MULTI-CHAMBER PCR CHIP WITH SIMPLE LIQUID INTRODUCTION UTILIZING THE GAS PERMEABILITY OF POLYDIMETHYLSILOXANE

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Abstract

On-chip polymerase chain reaction (PCR) is beginning to provide a viable alternative to conventional genetic profiling and amplification devices through minimal reagent use, short time, high detection resolution and potential high-throughput parallel testing of genetic materials. Despite the advantages, there are many challenges to overcome in accurate control and manipulation of fluid, circumventing bubble formation and inhibiting sample loss during PCR thermal cycling for successful PCR. In this research, gas permeability of Polydimethylsiloxane (PDMS) was employed for liquid sample introduction into PDMS multi-chamber PCR chip, avoiding trapped bubbles in the reaction chambers. This method is simpler and more reliable compared to the other reported methods where integration of many complicated components, such as micropumps and micromixers on the chip for both sample loading and mixing are necessary. The sample evaporation and bubble formation on chip were controlled by using glycerol as a vapor pressure modifier. With this device, successful amplification of human beta-Actin gene was demonstrated. This approach will be applicable in developing chip devices for multi-target sample amplification for diagnostic purposes.

Keywords: PCR in chip, PDMS gas permeability, evaporation suppression, bubble elimination, fluid manipulation.

1. Introduction

Advent of micro electro mechanical system (MEMS) technology initiated the development of miniaturized PCR chips which provide many advantages over classical PCR in terms of higher throughput, shorter amplification time, minimum human/world-to-PCR intervention, and reduced contamination. On-chip polymerase chain reaction (PCR) amplifies a piece of DNA by in vitro enzymatic replication. Currently this method is used in many applications such as virus detection [1, 2], disease diagnosis [3 - 8], gene expression analysis [9 - 11], environmental testing, and food safety testing [12].

Until today, PCR microfluidics of varying designs have been developed by many researchers for effective and fast DNA amplification, for example, chamber stationary PCR and flow through PCR, thermal convection-driven PCR etc. The chamber stationary PCR microfluidics can be separated into two groups, single chamber [13, 14] and multi-chamber [6, 32]. Single chamber PCR chips perform well in terms of fluidics and thermal control, but they are not suitable for high throughput PCR. In order to improve the PCR throughput and reduce the analysis time, multi-chamber stationary PCR microfluidics on a single chip has been explored. There are many challenges to be overcome for efficient PCR on chip. Because of the small volume of reaction chamber, the evaporation of reaction mixture during PCR must be considered seriously. In addition, bubble formation during PCR should also be controlled carefully to facilitate amplicon production in the most effective way [12].

Micro pumps and valves were integrated into chips for sample loading [15]. The integration of many components in a chip made it complicated in terms of fabrication and operation. There has been a report on the utilization of capillary force for loading sample into the reaction chambers [16] and surfactant was used with the PCR mixture in order to minimize the contact angle between PCR mixture and PDMS chip. The addition of surfactant above the minimal concentration produces undesirable effects on PCR amplification efficiency. Both these methods are good for sample loading purposes other than for PCR. Since high temperature is needed to perform PCR, during sample loading it becomes difficult to avoid formation of air bubbles adjacent to the wall of reaction chambers with rough wall-surface. At high temperature, the trapped air bubbles expand and lead to the expulsion of PCR solution out of the reaction chambers. Our previous report showed the prevention of air bubbles by introducing fluorinated oil - an inert and highly viscous liquid - before the introduction of the sample solution. This helped to increase the pressure of the sample solution in the micro channels [36]. However this

method is only suitable for continuous-flow PCR chip. It cannot be used for dead-end PCR reaction chamber.

In this research, we have introduced a new method for loading the PCR reagents into the reaction chambers of PDMS chip. The air was not trapped to form bubbles at high temperature, even though the inner surfaces were presumably unsmooth. Our sample loading method relies on the gas permeability of PDMS due to its intrinsic porosity. Compared to previous methods, our approach is simpler and more reliable, due to its easy performance and applicability. Using this technique, we could simplify the structure of high throughput PCR chip and ensure the same amplification rate for all the reaction chambers in a chip under unique condition. In this work we have also showed the way to minimize the sample loss for PDMS chips. Sample loss due to evaporation during PCR thermal cycling at high temperature can be eliminated by changing the vapor pressure of the PCR mixture with glycerol, and by fabricating a thin Parylene-C film between the membrane for valve and air layer. Finally, PCR amplification was successfully performed on our chip. The fluorescence intensity of PCR amplicons in the reaction chambers was clearly distinguishable. The proposed microfluidic PCR system may provide a promising platform to diagnose multiple biomarkers associated with diseases.

2. Material and methods

2.1. Reagents and sample preparation

PDMS and curing agent (Dow Corning, Toray Co., Japan) were used for chip fabrication. All chemicals used for DNA amplification in this research were from Applied Biosystems (USA). Nuclease-free water and glycerol were bought from Invitrogen (USA) and Wako (Japan), respectively.

On the chip, a 295-bp segment of human β-Actin was amplified to evaluate the performance of the DNA amplification. The primers and probe sequence specific to the β-Actin gene were: forward, 5′-TCA CCC ACA CTG TGC CCA TCT ACG A-3′; reverse, 5′-CAG CGG AAC CGC TCA TTG CCA ATG G-3′; probe, 5′-ATG CCC ***T***CC CCC ATG CCA TCC TGC GT-3′. This probe was labeled at the 5′ end with the fluorescent reporter FAM and at the bold and italic base (***T***, thymine) with the quencher TAMRA. The reaction mixtures consisted of 10 × GeneAmp PCR buffer, 0.2 mM each dNTP, 3.5 μM $MgCl_2$, 0.3 μM for each forward primer and reverse primer, 0.2 μM beta-Actin TaqMan probe, 0.025 – 0.25 U/μl of

AmpliTaq Gold DNA polymerase, 0.2 ng/μl of human template DNA, and desired concentration of Glycerol for each experiment.

2.2. Chip structure and fabrication

2.2.1. Chip structure

The PCR chip, made of silicone elastomer PDMS, is composed of three layers: air layer, flow layer, and a thin layer of hybrid Parylene-C - PDMS membrane as shown in Figure 1. PDMS was chosen because it is inert, easy to pattern by soft lithography, optically transparent, flexible, gas-permeable, stable, cheap and does not have fluorescence property itself.

The flow layer of the chip contained an array of circular-shaped reaction chambers, flow and air channels. All the reaction chambers were 1 mm in diameter and 200 μm in height, accommodating about 150 nl of PCR solution. The reaction chambers were connected with the sample inlet reservoir through the flow channel. Air jackets were incorporated with the flow layer encompassing the reaction chambers and then connected to the vacuum suction port through the air channel. The channels in the flow layer were 200 μm in width and 200 μm in height.

The air layer contained an array of valves respective to the reaction chambers on the flow layer. Each valve was 600 μm in width, 600 μm in length and 200 μm in height. All the valves were connected with each other through one air channel. Air layer was bonded with hybrid membrane to form an array of valves. By controlling the air pressure, the membrane could be deformed up/down to open/close the valves for sample flow in microfluidic chip. This is illustrated in Figure 2.

2.2.2. Chip fabrication

The Microfluidic device with flow layer and air layer was fabricated using PDMS by standard soft-lithography techniques [17].

Thin membrane of PDMS for valves was fabricated on Petri dish by spin coating the PDMS pre-polymer at 4000 RPM for 30 sec using a spin coater. After curing at 65°C for 2.5 hrs, a thin layer of Parylene-C, up to 2 μm in thickness, was then deposited on the PDMS membrane by chemical vapor deposition to obtain the hybrid Parylene-C - PDMS membrane.

The air layer was punched to create a 500 μm diameter hole for valve control port, then by oxygen plasma treatment it formed a multilayer with the thin hybrid Parylene-C - PDMS

membrane. The multilayer was cured in a convection oven at 65^0C for 30 min and left overnight at room temperature. Finally, the multilayer was peeled off the Petri dish by cutting along its edge with a razor blade.

Two more holes, 5 mm and 500 μm in diameter, were punched through the air layer and hybrid membrane for the sample inlet reservoir and vacuum suction port, respectively. Then the multilayer was attached to the flow layer by using oxygen plasma. Fluorinated Ethylene Propylene (FEP) tube (0.15±0.05 mm i.d.) (BAS Inc., Tokyo, Japan) was inserted into the valve control and the vacuum suction ports. These ports were then sealed with a small amount of PDMS to prevent leakage.

2.3. Sample loading into the multi-chamber PCR chip utilizing the gas permeability of PDMS

On-chip PCR, sample loading process is one of the main reasons for the bubble formation. If the chip is poorly designed and/or fabricated, air can easily get trapped in the micro-cavities of the reaction chamber, generating bubbles which may cause PCR failure [18]. In this research, loading sample from the inlet reservoir into every reaction chamber on the chip was achieved using the gas permeable property of PDMS [19, 34].

A software-controlled pressure system (made by our group) was used to control the pressure on the vacuum suction port and the valve control port for chip manipulation. Positive pressure (inlet pressure) and negative pressure (suction pressure) were created by compressing air and sucking air respectively through the ports. The pumping program can be set up easily by changing the parameters such as positive and negative pressures, required time for each cycle, and number of repeated cycles. Before use, the program was saved in system memory. Then the pressure system ran automatically.

2.4. DNA amplification in chip

To verify the performance of the PCR in chip, 10 μl of PCR mixture was injected into the inlet reservoir. Upon the completion of sample loading, the device was placed on the flat surface thermocycler (ASTEC) for DNA amplification. Heat transfer between the hot plate and the chip was improved by applying a thin layer of mineral oil between them. The thermal cycling program for housekeeping beta-Actin gene amplification was commenced by heating at 95°C for 10 minutes to activate the polymerase and denature the initial DNA, followed by thermal conditions consisted of denaturing at 95°C for 15 sec, and annealing and extension at

65°C for 1 minute. Upon completion of up to 30 thermal cycles, the chip was kept at 25°C for fluorescence intensity measurement. The negative control experiment was conducted by replacing the template genomic DNA with nuclease-free water.

2.5. Fluorescence based DNA detection on chip

Fluorescence based DNA detection method was applied for on chip PCR amplicon detection. It is a powerful technique used for single cell or molecular analysis [1, 10, 20]. In this research, TaqMan probe was used for the end-point detection of amplicon when the DNA template was successfully amplified on chip. This method uses internal probes specifically hybridizing with the target to generate fluorescent signal to reduce background and false positives significantly. Compared to SYBR green I (a double stranded DNA binding fluorescence dye) TaqMan probe is more advantageous for its high specificity, sensitivity, and ease of use [21].

A fluorescence microscope (Leica) with image-processing software was used for the detection of fluorescence intensity in the reaction chambers. The excitatory light from mercury vapor lamp passed through the filter which only lets through radiation with the desired wavelength matching the fluorescing sample. The excitation and emission wavelengths for the reporter dye FAM are 494 nm and 518 nm, respectively. The emitted light was separated from much brighter excitation light in the second filter. Finally, the fluorescence image was captured by CCD camera connected to the computer with image analysis software.

3. Results and discussion

3.1. Sample loading into the chip

The valve control and vacuum suction ports were connected to two pressure-controlled outlets of the system for loading the sample into the chip. Before loading process, positive pressure and negative pressure were applied to the valve control and vacuum suction ports respectively, and maintained for 30 seconds to close the valves. The fluid sample was loaded into the inlet reservoir. The valves were then opened by applying negative pressure to the valve control port, while the vacuum suction port was continuously sucking during this step. The principle of this technique can be explained as follows. During evacuation, air in the reaction chambers penetrated into air jackets through a PDMS gas permeable wall so that the pressure inside the reaction chambers decreased with evacuation time. As a result, sample in the inlet reservoir moved along the flow channel and entered every reaction chamber. After all the

reaction chambers were completely filled with fluid sample, and no trapped air remained in the reaction chambers, air was compressed through the valve control port to close the valves. This ensured the complete isolation among the chambers and flow channel on the chip.

In this experiment, the positive pressure and the negative pressure were set at 480 mmHg and -400 mmHg, respectively. The reaction chambers were gradually filled with PCR mixture while the air was being sucked through the air jacket. Figure 3 clearly shows that the entire reaction chamber was filled with the sample and no trace of air bubbles was observed after completion of the loading process.

Sample loading rate was closely related to the porosity of material, the vacuum pressure used, and the thickness of PDMS wall between the reaction chamber and the air jacket. High-porosity structure of PDMS was favorable for sample loading, but it also led to sample evaporation in PCR chip. Among the three options, thickness of the permeable PDMS wall could be minimized to increase the sample loading velocity. We performed sample loading process in chip with different thickness of PDMS wall, as shown in Figure 4. Reduction of PDMS wall thickness enhanced sample loading rate. When the thickness of the permeable wall was 100 μm, sample from the reaction chamber easily seeped out into the air jacket during suction process or thermal cycling at high temperature, due to leakage by deformation of PDMS. In the case of 200 μm wall thickness, no sample leakage was observed (data not shown).

3.2. Circumventing air bubbles and sample evaporation

As mentioned above, highly porous structure of PDMS inherently leads to the problems of sample loss and bubble formation during thermal cycles due to evaporation. Those lead to failed or inaccurate PCR. In order to solve the problem, we used glycerol, a high boiling point PCR compatible substance [12, 29, 35], in combination with a 2 μm Parylene-C film on the upper side of PDMS membrane to block the vertical evaporation from the PDMS chip.

The sample evaporation and bubble formation in chip were investigated with a mixture of 20% (w/w) glycerol and methyl green solution in water. The mixture was loaded into the reaction chambers as described earlier, followed by 30 thermal cycles of PCR, while using the microscope to monitor what was happening inside the reaction chambers. Neither bubble formation nor evaporation occurred in PCR chip even after 30 thermal cycles of PCR. The sample volume remained the same in the reaction chamber, as shown in Figure 5. Without the

Parylene-C film, about half of the volume of the solution in PCR chamber was lost after 30 thermal cycles (data not shown). These results show that our method is effective in suppressing bubble formation and sample loss during the PCR.

There are three main causes of the sample loss. The first one is the air trapped in the reaction chamber during sample loading. The air expands at high temperature to push out the sample solution from the chamber, which leads to PCR failure. Many reports tried to solve this problem in different ways, such as changing the chamber shape or/and the surface wetting property. They found that chips with the hexagonal or rhomboidal-shaped chambers have less risk of bubble entrapment during the sample loading process [3, 22]. Surface modification of PDMS also reduces the risk of air trapping. When the chamber surface is highly hydrophilic, the PCR sample can flow smoothly and rapidly into the chamber without tiny trapped air [9, 23, 24]. However, those approaches still have some risk to retain bubbles in the reaction chamber. In this research, such risk was completely removed by evacuation in the sample loading process, as mentioned above in section 3.1.

The second cause of sample loss is the dissolved gas in both PCR mixture solution and PDMS. At high temperature during PCR, those gases generate bubbles leading to the expulsion of PCR mixture from the chamber. Degasification of the PCR mixture before loading into the chip prevents bubble formation at high temperature [18]. Coating the wall of the reaction chamber with gas tight polymer such as Parylene can also prevent bubble generation caused by the porosity of PDMS [37]. In this research, this second type of bubble is also expected to be reduced by evacuation for the sample loading, because the dissolved gas in PDMS and PCR mixture is removed to some extent by evacuation.

The third reason, as mentioned before, is the evaporation of water from PCR mixture through PDMS at high temperature during thermal cycles. It is well known that the vapor loss due to gas permeability of PDMS changes the concentration of PCR reagents, and sometimes leads to a complete drying out [37]. This is the usual cause of unsuccessful gene amplification in stationary PCR chip. A number of measures were employed to minimize such evaporation, and all had their advantages and disadvantages. A layer of mineral oil was frequently used as a liquid vapor barrier to prevent evaporation [25, 26] because it has a boiling point far above 100°C and density below 1.0 g/cm^3. This method is adaptable to open air PCR chip, in which spotting method is used for sample loading. However, its applicability is problematic for highly integrated closed PCR systems. It is impossible to simultaneously load an equal amount of PCR

mixture and mineral oil into every reaction chamber in chip with nanolitre volume range. Gas tight polymer was also used for preventing evaporation in chip from the roof and side wall of reaction chamber [27,37]. Some authors added a fluid reservoir in the vicinity of reaction chambers to increase the water vapor in chip, thus the sample loss during PCR at high temperature was reduced [28]. However, the integration of many fluid reservoirs on chip not only makes the chip structure complicated in terms of fabrication and operation, but also decreases the space used for setting reaction chamber. In this research, we can overcome these troubles by adding glycerol in PCR mixtures in combination with fabrication of a thin Parylene-C film on the upper side of PDMS membrane.

3.3. PCR optimization for the Fluorescence detection

There are many methods for PCR amplicon detection in chip such as slab gel, fluorescence based detection and local surface plasmon resonance detection [33]. Among these methods, fluorescence-based technique is the most powerful and widely applicable, as it can be applied for both real-time and end-point detections [30]. In this research, we applied the fluorescence based end-point detection method, and we measured the intensity of each reaction chamber after completion of the PCR thermal cycling. The difference in fluorescence intensity between positive and negative samples helped us to determine whether chip-PCR was successful or not.

The experiment was performed using bench-top PCR system to find the optimal DNA polymerase concentration yielding the best fluorescence intensity after amplification. PCR mixture was prepared with different final concentrations of Taq polymerase, ranging from 0.025 U/μl to 0.25 U/μl. PCR product after 30 thermal cycles was diluted 40 × in distilled water. The fluorescence intensity measurement was performed using the F-4500 fluorescence spectrophotometer Hitachi (Japan). Figure 6 indicates increasing fluorescence intensity with higher polymerase concentration, ranging from 0.025 U/μl to 0.125 U/μl, and it saturates at 0.125 U/μl polymerase concentration. Thus we considered 0.125 U/μl of Taq polymerase as the optimal final concentration for fluorescence detectable PCR on our chip.

3.4. PCR with Glycerol

Bench-top real time PCR (7500 Real-Time PCR-Applied Biosystem, USA) was applied to observe the influence of glycerol on PCR. PCR mixtures with different concentrations of glycerol (0%, 5%, 10%, 15% and 20% (w/w)) were used. We observed no interference of glycerol on PCR efficiency, even at 20% (w/w) concentration. The fluorescence intensity and

the PCR cycle threshold (C_T) value were not significantly different among the five samples, as shown in Figure 7. Thus in our chip we have performed PCR using the 20% (w/w) of glycerol to induce low vapor pressure, which ultimately prevented the evaporation of reaction mixture.

3.5. On-chip PCR

On-chip PCR, bubble formation and sample evaporation are not the sole reasons for PCR amplification failure; the adsorption of polymerase enzyme onto the chip polymer surface also plays a key role in inhibiting the amplicon production. Previous studies showed the effectiveness of bovine serum albumin (BSA) to alleviate such problem [1, 31]. In this experiment, we coated the reaction chambers and the flow channels with 0.1% BSA solution for 4 hours.

The bubble formation and sample evaporation were circumvented by applying the combined efforts of changing the total vapor pressure and limiting the vapor run off through the PDMS. The use of glycerol with the PCR reaction mixture and a polymeric thin layer of Parylene-C on the interface of membrane for valve and air layer helped to reduce such bubble generation and sample evaporation.

The PCR mixtures with final concentrations of each component were as follows: 10 × PCR buffer solution, 200 μM of each dNTP, 3.5 μM of $MgCl_2$ solution, 300 nM of beta-Actin reverse primer, 300 nM of beta-Actin forward primer, 200 nM beta-Actin TaqMan probe, 0.125 U/μl of AmpliTaq Gold DNA polymerase, 20% (w/w) glycerol and 0.2 ng/μl human template DNA.

The sample was loaded into the reaction chambers and DNA amplification was performed. During PCR process, the valves were kept closed firmly to avoid sample leakage and cross contamination among the reaction chambers.

After 30 cycles, fluorescence images were taken from all the reaction chambers in chip. The obtained images were quantified by using free ImageJ software. Differences in fluorescence intensities were evident between positive and negative control, as shown in Figure 8.

Conclusions

We have developed a novel method for loading PCR samples into multi-chamber PCR chip without consideration of the shapes of reaction chamber and air trap during the sample loading

process. Compared to other methods, this method is very simple and applicable for practical use because of its ability to eliminate trapped air from the reaction chambers while loading the sample concurrently, without the need to integrate any complicated part into the chip.

By using glycerol, we successfully controlled sample loss through evaporation and bubble formation in our multi-chamber PDMS based PCR chip. Finally, PCR was successfully demonstrated on our chip using fluorescence microscopy as offline detection equipment.

This is, to the best of our knowledge, the first report on PDMS based PCR chip which took advantage of the gas permeability of PDMS for accurate sample loading combined with controlling the evaporation from small reaction volume in chip by using glycerol and Parylene-C coating. This approach can be applied to any chemical and biological analysis devices made of PDMS for liquid sample distribution.

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Figure Captions

Figure 1. The multi-chamber PCR chip platform; (a) On the left side, the structure of PCR chip with three layers: the air layer on the top, the thin Parylene-C - PDMS hybrid membrane for valve in the middle, and the flow layer at the bottom formed the PCR chip as shown on the right side; (b) The complete PDMS chip after fabrication; (c) The magnified reaction chamber with air jacket on the flow layer in chip.

Figure 2. Illustration of valve operation in chip. (a) The valves open as air is sucked out, the hybrid membrane gets vertically deformed and the sample moves from the inlet reservoir into the reaction chambers. (b) The valves close as air is compressed through the valve control port, and the hybrid membrane is bent down to separate the reaction chambers from the inlet reservoir.

Figure 3. Sample loading step in PCR chip under evacuation; air from the reaction chambers is sucked out through the PDMS wall, and the sample gradually flows into the reaction chambers to replace the lost air with time.

Figure 4. The dependence of loading rate of PCR mixture on the thickness of the PDMS wall between the reaction chamber and the air jacket.

Figure 5. Sample in reaction chamber ≈150 nl in volume (1mm in diameter and 200 μm in height) after 30 cycles of PCR run; (a) and (b) are both 20% by weight of glycerol in methyl green solution, before and after 30 cycles of PCR, respectively. The images were taken with Leica Microscopy at 10 × objective magnification.

Figure 6. The dependence of TaqMan probe based fluorescence intensity on polymerase concentration in tube PCR after 30 thermal cycles; (a) The fluorescence intensity was measured

from 40 × diluted PCR solution using fluorescence spectrophotometer; (b) Gel electrophoresis of PCR products with different polymerase concentrations.

Figure 7. Real time bench-top PCR with TaqMan probe under different concentrations of glycerol; (a) Delta Rn vs PCR Cycle number of negative control sample and positive control samples at 0%, 5%, 10%, 15% and 20% glycerol by weight. The C_T value remains nearly unchanged with different glycerol concentrations; (b) Gel electrophoresis of real-time PCR products with different glycerol concentrations after 40 thermal cycles.

Figure 8. Fluorescence intensity with TaqMan probe of negative sample (template DNA was replaced by nuclease-free water) and positive sample in chip after 30 thermal cycles of PCR; (a) and (b) represent negative and positive samples in chip respectively.

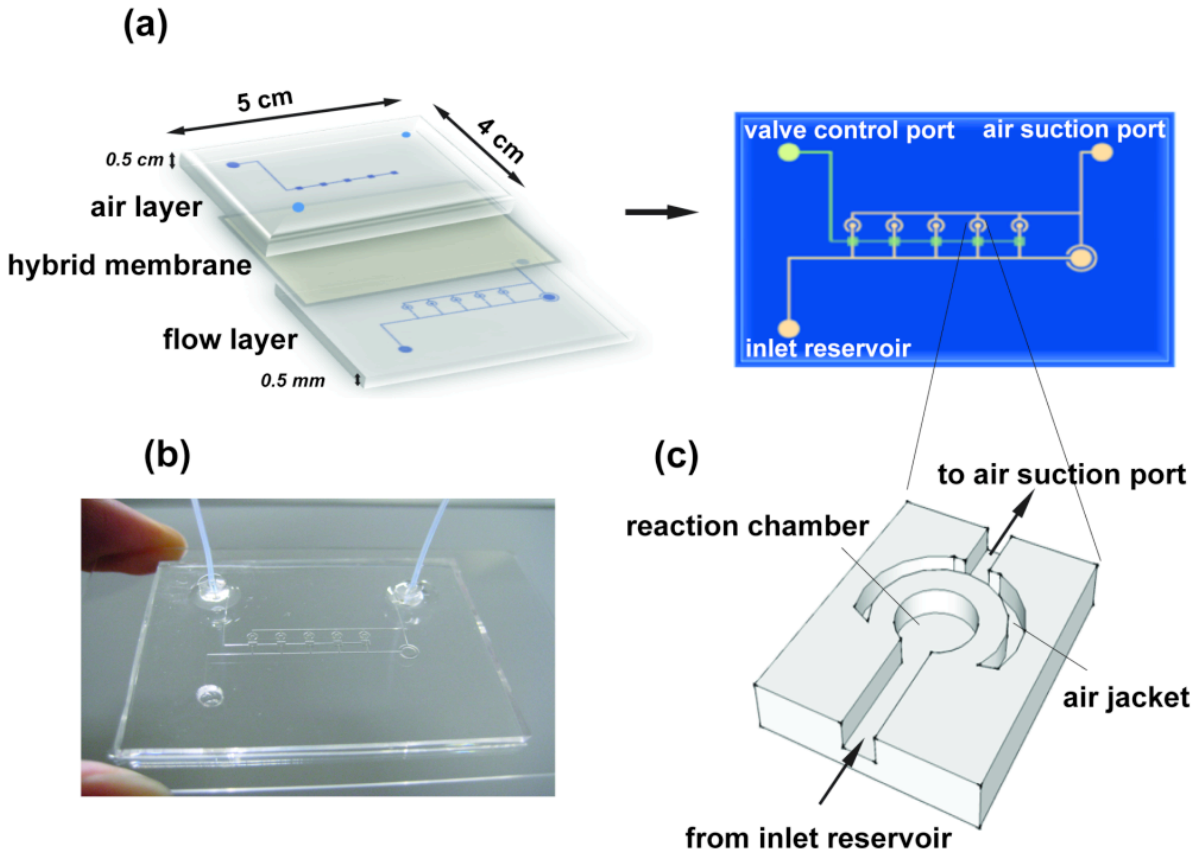
(a)
5 cm
4 cm
0.5 cm
air layer
hybrid membrane
flow layer
0.5 mm
valve control port
air suction port
inlet reservoir
(b)
(c)
to air suction port
reaction chamber
air jacket
from inlet reservoir

figure 1

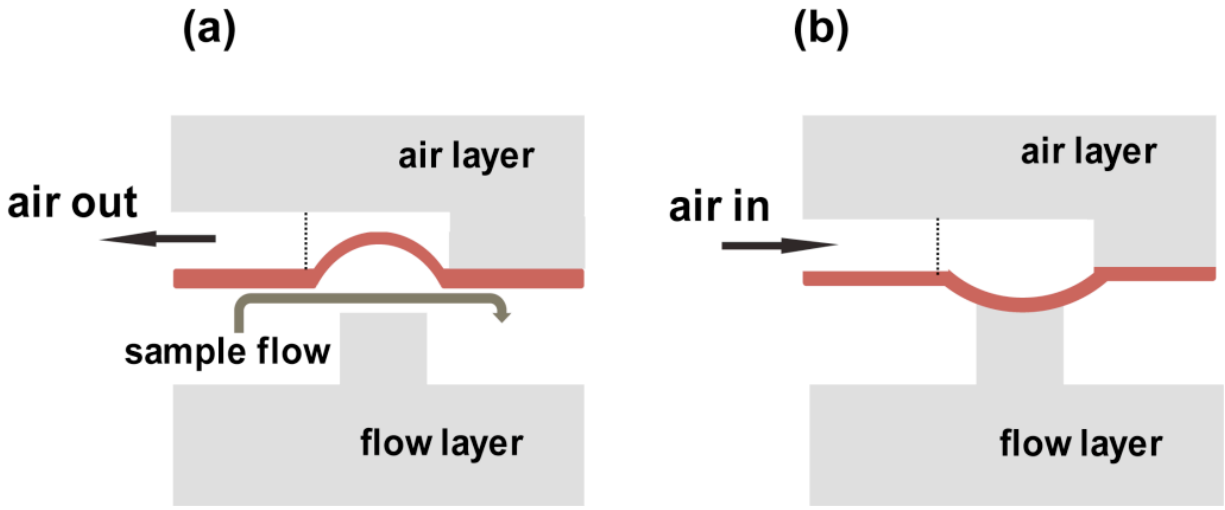
(a)
air layer
air out
sample flow
flow layer
(b)
air layer
air in
flow layer

figure 2

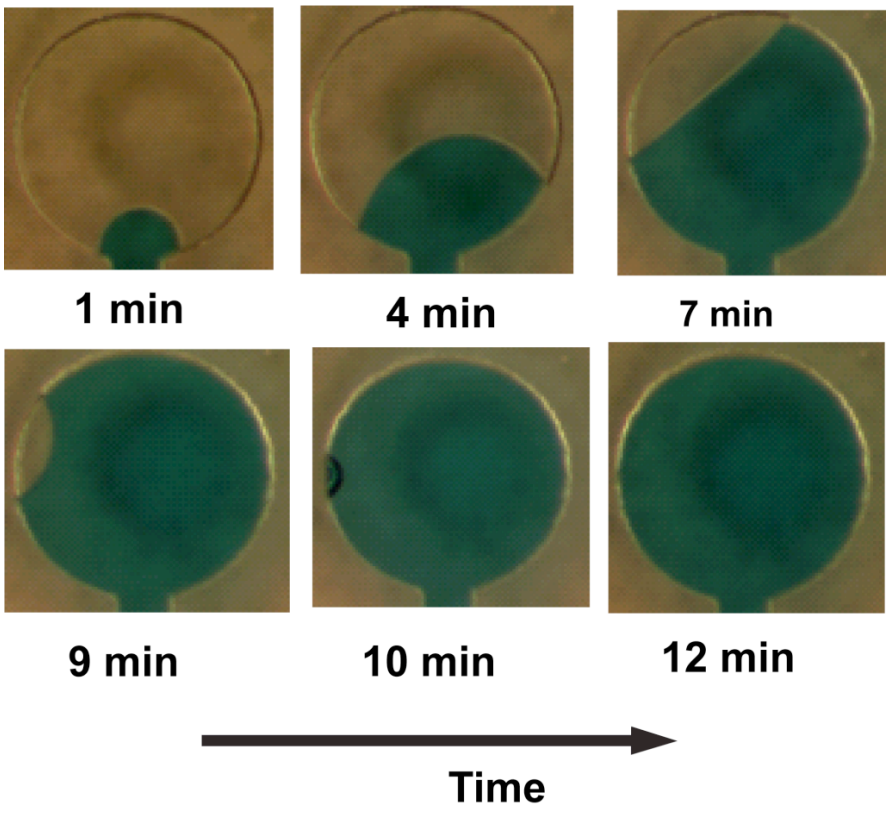

figure 3

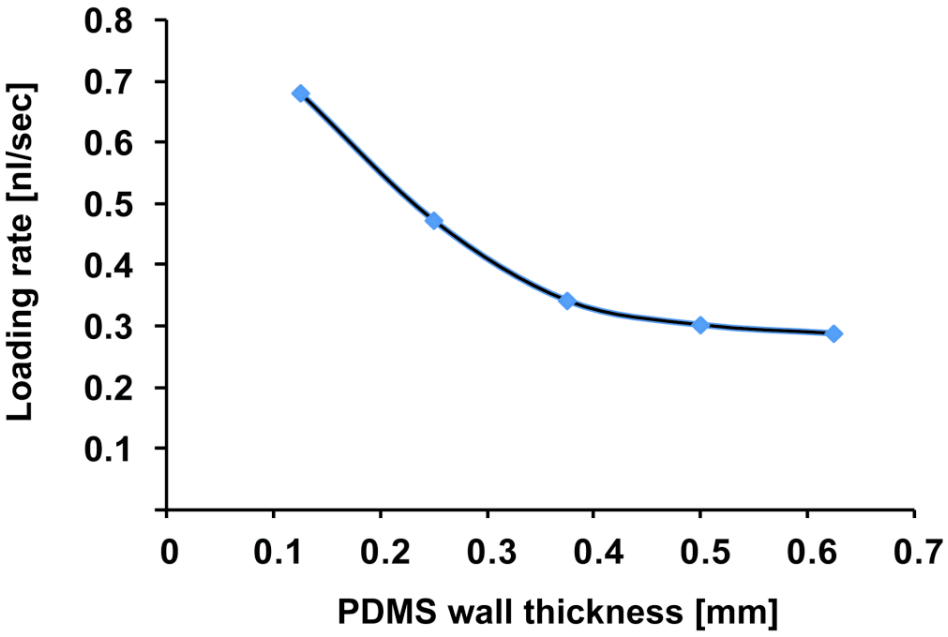
Loading rate [nl/sec]
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
PDMS wall thickness [mm]

figure 4

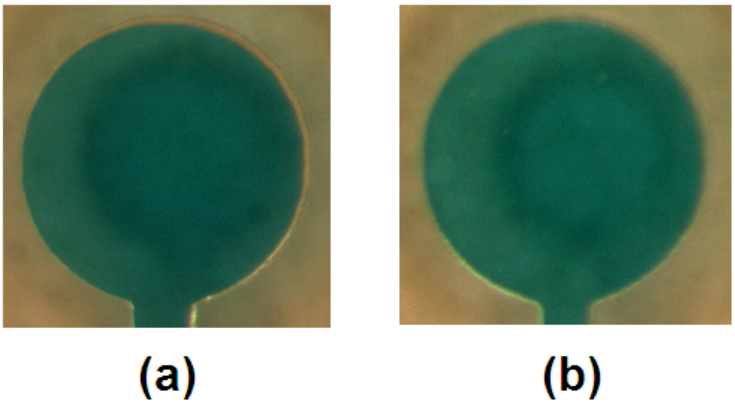

figure 5

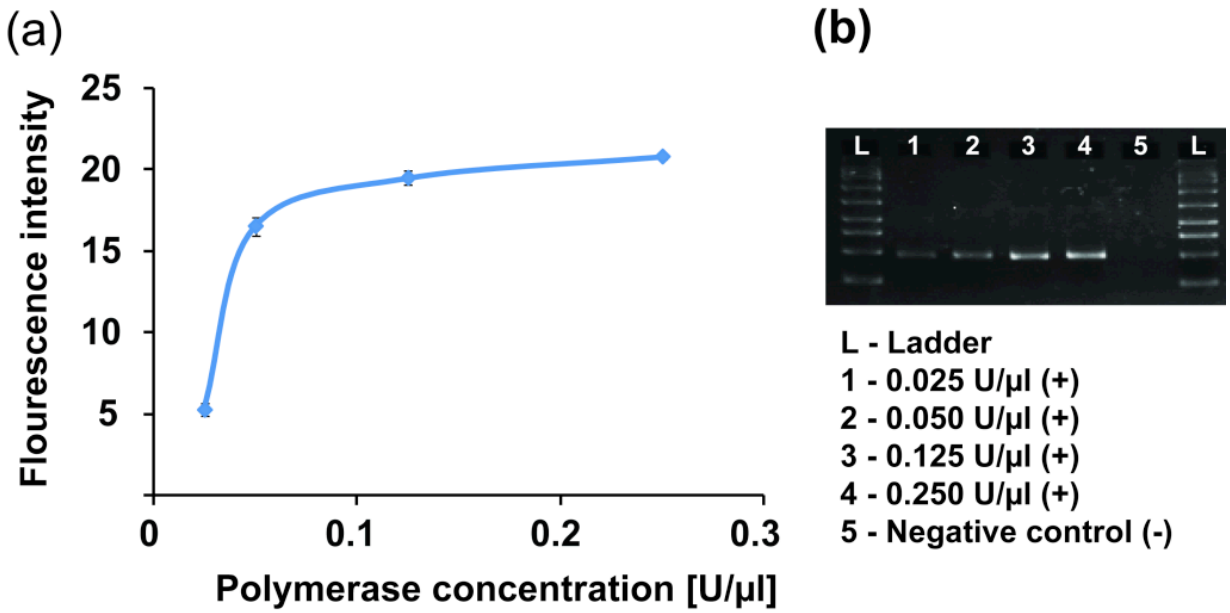
(a)
Flourescence intensity
25
20
15
10
5
0
0.1
0.2
0.3
Polymerase concentration [U/µl]
(b)
L 1 2 3 4 5 L
L - Ladder
1 - 0.025 U/µl (+)
2 - 0.050 U/µl (+)
3 - 0.125 U/µl (+)
4 - 0.250 U/µl (+)
5 - Negative control (-)

figure 6

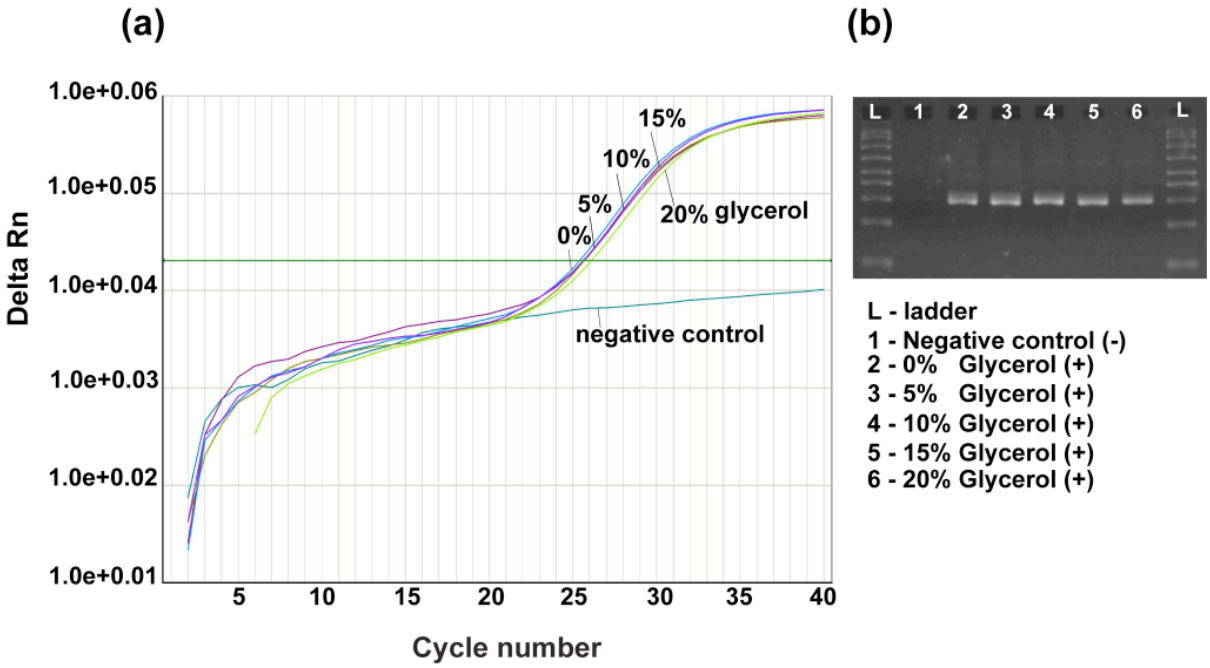
(a)
(b)
Delta Rn
1.0e+0.06
1.0e+0.05
1.0e+0.04
1.0e+0.03
1.0e+0.02
1.0e+0.01
5
10
15
20
25
30
35
40
Cycle number
15%
10%
5%
0%
20% glycerol
negative control
L 1 2 3 4 5 6 L
L - ladder
1 - Negative control (-)
2 - 0% Glycerol (+)
3 - 5% Glycerol (+)
4 - 10% Glycerol (+)
5 - 15% Glycerol (+)
6 - 20% Glycerol (+)

figure 7

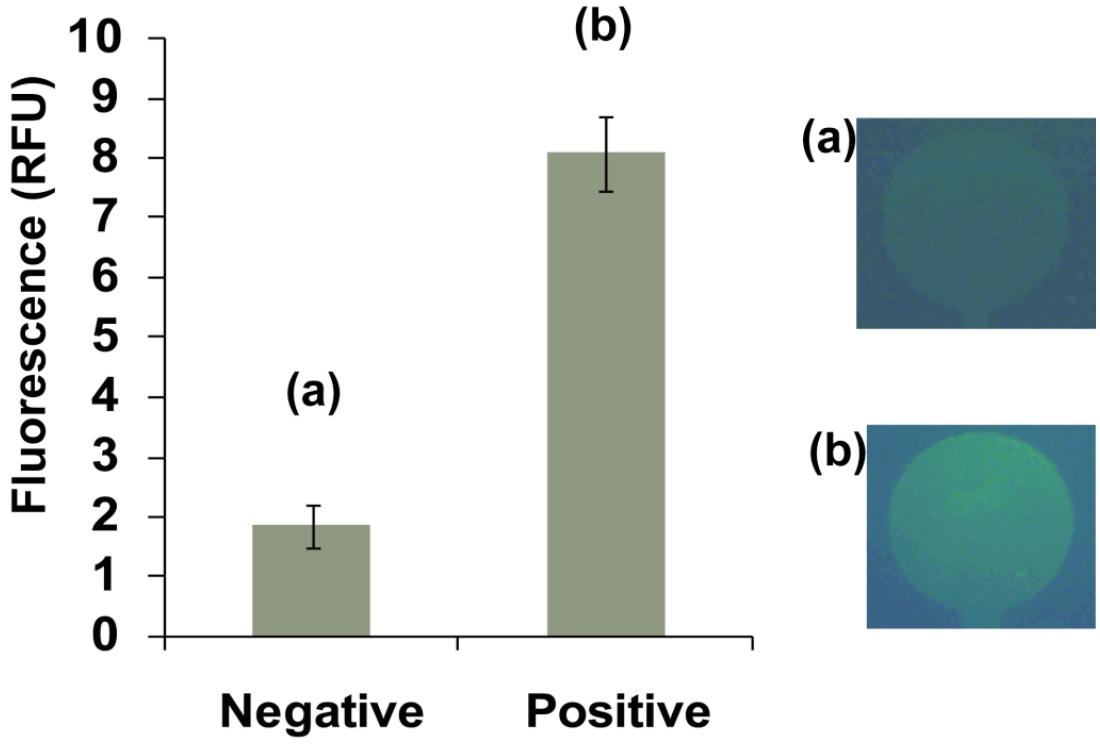

figure 8