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(71) Applicant (for all designated States except US): **THE GOVERNORS OF THE UNIVERSITY OF ALBERTA** [CA/CA]; c/o TEC Edmonton 4000 TEC Center, 10230 Jasper Avenue, Edmonton, Alberta T5J 4P6 (CA).

(72) Inventors; and

(75) Inventors/ Applicants (for US only): **ATRAZHEV, Alexey** [CA/CA]; W6-070 ECERF, 9107 - 116 Street, Edmonton, Alberta T6G 2V4 (CA). **ACKER, Jason** [CA/CA]; 331 Canadian Blood Services Building, University of Alberta, Edmonton, Alberta T6G 2R8 (CA).

(74) Agent: **SHERBURNE, Craig, K.**; #236, 6030 - 88 Street, Edmonton, Alberta T6E 6G4 (CA).

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(57) Abstract: Methods for detecting nucleic acid molecules comprising the use of polyacrylamide gel and PGR reagents photopolymerized together in a mold to create an array of semisolid posts that serve as reaction vessels for parallel, small volume PGR amplification of template DNA. Addition of a fluorescent marker prior to photopolymerization allows for the acquisition of real-time PGR data and melting curve analysis directly from the mold.



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SOLID GEL AMPLIFICATION METHOD AND APPARATUS FOR GENOTYPING AND PATHOGEN DETECTION

CROSS-REFERENCE TO RELATED APPLICATIONS

The present Application claims priority from U.S. Provisional Patent Application No.

- 5 61/362,878 filed July 9, 2010 such application is expressly incorporated by reference herein for all purposes.

FIELD OF THE INVENTION

The present invention pertains to the field of macro- and microfluidic devices and methods for detection of nucleic acids

10 BACKGROUND OF THE INVENTION

All of the publications, patents and patent applications cited within this application are herein incorporated by reference in their entirety to the same extent as if the disclosure of each individual publication, patent application or patent was specifically and individually indicated to be incorporated by reference in its entirety.

- 15 There is an increasing demand for a small scale array-based and/or microfluidic device that processes micro- or nano-volumes of sample, with time and cost savings arising from miniaturization. Prior art approaches to miniaturised polymerized chain reactions ("PCR") make use of open or enclosed chambers or flow through zones/channel networks with appropriate temperature regulation; some have on-board silicone rubber-based or magnetic-
- 20 based valving and/or pumping. Although potentially powerful approaches, challenges may arise of pressure seal and/or evaporation, pressure buffering, as well as others such as chemical interference through surface interactions, and evaporation/contamination via the porous, gas permeable membranes used in pumps and valves.

- Performing PCR in a colloidal hydrogel matrix (hereafter termed "gel") may confer a
- 25 multitude of advantages. For example, the DNA, polymerase enzyme and other PCR reagents a) have reduced access to the device materials' surfaces where they may be adsorbed,

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absorbed, poisoned or otherwise rendered inactive and b) are kept within close proximity to each other without the need for valves or pumps. Likewise, any contaminant solutes from device materials have reduced access to the PCR reaction.

- 5 Gels provide a successful medium for PCR, as first introduced by Chetverin et al, see for example U.S Pat. No. 5,616,478; which is herein incorporated by reference in its entirety. PCR was confined to circular spots in a gel sheet where the initial DNA or RNA templates, formed "molecular colonies" (short for polymerase colonies), named for their similarity to the growth of bacterial colonies in agar; the initial amount of DNA can be accurately estimated by counting the number of colonies. Mitra et al. (Mitra, R. D. et al; *Nucleic Acid Research* 1999, 10 27, e34) performed DNA amplification in a thin acrylamide film polymerized with all the reagents along with plasmid DNA as their template. In an alternate approach, Strizhkov et al. (Strizhkov, B. N. Et al; *Biotechniques* 2000, 29, 844-857) used nanoliter gel pads to immobilize primers for PCR. Single Nucleotide Polymorphisms (SNPs) in cDNA were detected with polony technology by Butz et al. (Strizhkov, B. N. et al; 2000, 29, 844-857)
- 15 Absent the use of immobilized primers within the gel, previous instances of in-gel PCRs were performed in a defined chamber with relatively large volumes (62-65 μL). The present art is in need of a means to perform post PCR analysis of amplicons, such as melting curve analysis ("MCA") without imposing additional steps for the transfer or handling of PCR product and in small volumes normally associated with microfluidic reactions (sub 100 μL).

20 SUMMARY OF THE INVENTION

The present art has suffered from an inability to perform PCR and MCA within an array of defined spaces of microfluidic volumes absent the immobilizing of at least one of the primers involved in the PCR.

- 25 In one aspect, the present invention provides for a method for detecting a nucleic acid molecule, including DNA, cDNA or RNA, within a hydrogel post array comprising providing a hydrogel post array of 2x1 or greater containing a cell-free, enzymatic, nucleic-acid

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amplification system; applying to at least one of said hydrogel posts nucleic acid molecules, at least one of which may comprise a template for said amplification system; and incubating said hydrogel posts under conditions promoting the synthesis of an amplified nucleic acid product by said amplification system from said at least one template; wherein said amplification
5 system comprises at least two non-immobilized nucleic acids capable of promoting synthesis of amplified nucleic acid product from said template.

In a further aspect, the hydrogel posts contain a fluorescent marker, wherein said fluorescent marker has different fluorescence properties when interacting with double-stranded nucleic acids than with single-stranded nucleic acids and in a still further aspect said fluorescent
10 marker is selected from the group comprising LC Green, SYBR Green and SYTO 62. In further aspect, PCR products can be detected by any agent or characteristic that has a measurable property that differs between forms of nucleic acid, by way of non-limiting example, between single stranded and double stranded nucleic acid.

In another aspect, the hydrogel post is comprised of polyacrylamide of 2.0-3.1% weight per unit volume, and photopolymerized in the absence of APS. In another aspect the
15 polyacrylamide is 2.0-12% weight per volume. In another aspect the template is included in said hydrogel posts. In another aspect the template is provided externally to said hydrogel posts.

In an additional aspect, the present invention provides for a method for detecting a nucleic acid molecule, including DNA, cDNA or RNA, within a hydrogel post array comprising
20 providing a hydrogel post array of 2x1 or greater containing a cell-free, enzymatic, nucleic-acid amplification system; including nucleic acid molecules in at least one of said hydrogel posts, at least one of which may comprise a template for said amplification system; and incubating said hydrogel posts under conditions promoting the synthesis of an amplified
25 nucleic acid product by said amplification system from said at least one template; wherein said amplification system comprises at least two non-immobilized nucleic acids capable of promoting synthesis of amplified nucleic acid product from said template.

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In a further aspect, the hydrogel posts contain a fluorescent marker, wherein said fluorescent marker has different fluorescence properties when interacting with double-stranded nucleic acids than with single-stranded nucleic acids and in a still further aspect said fluorescent marker is selected from the group comprising LC Green, SYBR Green and SYTO 62. In

5 further aspect, PCR products can be detected by any agent or characteristic that has a measurable property that differs between forms of nucleic acid, by way of non-limiting example, between single stranded and double stranded nucleic acid

In another aspect, the hydrogel post is comprised of polyacrylamide of 2.0-3.1% weight per unit volume, and photopolymerized in the absence of APS. In another aspect the

10 polyacrylamide is 2.0- 12% weight per volume. In another aspect the template is included in said hydrogel posts. In another aspect the template is provided externally to said hydrogel posts.

In an additional aspect, the present invention provides for a method for detecting a nucleic acid molecule, including DNA, cDNA or RNA, within a hydrogel post array comprising

15 providing a hydrogel post array of 2x1 or greater containing a cell-free, enzymatic, nucleic-acid amplification system; introducing nucleic acid molecules in a solution in fluid communication with at least one of said hydrogel posts, at least one of which may comprise a template for said amplification system; and incubating said hydrogel posts under conditions promoting the synthesis of an amplified nucleic acid product by said amplification system

20 from said at least one template; wherein said amplification system comprises at least two non-immobilized nucleic acids capable of promoting synthesis of amplified nucleic acid product from said template.

In a further aspect, the hydrogel posts contain a fluorescent marker, wherein said fluorescent marker has different fluorescence properties when interacting with double-stranded nucleic

25 acids than with single-stranded nucleic acids and in a still further aspect said fluorescent marker is selected from the group comprising LC Green, SYBR Green and Syto62. In further aspect, PCR products can be detected by any agent or characteristic that has a measurable

- 5 -

property that differs between forms of nucleic acid, by way of non-limiting example, between single stranded and double stranded nucleic acid

In another aspect, the hydrogel post is comprised of polyacrylamide of 2.0-3.1% weight per unit volume, and photopolymerized in the absence of APS, In another aspect the

- 5 polyacrylamide is 2.0-12% weight per volume. In another aspect the template is included in said hydrogel posts. In another aspect the template is provided externally to said hydrogel posts.

The accompanying description illustrates preferred embodiments of the present invention and serves to explain the principles of the **present** invention

10 BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1 shows a schematic diagram of gel posts of 1 mm diameter and 1.1 mm in height with (a) the 9x9 array of gel posts and (b) an enlarged diagram of four posts in the array;

FIGURE 2 shows the stages in preparation and use of gel post arrays (a) the mould, (b) 9x9 array of polymerized gel posts, and (c) fluorescent image of the gel posts;

- 15 FIGURE 3 shows a schematic diagram of the instrument used for performing PCR and MCA;

FIGURE 4 shows real-time PCR in gel posts arrays with (a) raw fluorescence data obtained by CCD image, (b) processed data as contemplated herein and (c) C_p values obtained for each post;

- 20 FIGURE 5 shows product detection in gel post arrays using melting point analysis with (a) melting curves of BKV amplicons in gel posts represented in Fig. 4, (b) the negative derivative of fluorescence versus temperature showing the melting point of the PCR product and (c) part of the sequence of the product;

FIGURE 6 shows amplification of template exogenous to the gel posts in a 2.8%

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polyacrylamide gel post array with (a) normalized real-time data and (b) melting curves of the BKV amplicons;

FIGURE 7 shows a comparison of BKV DNA PCR in a 2.8% polyacrylamide gels performed in a Lightcycler® (a-c) or gel posts (d-f) in particular (a) Lightcycler® real-time PCR

5 intensity (b) Lightcycler® Cp values versus logarithm of DNA quantity per 0.85 mL reaction, (c) size confirmation of the Lightcycler® amplified products in a vertical polyacrylamide gel, (d) gel post real-time PCR intensity (e) gel post Cp values versus logarithm of DNA quantity per 0.85 μ L reaction, (f) size confirmation of the gel post amplified products in a vertical polyacrylamide gel;

10 FIGURE 8 shows amplification of human genomic DNA by PCR in 2.8% polyacrylamide gel posts with (a) real-time PCR curves for HPA1, (b) melting curve analysis for HPA1, (c) real-time PCR curves for FGFR2 and (d) melting curve analysis for FGFR2; and

FIGURE 9 shows shows (a) the formula of cross-linked polyvinylpyrrolidone (b), real-time PCR of BKV template in xPVP posts array (c), Cp values and (d) melting point analysis of

15 BKV amplicons by means of negative derivative of fluorescence versus temperature showing the melting point of the PCR product .

DETAILED DESCRIPTION OF THE PRESENT INVENTION

The novel method and system described herein provides for the performance of PCR or other amplification or gene detection method in a gel medium less than 1 μ L in volume, obtaining

20 real-time data *in situ* by detecting the fluorescence of DNA in the presence of an intercalating dye or other means of product or amplicon detection. By performing replicate PCRs in multiple gel posts, statistical data to confirm a result can be obtained. The method of the present invention can be implemented for detection in the same sample of multiple nucleic acids, mutations/polymorphisms contained within a heterogeneous nucleic acid population, or

25 multiple organisms, pathogens, bacteria or viruses within a single sample. As used herein, "post", "posts", "hydrogel post" or "hydrogel posts" refer to any shape or form of a hydrogel

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such that it is spatially separated from at least one other form of hydrogel. In particular, the present invention contemplates hydrogel forms in the general shape of cylinders, cones, spheres, prisms of any number of sides greater than two, or pyramids of any number of sides greater than two, or portions thereof as well as more amorphous or irregular shapes. The
5 hydrogel posts of the present invention are advantageously of small volumes, less than 100 μl , thereby allowing duplicate reactions within a reduced space and all, advantageously, under the field of view of commercially available cameras or visualizers. The present invention contemplates arrays of any dimension and alignment.

The system described herein is designed to facilitate performance of diagnostic tests in
10 parallel on the same sample, using different posts in the same array. A non-limiting example of the utility of this platform is testing of patients as the patient presents in the clinic, for more rapid results, rather than transport of patient samples to a distant or centralized laboratory. The ability to acquire real-time PCR and MCA using the method and system of the present invention expands the use of this technique to applications such as asymmetric PCR for
15 mutation scanning and genotyping performed with unlabelled probes.

The novel in-gel PCR system of the present invention can perform PCR, melt curve analysis and real time quantitative PCR, with an output that compares favourably with conventional systems representing a "gold standard". Templates from a viral genome and from human genomic DNA are successfully amplified in the gel posts, with BK virus ("BKV"), by way of
20 non-limiting example, readily detected in unprocessed sub-microliter volumes of urine from patients with BK viruria. Further, it is contemplated by the present invention that both processed and unprocessed clinical samples other than urine may be used with the present method and system, including but not limited to, serum, plasma, whole blood, sputum, mucous, aspirates, debrided tissue, scrapings and lymphatic fluid. Further, the present
25 invention is not limited to use with only clinical samples, as the systems and methods described herein may use any sample which may contain a template for the amplification or detection as contemplated herein. The present invention contemplates use of methods for detecting a gene or transcript other than PCR and one skilled in the art would be aware of the

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variations of PCR and other gene or transcript detection systems.

The present invention provides a method of performing real-time PCR in gels with MCA in an array of cylindrical shaped self-standing gel posts ($\sim 0.86 \mu\text{L}$ per post). In a preferred embodiment the PCR and post-PCR analysis of the resulting amplified nucleic acid (if any) was performed in microfluidic volumes utilizing, in one embodiment, a 9x9 pattern of posts (Fig. 1). An inexpensive prototype heating device with a Peltier element was used for performing PCR and MCA, a diode laser for excitation of fluorescence, and detection optics containing a CCD, all of which controlled by a micro-controller. As well, the present invention provides the novel and desirable performance of in-gel amplification of templates from human genomic DNA ("gDNA") or from cDNA or RNA.

The gel of the present invention is contemplated to hydrophilic polymers forming colloidal hydrogel matrixes which result in similar mobilities of the nucleic acids of s^{2es} contemplated by the present invention as in the specifically described gels herein, by way of non-limiting example, polyacrylamide and polyvinylpyrrolidone ("xPVP") cross-linked with PEG-diacrylate resulting from the photopolymerization of 3.3% vinylpyrrolidone with 0.7% Polyethyleneglycol-diacrylate.

The posts contemplated by the present invention may be cylindrical, spherical, or any other shape and dimension, so long as the posts of the array are physically separated, though they may be in fluid communication or in a common fluid substrate. The size and shape of the posts presented herein are presented as exemplar structures, and it is contemplated that a variety of moulds and therefore post shapes are possible. Also contemplated are inverted shapes placed on a planar surface, for example wells or depressions comprised of a hydrogel. Also contemplated are wells or depressions made within a structure, for example plastic, glass or other materials, filled with hydrogel.

Example 1: Gel Polymerization

The polymerization of acrylamide gel for gel PCR can be initiated either by a photochemical

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method or by using peroxide. Adding ammonium persulfate (APS) as the initiator peroxide is the widely used method (Sambrook, J. & Russel, D. W.; *Molecular Cloning*, 3rd Edition ed.; CSHL Press, 2001). For photochemical polymerization, 'azobis⁵(2,2'-azobis(2-methyl-N-(2-hydroxyethyl) propionamide)) or riboflavin is added to the gel mix and the photochemical

5 reaction is started by exposing the gel mix to ultraviolet light. It was noted that the polymerization initiator, APS, inactivated or inhibited the fluorescent dyes such as SYBR Green I and LC Green Plus that are needed for subsequent product detection by MCA, precluding addition of dye prior to the polymerization if the APS is used as the polymerization initiator. To circumvent this problem, gel posts were made by photo-

10 polymerizing the PCR reaction mix with LC Green Plus, with or without template DNA, along with the acrylamide gel reagents in a glass mould having a 9x9 array of wells (**Fig. 1**). An alternate embodiment is to introduce the intercalating dye or other agent after completion of PCR or other amplification reaction. The wells were then sealed with a cover slip treated to promote gel adhesion. Once gel polymerization had occurred, the cover slip was detached

15 from the mould along with the array of gel posts, and immersed in mineral oil to minimize evaporation, as described below.

Example 2: Mould & Cover slip preparation

The mould, approximately 20 mm x 20 mm, is made with a 1.1 mm thick glass microscope slide permanently bonded to another 1.1 mm thick microscope slide with a 9 x 9 array of

20 holes as shown in **Fig. 2(a)**. **Fig. 2(b)** shows an image of the 9x9 array of polymerized gel posts, while **Fig. 2(c)** shows a fluorescing image of the gel posts. Embodiments incorporating other types of arrays are also possible. Post arrays may be removed from the mould for use as reaction vessels, or in an alternate embodiment may remain within the mould for an enclosed reaction. The scale bar shown in **Fig. 2** is 4 mm in length. It is notable that the fluorescence

25 of the gel posts was always uniform throughout any given post, as imaged by CCD; and in particular none of posts show any distinct "colonies". These 2.8% polyacrylamide gels are the softest gels reported for in-gel PCRs. In addition, gel posts are 3 D structures with in depth fluorescence that may obscure boundaries of any individual colonies. Each hole is 1 mm in

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diameter.

To prepare the surface of the mould so that it would not adhere to the gels, a thin layer of Sigma Coat (Sigma, St. Louis, MO, cat# SL2) was spread onto the surface of the mould and left to dry. The mould was then washed with n-heptane (Applied Bio Systems, Foster City, CA, cat # 400079) and blown dry with air. In contrast, the surface of the cover slips (22 mm x 22 mm, Fisher, Fair Lawn, NJ, cat#12-54B) were treated to enhance adherence to the gel by immersing them in a mixture of 40 mL of 95% ethanol, 1 mL of 100% acetic acid (Fluka, Buchs, cat# 45725), 8.9 mL of water, 100 μ L of 3-(trimethoxysilyl)propyl methacrylate (Sigma, cat# 440159) for 1 hour followed by washing with isopropanol (2-propanol). After casting a gel, the mould can be washed and reused for subsequent gel casting or the mould can remain permanently in contact with the gel. In another embodiment, the gel post array may be fully enclosed with a port for introduction of template and/or other reagents..

Example 3: PCR/MCA Process, BKV PCR

The gel can be polymerized with or without template DNA included in the polymerization mixture. In the latter case, the DNA can be added atop the gel posts where it diffuses into the gel before PCR is performed. 100 μ L PCR gel mix contained 47 μ L PCR reagents, 10 μ L gel reagents and 43 μ L water. The 47 μ L PCR reagents were: 20 μ L of 5 x PCR buffer (333 mM tris-sulphate, pH 8.6, 83 mM (NH₄)₂SO₄ (Sigma); and 40% sucrose (Sigma)), 4 μ L of 50 mM MgCl₂ (Fluka), 2 μ L of 10 mM [dNTP] (Sigma), 2 μ L of 1% BSA (Sigma), 2 μ L of 10 μ M primer solution (Integrated DNA technologies, San Diego, CA) for each of 2 primers to produce 100 base pair ("bp") product, 2 μ L BKV template DNA, 10 μ L of lOxLC Green Plus (Idaho Technology Inc., Salt Lake City, Utah) and 3 μ L of Taq polymerase (20 units/L). The 10 μ L of gel reagents were: 7 μ L of a 40% acrylamide (Sigma, cat # A9099) + 4% bis-acrylamide aqueous solution (N,N-methylene bisacrylamide, BioRad, Hercules, CA, cat# BA05-1610201), 2 μ L of 3% azobis (Wako, Richmond, cat# VA-086), and 1 μ L of 10% TEMED (N,N,N',N'-tetramethylethylenediamine, Sigma, cat# T7024). This mixture was

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degassed in vacuum and pipetted into the wells in the mould. Once all the wells were full, a 22 mm x 22 mm cover slip treated, as noted above, for adherence to the acrylamide was slipped atop the wells. The mould with the cover slip atop was then exposed to the 405 nm laser (~4 mW/cm² on the posts) for 25 min in order to photo-polymerize the acrylamide mix.

- 5 The cover slip with the attached posts was then slowly lifted from the mould (Fig. 2(b)), immediately immersed in mineral oil (Sigma, cat# M5904) in a shallow anodized aluminum 23 mm x 23 mm pan (posts facing up), and placed on the Peltier element. Thermal cycling was performed with an initial denaturation of 30s at 94°C followed by 50 cycles of denaturation at 94°C for 15s, annealing at 52°C for 30s, and extension at 72°C for 30s, and
- 10 ending with an extension step of 72°C for 60s. After completion of PCR, MCA was performed (Fig. 2(c)). To determine the threshold for BKV amplification, BKV PCRs were performed with 34 to 8640 BKV copies/post. Overall, a total of 52/52 independent experiments to amplify BKV were successfully performed on gel posts, confirming reproducibility of the method.
- 15 In order to show that PCR can be performed with unprocessed samples, PCR was performed with 1.5 µL of raw urine added to the PCR reaction mix prior to the polymerization. All the PCR parameters are similar to the BKV DNA PCR other than the PCR cycle number was reduced to 35.

- For addition of template after polymerization of gel posts, a similar PCR gel mix (as above)
- 20 was made without BKV DNA and polymerized. After the gel was detached from the mould, a 14 µL BKV template (2.86×10^7 copies/mL) was added atop the gel posts and the DNA allowed to diffuse for 30 min in a covered Petri-dish before performing PCR with the same thermal cycle conditions as above. If the DNA added atop the gel was uniformly absorbed, the amount of template DNA was 4900 BKV copies/post. In order to study the size limitation of
- 25 the product that could be amplified in a given gel concentration, we have also performed BKV PCR (17,280 BKV copies per post) with a series of different primers (Table 1), with the template DNA polymerized in the gels as indicated above.

Table 1: Primer sequences for BKV and HPA1 amplification by PGR

Primer description	SEQ ID	Product length (bp)	Sequence
BKV reverse	SEQ ID NO 1		5'-aaacaccctaacctctctac-3'
BKV forward	SEQ ID NO 2	100	5'-ttccttttgctaagtgacc-3'
	SEQ ID NO 3	150	5'-tattttaagatccgcctga-3'
	SEQ ID NO 4	200	5'-gcctgtttactaacagctctg-3'
	SEQ ID NO 5	250	5'-gcctctttgtaaagctgtag-3'
	SEQ ID NO 6	300	5'-catgtgaccaacacagctac-3'
	SEQ ID NO 7	350	5'-ctaggtaatttgggacttca-3'
	SEQ ID NO 8	400	5'-tgcttatccagttgagtgc-3'
	SEQ ID NO 9	450	5'-ccagtcaccaggtaatgaatac-3'
	SEQ ID NO 10	500	5'-gaattacagggtcaaagtaccc-3'
	SEQ ID NO 11	600	5'-gtgcatgagcatgggtga-3'
	SEQ ID NO 12	800	5'-aagctaagtgtgaaaatgac-3'
	SEQ ID NO 13	1000	5'-cccaaccaaaagaaaagg-3'
HPA1 reverse	SEQ ID NO 14		5'-cacagcgagggtgagcc-3'
HPA1 forward	SEQ ID NO 15	42	5'-ctcctgtcttacaggccc-3'

Example 4: PCR/MCA Process, Genomic DNA PCR

- Similar PCRs were performed with purified gDNA added to the gel before or after the polymerization. Overall, gDNA has been successfully amplified in gel posts for 27/27 independent experiments. For the PCR performed with gDNA template polymerized in the gel, a 42 bp product from human HPA1 (human platelet antigen 1) was amplified. Except for the template and oligonucleotide primers (in Table 1), the PCR reaction mix was similar to that for the BKV PCR. 225 ng of gDNA was added to the 100 μ L mix (-2.2 ng per post).
- PCR thermal cycling conditions were as indicated for the BKV PCR.

For the PCR performed with gDNA added atop the polymerized gel, a 71 bp product from the FGFR2 gene from human gDNA was amplified. Primers used for FGFR2 amplification were forward primer SEQ ID No. 16 (5' "CAGAAGTTTTTGAGAGTGGCATGATG") and

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reverse primer SEQ ID NO 17 (5' "GCTGACTTCTATTTATATAACTTCAAGC"). Fourteen μL of gDNA (30 ng/ μL) was pipetted onto the whole array of posts and was left in a covered Petri-dish for 30 min to allow diffusion of gDNA into the gel. If all the gDNA was uniformly absorbed, a DNA concentration of ~ 5 ng/post is predicted.

5 Example 5: PCR/MCA Instrumentation

An inexpensive prototype instrument (shown in **Fig. 3**) is used to perform the PCR reaction in the gel posts. This instrument uses Motorola 68332 microprocessor **307** to control Peltier element **304** (XLT2398-01L, Marlow Industries, Dallas, TX) to perform heating and cooling for PCR and MCA, where Peltier element **304** is placed in thermal communication with

10 hydrogel array **310** and heatsink **305**. A charge-coupled device ("CCD") camera **301** (Deep Sky Imager, Meade, Irvine, CA) is mounted above Peltier element **304** as well as hydrogel array **310**. 65 mW 405 nm laser diode **306** (DL-7146-10 IS, Sanyo) is mounted at a 70 degree angle to horizontal for a fluorescence excitation source. Laser diode **306** delivers an average of 32 μW of excitation power to each post within hydrogel array **310**. 50 nm wide band-pass

15 interference filter centered at 510 nm **302** (BP5 10/50, Chroma Technology, Bellows Falls, VT) is mounted in front of the camera to attenuate excitation light. Biconvex lens **303** (KBX046, Newport, effective focal length 25.4 mm) is mounted between filter **302** and hydrogel array **310**. Camera parameters such as the exposure time, light and dark levels are set by the user on PC computer **308**, in electronic communication with microprocessor **307**.

20 During PCR, once the extension temperature is reached, laser diode **306** is switched on and a fluorescent image of the gel posts is taken by CCD camera **301** and stored by computer **308**. During the MCA, laser diode **306** is left on continuously and an image is taken by CCD camera **301** and stored by computer **308** at each degree from 50 °C to 95 °C, once the chip has been stabilized at a particular temperature. The system is calibrated by placing calibrated

25 thermocouple **309** (5TC-TT-K-40-36, Omega Engineering Inc., Stamford, CT) between the hydrogel posts within hydrogel array **310**, under the oil. The settings on the system are then correlated to the observed temperatures of the calibrated thermocouple. Microprocessor **307** is in electronic communication with, and controls, Peltier element **304**, temperature sensor **309**

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and laser diode 306.

Example 6: PCR and Melting Curve Analysis

The CCD images acquired at the extension step of each PCR cycle (total of 50 images) were analysed with ImageJ software (National Institutes of Health, USA) using the MicroArray
5 Plug-in (Dr. Robert Dougherty, OptiNav Inc., Redmond, WA) that can be used to plot the cycle number vs. the fluorescence intensity of each post. Even though the mould disclosed herein creates a 9 x 9 array of posts, optical limitations of the CCD assembly allow image acquisition for only a 6x8 array. In order to determine the efficiency of the PCR, most
10 commercial real-time quantitative PCR instruments embed some proprietary version of data processing in their software. All reported methods characterize the real-time PCR curves by applying curve fitting and determining the threshold values where the fluorescence of the PCR begins to rise above the background signal and the DNA copy numbers can be seen to increase exponentially.

Therefore a sigmoid was fitted to the real-time PCR curves in order to find the exponential
15 region and to find the threshold value, termed the "crossing point" (CP). A Linear Regression of Efficiency or LRE method (Rutledge, R. G. & Stewart, D. BMC *Molecular Biology* 2008, 9) modified with a linear baseline correction (Rebrikov, D. V. & Trofimov, D. Y. *Applied Biochemistry and Microbiology* 2006, 42, 455-463) to fit the sigmoid.

With LRE, the fluorescence of the DNA, F_c at cycle c of the PCR can be written as

$$F_c = \frac{F_{Max}}{1 + \left(\frac{F_{Max}}{F_0} - 1\right)(E_{Max} + 1)^{-c}} + F_B + cF_K \quad \text{Formula 1}$$

where F_0 , F_{Max} , F_B and F_K are the fluorescence values for the initial reaction, endpoint reaction, constant background and variable background, and E_{Max} is the maximum amplification efficiency. A linear baseline to the equation was used to facilitate baseline subtraction with the last two terms of the equation, F_B and cF_K . The maximum of the second

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derivative of the sigmoid is determined to calculate the C_p value, representing the cycle number at which the fluorescence has risen above the background level and the exponential growth of the PCR is at a maximum.

- The algorithm of **Formula 1**, was **implemented** into computer code using visual basic for applications 6.3 in Microsoft Excel, which receives the fluorescence intensities obtained from the text file output by the ImageJ software and returns multiple plots including the raw, fitted, and normalized data. C_p -values for each PCR reaction are also calculated for each post. **Fig. 4(a)** shows real-time data (experimental data points connected by interpolated lines) for 36 posts that were obtained after PCR performed with 3,456 starting copies of BKV DNA template per post in a 2.8% polyacrylamide gel. Insets in **Fig. 4(a)** show CCD images of the gel array at the 1st cycle, 30th cycle, and 50th cycle. These results were confirmed in more than 10 independent experiments and no fluorescence above background was detected in the negative controls **Fig. 4(b)** and **Fig. 4(c)** show the plots produced by the algorithm and the C_p values for each post respectively.
- There is spatial variation in the illumination of the post array due to the oblique incident angle and intentional optical diffusion of the laser. As a result, fluorescence excitation is not uniform on all posts, and thus each real-time PCR curve starts at a different intensity level as seen in **Fig. 4(a)**. These background variations are removed by data processing to produce the normalised curves of **Fig. 4(b)**. Considerable background light between posts is observed, as shown in the inset post array image for the 50th cycle in **Fig. 4(a)**. This background is due to a thin film of gel that remains between the posts as the gel post array is assembled, and where PCR also occurs. One skilled in the art would recognize that modification of assembly protocols would remove or reduce this thin film, and it is contemplated that the present invention also encompasses such modified assembly protocols. Despite the presence of the thin gel layer, fluorescence data is largely independent of the background as they result from the summation of pixels entirely within each post. As disclosed herein, the present invention does not suffer from "cross-talk" between posts.

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Melting of the DNA was performed immediately after the PCR was completed. The melting curves were obtained by measuring the fluorescence in the CCD images obtained at each degree from 50 °C to 95 °C as seen in Fig. 5(a). The negative derivative of the fluorescence with respect to the temperature was plotted in Fig. 5(b) and allowed the melting temperature of the PCR products (T_m) to be determined as the temperature at the peak 31. The melting temperature for BKV amplicons (average $T_m \pm 1\sigma$ for all 36 traces) was 82.6 ± 0.4 °C. The sequence of BKV PCR product was confirmed by sequencing the DNA from one post. Part of the sequence is shown in Fig. 5(c) with sequencing performed with AB1 3130x1 DNA capillary analysis system (Applied Biosystems, Foster City, CA). As for the real-time PCR traces in Fig. 4(a), the melting curve baselines of Fig. 5(a) are influenced by the heterogeneous laser illumination; this bias is removed through the data differentiation used to produce Fig. 5(b). Also in keeping with Fig. 4(a), the inset image for 75°C shows considerable background fluorescence, owing to the thin layer of gel that remains between posts. Insets in (a) show the CCD images of the gel array at 75° C and 85° C. These results were confirmed in more than 10 independent experiments.

In gel PCR can also be performed using unprocessed clinical samples. The real time PCR curves for the amplification of BKV in unprocessed urine of patients with viruria are shown with real-time data shown in Fig. 6(a) and melting curve analysis is shown in Fig. 6(b). Other embodiments include use of unprocessed blood, saliva, buccal swabs, genital swabs, sputum or other body fluids or tissues.

The results shown in the Fig. 4 and Fig. 5 were acquired with the gels polymerized with the BKV DNA template inside. However, if this technology is to be applied to real-world medical diagnostics, adding clinical samples to the pre-cast gel is likely to be a better approach. PCR with a BKV DNA template that was allowed to diffuse into a pre-cast gel matrix was performed and real-time PCR curves obtained, as shown in Fig. 6(a). This confirms that exogenous template DNA can successfully enter the gel and interact with the embedded PCR components. The melting curve analysis data is shown in Fig. 6(b); for the experiment shown, the average melting temperature was 82.8 ± 0.6 °C.

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Example 7: Effect of the gel concentration on the PCR product size

In order to study the limitations of PCR product lengths in polyacrylamide gels, a series of BKV products with lengths from 100 to 1000 bp in 5 different gel concentrations were amplified, with template DNA added prior to polymerization. Table 2 shows PCR

5 amplification of different lengths of BKV template in different polyacrylamide gel concentrations, with a (+) sign indicating that PCR product was detected. Thirteen different primer sets were used to amplify different sized segments of a BKV template, using 5-6 different primer sets per gel post array (see Table 1). Primers were added to the moulds prior to polymerization. Results were confirmed with at least two experiments for each primer set

10 and the sizes of the PCR products were confirmed by running vertical in situ gel electrophoresis on each post. Each array included several different primer sets for distinct sets of gel posts, demonstrating simultaneous multiparameter testing. The results demonstrate that the gel limits the size of the product that can be amplified. As the gel concentration increases, the maximum product size that can be amplified decreases suggesting that the smaller pore

15 size of harder, high concentration gels restricts movement of the larger reagent molecules (DNA template, polymerase etc.) inside the gel as compared to their movement in lower concentration, softer gels.

Table 2; BKV template amplification length, gel concentration and cross-talk controls.

Size of Amplicon (bp)	Concentration of Polyacrylamide				
	4%	6%	8%	10%	12%
100	+	+	+	+	+
150	+	+	+	+	+
200	+	+	+	+	+
250	+	+	+	+	+
300	+	+	+	+	+
350	+	+	+	+	+
400	+	+	+	+	+
450	+	+	+	-	-
500	+	+	-	-	-
550	+	-	-	-	-
600	+	-	-	-	-
800	-	-	-	-	-
1000	-	-	-	-	-

- In order to show that the primers do not diffuse from one post to another, a separate experiment was performed in which a single primer set was added to some but not all wells prior to the addition of the gel polymerization mix to create a checkerboard of adjacent positive and negative controls. Table 3 shows positive posts have PCR amplification of 100 bp PCR fragment in 6% polyacrylamide gel while negative posts lacked the primer to allow

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amplification. The lack of amplification observed confirms that cross-talk is suppressed. No product was obtained for posts lacking primers, indicating that cross-contamination by primers does not occur. Diffusion of PCR components between posts was not detected

Table 3: Cross-talk controls via checkerboard of alternating positive and negative posts.

5

posts with primers (100 bp)	posts without primers
+	-

Example 8: Quantitative PCR

In order to characterize quantitative real-time PCR in gel posts, different amounts of BKV DNA were tested under the same PCR conditions to amplify a product of 100 bp in 2.8% polyacrylamide gel posts. For comparison, conventional real-time PCR with the same template was carried out in the Lightcycler®, an instrument that is routinely used for melt curve analysis in clinical diagnostic laboratories and provides a clinically relevant "gold standard". For the Lightcycler® PCR, PCR reaction/gel mixes were polymerized in capillaries in order to mimic the PCR in the gel posts and held a total volume of 0.84 µL per reaction, similar to gel posts. **Fig. 7(a-c)** shows real-time PCR data generated by the Lightcycler®, relationship of C_p values versus $\log [DNA]_{\text{initial}}$ and the confirmation of the product size by vertical gel electrophoresis. The analogous results of **Fig. 7(d-f)** obtained with the in-gel post PCRs mirror those of **Fig. 7(a-c)** from the Lightcycler®. Each post was picked up individually and placed above the gel before running electrophoresis as shown in **Fig. 7(f)**, with a 100 bp DNA ladder shown in the middle in **Fig 7(f)** and on the left in **Fig. 7(c)**. The results below confirm that melt curve analysis of PCR in gel posts matches that from gold standard testing.

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Fig. 7(b) and **Fig. 7(e)** show that, as expected, the C_p values decrease linearly with the logarithm of increasing template DNA copy number for the Lightcycler® and gel post array, respectively, and that the relationship is comparable in the two systems (within ~1 cycle).

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- 20 -

The melting temperature of the products in the Lightcycler® is 81.5°C which agrees with the gel posts value of 82.6°C. Both real-time PCR and MCA validate the PCR conditions in gel posts. The inset in **Fig. 7(f)** is an enlarged section of the gel band, showing individual bands from each gel post. The samples loaded to electrophoresis lanes shown in **Fig. 7(f)** were from
5 PCR performed with an initial 3456 BKV DNA templates per post.

Fig. 6 shows successful BKV PCR when template was added after the gel was polymerized. Assuming that the DNA was uniformly absorbed by all the 81 posts, each post would have ~4900 copies of BKV template. The C_p value of the PCR shown in **Fig. 6(a)** is 28.56 ± 0.97 . According to the **Fig. 7(e)** (where template DNA was added prior to the polymerization), the
10 calculated C_p value for a PCR performed with the 4900 copies/post would be 28.86. This calculated value is very similar to the experimental C_p value, 28.56 ± 0.97 that was obtained for the PCR where template DNA was introduced to pre-cast gel posts, confirming that PCR amplification of exogenous DNA template is as efficient as that performed with the template DNA polymerized in the gel. This also indicates that almost all the exogenous DNA placed
15 atop the gel must have diffused into to the gel. The results herein show the feasibility of applying this technique to medical diagnostics where adding clinical sample to pre-cast gel posts is the only feasible approach.

Example 9: Genomic DNA PCR

Human gDNA is made of 3 billion base pairs of DNA, as compared to viral DNA, or plasmid
20 DNAs that are only a few thousand to hundreds of thousands of base pairs in size. During PCR, there is a great deal of heterogeneous non-target DNA present in the long gDNA compared to the uniformity of short plasmid DNA, suggesting that the efficiency of the gDNA PCR is less than that of plasmid DNA. The prior art with respect to gel PCRs used plasmid DNA or cDNA as the template but not gDNA. Two gDNA PCR were undertaken in
25 2.8% polyacrylamide gel, one with the gDNA polymerized in the gel and one with the gDNA added after polymerization of the gel.

gDNA was subjected to PCR to amplify a 42 bp product containing a known SNP from the

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human HPA1 gene in the gDNA template polymerized inside the gel, using SEQ ID NOs 14 and 15 as primers; as otherwise shown in Table 1. The template was chosen in anticipation of future genotyping with the gel posts using e.g. allele specific PCR as previously shown. The processed real-time PCR curves and the melting curve analysis data are shown in Fig. 8(a) and Fig. 8(b) respectively. The amount of genomic DNA in the HPA1 PCR is ~ 2.2 ng per post. PCR was then performed with gDNA template (~5 ng/post) added after the gel polymerization, the gDNA allowed to diffuse into the gel matrix. For the latter approach, the FGFR2 gene from human genomic DNA, with amplification of a 71 bp product also containing a known SNP using SEQ ID NOs 16 and 17 as primers. Fig. 8(c) shows the processed real-time PCR curves while Fig. 8(d) shows the melting curve data for the FGFR2 PCR. Both HPA1 and FGFR2 product sizes were confirmed by vertical gel electrophoresis. This is the first use of gDNA for gel PCR, where gDNA is introduced to the gel mix either prior to or after the polymerization.

Example 10: xPVP hydrogel

A hydrogel other than polyacrylamide, namely cross-linked polyvinylpyrrolidone (xPVP), was used as gel matrix for the posts. It was photo-polymerized with azobis catalysis in the presence of all other ingredients necessary for PCR of BKV template as described in Example 3. The 10 μ l of gel reagents contained 33% n-vinylpyrrolidone (Sigma-Aldrich cat# V3409) and 7% of polyethylene glycol diacrylate (Sigma-Aldrich cat#437441) in water. The qPCR and MCA conditions were same as described Example 3. The results are shown on Fig.9

The prior art has used APS as the polyacrylamide polymerization initiator, precluding the use of in situ real-time detection techniques such as MCA. In contrast, the polymerization initiator used in our photo-polymerization technique, azobis, did not inhibit or inactivate the dye allowing the addition of LC Green Plus dye prior to the polymerization and the acquisition of real-time PCR data at each cycle without disturbing or moving the gel, thus eliminating the need for post PCR staining or the use of expensive and cumbersome hybridization or probe techniques. Alternate dyes are contemplated, including by way of non-limiting example,

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Styo62 and SYBR Green.

While particular embodiments of the present invention have been described in the foregoing, it is to be understood that other embodiments are possible within the scope of the invention and are intended to be included herein. It will be clear to any person skilled in the art that

5 modifications of and adjustments to this invention, not shown, are possible without departing from the spirit of the invention as demonstrated through the exemplary embodiments. The invention is therefore to be considered limited solely by the scope of the appended claims.

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[0001] SEQUENCE LISTING

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What is claimed is:

1. A method for detecting a nucleic acid molecule within a hydrogel post array comprising
 - a) providing a hydrogel post array of 2x1 or greater containing a cell-free, enzymatic,
5 nucleic-acid amplification system;
 - b) applying to at least one of said hydrogel posts nucleic acid molecules of, at least one of which may comprise a template for said amplification system;
 - c) incubating said hydrogel posts under conditions promoting the synthesis of an amplified nucleic acid product by said amplification system from said at least one template;
- 10 wherein said amplification system comprises at least two non-immobilized nucleic acids capable of promoting synthesis of amplified nucleic acid product from said template.
2. The method of claim 1 wherein the hydrogel posts contain a marker, wherein said marker has different properties, capable of being measured, when interacting with double-stranded nucleic acids than with single-stranded nucleic acids.
- 15 3. The method of claim 2 wherein said measurement is in real-time and the fluorescent marker is selected from the group comprising LC Green, SYBR Green or SYTO 62.
4. The method of claim 3 wherein said hydrogel post is comprised of polyacrylamide of 2.0-12% weight per unit volume, and photopolymerized in the absence of APS.
5. The method of claims 1 through 4 wherein said template is included in said hydrogel
20 posts,
6. The method of claims 1 through 4 wherein said template is provided externally to said hydrogel posts.

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7. The method of claim 1 wherein the template is between 3,800 and 250,000,000 base pairs in size.
8. A method for detecting a nucleic acid molecule within a hydrogei post array comprising
- 5 a) providing a hydrogei post array of 2x1 or greater containing a cell-free, enzymatic, nucleic-acid amplification system;
- b) including nucleic acid molecules in at least one of said hydrogei posts, at least one of which may comprise a template for said amplification system;
- c) incubating said hydrogei posts under conditions promoting the synthesis of an
- 10 amplified nucleic acid product by said amplification system from said at least one template;
- wherein said amplification system comprises at least two non-immobilized nucleic acids capable of promoting synthesis of amplified nucleic acid product from said template.
9. The method of claim 8 wherein the hydrogei posts contain a marker, wherein said marker has different properties, capable of being measured, when interacting with double-
- 15 stranded nucleic acids than with single-stranded nucleic acids.
10. The method of claim 9 wherein said measurement is selected from the group comprising LC Green, SYBR Green or SYTO 62.
11. The method of claim 10 wherein said hydrogei post is comprised of polyacrylamide of 2.0-12% weight per unit volume, and photopolymerized in the absence of APS.
- 20 12. The method of claims 8 through 11 wherein said template is included in said hydrogei posts.

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13. The method of claims 8 through 11 wherein said template is provided externally to said hydrogel posts.

14. The method of claim 8 wherein the template is between 3,800 and 250,000,000 base pairs in size.

5 15. A method for detecting a nucleic acid molecule within a hydrogel post array comprising

a) providing a hydrogel post array of 2x1 or greater containing a cell-free, enzymatic, nucleic-acid amplification system;

b) introducing nucleic acid molecules in a solution in fluid communication with at least one of said hydrogel posts, at least one of which may comprise a template for said
10 amplification system;

c) incubating said hydrogel posts under conditions promoting the synthesis of an amplified nucleic acid product by said amplification system from said at least one template;

wherein said amplification system comprises at least two non-immobilized nucleic acids capable of promoting synthesis of amplified nucleic acid product from said template.

15 16. The method of claim 15 wherein the hydrogel posts contain a marker, wherein said marker has different properties, capable of being measured, when interacting with double-stranded nucleic acids than with single-stranded nucleic acids.

17. The method of claim 16 wherein said measurement is selected from the group comprising LC Green, SYBR Green or SYTO 62.

20 18. The method of claim 17 wherein said hydrogel post is comprised of polyacrylamide of 2.0-12% weight per unit volume, and photopolymerized in the absence of APS.

19. The method of claims 15 through 18 wherein said template is included in said hydrogel posts.

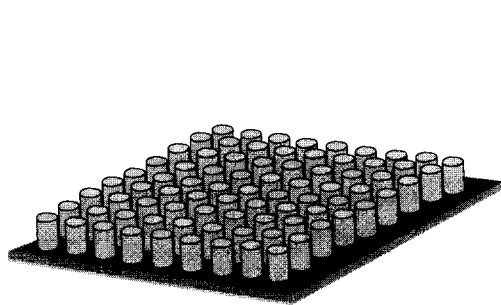
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20, The method of claims 15 through 18 wherein said template is provided externally to said hydrogel posts.

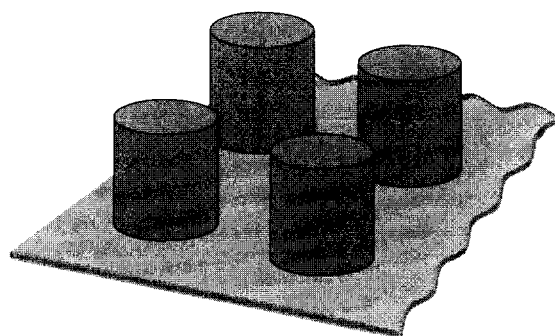
21. The method of claim 15 wherein the template is between 3,800 and 250,000,000 base pairs in size.

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Figure 1



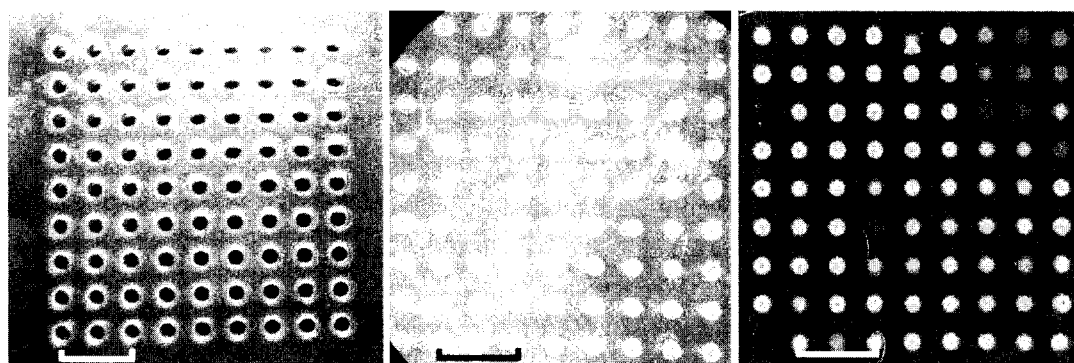
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(b)

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Figure 2



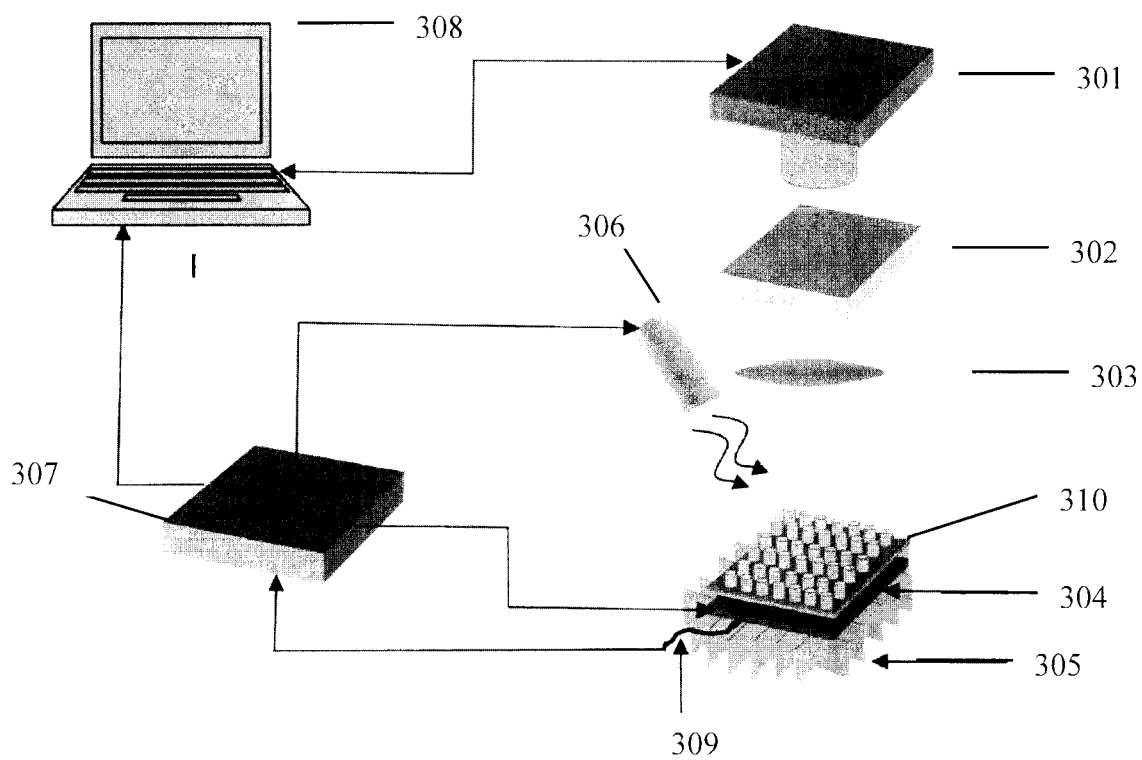
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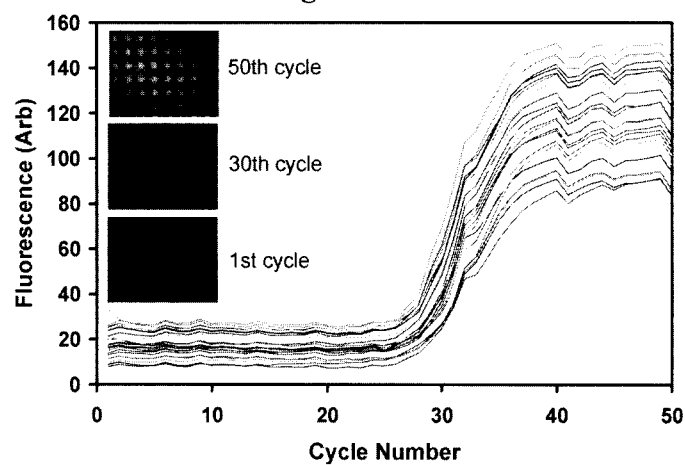
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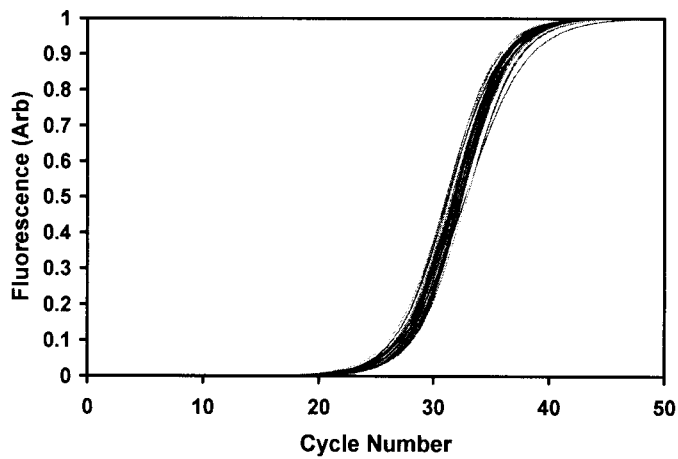
Figure 3



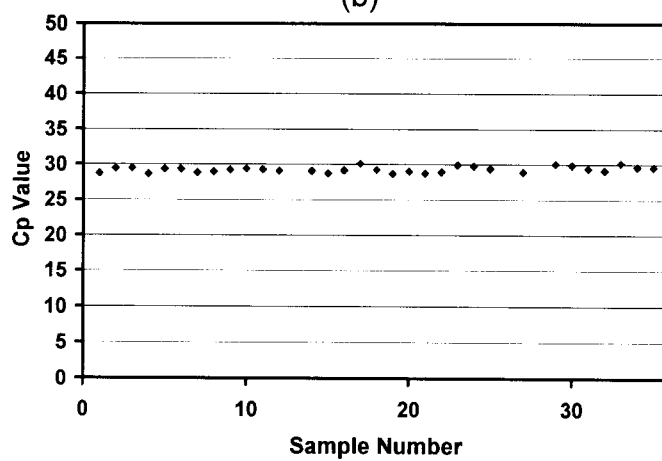
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Figure 4



(a)

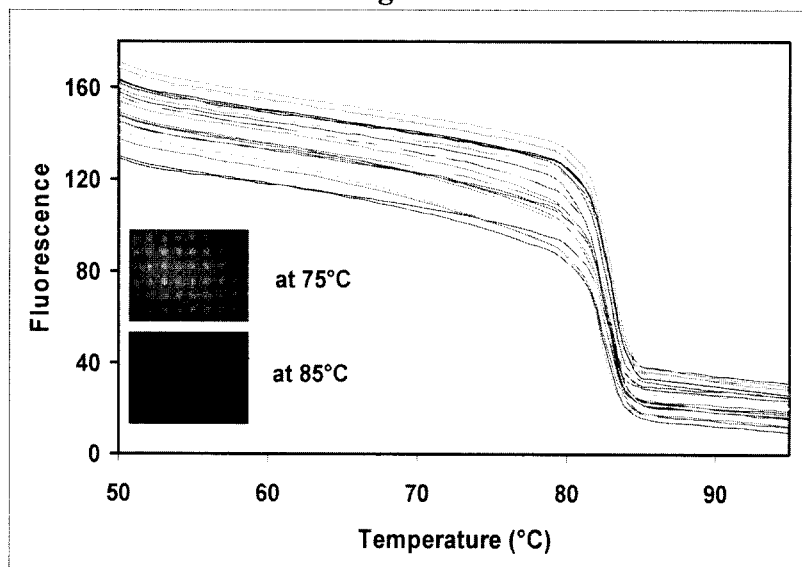


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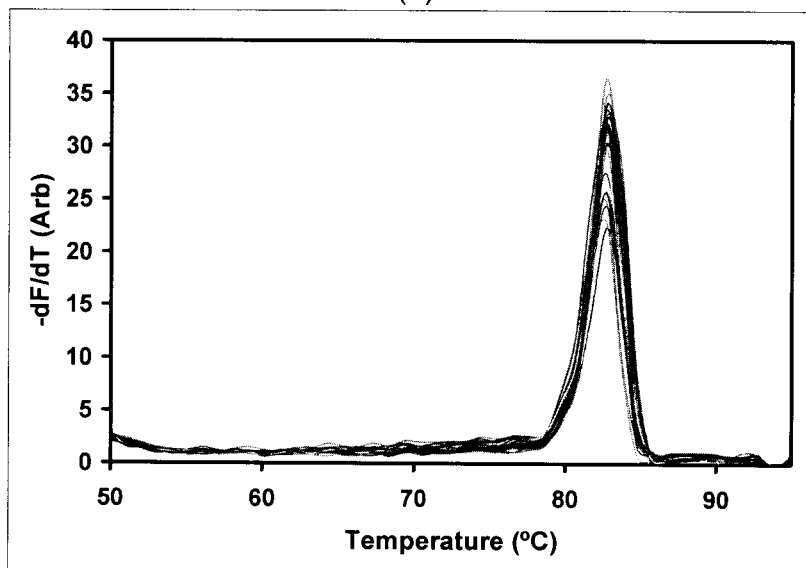


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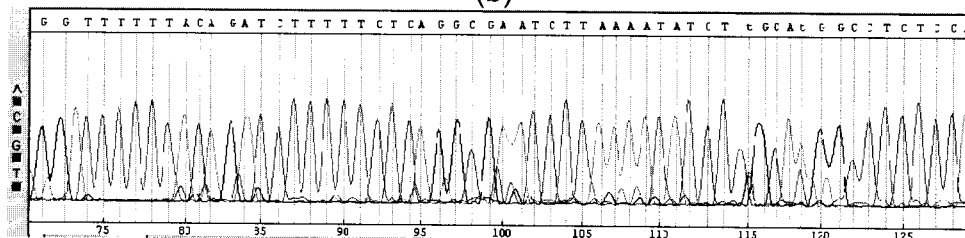
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Figure 5



(a)



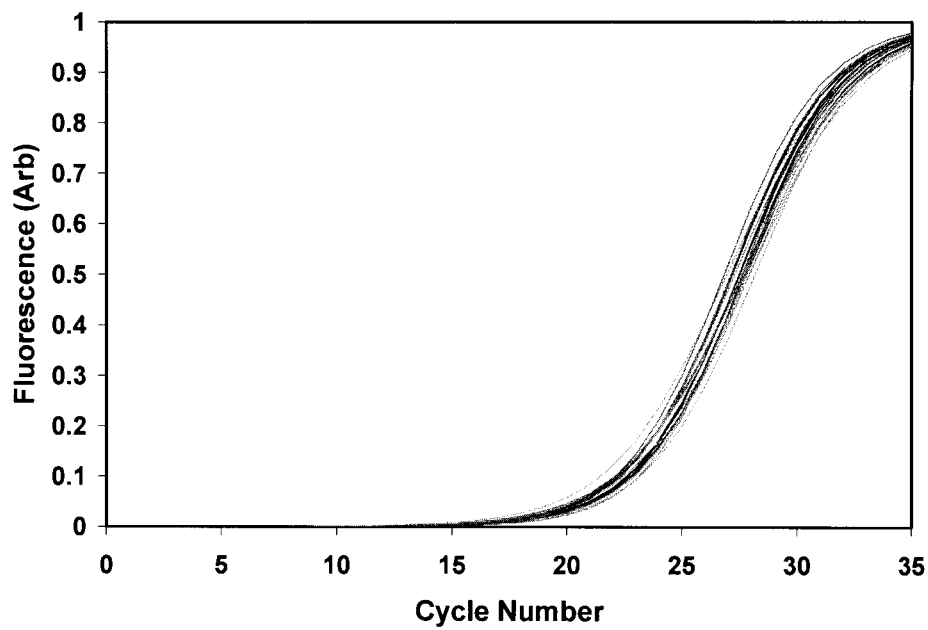
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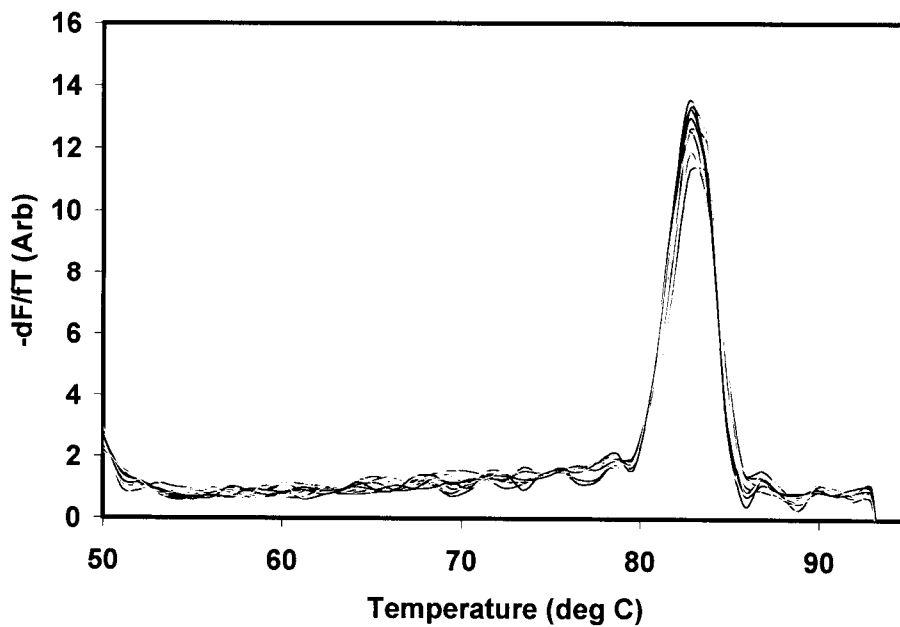
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Figure 6



(a)



(b)

Figure 7

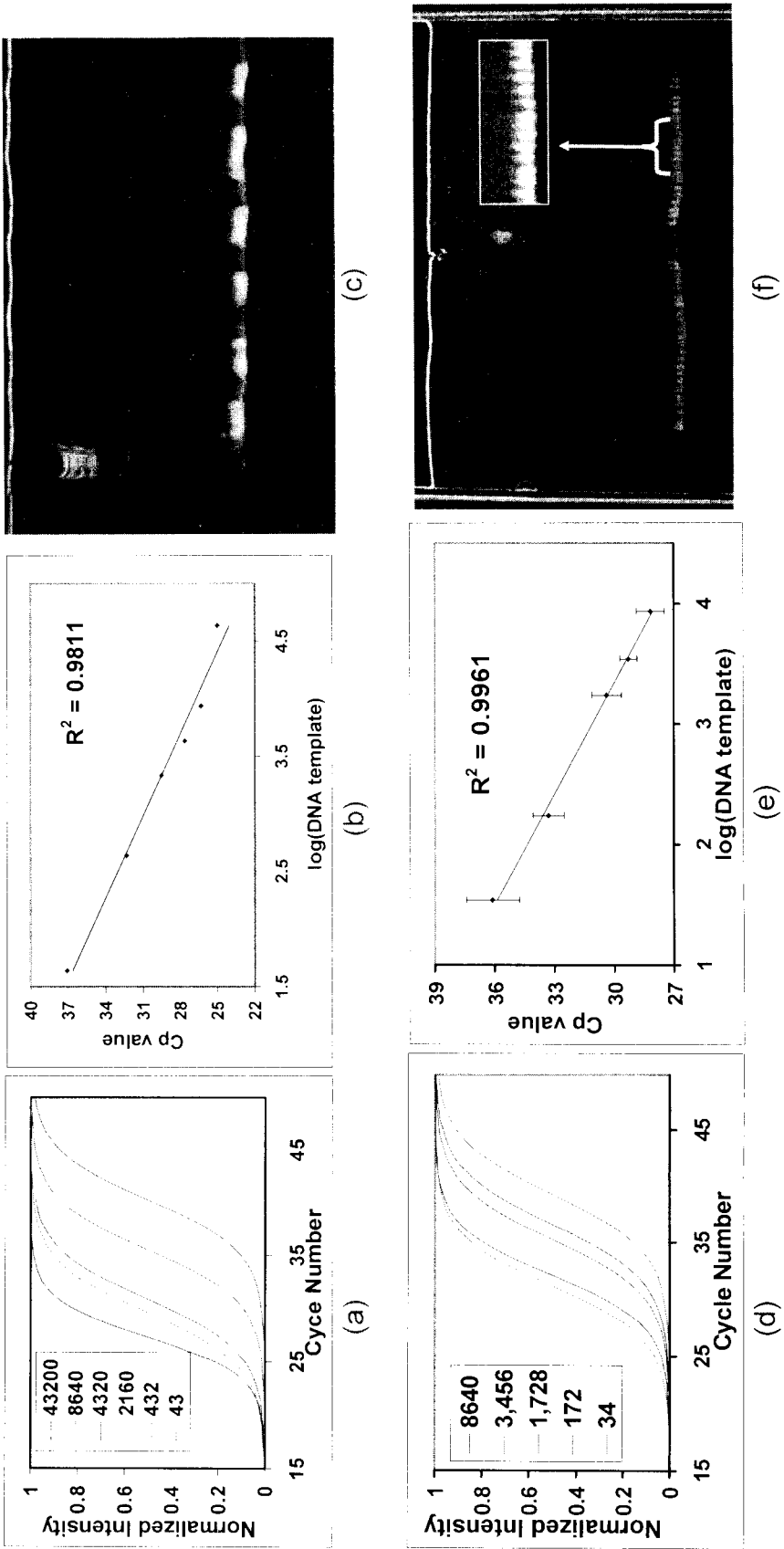
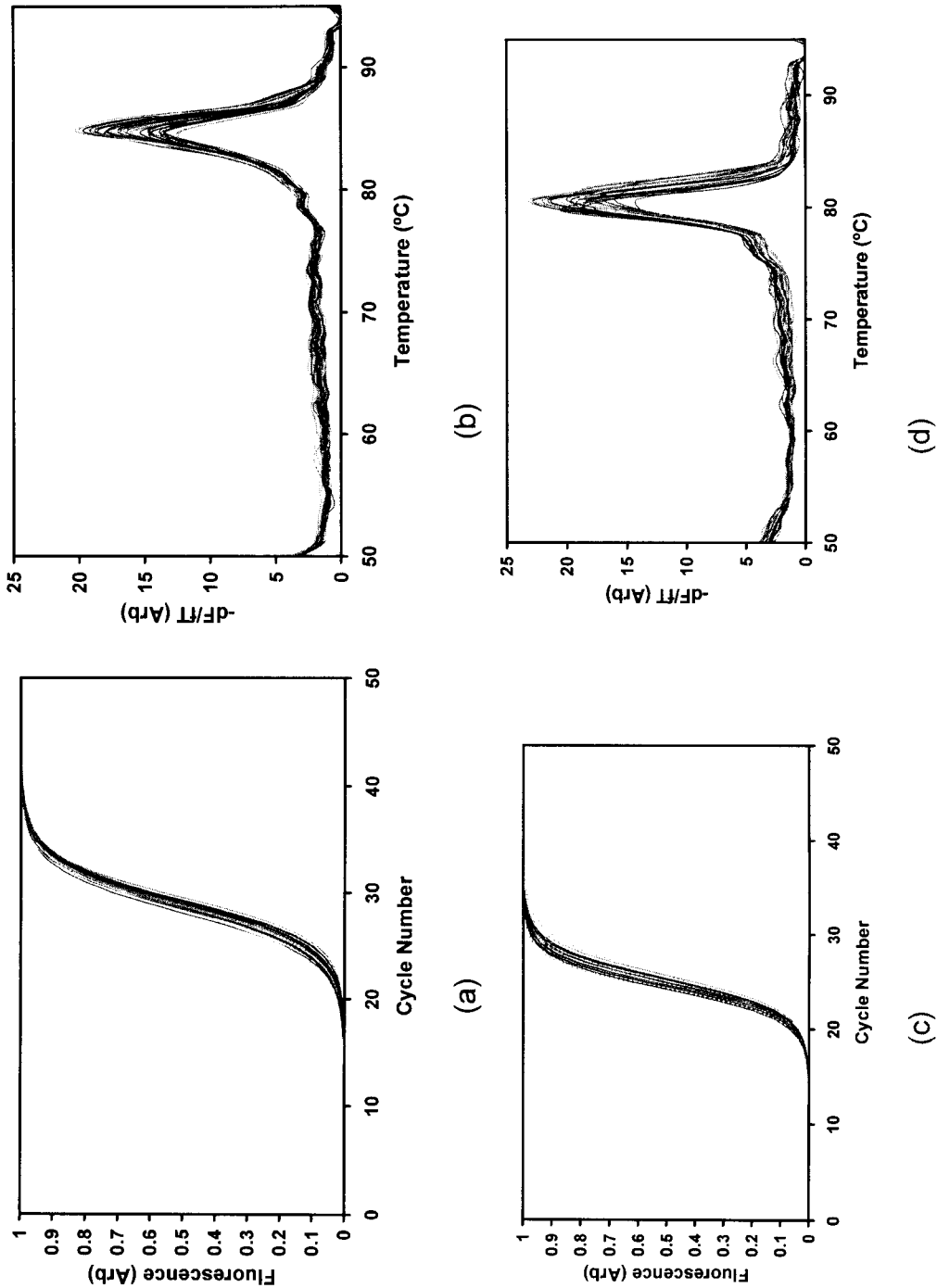
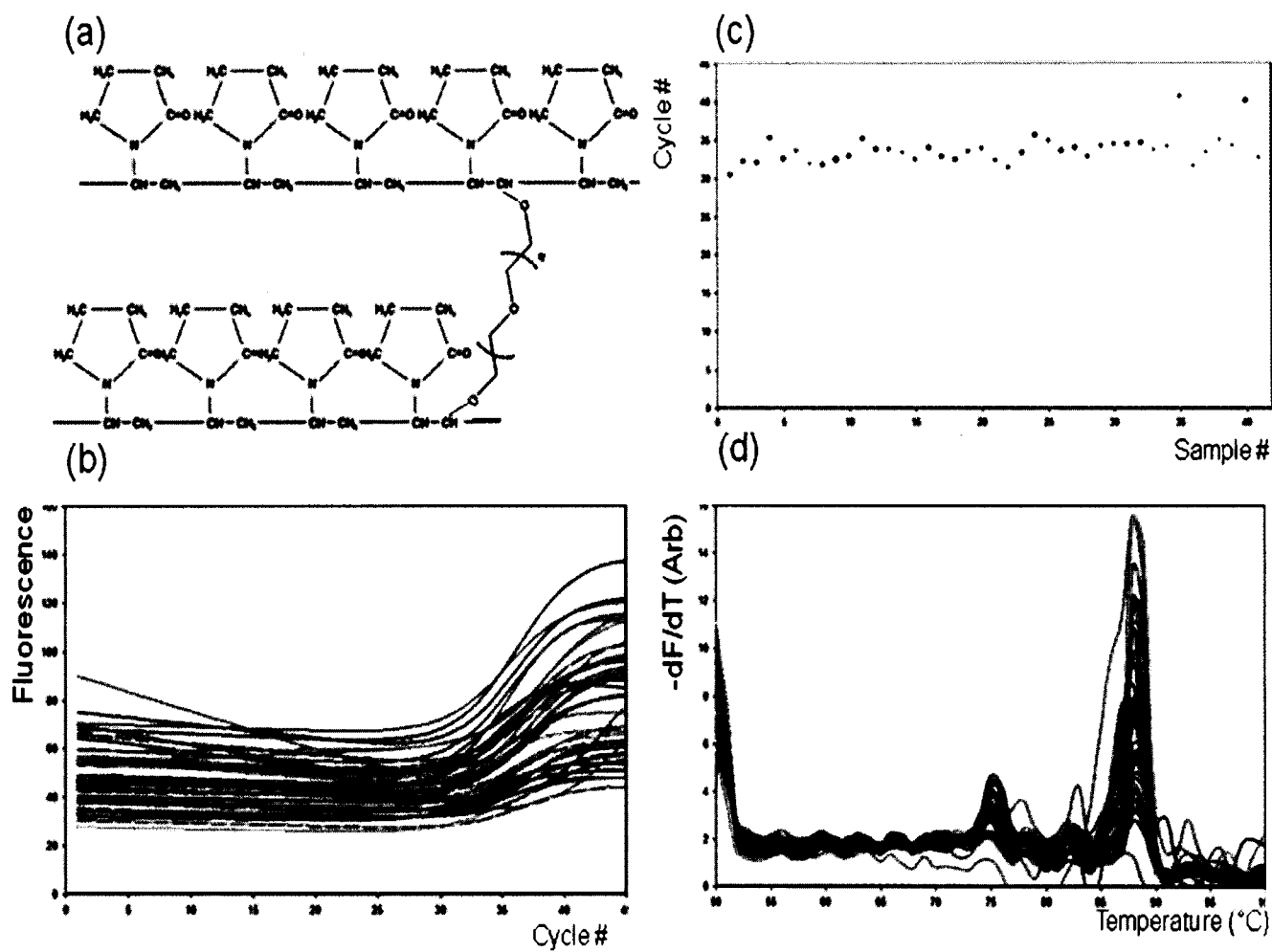


Figure 8



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Figure 9



INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA20 11/000789

A. CLASSIFICATION OF SUBJECT MATTER

IPC: **C12Q 1/68** (2006.01) , **C12M 1/40** (2006.01) , **CUP 19/34** (2006.01) , **C12M 1/38** (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12Q 1/68 (2006.01) , **C12M 1/40** (2006.01) , **C12P 19/34** (2006.01) , **C12M 1/38** (2006.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
Author and keyword (In-gel, PCR, array, polyacrylamide, solid matrix, photopolymerization, DNA amplification, pathogen detection, microfluidic PCR) searches using Canadian Patent Database, Medline and TotalPatent.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO2007/1 11639 A1 (CHETVERIN et al.) 4 October 2007	
A	US56 16478 (CHETVERIN et al.) 1 April 1997	
A	MITRA and CHURCH, "In situ localized amplification and contact replication of many individual DNA molecules", NUCLEIC ACIDS RESEARCH, 15 December 1999, Vol 27, No. 4, page e34. ISSN 0305-1048	
A	CHETVERJNA et al., "Molecular colony diagnostics: detection and quantitation of viral nucleic acids by in-gel PCR", BIOTECHNIQUES, July 2002, Vol. 33, No. 1, pages 154-156. ISSN 0736-6205	
A	STRIZHKOV et al., "PCR amplification on a microarray of gel-immobilized oligonucleotides: detection of bacterial toxin- and drug-resistant genes and their mutations", BIOTECHNIQUES, October 2000, Vol. 29, No. 4, pages 844-857	
P, X	ATRAZHEV et al., "In-gel technology for PCR genotyping and pathogen detection", ANALYTICAL CHEMISTRY, October 2010, Vol. 82, No. 19, pages 8079-87. ISSN 0003-2700	1-21

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents :	"Y" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance ; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 29 September 2011 (28-09-2011)	Date of mailing of the international search report 12 October 2011 (12-10-2011)
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476	Authorized officer Micheal O'Hare (819) 994-416 1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/CA20 11/000789

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
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		WO20071 11639A1	04-10-2007
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