

A portable DNA detector based on a multiple polymerase chain reaction (PCR) chip

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Abstract

This paper deals with the design of a multi-reaction PCR chip and a real-time quantitative PCR thermocycler instrument, as well as the evaluation of the instrument using IR-mediation for chip heating and cool air for chip cooling for the DNA amplification on the PCR chip. The fabrication of the PCR chip and the liquid loading procedure for the PCR mixture were investigated, and experimental tests DNA detection were carried out to evaluate the performance of the PCR thermocycler and multi-reaction chip in DNA amplification using both fluorescence detection and gel electrophoresis.

Keywords: Real-time PCR, DNA detector, PCR chip, polymerase chain reaction

1 Introduction

Nucleic-acid analysis using polymerase chain reaction (PCR) is one of the indispensable and powerful methods used in the areas of molecular and clinical diagnostics and forensic applications. Rapid advances in micromachining techniques promise better miniaturized, integrated, portable and high throughput PCR-based point-of-care devices. Currently the commercial thermal cycling instruments for PCR are restricted for laboratory use because of their bulky system design and large PCR mixture volumes used with the system. There is a great demand for portable real-time PCR based point-of-care devices which can perform rapid thermocycling at point of care and for field use. Recent development of miniaturized PCR instrument using biochips made the portable PCR instrument possible with a rapid PCR cycling due to reduced thermal mass [1, 2]. A portable nucleic acid analytical instrument based on micromachined silicon reaction chambers was first reported in 1998 [3], in which human β -globin gene was PCR amplified using fast cycling conditions (3.5 cycles/min). Most of these systems used thin film resistive heaters to heat the PCR mixture and the large thermal mass of these heating/cooling blocks, which limited the heating and cooling rates. A noncontact PCR thermocycling approach based on infrared (IR) radiation was first reported by Landers [4], in which 500 bp λ -phage DNA sequence was PCR amplified.

In this study, we describe the development of a portable DNA detector using a real-time quantitative PCR chip. A rapid thermocycling in our real-time PCR instrument was achieved by a combined use of an infrared (IR) radiation source and cool air flow. The performance of our IR-based real-time PCR instrument was evaluated by amplifying human β -actin gene fragment on the PCR chip.

2 Materials and methods

2.1 PCR Chip design

The proposed Polymerase Chain Reaction (PCR) chip design is mainly an array of multi-reaction thin-wall borosilicate glass capillaries. Capillaries offer a low-cost and easily available alternative for PCR mixture on-chip containment as opposed to PCR reaction chambers fabricated on chip by other means such as MEMS fabrication processes. Since capillaries can be disposable, the risk of contamination is further reduced. Depending on the diameter of capillary used, a single PCR chip can accommodate more than 30 capillaries. Sample volume ranges from 1 to 12 μ l, depending on the type of capillary used. Since the developed PCR thermocycler is suitable for any PCR chip of the standard glass slide format, hence the proposed design for the PCR chip is identical to the size of a glass slide.

The schematic layout of the PCR chip is shown in Figure 1. The capillaries have an internal diameter of 0.75 mm and have to be cut to the desired length of 18 mm. Therefore, each capillary contains approximately 7 μ l of PCR mixture.

2.2 PCR chip fabrication

A prototype of the PCR chip is fabricated, comprising of an array of 12 thin-wall capillaries (World Precision Instruments Inc., Sarasota, U.S.A). Even though the capillaries in an unopened vial are sterilized, additional preparations are required to remove organic impurities which may inhibit PCR. The glass capillaries were cleaned in piranha acid to remove metal ions and organic impurities present on the surface of the capillaries. Borosilicate glass capillary (World Precision Instruments Inc., Sarasota, USA) was first washed with acetone. Following this,

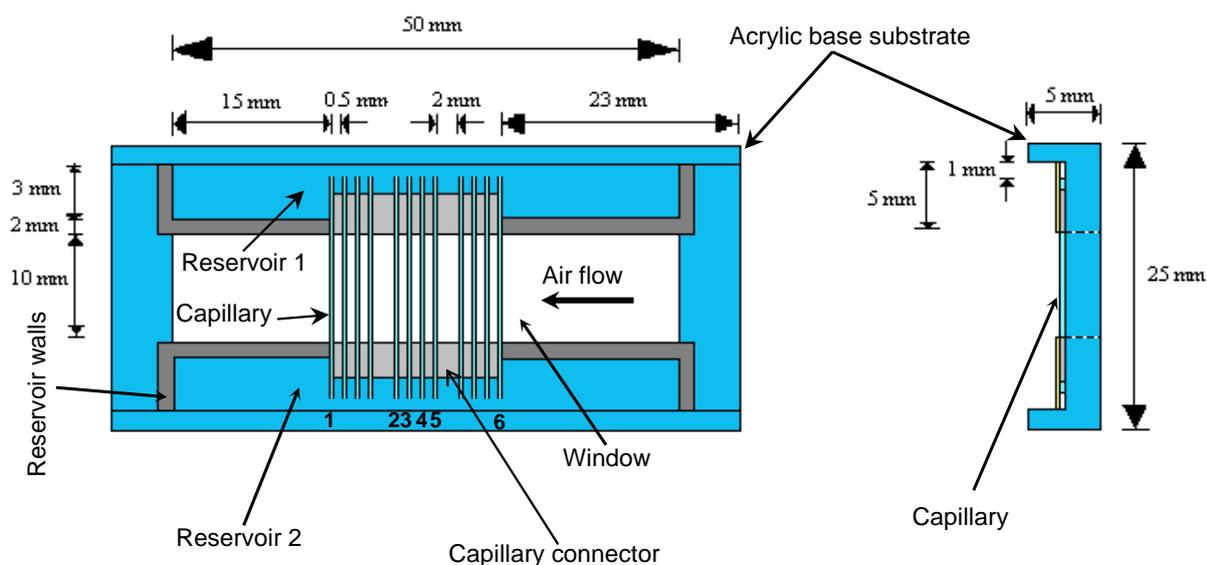


Figure 1: Schematic layout of PCR chip fabricated on an acrylic substrate.

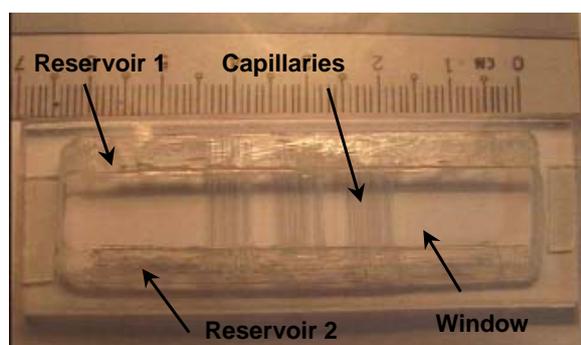


Figure 2: Photograph of the PCR chip showing channel 1 and 2 containing an array of 12 capillaries.

the capillaries were soaked in piranha solution (concentrated sulfuric acid mixed with Hydrogen peroxide at a volumetric ratio of 70:30) boiling at 120°C for 4 hours in the fume hood. The capillaries were then washed with de-ionized water overnight and then dried in an oven at 110°C.

After the surface treatment, the capillaries were ready for the fabrication of the PCR chip. Acrylic was chosen for the base substrate material, as it can easily machined and is commonly available. In addition, acrylic is a good thermal insulator and reduces thermal cross-talk between the capillaries. Figure 1 presents the dimensions for the base substrate. The substrate was fabricated by milling. Since the IR-transmitting lens is 50mm long and the diameter of the halogen tungsten light bulb is 10mm, an effective area of 50mm long and 10mm wide on the substrate can be heated by the light bulb. A window of the same size was cut from the substrate to allow optical detection of fluorescence emission from the PCR mixture by a linear image sensor. The window also facilitates the hot and cool airflow below the

capillaries. Before the positioning of the capillaries, the substrate was washed thoroughly with ethanol to remove any grease and other surface contaminants. Based on our numerical simulation, the capillary-to-capillary spacing of 0.5mm or less is preferred to achieve a good heating and cooling during the air thermocycling. Also, this spacing can depend on the intended user and be reduced to less than 0.5mm to accommodate more capillaries.

A 3M clear double-sided tape was applied to form the walls of the reservoirs on the chip, as shown in Figure 1. This tape was tested and it was able to retain its adhesion when in contact with water-based PCR solution and during elevated PCR temperatures. In addition, a small amount of silicone sealant was applied between the capillaries to form the capillary connectors. These connectors connected the capillaries to form a capillary array for multiple PCR and serve as the walls for reservoir 1 and 2 to prevent leakages. Lastly, a thin glass plate (20mm x 5mm x 0.1mm) was adhered to the top of the capillary using silicone sealant. Caution had to be exercised to prevent any sealant from entering the ends of the capillaries prior to sample loading. A photograph of the fabricated PCR chip is shown in Figure 2.

2.3 Operation of PCR chip

The PCR mixture was initially dispensed into the reservoir 1. Capillary action caused the PCR mixture to flow into the all capillaries in 2 minutes. During the PCR thermocycling, in the event if the capillaries end were not sealed properly, a loss of PCR mixture may occur by evaporation, or a cross-contamination between adjacent capillaries. Hence, a sealant is required to effectively seal the ends of the capillaries while capable of enduring intense thermal cyclic stresses from the PCR thermocycling.

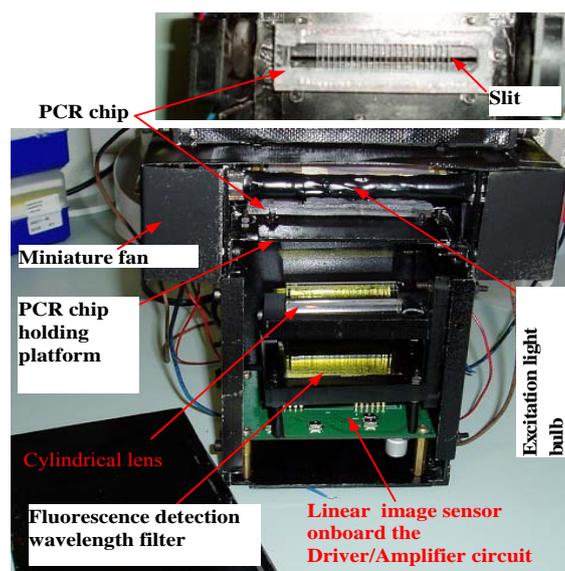


Figure 3: Photograph of the portable real-time quantitative PCR machine with the thermocycling and fluorometric module. The view of other components such as excitation wavelength filter, IR-transmitting lens holding assembly, cooling miniature fans and the halogen tungsten light bulb (heat source) are obstructed and are not seen in the photograph.

Polydimethylsiloxane (PDMS) (Dow Corning Corporation Midland, USA) elastomer was used to fulfill this function. a liquid containing 10:1 PDMS Sylgard Silicone Elastomer 184 and Sylgard Curing Agent 184 was mixed thoroughly and degassed in a vacuum chamber.

After the PCR mixture was loaded into the capillary array, the liquid PDMS was pipetted into the reservoir 1. The PDMS began to displace the PCR sample in the reservoir, subsequently the PDMS was pipetted into the reservoir 2 after 15 seconds. We found that the PDMS was unable to flow into the capillaries as the PCR mixture already occupied the capillaries. The PDMS sealing process completed in about 13 minutes and 33 seconds. Hence, it effectively sealed the capillary ends in reservoir 1. On the other hand, the PDMS was able to flow faster in reservoir 2, as there was no PCR mixture impeding its passage. Reservoir 2 was filled entirely with PDMS in 4 minutes and 1 second. To effectively seal the capillaries, the liquid PDMS sealant was partially cured at 65°C for 20 minutes.

2.4 Real-time quantitative PCR instrument

A prototype real-time PCR instrument for the PCR chips as shown in Figure 3 was constructed and tested; using an infrared (IR) mediated thermocycling. The PCR thermocycler is an automated and integrated system with multiple functions including thermal cycling control, real-time fluorescence detection, and data processing for real-time data analysis. Heating and cooling are realized by alternating infrared (IR) radiation heat transfer and forced convective cooling by a set of miniature fans. The thermocycler system

consists of two modules, which are the thermocycling and fluorometric module as well as the temperature control and data acquisition module. The entire system weighs less than 10 kg and is several times more compact as compared to many commercial systems.

2.4.1 PCR thermocycling and fluorometric module

This module comprises of the heating and cooling elements for PCR rapid thermocycling and the real-time fluorescence detection/imaging system. The photograph of this module is shown in Figure 3. The PCR mixture inside the capillaries was heated using a 500W Tungsten-halogen lamp (OSRAM HALOLINE, Philips Singapore). An IR-transmitting filter was used to reduce the occurrence of temperature gradients in the PCR mixture and also to prevent partial boiling of the PCR mixture. The PCR sample was cooled using fans. The optics of our real-time PCR was designed to fluorescense of SYBR Green I, a DNA intercalating dye. The SYBR Green I fluorophore was excited using another 150W Tungsten-halogen lamp (OSRAM HALOLINE, Philips Singapore). The long cylindrical design of the excitation source ensured uniform illumination of the PCR chip surface. The excitation light from the 150W Tungsten-halogen lamp was filtered using a narrow bandpass interference filter (488nm, Edmund Industrial Optics, Singapore), and the emission light from every capillaries was filtered using a narrow bandpass interference filter (520 nm, Edmund industrial Optics, Singapore) before being detected simultaneously by a NMOS linear image sensor (Hamamatsu Photonics, Japan). The NMOS linear

Plot of fluorescence intensity (FI) vs cycle number

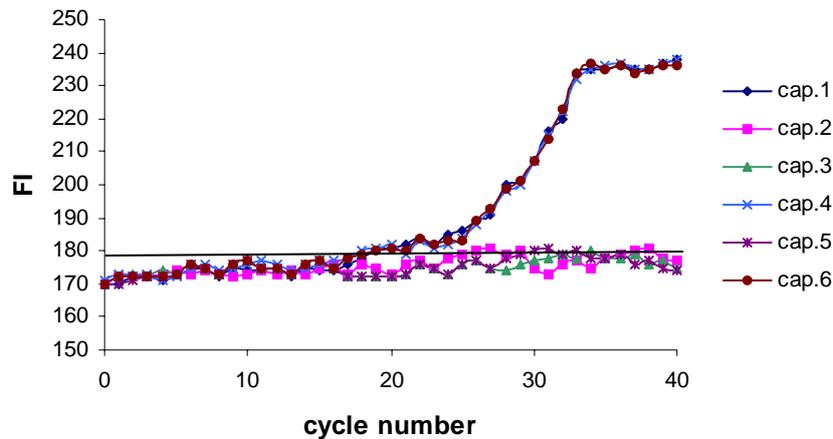


Figure 4: Amplification plot for β -actin gene fragment on the fabricated PCR chip using IR-based thermocycler. Capillaries at position 1 (Cap. 1), 4 and 6 contained positive PCR control. Capillaries at position 2, 3 and 5 contained negative PCR control without DNA template, without primer pair and without Taq DNA polymerase, respectively.

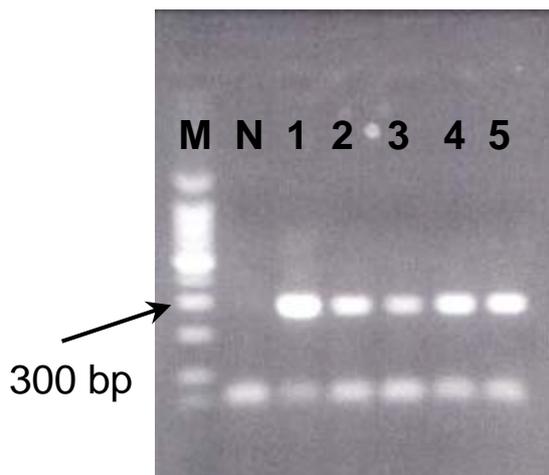


Figure 5: Agarose gel (1%) electrophoresis of amplification product from PCR in capillary at position 1 (lane 1), 4 (lane 2) and 6 (lane 3). For comparison, β -actin gene fragment from human genomic DNA was amplified on a commercial PTC-100 thermocycler in duplicates (lane 4 and 5). M is 100 bp molecular weight marker and N is negative control without DNA template.

image sensor is a self-scanning photodiode array designed specifically as detectors for multi-channel spectroscopy. The scanning circuit is made up of N-channel MOS transistors, operates at low power consumption and is easy to handle. Each photodiode has a large active area, high UV sensitivity yet low noise, delivering a high S/N even at low light levels. The current output type NMOS linear image sensors also feature excellent output linearity and wide dynamic range. The photodiode array sensor has a quantity of imaging 1024 pixels. A long slit (2mm

wide and 70mm long) was machined into the PCR chip holding platform (Figure 3) and serve as the detection window for fluorescence detection by the linear image sensor. The sensor is located beneath the platform and detects the fluorescence emission from the capillaries through the slit. A narrow slit also helps to ensure that the main bulk of light detected by the linear sensor are from the capillary array and interference from unwanted light sources are avoided. The detection window is situated at a distance of 3mm below the capillaries, which aids to ensure maximum intensity of the fluorescence to pass through the window.

2.4.2 Temperature control and data acquisition module

This module comprises of the PID temperature controller, the circuitry of solid-state (SS) relays and DC power supply, and lastly, the data acquisition and processing unit. The Watlow Series 982 vertical 1/8 DIN-ramping temperature controller (Watlow Electric, Missouri, USA) was selected as part of the temperature control and data acquisition module. Its primary analog input accepts up to 11 different thermocouple types, RTD or scalable process inputs. With a maximum of two event inputs, the SERIES 982 is capable of remote program start or hold functions and allows the operator to program a wait-for event.

A data processing unit (C8799 from Hamamatsu Photonics, Japan) converts video signals from the NMOS image sensor driver circuit into digital signals and transfers them to a PC. It has USB interface which allows easy connection to a PC for high-speed data communications at 12 Mbps. A solid-state relay

SSR) was used to conduct load current to the device, when the SSR is energized by the control output. The SSR can achieve very short cycle time of between ½ to 1 second. This will allow the PID controller, such as the Watlow ramping controller, to react rapidly to temperature fluctuations. In addition, without moving parts, the SSR is less likely to experience mechanical breakdown and generate less electrical noise during operations.

2.5 PCR experiment

A 300 bp of β -actin gene fragment was PCR amplified in the fabricated PCR chip. The sequences of forward and reverse primers were 5'-TCA CCC ACA CTG TGC CCA TCT ACG A-3' and 5'-CAG CGG AAC CGC TCA TTG CCA ATG G-3' respectively. The PCR mixture contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.2 mM each dATP, dCTP, dTTP and dGTP, 2 mM MgCl₂, 0.5 μ M each forward and reverse primer, 1 μ g/ μ l of BSA, 0.5 U/ μ l of Platinum Taq DNA polymerase (Invitrogen Singapore Pte. Ltd., Singapore), 4X SYBR Green I (Cambrex Biosciences, Maine, USA) and 0.37 ng/ μ l of human genomic DNA (Promega, Madison, USA).

To compare and verify the DNA amplification in the fabricated PCR chip, β -actin gene was PCR amplified on a commercial PTC-100 thermocycler instrument (MJ Research Inc., Waltham, USA) with the following thermocycling profile: Initial denaturation at 95°C for 2 minutes followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 55°C for 15 seconds and extension at 72°C for 15 seconds and a final extension step at 72°C for 10 minutes. The fluorescence of the SYBR Green I dye was measured at the extension step of every PCR cycle. The desired PCR product was confirmed by agarose gel electrophoresis.

3 Results and discussions.

In addition to the loading of the PCR mixture into reservoir 1, as described in section 2.3, we also conducted experiment to evaluate the effective area for temperature uniformity, PCR efficiency across the chip. In this experiment, the capillaries containing positive PCR control were placed at position 1, 4 and 6 (Figure 1) at both sides of the effective area (40 mm apart). The capillaries placed at position 2, 3 and 5 contained negative PCR control without DNA template, without primer pair and negative PCR control without Platinum Taq polymerase enzyme, respectively. To achieve the PCR mixture loading, the capillaries were preloaded with their respective PCR mixture before being fixed onto the PCR chip substrate. Following PCR solution loading into the capillaries, silicone elastomer was loaded into channel 1 to microfluidically seal all the capillaries under capillary action.

The complete filling of PCR mixture in the capillaries prevented PDMS from entering the capillaries. In addition to this, the geometric flow restriction at the end of the capillaries prevented the PCR mixture to flow into reservoir 2. The PCR chip was incubated at 65°C for 20 minutes to cure the PDMS sealant and rigidly seal the capillaries. The PCR chip was finally thermocycled on the fabricated IR-mediated thermocycler.

The DNA amplification in our chip was successful for positive PCR controls (Figure 4). The threshold cycle value (Ct) for amplification of the β -actin gene fragment is 17 cycles (Figure 4). It can be concluded from Figure 4 that the temperature is uniform across the capillaries from position 1 to 6. The capillary containing negative PCR control reaction (at position 2, 3 and 5) did not show any increase in fluorescence value, which indicates that there was no cross-contamination between adjacent capillaries during PCR process. For comparison, the β -actin gene fragment was PCR amplified on a commercial PCR instrument (MJ Research Inc. Waltham, USA) and the PCR product was analyzed using 1% agarose gel electrophoresis (Figure 5).

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5 References

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