eventually cutting off the flow of the first phase into the channel and reversing the process." Ex. 1007 at 11. Fig. 3 shows oil-in-water slug generation and Fig. 4 shows water-in-oil slug generation. *Id.* at 11. In Burns' Fig. 4 embodiment, an aqueous plug of reagents is formed in a continuous oil phase. The continuous flow plug formation used by Burns was a well-known alternative to periodically injecting a measured slug of reactant into the main channel. See Ex. 1010; 1027; Ex. 1025 at 23:2-8. Burns I demonstrates that making slugs or plugs with continuous flows of immiscible fluids was a mere design choice as of 2001.

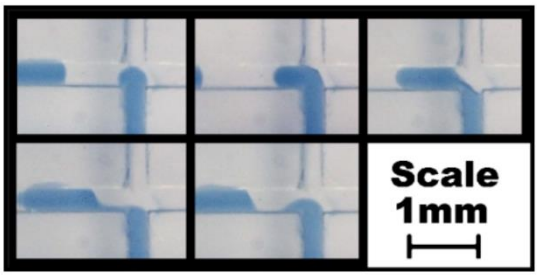


Fig. 3 Typical slug generation in glass device with 380 μm wide channels.

Turning to the recitation "each having a substantially uniform size of about 200 μm or less," Burns I discloses that the droplets are 100 μm to 400 μm in diameter. Ex. 1007 at 10. Moreover, it was well known that droplets smaller than 200 μm could be advantageously used in droplet generators. Quake teaches that "the droplets have a diameter that is smaller than the diameter of the microchannel; i.e., preferably less than 60 μm." Ex. 1034 at 27:29-30. Fig. 21 of Quake shows the droplets sizes generated by the Quake droplet generator were all less than 60 μm. Burns II (1996) similarly discloses uses of 60 nl droplets (which equates to a droplet

diameter of about 62 μm). Ex. 1008 at 5558. Accordingly, it would have been obvious to a skilled artisan that as of the earliest claimed priority date (2002) droplets below 200 μm in diameter could, and should, be used in order to reduce reagent usage and reaction time and achieve the various other benefits described above with the combination. Ex. 1004 ¶36, 66.

This leaves the recitation that "the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule." As discussed above, in the combined method of Quake, Corbett and Lagally, the samples are separated into a number of discrete droplets. That has the advantages of i) overcoming the signal-to-noise limitations associated with certain other PCR techniques and ii) enabling the detection of relatively rare mutations, dislocations and allelic imbalances. Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Lagally expressly teaches that "[t]he event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a **Poisson** distribution, demonstrating that we are operating at the single-molecule level." Ex. 1028 at 566 [emphasis added]. Vogelstein likewise teaches that the aforementioned advantages are realized by **reducing the number of sample molecules per droplet to zero or one.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶82**.** That ensures that any amplification that occurs in a given droplet can be readily identified as containing the target sequence. *Id.* Thus, in the combined method the droplets have either one or zero template molecules.

As suggested by Lagally, any process which is generating droplets containing about one template molecule per droplet is expected to conform to a random, or Poisson, distribution. Ex. 1028 at 566; Ex. 1004 ¶48. It was known in the art that "[i]f a solution containing x molecules of

enzyme per ml is dispersed into droplets of v ml volume, the average number of enzyme molecules per droplet n is equal to vx. Because of statistical fluctuations each droplet will not have exactly n enzyme molecules. The probability p(r) of finding 0, 1, 2, 3, . .. , r enzyme molecules in a droplet **is given by the Poisson law**." Ex. 1112 at 1985 [emphasis added]; see also Ex. 1111 at 1667 ("Spectra observed in a time sequence for an average of 0.6 dye molecule in the probed volume exhibited the expected Poisson distribution for actually measuring 0, 1, 2, or 3 molecules.").

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1078.

Claim 1[d]: providing conditions suitable for a polymerase-chain reaction in at least one plug of the plurality of plugs such that the target DNA or RNA is amplified

Burns I discloses that "[t]he high heat and mass transfer capability offered by capillary microreactors allows high intensity reactions to be precisely controlled for optimum yield and selectivity." Ex. 1007 at 10. "The internal circulation, which is stimulated within the slugs by their passage along the channel, is responsible for a **large enhancement** in the interfacial mass transfer and the **reaction rate**." Ex. 1007 at 10 [emphasis added].

As discussed above in connection with element 1[b], in the instant combination Burns I is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[137] These limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in

[137] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

Burns I) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1078.

Thus, claim 1 is shown to be rendered obvious by the combination of Burns I, Kopp and Burns II.

Dependent Claim 2

Claim 2 of the '148 patent recites "wherein the step of providing conditions includes heating."

As discussed above in connection claim elements 1[b] and 1[c], in the combined method (and in virtually any other PCR reaction) the DNA and polymerase undergo polymerase chain reaction when heated. Ex. 1004 ¶32; Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1078.

Thus, claim 2 is shown to be rendered obvious by the combination of Burns I, Kopp and Burns II.

Dependent Claims 3-5

Claim 3 of the '148 patent recites "providing a detector to detect, analyze, characterize, or monitor the target DNA or RNA molecule in the at least one member of the plurality of plugs during and/or after the reaction has occurred." Claim 4 of the '148 patent further recites "wherein the at least one other molecule includes one or more fluorescent labels and the detector is configured to monitor progress of the reaction by detecting fluorescence emissions from the

one or more fluorescent labels." Claim 5 recites "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels."

Burns II teaches that "[c]apillary gel electrophoresis of DNA samples was performed using a Beckman P/ACE instrument with a laser-induced fluorescence detector and 37 cm length, 100 μm diameter, linear polymerase gel capillary according to manufacturer's recommendation." Ex. 1008 at 5558. The results of capillary electrophoresis were generally detected optically at the time of filing. Ex. 1011 at 46 (Dolnick). "The amplified product, labeled with an intercalating **fluorescent** dye, is directly injected into the gel-filled capillary channel for electrophoretic analysis." Ex. 1028 at 1, [emphasis added].

Turning to claim 5, under the broadest reasonable interpretation the phrase "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels" requires simply that at least some of the droplets or plugs are transmitted to an outlet or other channel. Burns I teaches an outlet channel downstream of the droplet generating junction and thus meets claim 5 under the broadest reasonable interpretation. Ex. 1007 at 11. If claim 5 is more narrowly interpreted as requiring selective diversion of the droplets, that was well known. For instance, Quake teaches that "[a] preferred 'forward' sorting algorithm consists of running molecules, cells or virions from the input channel to the waste channel, until a molecule, cell or virion is identified to have an optically detectable signal (e.g. fluorescence) that is above a pre-set threshold, at which point voltages are temporarily changed to electro-osmotically divert the molecule [] to the collection channel." Ex. 1034 at 20:22-26. Fu likewise discloses a microfabricated fluorescence-activiated cell sorter for use in connection with emulsion or droplet based systems. Ex. 1113 at 1109. A skilled artisan would have considered it obvious to employ such a technique to sort droplets after they are subjected to the optical

detection. Ex. 1004 ¶68,69. For instance, a skilled artisan would have found it advantageous to sort and collect droplets which tested positive or negative for the presence of a certain substance or property, especially in the context of droplet generators which are used to encapsulate on average one template molecule per droplet. *Id.* ¶82.

In the combined method of Burns I, Kopp and Burns II, microfluidic droplet reactors are used to perform PCR reactions after which the reaction products are detected optically and sorted by diverting certain droplets into collection channels. Ex. 1004 ¶69.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1078.

Thus, claims 3-5 are shown to be rendered obvious by the combination of Burns I, Kopp and Burns II.

R. The Combination of Burns I, Kopp and Vogelstein Renders Obvious Claims 1-5 of the '148 Patent Under (pre-AIA) 35 U.S.C. § 103(a)

The prior art submitted herewith shows that i) droplet micro reactors that formed plugs from continuously flowing immiscible fluids were well known (Burns I), ii) microfluidic PCR devices were well known (Kopp and Vogelstein), and iii) there were compelling reasons to use the microfluidic droplet reactors to conduct microfluidic PCR (Kopp, Burns II, Lagally and Vogelstein).

Burns I teaches that plugs in the channels of a microreactor by the continuous flow of fluids at an intersecting channel. Burns I explained that "various methods may be used to generate slugs of liquid within a microreactor." Ex. 1007 at 10. Burns I chose a method in which "the continuous flow of both phases through T or

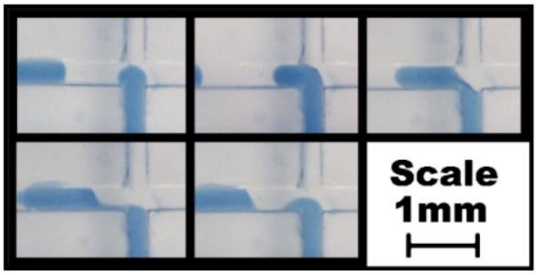


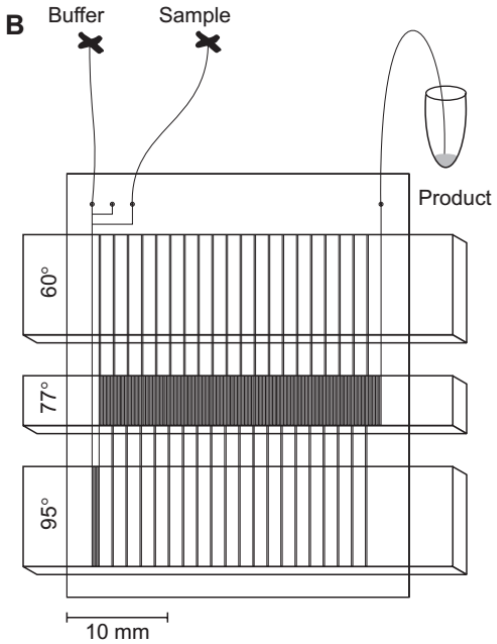
Fig. 3 Typical slug generation in glass device with 380 μm wide channels.

cross-shaped intersections" and "[s]lugs are generated by the action of one phase flowing into the channel whilst the other phase moves into the intersection, eventually cutting off the flow of the first phase into the channel and reversing the process." Ex. 1007 at 11 [emphasis added]. Fig. 3 shows oil-in-water slug generation and Fig. 4 shows water-in-oil slug generation. *Id.* at 11. In Burns' Fig. 4 embodiment, an aqueous plug of reagents is formed in a continuous oil phase. The continuous flow plug formation used by Burns was a well-known alternative to periodically injecting a measured slug of reactant into the main channel. See Ex. 1010; 1027; Ex. 1025 at 23:2-8. Burns I demonstrates that making slugs or plugs with continuous flows of immiscible fluids was a mere design choice as of 2001.

As of the earliest claimed priority date microfluidic devices were routinely being used to perform high-throughput PCR, as demonstrated by Kopp and Vogelstein. By the late 1990s Kopp *et al.* had successfully implemented continuous flow PCR on a chip. In his 1988 article (not previously presented to the USPTO), Kopp explained that "[a] wide range of microreactors, microcapillary electrophoresis devices, and microcell manipulation devices have been described in recent years." Ex. 1027 at 1046. Building on that body of work, Kopp's group developed

> a] micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in **continuous flow** at high speed. The device is analogous to an electronic amplifier and relies on the movement of sample through thermostated temperature zones on a glass microchip. Input and output of material (DNA) is **continuous**, and amplification is independent of input concentration. . . .
>
> The results demonstrate that with a very simple device and virtually no optimization, PCR can be performed in **continuous flow**, yielding product quality and quantity comparable to standard thermal cycling methods (Fig. 2B). . . .

> We suggest that cross-contamination in a **continuous-flow** format may be much less of a problem than in a stationary-tube PCR format. Thus, a flow-through PCR chip can be reused for extended periods without major cleaning. . . .



Ex. 1027 at 1046-1047 [emphasis added]. Kopp *et al.* also explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications:

> To build a complex system analogous to an electronic integrated circuit, it is necessary to integrate a variety of other continuously working parts. A cursory glance at developments in miniaturized total analysis systems (uTAS) during the past 5 years reveals that most of the relevant components, such as **continuous-flow mixers** (8), **continuous-flow microreactors** (9), **continuous separations** (10), and high-speed capillary gel electrophoresis (as a conventional batch process), have already been presented (11). Accordingly, the **continuous-flow microamplifier** should allow the creation of highly elaborate analysis and synthesis systems.
>
> Although chemical amplification reactors will obviously not be applicable to all reactions, neither will they be limited to PCR. Devices of

> this kind open up whole new areas of application in medical diagnostics. For example, the online amplification and monitoring of a specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy.

Ex. 1027 at 1047 [emphasis added].

By 1999, Vogelstein had reported **various applications for single-molecule microfluidic PCR**. Ex. 1044 at 9241. In a seminal article which has been cited over by over 1,000 other publications, Vogelstein explained that PCR amplification of small aliquots or droplets containing single molecules was effective for "detection of a small number of mutant-containing cells among a large excess of normal cells." *Id.* at 9236. In Vogelstein's method, the DNA was diluted into 7 µl volumes such that, on average, there was one template molecule for every two wells (i.e., approximately half of the wells contained no DNA template molecule and approximately half contained a single DNA template molecule). *Id.* at 9236, 9237. This avoided the problem of background noise impairing the determination of whether each volume contained the mutant sequence. *Id.* By counting the number of volumes which indicated wild type or mutant templates, the presence or absence of the mutation (e.g., a sequence associated with colorectal cancer) can be determined. *Id.* at 9238. The accuracy of the test is a function of the number of discrete volumes into which the sample is diluted. *Id.* at 9239. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. *Id.* at 9239. ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude.") *Id.* 9239. The article explained that the same technique would be useful for **detecting a wide array of mutations, dislocations and allelic imbalances**, as summarized in Table 1. *Id.* at 9241. Vogelstein thus taught that microfluidic PCR

had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby overcoming the signal-to-noise limitations associated with certain other PCR techniques and enabling the detection of relatively rare mutations, dislocations and allelic imbalances. Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84.

In sum, Kopp and Vogelstein show that microfluidic PCR devices had reached a state of scientific maturity and were in widespread use as of the earliest claimed priority date.

In the instant combination, the microfluidic droplet reactor of Burns I is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[138] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Burns I) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I, to conduct microfluidic PCR as taught by Kopp and Vogelstein. A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic reactor of Burns I because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and**

[138] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

reagents needed." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The modification of Corbett to microfluidic dimensions would have reduced the amounts of reagents used, which would in turn **decrease operating costs.** Ex. 1004 ¶105, 107. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Burns I to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the droplet reactors of Burns Iwould **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the

limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each

contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude."). Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Burns I to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Kopp or Vogelstein) to incorporate a droplet reactor (Burns I). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Kopp or Vogelstein. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to droplets

produced using injection of an aqueous fluid into an immiscible fluid (e.g., Kopp). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize

> noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a Poisson

> distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at

1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactor of Burns I could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful modifying Kopp or Vogelstein to work in the microfluidic reactor of Burns I, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[139] As

[139] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also

discussed in Section VI.A.3, above, Higuchi I-III thus demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained in more detail below, in the accompanying claim chart (Ex. 1079) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I to conduct microfluidic PCR as taught by Kopp and Vogelstein.

Independent Claim 1

Claim 1[a]: A method comprising the steps of: providing a microfluidic system comprising one or more channels

Burns I discloses "[a] multiphase microreactor based upon the use of slug flow through a narrow channel.…" Ex. 1007 at 10. "The experimental work discussed here is based on the use of alternating slugs of two liquid phases to provide the environment for mass transfer and reaction within the microreactor." *Id.*

Burns I explains that "[t]he method discussed in this work **uses the continuous flow of both phases through T or cross-shaped intersections**. Slugs are generated by the action of **one phase flowing into the channel whilst the other phase moves into the intersection,** eventually cutting off the flow of the first phase into the channel and reversing the process." Ex. 1007 at 11 [emphasis added].

Additional correspondence between this claim element and the cited references is shown

relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

in the claim chart submitted herewith as Exhibit 1079.

Claim 1[b]: providing within the one or more channels a continuously flowing carrier fluid comprising an oil and a continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule in the fluid that can react with the target DNA or RNA molecules under conditions in which the target DNA or RNA molecules and the other molecules in the fluid do not react with each other

As noted above, Burns I teaches that "[t]he method discussed in this work uses the continuous flow of both phases through T or cross-shaped intersections. Slugs are generated by the action of one phase flowing into the channel whilst the other phase moves into the intersection, eventually cutting off the flow of the first phase into the channel and reversing the process." Ex. 1007 at 11 [emphasis added]. Figure 1 shows that the reactant slug is composed of an "**aqueous phase**." *Id.* at Figure 1 [emphasis added]. Fig. 3 shows oil-in-water slug generation and Fig. 4 shows water-in-oil slug generation. *Id.* at 11. In Burns' Fig. 4 embodiment, an aqueous plug of reagents is formed in a continuous oil phase. The continuous flow plug formation used by Burns was a well-known alternative to periodically injecting a measured slug of reactant into the main channel. See Ex. 1010; 1027; Ex. 1025 at 23:2-8. Burns I demonstrates that making slugs or plugs with continuous flows of immiscible fluids was a mere design choice as of 2001.

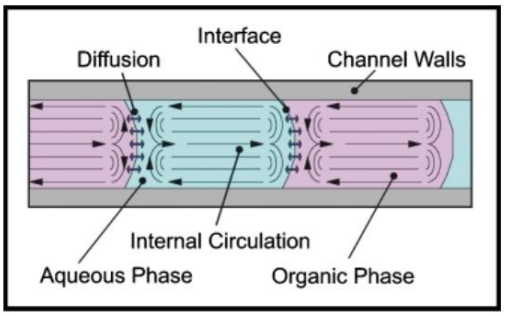


Fig. 1 Illustration of internal circulation generated within immiscible slug flow.

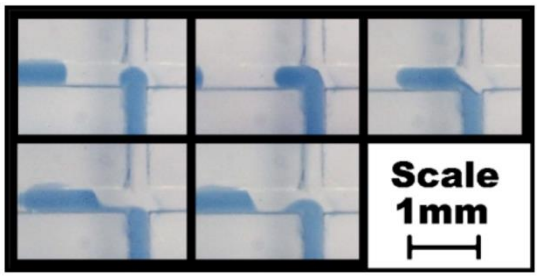


Fig. 3 Typical slug generation in glass device with 380 µm wide channels.

In the instant combination, the microfluidic droplet reactor of Burns I is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[140] Ex 1001 at 78:22-24. In the instant combination, these limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Burns I) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

A skilled artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I to conduct microfluidic PCR as taught by Kopp and Vogelstein. A skilled artisan would have been strongly motivated to perform such PCR reactions in the microfluidic droplet reactor of Burns I because doing so have provided the substantial benefits known to be associated with microfluidic droplet reactors. As noted by Lagally, "[t]he advent of biological microdevices allows one to consider conducting bioanalytical assays such as PCR at very small volumes to **increase the speed** of these assays and to **reduce the amount of material and reagents needed**." Ex. 1028 at 565 [emphasis added]. Burns II similarly explained that "[t]he combination of pump and components into self-contained miniaturized devices may provide significant improvements in DNA analysis **speed, portability, and cost**." Ex. 1008 at 5556 [emphasis added]. The reduction of fluid plug size would also have increased the rate at which the droplets or slugs could be thermocycled, thereby increasing the speed of the overall process. Ex. 1004 ¶47; Ex. 1008 at Abstract; Ex. 1027.

[140] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

It was also well known that miniaturization of PCR reactions to the microfluidic level provided the **substantial advantage of making PCR reactors highly portable** (sufficient to provide point of care testing)**.** Ex. 1027 at 1047; Ex. 1004 ¶103. Kopp explained that such portable PCR microreactors could enable "on-site analysis of patient samples [which] could demonstrate the presence of bacterial DNA and any susceptibility to antibiotic treatment." Ex. 1027 at 1047. Moreover generally, it was known that portable PCR reactors were useful to aid physicians in development of treatment of various conditions. Ex. 1004 ¶108. For instance, identification of a "specific gene could show the patient's ability to metabolize a given drug so as to determine the ideal course of therapy." Ex. 1027 at 1047. It was thus known that using the droplet reactor of Burns I to perform PCR advantageously would have allowed PCR reactions to be performed in point of care diagnostic applications. Ex. 1004 ¶99,103.

Skilled artisans would also have recognized that performing Corbett's PCR reaction in the droplet reactors of Burns I would **enhance the precision of the PCR reaction with a given primer and reduce the production of non-specific products** relative to then-traditional approaches. As of the filing date it was well known that PCR reactions suffered from the limitation that the primers could bind to non-specific sequences, including each other, leading to production on non-specific products. Ex. 1004 ¶33,36; Ex. 1032 (Parsons) at 106-07. In larger volume PCR reactions, the temperature of the reactants ramps up and down gradually as the reaction mixture is thermocycled. Ex. 1004 ¶91,92. If the ramp speed of the thermocycler is too slow, spurious annealing may occur due to lower temperature and sufficient time for nonspecific binding. *Id.* These long ramping periods thus increase the likelihood of off-target effects, such binding to non-target sequences and the production of primer dimers. *Id.* Rapid ramp times of the temperature-dependent reactions in PCR amplification can improve product specificity

significantly while decreasing the cycle time by an order of magnitude. Ex. 1045 at Abstract (Wittwer). It is therefore highly desirable to have short heating and cooling times to achieve more precise and efficient reactions with fewer off-target reaction products. Ex. 1004 ¶96. Accordingly, at the time of filing it was well-known that, for a given primer, the PCR reaction is more efficient and precise where the temperature ramping time is minimized. *Id.*

Moreover, using the microfluidic droplet reactors for PCR reactions would have **substantially increased the ability to successfully utilize degraded or low-level DNA** in a sample. For example, the fragmentation of DNA derived from aged or degraded tissue severely reduces the efficiency of the PCR. Using conventional PCR on such a degraded or aged sample leads to competition for enzyme due to template reconstruction and resulting depletion of dNTPs, both of which hinder the efficient amplification of a target product. Ex. 1015 at 5026 (Golenberg). Thus, at the time of filing, skilled artisans understood that using a microfluidic droplet system would prevent such template reconstruction and dNTP depletion.

Vogelstein taught that microfluidic PCR had the substantial advantage that it enabled a sample to be diluted into thousands or tens of thousands of discrete reaction volumes that each contained either zero or one DNA template molecule, thereby **overcoming the signal-to-noise limitations** associated with certain other PCR techniques and **enabling the detection of relatively rare mutations, dislocations and allelic imbalances.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Vogelstein taught that diluting the sample into ~1,500 discrete volumes allowed sensitivities up to about 0.1% and that further miniaturization would dramatically increase the assay sensitivity. Ex. 1044 at 9239 ("It is also possible that Dig-PCR can be performed in microarray format, potentially increasing the sensitivity by another order of magnitude.").

Vogelstein article explained this technique was useful for detecting an array of mutations, translocations and other rare genetic conditions:

Table 1. Potential applications of Dig-PCR

Application	Example	Probe 1 detects	Probe 2 detects
Base substitution mutations	Cancer gene mutations in stool, blood, and lymph nodes	Mutant or WT alleles	WT PCR products
Chromosomal translocations	Residual leukemia cells after therapy (DNA or RNA)	Normal or translocated alleles	Translocated allele
Gene amplifications	Determine presence or extent of amplification	Sequence within amplicon	Sequence from another part of same chromosome arm
Alternatively spliced products	Determine fraction of alternatively spliced transcripts from same gene (RNA)	Minor exons	Common exons
Changes in gene expression	Determine relative levels of expression of two genes (RNA)	First transcript	Reference transcript
Allelic discrimination	Two different alleles mutated vs. one mutation in each of two alleles	First mutation	Second mutation
Allelic imbalance	Quantitative analysis with nonpolymorphic markers	Marker from test chromosome	Marker from reference chromosome

Id. at 9241. A skilled artisan would thus have been strongly motivated to use droplet reactors such as those taught in Burns I to dilute the samples and thereby enable the detection of mutations, translocations and other rare genetic conditions by PCR as taught in Vogelstein. Ex. 1044 at 9236, 9241.

A skilled artisan would also have been strongly motivated to combine the references in the "opposite" direction, i.e., modifying the microfluidic PCR apparatus (Kopp or Vogelstein) to incorporate a droplet reactor (Burns I). Using two continuously flowing immiscible fluids to create droplets would have further enhanced the benefits provided by the fluid microfluidic PCR devices taught in Kopp or Vogelstein. The use of two continuously flowing, immiscible fluids creates droplets which in turn **further reduces the volumes of reagent** compared to droplets produced using injection of an aqueous fluid into an immiscible fluid (e.g., Kopp). Droplet reactors also **decrease contamination and increase the number of reactors** that can be created on a given chip. Moreover, the smaller reaction volumes would **enhance temperature control and reaction specificity.** Ex. 1004 ¶¶92, 93.

Moreover, a skilled artisan would have **fully expected the combination to be successful**. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR on a microfluidic chip. Ex. 1027 at 1046 ("A micromachined chemical amplifier was

successfully used to perform the polymerase chain reaction (PCR) in continuous flow at high speed."). Kopp noted that all of the underlying components (continuous-flow mixers, continuous-flow microreactors, continuous separations and high speed gel electrophoresis) were already known and predicted that "the continuous-flow microamplifier should allow the creation of highly elaborate analysis and synthesis systems." *Id.* 1047. In 1999 and 2000, continuous flow PCR microreactors underwent substantial evolution. In a 2001 article Lagally *et al.* provided an overview of that evolution:

> The advent of biological microdevices[6] allows one to consider conducting bioanalytical assays such as PCR at very small volumes to increase the speed of these assays and to reduce the amount of material and reagents needed. Our own work in this area has included DNA fragment sizing and sequencing on capillary and capillary array electrophoresis microdevices,[7,8] integrated electrochemical detection,[9] and amino acid chirality analysis.[10] The first microfabricated PCR reactors were constructed from silicon and glass and amplified DNA from template concentrations down to roughly 2000 copies/μL, in volumes down to 1 μL.[11] Since then, stand-alone PCR reactors have been constructed in silicon,[12-15] glass,[16-18] and fused-silica capillaries.[19] Most designs use resistive heaters surrounding the chambers, but some designs utilize noncontact methods[16] or **continuous flow** through three differentially heated regions.[18] Unfortunately, most of these designs either require bulk heating,[16,17] making parallel analyses with different thermal cycling profiles difficult, or require large starting concentrations or large sample volumes.[18] Furthermore, typically the sample must be manually transferred to an analysis device, introducing external contamination and increasing the time needed for the assay.
>
> Integrated systems combining rapid thermal cycling PCR amplification with capillary electrophoretic (CE) analysis address most of the problems outlined above. In previous work, we developed two generations of small-

> volume PCR chambers integrated with glass CE microdevices.[20,21] The first integrated PCRCE device used 10-μL silicon chambers with polysilicon heaters that were attached to a glass CE microchannel.[21] This device was capable of amplifying 4×10^5 template copies/μL in a time of 15 min. Later work from other laboratories utilizing Peltier elements for thermal cycling have been successful, but have not shown a significant time or sensitivity advantage over conventional thermal cycling systems. An integrated microdevice utilizing submicroliter sample volumes with sensitivity to the single-molecule limit would avoid these shortcomings and could serve as a platform for high-throughput parallelization.

Ex. 1028 at 565-566.

In 2001, Lagally further improved on the state of the art by describing a microfluidic reactor that was successfully used to conduct **single-molecule** DNA amplification by PCR:

> Here we present an extensive series of PCR-CE chip experiments at very low concentrations of target to explore the stochastic amplification of single DNA molecule templates. A statistically significant number of trials are performed and analyzed with calculations of normalized peak areas between the single-molecule product and an internal amplification control. The event amplitudes and frequencies at a mean occupancy of ∼1 template molecule in the reactor are found to conform to a Poisson distribution, demonstrating that we are operating at the single-molecule level. The capability of amplifying single-copy templates combined with microfluidic integration will facilitate the development of enhanced microfluidic diagnostic devices as well as studies of single-cell genetic phenomena and expression variation. . . .
>
> The ability of our device to amplify single DNA molecules provides an unprecedented molecular limit of detection using microfabricated PCR reactors, and this coupled with the integrated capillary electrophoretic analysis forms the basis of a powerful device for high-sensitivity detection of DNA targets. On one hand, this accomplishment will lead to the

> development of more complex high-density, low-volume microfluidic diagnostic devices even if they do not operate at the single-molecule limit. On the other hand, single-molecule nucleic acid analyses should facilitate studies of expression from individual cells in a population as well as genetic heterogeneity in cases where population averages mask important biological complexity and variation.

Ex. 1028 at 566, 570.

Further, at the time of filing it was **well-known that PCR could be performed in microfluidic plugs or droplets**. More than ten years before the earliest claimed priority date, Corbett reported that PCR was successfully performed in aqueous slugs. Ex. 1010 6:60-62. In 1996, Burns II likewise reported that PCR was successfully performed in aqueous droplets and further explained that "[n]o new biochemistries or DNA detection methods are required" and that "the components simply reproduce current laboratory procedures in a micron-sized environment." Ex. 1008 pp. 5558-59. In 1998, four years prior to the earliest claimed priority date, Kopp reported continuous flow PCR in microfluidic plugs and explained that because all the relevant underlying components and technologies had already been developed, the lab-on-a-chip concept was applicable to a wide range of diagnostic and medical applications. Ex. 1027 at 1047. As of 2002, there was nothing new or unpredictable about performing PCR in microfluidic plugs or droplets. Consistent with this fact, the **'148 patent provides no teaching whatsoever as to how to conduct the claimed method of performing PCR in a microfluidic droplet** (claim 1). Ex. 1001 at 44:58-60 (simply stating that "[a]nother example of an autocatalytic reaction is the polymerase-chain reaction (PCR), which is a very effective amplification method that has been widely used in the biological sciences.").

Accordingly, the prior art shows that many forms of PCR had been successfully achieved in microfluidic devices in the years leading up to the earliest claimed priority date. Consistent

with the observations of Kopp and Lagally, a skilled artisan would have expected that the microfluidic droplet reactors Burns I could be successfully modified to perform PCR reactions.

As further evidence that a skilled artisan would have expected to be successful in modifying Vogelstein or Kopp to work in the microfluidic droplet reactor of Burns I, the new teachings set forth above must be considered in light of the previously undisclosed fact that the same invention was contemporaneously and independently developed by others. In early 2001 a group from the University of Tokyo developed a droplet reactor that falls within the claims of the '148 patent at least as early as February 23, 2001, more than a year prior to the '148 patent's earliest claimed priority date. Ex. 1018-20 (Higuchi I, II and III). The group's work was published in a patent application (Higuchi I, Ex. 1018) that would qualify as prior art to the '148 patent but for the fact that the PCT was published in Japanese rather than English. Pre-AIA 35 U.S.C. §102(e). Accordingly, the Higuchi references are offered as evidence of the level of skill in the art.[141] As discussed in Section VI.A.3, above, Higuchi I-III demonstrate that the use of microdroplet reactors to create droplets from continuously flowing streams of water and oil was within the level of skill in the art as of the earliest effective priority date.

As explained further in the accompanying claim chart (Ex. 1079) and the declaration of Dr. Shaqfeh (Ex. 1004), the prior art and evidence submitted herewith demonstrates that a skilled

[141] "In *Monarch Knitting Machinery Corp. v. Sulzer Mor*at *GmbH*, 139 F.3d 877 (Fed. Cir.1998), the Federal Circuit considered two particular references before it that were submitted as evidence of contemporaneous independent development, and determined that while the references 'do not qualify as `prior art' under [§ 102 or § 103(A)],' the references were nonetheless 'relevant to obviousness as a secondary consideration.' *See* 139 F.3d at 884. In so ruling, the Federal Circuit also noted that evidence of contemporaneous independent invention is also relevant to the level of ordinary skill in the art insofar as the obviousness inquiry is concerned. See id. at 883 ('Although this court has noted the relevance of contemporaneous independent invention to the level of ordinary knowledge or skill in the art, it has also acknowledged the view that this evidence is relevant as a secondary consideration') (citations omitted); see also Stewart-Warner Corp. v. City of Pontiac, Mich., 767 F.2d 1563, 1570 (Fed.Cir.1985) ('Development by others may also be pertinent to a determination of the obviousness of an invention'). *Stewart-Warner Corp. v. City of Pontiac, Michigan*, 767 F. 2d 1563 (Fed.Cir. 1985)

artisan would have seen compelling reasons to modify the microfluidic droplet reactor of Burns I to conduct microfluidic PCR as taught by Kopp and Vogelstein.

With regard to the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other," this is merely a description of a PCR reaction. Ex. 1004 ¶31,32. As explained in Corbett, Kopp, Burns II, and Lagally, in a PCR reaction the sample DNA or RNA is admixed with various reagents including polymerase under conditions in which no reaction occurs; only when heat is applied does the PCR reaction occur. Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68. In the PCR of the combined method, as in virtually any PCR method, the DNA and polymermase do not react until mixture of reagents is heated. *Id*.; see also Ex. 1004 ¶32. The combined method thus meets the recitation that the fluids are provided under conditions in which "the target DNA or RNA molecules and the other molecules in the fluid do not react with each other" this is merely a description of a PCR reaction. Ex. 1004 ¶31,32.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1079.

Claim 1[c]: controlling flow rates of said aqueous fluid and said carrier fluid to partition the continuously flowing aqueous fluid with the continuously flowing carrier fluid to form a plurality of plugs of the aqueous fluid, each having a substantially uniform size of about 200 µm or less, wherein the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule

Burns I teaches that plugs in the channels of a microreactor by the continuous flow of fluids at an intersecting channel. Burns I explained that "various methods may be used to generate slugs of liquid within a microreactor." Ex. 1007 at 10. Burns I chose a method in which "the continuous flow of both phases through T or cross-shaped intersections" and "[s]lugs are generated by the action of one phase flowing into the channel whilst the other phase moves into the intersection, eventually cutting off the flow of the first phase into the channel and reversing the process." Ex. 1007 at 11. Fig. 3 shows oil-in-water slug generation and Fig. 4 shows water-in-oil slug generation. *Id.* at 11. In Burns' Fig. 4 embodiment, an aqueous plug of reagents is formed in a continuous oil phase. The continuous flow plug formation used by Burns was a well-known alternative to periodically injecting a measured slug of reactant into the main channel. See Ex. 1010; 1027; Ex. 1025 at 23:2-8. Burns I demonstrates that making slugs or plugs with continuous flows of immiscible fluids was a mere design choice as of 2001.

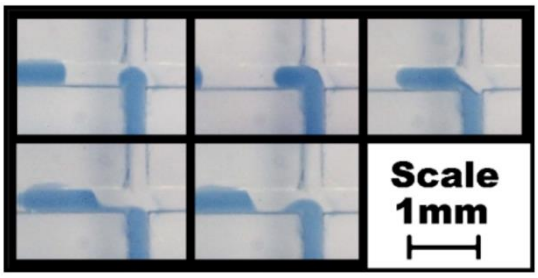


Fig. 3 Typical slug generation in glass device with 380 μm wide channels.

Turning to the recitation "each having a substantially uniform size of about 200 μm or less," Burns I discloses that the droplets are 100 μm to 400 μm in diameter. (Ex. 1007 at 10.) Moreover, it was well known that droplets smaller than 200 μm could be advantageously used in droplet generators. Quake teaches that "the droplets have a diameter that is smaller than the diameter of the microchannel; i.e., preferably less than 60 μm." Ex. 1034 at 27:29-30. Fig. 21 of Quake shows the droplets sizes generated by the Quake droplet generator were all less than 60 μm. Burns II (1996) similarly discloses uses of 60 nl droplets (which equates to a droplet diameter of about 62 μm). Ex. 1008 at 5558. Accordingly, it would have been obvious to a

skilled artisan that as of the earliest claimed priority date (2002) droplets below 200 μm in diameter could, and should, be used in order to reduce reagent usage and reaction time and achieve the various other benefits described above with the combination. Ex. 1004 ¶36, 66.

This leaves the recitation that "the target DNA or RNA in said plurality of plugs represents a Poisson distribution, and at least one member of said plurality comprises a single target DNA or RNA molecule and at least one of the other molecules that can react with the target DNA or RNA molecule." As discussed above, in the combined method of Quake, Corbett and Lagally, the samples are separated into a number of discrete droplets. That has the advantages of i) overcoming the signal-to-noise limitations associated with certain other PCR techniques and ii) enabling the detection of relatively rare mutations, dislocations and allelic imbalances. Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶84. Lagally expressly teaches that "[t]he event amplitudes and frequencies at a mean occupancy of ~1 template molecule in the reactor are found to conform to a **Poisson** distribution, demonstrating that we are operating at the single-molecule level." Ex. 1028 at 566 [emphasis added]. Vogelstein likewise teaches that the aforementioned advantages are realized by **reducing the number of sample molecules per droplet to zero or one.** Ex. 1044 at 9236, 9239, 9241; Ex. 1004 ¶82**.** That ensures that any amplification that occurs in a given droplet can be readily identified as containing the target sequence. *Id.* Thus, in the combined method the droplets have either one or zero template molecules.

As suggested by Lagally, any process which is generating droplets containing about one template molecule per droplet is expected to conform to a random, or Poisson, distribution. Ex. 1028 at 566; Ex. 1004 ¶48. It was known in the art that "[i]f a solution containing x molecules of enzyme per ml is dispersed into droplets of v ml volume, the average number of enzyme

molecules per droplet n is equal to vx. Because of statistical fluctuations each droplet will not have exactly n enzyme molecules. The probability p(r) of finding 0, 1, 2, 3, . .. , r enzyme molecules in a droplet **is given by the Poisson law**." Ex. 1112 at 1985 [emphasis added]; see also Ex. 1111 at 1667 ("Spectra observed in a time sequence for an average of 0.6 dye molecule in the probed volume exhibited the expected Poisson distribution for actually measuring 0, 1, 2, or 3 molecules.").

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1079.

Claim 1[d]: providing conditions suitable for a polymerase-chain reaction in at least one plug of the plurality of plugs such that the target DNA or RNA is amplified

Burns I discloses that "[t]he high heat and mass transfer capability offered by capillary microreactors allows high intensity reactions to be precisely controlled for optimum yield and selectivity." Ex. 1007 at 10. "The internal circulation, which is stimulated within the slugs by their passage along the channel, is responsible for a **large enhancement** in the interfacial mass transfer and the **reaction rate**." Ex. 1007 at 10 [emphasis added].

As discussed above in connection with element 1[b], in the instant combination Burns I is relied upon to meet all of the limitations of the independent claims of the '148 patent except the PCR-related limitations (e.g., "continuously flowing aqueous fluid comprising target DNA or RNA molecules and at least one other molecule").[142] These limitations are met by using the microfluidic droplet reactors with two continuously flowing immiscible fluids (as taught in Burns I) to perform PCR reactions, wherein each droplet or slug includes an aqueous mixture of DNA, polymerase, primers, and other reagents. Ex. 1004 ¶¶73,75,89.

[142] Requestor notes that Quake does disclose use of PCR (Ex. 1033 ¶¶6, 80), although that disclosure is not expressly relied upon herein as satisfying any PCR-related limitations.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1079.

Thus, claim 1 is shown to be rendered obvious by the combination of Burns I, Kopp and Vogelstein.

Dependent Claim 2

Claim 2 of the '148 patent recites "wherein the step of providing conditions includes heating."

As discussed above in connection claim elements 1[b] and 1[c], in the combined method (and in virtually any other PCR reaction) the DNA and polymerase undergo polymerase chain reaction when heated. Ex. 1004 ¶32; Ex. 1027 (Kopp) at 1046; Ex. 1010 (Corbett) at 8:37-51; Ex. 1008 (Burns II) at 5557-58; Ex. 1028 (Lagally) at 567-68.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1079.

Thus, claim 2 is shown to be rendered obvious by the combination of Burns I, Kopp and Vogelstein.

Dependent Claims 3-5

Claim 3 of the '148 patent recites "providing a detector to detect, analyze, characterize, or monitor the target DNA or RNA molecule in the at least one member of the plurality of plugs during and/or after the reaction has occurred." Claim 4 of the '148 patent further recites "wherein the at least one other molecule includes one or more fluorescent labels and the detector is configured to monitor progress of the reaction by detecting fluorescence emissions from the one or more fluorescent labels." Claim 5 recites "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels."

Vogelstein teaches that the "[r]eactions were analyzed immediately or stored at room temperature for up to 36 h before fluorescence analysis." Ex. 1044 at 9236.

Turning to claim 5, under the broadest reasonable interpretation the phrase "directing a subset of the plurality of plugs to a first channel in fluid communication with the one or more channels" requires simply that at least some of the droplets or plugs are transmitted to an outlet or other channel. Burns I teaches an outlet channel downstream of the droplet generating junction and thus meets claim 5 under the broadest reasonable interpretation. Ex. 1007 at 11. If claim 5 is more narrowly interpreted as requiring selective diversion of the droplets, that was well known. For instance, Quake teaches that "[a] preferred 'forward' sorting algorithm consists of running molecules, cells or virions from the input channel to the waste channel, until a molecule, cell or virion is identified to have an optically detectable signal (e.g. fluorescence) that is above a pre-set threshold, at which point voltages are temporarily changed to electro-osmotically divert the molecule [] to the collection channel." Ex. 1034 at 20:22-26. Fu likewise discloses a microfabricated fluorescence-activiated cell sorter for use in connection with emulsion or droplet based systems. Ex. 1113 at 1109. A skilled artisan would have considered it obvious to employ such a technique to sort droplets after they are subjected to the optical detection. Ex. 1004 ¶68,69. For instance, a skilled artisan would have found it advantageous to sort and collect droplets which tested positive or negative for the presence of a certain substance or property, especially in the context of droplet generators which are used to encapsulate on average one template molecule per droplet. *Id.* ¶82.

In the combined method of Burns I, Kopp and Vogelstein, microfluidic droplet reactors are used to perform PCR reactions after which the reaction products are detected optically and sorted by diverting certain droplets into collection channels. Ex. 1004 ¶69.

Additional correspondence between this claim element and the cited references is shown in the claim chart submitted herewith as Exhibit 1079.

Thus, claims 3-5 are shown to be rendered obvious by the combination of Burns I, Kopp and Vogelstein.

VIII. CONCLUSION

Based on the above, the accompanying references show that there exists at least one substantial new question of patentability as to claims 1-8 of the '148 patent. For the reasons set forth in this Request, it is respectfully requested that the *ex parte* reexamination of the '148 patent be ordered.

Respectfully submitted,

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