Regeneratable PCR array chip

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**REGENERATABLE PCR ARRAY
CHIP**

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**SCHOOL OF MECHANICAL & AEROSPACE
ENGINEERING**

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REGENERATABLE PCR ARRAY CHIP

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SUMMARY

The development and applications of polymerase chain reaction (PCR) for DNA detection and quantification are becoming increasingly important in molecular diagnostics and drug development processes. Comparing to conventional PCR instruments, PCR microfluidic devices, or commonly known as PCR microchips, have many advantages such as reduced time, reduced sample and reagent consumption, high throughput, etc. However, most of the reported PCR microfluidic devices involved tedious PCR mixture loading process which required an expensive liquid dispensing robot. Furthermore, few efforts have been made to investigate the regenerative capability of PCR microfluidic devices which is crucial in continuous online detection of target samples. Thus a regeneratable PCR array chip was designed and tested in this thesis.

The regeneratable PCR array chip comprised of two key elements. The first element is an array of unsealed microreactors for simultaneous PCR analysis of multiple templates. Localized heating was used to contain the evaporative loss during PCR thermal cycling which reduced the complexity of using valves for the sealing of microreactors. The microfluidic operation of the PCR array chip was realized by capillary action, eliminating the need of liquid handling systems for liquid loading and microreactor isolation. Simultaneous PCR amplifications were performed on the chip using a pool of DNA templates. Detection limit of the PCR amplification on the PCR array chip was determined. Melting curve analysis and gel electrophoresis verified the successful amplifications of desired gene templates in the microreactors

without cross contamination.

The second element of regeneratable PCR array chip is the capability to allow repeated PCR amplifications in the microreactors on the PCR array chip by thoroughly eliminating carry-over contamination. In order to demonstrate the regenerative capability of the PCR array chip which is crucial to repeated online detection, the capillary-action based microfluidic operation of chip regeneration was proved to be feasible using a similar PCR array chip with two microreactors. The chip regeneration was realized by washing steps which decontaminated the microreactors between serial amplifications. Two washing buffers including DNA Away and propidium monoazide (PMA) were used in the washing steps. Experiments on the feasibility of chip regeneration using the above washing buffers were performed in PDMS-coated glass capillaries which simulated the microreactors of the regeneratable PCR array chip. Based on the developed cleaning protocols, both DNA Away and PMA were able to eliminate residual DNA from previous amplification thoroughly and thus eliminate the carry-over contamination without significant PCR inhibition. Repeated PCR amplifications and washing steps were performed and the robustness of the regeneration methods was demonstrated. Factors which could influence the cleaning efficiency such as DNA Away incubation time, PMA concentration, PMA dark incubation time and light exposure time were evaluated. Suggestions on future work were presented.

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Chapter 1 Introduction

1.1 Background

In the 1980s the concept of polymerase chain reaction (PCR) was first conceived by Kary Mullis and a team of researchers. This DNA amplification procedure allows the in vitro production of millions identical copies of a specific DNA in a simple enzymatic reaction. In 1991, Higuchi et al [1] firstly introduced the concept of real-time quantitative PCR in which a fluorescent reporter ethidium bromide (EtBr) was included in the amplification reaction. Its key feature is that the amplified specific sequence of a DNA sample is detected and quantified as the PCR reaction progresses. Since then, PCR has become an immensely powerful technique for providing essentially large quantities of precise genetic material that is needed in many bio-molecular applications such as DNA cloning for sequencing, functional analysis of genes, diagnosis of hereditary diseases, identification of genetic fingerprints and detection and diagnosis of pathogens. With the development of micro-electro-mechanical-system (MEMS) technology, miniaturization of PCR systems has been extensively studied. The miniaturized PCR instruments, or PCR microfluidic devices, are able to facilitate DNA amplification with much faster rates, lower reaction consumption and higher integration of heating/cooling, temperature sensing and detection devices.

1.2 Current problems

Most of the developed PCR microfluidic devices include sophisticated valves, pumps and liquid handling systems for the microfluidic operations such as

sample loading and microreactor isolation. Thus the complexity of device fabrication and operation is greatly increased. Another major disadvantage of most PCR microfluidic devices is lack of reusability. Carry-over contamination is the major cause that hinders the regeneration of PCR microchips, defying much of the advantages of miniaturized systems developed using expensive MEMS processing. The ability of continuous and repeated online detection using PCR microchips is also inhibited because of the lack of reusability.

1.3 Objective

The objective of this project is to design, fabricate and investigate a regeneratable PCR array chip which can facilitate automatic online monitoring of multiple genetic samples with the following features:

1. An array of PCR microreactors on a single chip which can achieve multiple PCR reactions of different target templates using different pre-deposited PCR primers simultaneously.
2. A network of microreactors and channels to implement capillary microfluidics for sample loading, microreactor isolation and microarray chip regeneration.
3. Regeneratable microreactors to allow continuous and repeated PCR amplifications on the same PCR array chip with no carry-over contamination.

1.4 Scope

In this project, a regeneratable PCR array chip has been developed and tested.

The scope of the project includes:

- Literature review on relevant PCR and MEMS technologies
- Design and fabrication of a regeneratable PCR array chip
- Evaluation of the performance of the regeneratable PCR array chip by specifically detecting DNA from a pool of templates
- Determination of the detection limit of sample DNA using the PCR array chip and real-time PCR system by Microgene
- Evaluation of the regenerative capability of the PCR array chip using washing buffers

1.5 Thesis organization

The thesis contains the following 6 parts. Chapter 1 presents an overall introduction of the entire project. Chapter 2 is dedicated to a literature review of PCR technology and PCR microfluidic devices. Chapter 3 presents the design and fabrication of the regeneratable PCR microarray chip. Methodology of PCR amplifications using the PCR array chip is also included. Chapter 4 illustrates microfluidic operation of chip regeneration and the methodology of using washing buffers to achieve regeneration. In Chapter 5, the results of PCR amplifications and evaluations of chip regeneration are shown. Chapter 6 concludes the current work and indicates the future direction of the project.

Chapter 2 Literature review

2.1 Introduction

Although technologies of PCR microarray chip are still in the research stage, their futures have been foreseen to be promising. This chapter will give a review from conventional PCR devices to the latest developments in PCR microarray technology and various regeneration techniques.

2.2 Polymerase Chain Reaction (PCR)

Deoxyribonucleic acid (DNA) is the genetic information in human stored in chromosomes [2]. The basic unit of DNA is nucleotide which consists of a base, a sugar linkage and a phosphate bridge. There are four types of nucleotides corresponding to four types of bases: adenine, guanine, cytosine and thymine, or known as A, G, C and T. Single-stranded DNA tends to link to another strand of complementary base pairs (A-T and G-C) through hydrogen bonds and thus a double DNA strand is formed. It is not uncommon to find that the concentrations of DNA samples are usually too low to be directly detected by any instrument [3]. Therefore, amplification before detection is a practical approach.

PCR is a technique to amplify a small number of DNA copies across several orders of magnitude, generating millions of copies of the same DNA sequence. The PCR method relies primarily on thermal cycling, which consists of cycles of repeated heating and cooling. As PCR progresses, the DNA generated is used as a template for replication itself, producing a chain reaction in which

the DNA template is exponentially amplified. Primers and DNA polymerase are indispensable in the amplification process. Primers, which are short DNA fragments containing complementary sequences to the target regions, initiate the DNA synthesis and determine the part of the target DNA to be amplified. DNA polymerase, such as Taq polymerase, assembles a new DNA strand from the nucleotides by using a single-stranded DNA as a template [4].

Most PCR methods use a number of alternating cooling and heating cycles, resulting in millions of identical DNA fragments [5]. Each PCR cycle usually has three temperature steps: denaturation step, annealing step and extension step. In denaturation step, reaction is heated to 94 °C to 98 °C, and hydrogen bonds between complementary bases are interrupted, yielding two single strands of DNA. In annealing step, the temperature is lowered to 50 °C to 65 °C to allow annealing of the primers to the single-stranded DNA template. The DNA polymerase then binds to the primer-template hybrid and begins DNA synthesis. During extension step, the temperature is usually around 72 °C, and the DNA polymerase synthesizes a new DNA single strand by adding dNTPs which are complementary to the template (Figure 2.1).

Figure 2.1 Principle of DNA replication in PCR.

At the end of PCR experiment, a sample of the reaction mixture is usually analyzed by agarose gel electrophoresis. Amplified DNA fragments can be visible as discrete bands staining with ethidium bromide.

There is an optimum temperature or temperature range for each stage of the PCR reaction depending on some factors such as template used, primer length and enzyme used. Precise maintenance of the optimum temperature is crucial to the success of PCR reactions. Insufficient heating is a common reason for PCR failure. Overheating during denaturing stage may cause the rapid evaporation of PCR mixture if the temperature is above the boiling point. During PCR thermal cycling, the annealing temperature is of great importance because it will influence the specificity of PCR reactions. If the annealing temperature is too high, primers and DNA templates will remain dissociated and hybridization will be inhibited, resulting in reduced yield of desired

product. On the other hand, if the annealing temperature is too low, mismatched hybrids will occur and the number of potential hybridization sites for each primer will increase. When the amplification occurs at non-target sites on template molecules, non-specific DNA fragments will be amplified. The ideal annealing temperature can be determined from the melting temperature of primer-template hybrid [6].

2.3 Real-time quantitative PCR

Conventional PCR has several limitations. In conventional PCR, gel electrophoresis is used for detection of PCR amplifications at the endpoint of PCR reactions. The endpoint detection is a very time consuming process. The results are based on size discrimination, which may not be very precise. The sensitivity of conventional PCR is low and the resolution of gel electrophoresis is poor. Thus conventional PCR is gradually replaced by real-time quantitative PCR which allows the detection of PCR amplification during the reaction and enjoys a higher precision of DNA quantification.

2.3.1 Fluorescence detection technology

Fluorescence detection technology is widely used in real-time quantitative PCR. Fluorescence is the molar absorption of light energy at one wavelength and its instantaneous emission at another wavelength [7]. There are several different methods of fluorescence detection in real-time quantitative PCR including SYBR Green method and TaqMan method.

SYBR Green is a dye that binds to the minor groove of double-stranded DNA.

When SYBR Green dye binds to double-stranded DNA, the intensity of the fluorescent emissions increases. As more double-stranded amplicons are produced, SYBR Green dye signal will increase. The increased fluorescence signal is analyzed against the background fluorescence level. Figure 2.2 below illustrates the SYBR Green working principle.

Figure 2.2 Principle of real-time PCR using SYBR Green.

As it does not need the specific designed probes, the SYBR Green method is an easy approach to real-time detection [8]. Melting curve analysis is often performed after the amplification.

TaqMan method, also referred to as 5'-Nuclease assays, is another widely used method of fluorescence detection in real-time PCR. TaqMan assays exploit the 5' to 3' exonuclease activity of Taq DNA polymerase. Each

reaction contains a gene specific primer and a fluorescently labeled TaqMan probe. The probe is designed to anneal the target sequence between the forward and reverse PCR primers. A fluorescent reporting dye is attached to the 5' end of the TaqMan probe and a quencher molecule is attached to the 3' end of the TaqMan probe. As the probes are usually less than 30 base pairs long, the reporter dye and the quencher are in close proximity and the quencher suppresses the fluorescence of the reporter dye. Thus almost no fluorescence can be detected. During amplification, Taq DNA polymerase cleaves the probe and displaces it from the target, allowing extension to continue. Cleavage of the probe separates the reporter dye from the quencher dye, resulting in an increase in fluorescence (Figure 2.3).

Figure 2.3 Principle of real-time PCR using Taqman method.

The increased fluorescence only occurs if the target sequence is amplified and is complementary to the probe, thus preventing detection of non-specific amplification. As the reporting dye is cleaved, melting curve analysis is not

possible. Comparing to SYBR Green method, Taqman probe assay has the advantages of higher specificity and lower detection limit [9].

2.3.2 DNA quantification by standard curve

The fluorescence is detected and quantified on real-time PCR. The intensity of the fluorescent signals is in proportion to the volume of the final PCR products at the end of each cycle. Thus, it is possible to monitor the PCR reaction at exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template by recording the amount of fluorescence emission at each cycle. In most cases, fluorescence is measured at the extension stage of the PCR cycle. After the curve of fluorescence vs. time is established, data is normalized to the account for differences in background fluorescence. One of the most important parameters is the cycle threshold (Ct) value. The Ct value is defined as the number of cycles required for the fluorescent signal to cross the threshold. Ct levels are inversely proportional to the amount of target nucleic acid in the sample. Larger Ct value suggests lower initial concentration of target and vice versa. With some known Ct values and corresponding target concentrations, a standard curve can be generated, from which the concentration of an unknown target can be determined.

2.3.3 DNA melting analysis

Melting curve analysis is an indispensable process which is usually performed after all the cycles of PCR are completed. The information gathered in melting

curve analysis can be used to infer the presence and identity of single-nucleotide polymorphisms. The energy required to break two complementary DNA strands depends on the strand length, GC content and their complementarities. The fluorescence decreases as the PCR products are heated at a constant rate of 0.1-0.2 °C/s in the melting curve analysis. The temperature at which the rate of change of fluorescence is greatest is defined as the melting temperature of the product [10]. By taking differential curves, melting temperature can also be represented as peaks. And different targets have their own specific melting temperature peaks. Thus melting curves can be used to examine the correct amplifications of desired templates.

2.4 PCR microfluidic devices

Conventional real-time PCR has some restrictions. The basis of most conventional real-time PCR is the intimate contact of PCR reaction tubes with a heating block. However, the heating block usually has a large heating capacity, which will limit the rate of sample heating and cooling. Generally speaking, conventional systems require a large volume of reaction mixture, usually bigger than 10 µl. Besides, reaction mixture is manually pipetted into PCR reaction tubes in conventional real-time PCR, and thus there is a high risk of contamination of samples. Furthermore, when dealing with a large number of samples, manual loading of samples could be tedious and time-consuming. Comparing to conventional PCR instruments, PCR microfluidic devices, or commonly referred as PCR microchips, have many improvements, including decreased time of DNA amplification, reduced consumption of biological samples, increased portability of PCR device and less cross contamination of the PCR reactions. In addition, PCR microchips allow large numbers of parallel

amplifications to be performed simultaneously and lead to more accurate information and greater understanding necessary for some particular bioassays which are difficult or unpractical to perform on conventional PCR devices [11].

2.4.1 Materials of PCR microfluidic devices

Most of the early PCR microchips were constructed from silicone as the substrate material. It is not surprising because the standard photolithography technique can be used conveniently to produce these substrates with microfluidic networks in order to perform PCR. In addition, the silicone material has superior thermal conductivity which allows rapid ramping of temperature. However, silicone as a substrate material will usually inhibit the PCR reaction by reducing the amplification efficiency and a thermal isolation of the silicone substrate is often needed to prevent the energy loss into surroundings due to its high thermal conductivity. Besides, silicone material is not transparent and thus limits the real-time optical detection of PCR reactions on the PCR microfluidic devices. Glass, on the other hand, has become an alternative substrate material in constructing PCR microchips. Glass possesses some advantages including superior optical transparency and well-defined surface chemistry [11]. However, PCR microchips manufactured from silicone or glass substrate usually have considerable cost of fabrication which hinders their uses in commercial applications. Currently many researchers have taken an interest in developing PCR microchips using polymer-based material, including polydimethylsiloxane (PDMS) [12]. There are several requirements for the satisfactory application of polymer substrates to the PCR microfluidics: Firstly, they must be thermally stable above 95 °C, as the PCR reaction has a

series of temperature cycles in the range of 55-95 °C. Secondly, they have good chemical resistance to some solvents necessary for a successful PCR reaction due to the high sensitivity and specificity of PCR reactions. Thirdly, they should not inhibit PCR reactions or eliminate PCR reagents by adsorption onto the surfaces of the reaction chambers. Among all the silicone rubber polymers, PDMS is one of the most promising materials and the soft lithography replica molding of PDMS devices is widely used in the PCR microchip fabrication [13].

2.4.2 Fabrication methods of PCR microfluidic devices

Fabrication of PCR microchips is closely related to the substrate materials. As a whole, fabrication methods can be classified into silicone/glass-based and polymer-based fabricating methods.

Most of the early works on PCR microchips employed the silicone/glass-based fabricating method which now has grown into more mature technologies. Fabrication of silicone/glass-based PCR microfluidics usually involves a series of micromachining process such as photolithography, film deposition, wet etching, etc. For example, Belgrader et al. [14] and Daniel et al. [15] used lithography and photolithography to construct networks of microfluidics on PCR microchips using silicone as substrates. Lao et al. [16] utilized etching and thermal oxidation to develop a PCR microfluidics using silicone substrate. Lagally et al. [17] and Fukuba et al. [18] employed etching and chemical vapor deposition to fabricate glass-based PCR microchips.

The polymer-based fabricating method is relatively new and is still developing. Generally speaking, all methods for fabricating polymers can be classified into two groups: replication methods and direct fabrication methods [19]. Replication methods include hot embossing, injection mould and casting. These methods normally make use of a template of master from which identical polymer microstructure can be made with precision. For example, Hong et al. [20] created SU-8 masters on glass wafers by an SU-8 thin film photolithographical technique and treated the master with fluorocarbon (CHF₃) in a reactive ion etching system with the purpose to facilitate the easy release of PDMS replica from the master after curing. PDMS microchips with chamber volume of 30-50 μ L were fabricated from the master. However, the limitation in height of SU-8 is a problem in developing deep reaction chambers. Yu et al. [21] developed PCR microchips with chamber volume of 25 μ L replicated from silicone master using PDMS injection molding. Cross contamination among chambers was avoided by inductively coupled plasma etching which created a deep 3-D silicone master. On the other hand, direct fabrication methods including laser ablation, plasma etching and X-ray lithography can direct construct desired microfluidic network on the polymeric surfaces. Yang et al. [22] and Liu et al. [23] used CO₂ laser ablation to fabricate PCR microchips directly on polycarbonate.

2.4.3 Bonding methods of microfluidic structures

All the fabrication methods mentioned above need the sealing of microchambers to form an enclosed structure. Various bonding techniques, such as anodic bonding, adhesive bonding and oxygen plasma bonding can be used to achieve the sealing of microchambers. Anodic bonding technique is a

method of permanently joining glass to silicone in the presence of an electrostatic field. This technique has the advantage of high stability at the interface of silicone and glass. Adhesive bonding is a process in which two materials are solidly and permanently assembled using an adhesive. This bonding method can be applied to silicone, glass and polymer-based substrates due to its advantages such as simplicity and low cost. Some widely used adhesives include epoxy, UV glue, PP tape, etc. The oxygen plasma bonding technique has been applied to enclose PDMS-based PCR microchips. This irreversible bonding is achieved by activation of the PDMS surface with oxygen plasma in reactive ion etcher (RIE) machine. PDMS has repeating units of $-\text{O}-\text{Si}(\text{CH}_3)_2-$. Polar groups, namely silanol groups ($\text{Si}-\text{OH}$), will be introduced by exposing PDMS to the oxygen plasma. These silanol groups can condense with other groups (for instance OH, COOH) on another surface when the two layers are in close proximity to each other [24]. The PCR microchips fabricated in this method can withstand high pressure.

2.4.4 Various designs of PCR microfluidic devices

In terms of PCR microchip design, most of the reported PCR microfluidics can be classified into two groups: flow-through PCR and chamber stationary PCR. In flow-through PCR microfluidics, the PCR solution continuously and repeatedly flows through three different temperature zones, which are necessary for PCR amplification. Chiou et al. [25] constructed a capillary based flow-through PCR microchip on which three heating blocks define denaturation, annealing and extension. 30 cycles of a 500 base-pair product were performed in 23min with 78% amplification efficiency. Although flow-through PCR microchips can complete PCR reactions faster due to less

cycling and heating time, there are some disadvantages including low efficiency and complicated microfluidic design. Another limitation of flow-through PCR microfluidics is the fixed number of thermal cycling which is dictated by the channel layout.

On the other hand, chamber stationary PCR microfluidics works in a similar manner as conventional PCR devices. The PCR mixture is kept stationary in the chambers and the temperature of the chambers is cycled. The first chamber stationary PCR microfluidic device was reported by Northrup et al. [26] where a microwell cavity structure acting as a PCR reaction chamber was fabricated by using silicone anisotropical wet etching and 20 thermal cycles were performed in the 50 μL microwell.

Most of the reported PCR-based microchips require tedious, manual PCR mixture loading into individual reaction chamber, and thus an expensive liquid dispensing robot is required. In order to achieve high throughput PCR on chip, Nagai et al. [27] developed a microchamber array on silicone substrate for picoliter PCR using manual loading sample step, and the amplification product was characterized by comparing the fluorescence intensity at the beginning and at the end of the PCR process. Matsubara et al. [28] developed a microchamber array in which DNA samples were loaded by dispensing robotic system and the PCR data was analyzed by using a DNA microarray scanner. Many studies in PCR microchips involve using of micropumps and valves for sample loading. Quake et al. [29] demonstrated the microfluidic distribution of 2 μL of PCR mixture into 400 independent chambers, using 2860 integrated hydraulic valves and pneumatic pumps. Mathies et al. [30] demonstrated multiple on-chip PCR amplifications with a microfluidic PCR mixture distribution using a mechanical

valve array for sample loading and chamber sealing. The using of valves and pumps on PCR microchips has greatly introduced complexity to the chip fabrication and operation processes. Each chamber on the PCR microchips needs to be sealed to prevent cross contamination. Although mineral oils and pressure-sensitive adhesive tapes have been successfully used to seal the chambers [31], most of the PCR microchips use valves to seal the microchambers [32] [33]. Thus, one of the major challenges in developing cost-effective, high throughput PCR microchips is to develop a non-robotic/micropump-less based manual liquid loading method to facilitate the development of point-of-care diagnostic device. The manual loading method includes the loading of DNA samples into reaction chambers in micro or nanoliter volume range and subsequent isolation of these chambers to prevent cross contamination. Ramalingam et al. [34] developed a PCR microarray harboring open or unsealed chambers and capillary microfluidics was implemented to load samples.

2.5 Regeneration of PCR microfluidic devices

Although PCR microchips have the advantages of low reagent consumption, high throughput and ability to integrate with various platforms, the microfluidic devices are usually fabricated using expensive technology, making them not affordable for single use. In addition, microfluidic device reusability is also a crucial part in achieving online continuous and automated detection of targets. Carry-over contamination in PCR microchips is the main reason to prevent reuse of the microfluidic devices. After DNA amplification, the chambers or microreactors of the microfluidic devices are contaminated with residual PCR products, and subsequent amplification on the same microfluidic device will be

contaminated, showing false-positive or false-negative results (a false positive results with the presence of nucleic acid contamination from previous amplified DNA while a false negative is the result of an unsuccessful PCR amplification due to contamination or inhibition from previous runs). In 2005, Dorfman et al. [35] demonstrated a high throughput system combined with reusability by a surfactant and oil emulsion technique. However, tedious reagent titration and expensive PCR protocol optimization were often required by using surfactants. Ranjit et al. [36] presented a PCR microchip fabricated on glass, and reusability was achieved by repeated surface silanizing of the glass PCR chamber to alter the glass surface from its virgin hydrophilic to hydrophobic. A ‘stripping and re-silanizing’ method was implemented to strip the silanization from the surface and re-silanize the exposed virgin hydrophilic glass surface to again form a hydrophobic coating between successive PCR runs.

Cynthia et al. [37] developed a renewable integrated microfluidic device for sample purification and subsequent PCR amplification. The renewable sample purification system utilized a rotating rod microcolumn to facilitate the purification of target DNA from sediment samples using microparticle matrix and solution from a kit (BIO 101 Inc., La Jolla, CA). After DNA purification, column packing materials including the microparticle matrix were ejected to waste and the renewable purification system was thoroughly cleaned by bleach and Roccoal detergent followed by Triton treatment. The PCR amplifications were performed within renewable Teflon PCR chambers and a cleaning protocol using DNA Zap (Ambion Inc., Austin, TX) and 0.1% Triton X-100 solution to decontaminate the chamber for serial amplifications was established. Carry-over contamination can be successfully contained and repeated amplifications up to 20 times can be performed on the chip.

So far there are few publications on the regeneration of PCR microreactors which are fabricated on PDMS substrate and employs simple microfluidic operations without the need of sophisticated liquid handling systems to perform parallel online and continuous detection of multiple target templates. Thus in this report, a regeneratable PDMS-based PCR array chip which harbored unsealed microreactors was designed and fabricated. Capillary microfluidics was implemented to facilitate the microfluidic operations and protocols for microreactor regeneration were established by using washing buffers.

Chapter 3 Methodology for PCR array chip design and operation

3.1 Chip material

As discussed in the previous chapter, there are different materials for fabricating PCR microfluidic devices. The regeneratable PCR array chip described in this thesis used polydimethylsiloxane (PDMS) as substrate material. PDMS is known to be one of the most attractive materials in fabricating microfluidic devices. It is transparent and has favorable optical properties for a fluorescence-based detection. PDMS is also cheap, biocompatible and non-toxic. Some of the important properties of PDMS are listed in the table below (Table 3.1):

Table 3.1 Important properties of PDMS [38]

Property	Value
Young's modulus	360-870 KPa
Poisson ratio	0.5
Tensile or fracture strength	2.24 MPa
Specific heat	1.46 kJ/kg K
Thermal conductivity	0.15 W/m K
Dielectric constant	2.3-2.8
Index of refraction	1.4
Electrical conductivity	$4 \times 10^{13} \Omega\text{m}$
Magnetic permeability	$0.6 \times 10^6 \text{ cm}^3/\text{g}$
Plasma etching method	$\text{CF}_4 + \text{O}_2$
Adhesion to silicon dioxide	Excellent

Biocompatibility	only mild inflammatory reaction when implanted
Hydrophobicity	Highly hydrophobic, contact angle 90-120 °
Freezing Point	< -25 °C

In this experiment, Sylgard 184 Silicon Elastomer obtained from Dow Corning Corporation, Midland, MI, USA was used to prepare PDMS. Curing agent and elastomer base were mixed thoroughly at 10:1 weight ratio. Then the PDMS prepolymer was vacuumed in a vacuum chamber to remove air bubbles. A piece of polymethyl methacrylate (PMMA), or common known as acrylic, was prepared and washed with ethanol. Vacuumed PDMS prepolymer was poured onto the acrylic sheet by control volume casting and a PDMS layer of 0.8mm was generated. Then the PDMS prepolymer was cured at 80 °C for 1 hour. The microfluidic channels were patterned on the PDMS layer by CO₂ laser ablation later as shown in Section 3.3.

3.2 PCR array chip design

The regeneratable PCR array chip described in this thesis comprises of 3 layers of PDMS bonded together. The median layer of PDMS, incorporating loading channels, microreactors, outlet channel, exit valves, primer loading channels, inlet bridges and outlet bridges, was fabricated on PDMS substrate by a laser cutting technique. A photograph of the prototype PDMS chip is shown in Figure 3.1(A). The chip contained six microreactors (microreactor 1, 2, 3, 4, 5, 6) connected to a common loading channel for target samples (positive control reactions), six microreactors (microreactor 7, 8, 9, 10, 11, 12) connected to another common loading channel for non-template control reactions (NTC), and a single microreactor for internal positive control reaction (IPC). The internal

positive control (IPC) reaction was included to identify PCR failures caused by failure of on-chip thermal cycling or optical instrument. Primer loading channels were connected to the microreactors to dispense primer pair liquors before the loading of PCR mixture. Different primer pair liquors corresponding to different target gene samples can be loaded into the microreactors and thus parallel detection can be performed simultaneously. The loading channels were used for distributing PCR mixture (without primer pairs) or washing buffers into the microreactors. During PCR mixture loading step, the mixture containing a pool of DNA templates flowed into the microreactors and mixed with the pre-loaded primer pair liquors. The outlet bridges were connected to the common outlet channel via exit valves which can prevent the liquid from entering the outlet channel during the PCR mixture or washing buffer loading step. After complete filling of the microreactors, the liquid samples inside the microreactors were isolated from each other by continuous removal of excess PCR mixture in the loading channels through sample removal ports. The outlet channel was used to remove the liquid inside the microreactors and bridge channels after the completion of PCR thermal cycling or washing step.

Figure 3.1(A) The structure layout of the regeneratable PCR reactor array comprising an array of thirteen microreactors. PCR mixture loading and isolation of the microreactors were achieved by capillary microfluidics.

The microreactors on the regeneratable PCR array chip were not sealed during PCR thermal cycling, and the sample evaporative loss in the unsealed reactors was well controlled by reactor design and localized heating of the PCR array chip. For the chip illustrated in Figure 3.1(A), all the channels and microreactors were 0.8mm high. The microreactors were 5mm long and 2mm wide, with volume of 8 μ l. The loading channels were 2mm wide and the outlet channel was 0.6mm wide. The inlet and outlet bridges were 0.75mm wide and 12mm long. The exit valves were 0.1mm wide at the opening to the outlet channel. The specific geometric design of the chip made it possible to use capillary action and passive valve effect to carry out the microfluidic

operations on the chip. The exit valve on the PCR array chip is designed based on the passive valve effect. The Hagen-Poiseuille equation for laminar flow governs the pressure drops in microfluidic systems. For a rectangular channel,

the pressure drop is governed by the equation: $\Delta P = \frac{12L\mu Q}{wh^3}$, where L is the

length of the microchannel, μ is the viscosity of the liquid, Q is the flow rate, w and h are the width and height of the rectangular microchannel, respectively.

For a given microchannel length L, a specific liquid with a certain viscosity μ and a fixed flow rate Q, varying the value of w and h can produce a pressure difference. In the microfluidic system used in this thesis, the microchannel height h is fixed. Thus an abrupt change in the width w of the microchannel causes a pressure drop at the point of restriction. For PDMS channel material (hydrophobic) used in this thesis, an decrease in channel width w causes a

positive pressure drop: $\Delta P = 2\Omega \cos \theta \left(\frac{1}{w_1} - \frac{1}{w_2} \right)$, where Ω is the surface

tension of the liquid, θ is the contact angle, and w_1 and w_2 are the widths of the two sections before and during the restriction. Therefore, by varying the microchannel width, the pressure drop can stop the liquid in hydrophobic microchannels demonstrated by the passive valve effect. In the PCR array chip described in the thesis, the width of the outlet bridge microchannel is 0.75mm and the width of the exit valve is 0.1mm. The vast width difference allows the liquid to stop at the exit valve. A detailed geometric dimension for one microreactor with bridges and channels were illustrated in Figure 3.1(B) below.

Figure 3.1(B) The schematic structure layout of one microreactor with detailed dimensions on the PCR array chip. All the measurements were in mm.

The width of the loading channel was bigger than the width of the inlet bridge and outlet bridge such that liquid would easily flow from loading channel into the inlet bridge and subsequently fill the microreactor by capillary action. The width of the exit valve was extremely small so that liquid will be stopped at the exit valve, without entering the outlet channel by passive valve effect. The width of the outlet channel was made smaller than the width of the inlet and outlet bridges so that liquid in the bridges and microreactor will be emptied first before it was emptied in the outlet channel during the liquid removal step by capillary action again.

3.3 Chip fabrication

The fabrication process of the PDMS array chip is elaborated in the following. Firstly, a PDMS median layer with a thickness of 0.8mm was prepared on an acrylic substrate by control volume casting (Figure 3.2A). A certain amount of PDMS prepolymer was weighed and degassed. From the density of PDMS from Table 3.1, the volume of the prepared PDMS was calculated. Depending on the acrylic dimension, the thickness of PDMS prepolymer was acquired when dividing the volume by the area of acrylic plate. Then the PDMS layer was cured at 80 °C for 1 hour. Secondly, a pulsed CO₂ laser (VersaLaser VLS 2.30) was used to generate the desired network of channels and reactors on the PDMS median layer (Figure 3.2B). Based on the thickness of PDMS layer, the laser power used was 0.6W, and the resolution setting was 300 pulses per inch (PPI). Higher laser power results in burning of the PDMS layer and lower laser power could not penetrate the PDMS layer thoroughly. Debris from the laser ablation was minimized by choosing the optimized PPI setting and removed by rinsing the PDMS layer with ethanol and DI water. After laser ablation, unwanted parts of the PDMS median layer were peeled off from the acrylic substrate. A 0.8mm thick PDMS cover layer was prepared and punched with holes by Harris Uni-Core puncher. The holes which were used for liquid loading had a diameter of 2mm. Next, a 20 µm thick layer of PDMS prepolymer was deposited on the PDMS cover layer by spin coating at 4000rpm for 1min (The spin coater was acquired from Laurell Technologie Corporation; Model Number WS-650S-LNPP/LITE). The cover layer was then bonded to the patterned median layer using the thin layer of PDMS adhesive by curing at 80 °C for 1 hour (Figure 3.2C). Then the acrylic substrate was removed (Figure 3.2D). A layer of PDMS prepolymer was

deposited on a piece of 0.1mm thick acid-washed borosilicate glass (Herenz Medizinalbedarf, Hamburg, Germany) by spin coating at 4000 rpm for 1min. The glass bottom layer was then bonded to the PDMS structure by curing at 80 °C for another 1 hour (Figure 3.2E). One important requirement of the chip fabrication is that the inner surface of the microreactors should be smooth. Otherwise air bubbles might be formed in the microreactors and purge the liquid out of the reactors due to bubble expansion during PCR thermal cycling. Thus PDMS prepolymer was used to coat the inner surface of the microreactors to prevent the bubble formation. PDMS prepolymer was loaded into the channels and microreactors by capillary action. After completely filling the channels and reactors, the PCR array chip was spun using the spin coater at 4000 rpm for 1min. A thin layer of PDMS layer was thus coated on the inner surface of the channels and microreactors (Figure 3.2F).

Figure 3.2 Fabrication of the PDMS-glass hybrid array chip. (A) Preparation of PDMS layer on acrylic substrate. (B) Fabrication of the patterned PDMS structure by laser ablation. (C) Bonding of cover layer and patterned median layer using spin coated PDMS prepolymer adhesive. (D) Removal of PDMS structure from acrylic substrate. (E) Bonding of PDMS structure and glass

bottom layer using spin coated PDMS prepolymer adhesive. (F) PDMS coating of the inner surface of microreactors.

As mentioned earlier, air bubble formation is a prominent problem with PCR chips during PCR thermal cycling. Once a bubble has been generated in the microreactor, in the DNA denaturation stage which has a temperature of 95 °C, the bubble can expand quickly and purge the PCR mixture out of the microreactor, causing PCR failure. Also bubbles inside the microreactor act as thermal insulators and introduce uneven temperature distribution in the sample.

The formation of air bubbles inside the microreactor is because of the presence of tiny air bubbles in the microreactor before thermal cycling. The presence of tiny air bubbles are related to the micro-features of microreactor surfaces which are determined by the chip fabrication processes. Generally speaking, tiny air bubbles are easily trapped inside the microreactors in the following two ways during PCR mixture loading process: Firstly, air bubbles are easily trapped in the wedges, which are formed by the PDMS sidewalls and flat glass bottom layer (Figure 3.3).

Figure 3.3 The formation of air bubbles which was caused by tiny bubbles trapped in a wedge of a microreactor. (A) A wedge was present at the bonding interface of PDMS sidewall and glass substrate. (B) Air bubbles were trapped in the wedge when the microreactor was loaded with PCR mixture. (C) Upon thermal cycling, air bubbles expanded and might eventually purge out the PCR mixture in the microreactor.

Secondly, air bubbles may be trapped inside the micro-cavities on the PDMS sidewalls of the microreactors (Figure 3.4). The rough PDMS surface including micro-cavities and pinholes on the PDMS sidewalls were generated during the laser ablation fabrication process.

Figure 3.4 The formation of air bubbles which was caused by tiny bubbles trapped in a micro-cavity on the PDMS sidewall of a microreactor. (A) A micro-cavity was present at PDMS sidewall of a microreactor. (B) Air bubbles were trapped in the micro-cavity when the microreactor was loaded with PCR mixture. (C) Upon thermal cycling, air bubbles expanded and might eventually purge out the PCR mixture in the microreactor.

When PCR mixture was loaded into the microreactors, air bubbles are trapped in wedges or micro-cavities. Upon heated, bubbles expand and purge the liquid out of the microreactors. Figure 3.3 schematically illustrates the formation of air bubbles which were caused by tiny bubbles trapped in a wedge. Figure 3.4 schematically illustrates the formation of air bubbles which were caused by tiny bubbles trapped in the micro-cavities on the PDMS sidewalls.

In order to inhibit air bubble formation, a bonding interface cladding technique and a surface coating method were used to reduce wedge formation and modify the surface of the microreactor. PDMS prepolymer was used as adhesive and spin-coated on the glass bottom layer. When PDMS layer and the glass layer were bonded, there was extra amount of PDMS prepolymer which acted as cladding material and filled the wedges of the microreactor to prevent the trapping of air bubbles (Figure 3.5).

Figure 3.5 PDMS cladding. (A) A wedge which was present at the corner of PDMS sidewall and glass substrate could introduce air bubbles. (B) Extra amount of PDMS prepolymer which acted as cladding material filled the wedge when a layer of PDMS prepolymer was used as adhesive to bond the glass substrate and PDMS structure.

The thickness of the spin-coated PDMS prepolymer was control by the spinning speed. If the speed was too low, the PDMS prepolymer (cladding material) became too thick, and the excessive cladding material might block the fine structures such as bridge channels. If the speed was too high, the PDMS cladding became too thin, and wedges were not filled completely. In the experiments described in this thesis, a 20 μ m thick PDMS cladding on the

spin-coated glass bottom layer was found suitable, which was obtained by spinning at 4000rpm for 1min at room temperature.

Surface coating was performed at the end of the fabrication process. The inner surface of the microreactors was coated by a layer of PDMS prepolymer. Micro-cavities and pinholes on the surface were filled. Thus the inner surface can be smoothened and the trapping of air bubbles can be avoided by this PDMS coating process. Figure 3.6 below illustrates the smoothening of PDMS sidewall by PDMS surface coating.

Figure 3.6 PDMS surface coating. (A) A micro-cavity which could introduce air bubbles was present on the PDMS sidewall of a microreactor. (B) Surface coating of the microreactor was performed using PDMS prepolymer and the PDMS sidewall was smoothened.

3.4 In-house real-time PCR instrument

A real-time PCR instrument Microgene for the PCR reactor array was used. The thermal cycling temperature of the PCR array chip was controlled by a thermal

electric cooler (TEC) with a dimension of 35mm * 35mm (Melcor Corp., Trenton, NJ, USA). A resistant temperature detector (RTD) which was mounted on the surface of the TEC was used as the feedback control to measure the temperature. The RTD was obtained from RS Components Singapore and had a probe dimension of 1.6mm*1.2mm. The thermal response time for the RTD was 0.1s. A 5mm wide and 4mm thick copper block was placed on top of the TEC surface to enable the localized heating. Mineral oil was used between copper block and TEC surface to facilitate heat conduction during PCR thermal cycling. A schematic drawing of the localized heating was illustrated in Figure 3.7.

Figure 3.7 The schematic drawing of TEC and PCR array chip to illustrate localized heating mechanism.

The optics of the instrument was designed to measure the fluorescence of SYBR Green dye used in the experiment. The SYBR Green fluorophore can be excited by using an array of blue LED (Marl International Ltd, Cumbria, UK) at 480nm and filtered by using a band pass filter (465-495nm, Chroma Technologies Corp, Brattleboro, USA). During the PCR amplification using the in-house real-time PCR system Microgene, the SYBR Green fluorophore was excited by the LED for 1.5 second in every PCR cycle and possible

photo-bleaching effect was minimal. The emission light from the microreactors was filtered by using another band pass filter (515-555nm, Chroma Technologies Corp, Brattleboro, USA) and detected by a CCD camera. Multiple functions such as thermal cycling control, data analysis and image processing were integrated in the system (Figure 3.8).

Figure 3.8 The schematic drawing showing various components of the in-house real-time PCR instrument Microgene.

A photograph shows the PCR instrument with a PCR array chip and a copper block placed on top of the TEC in Figure 3.9(A) and a close-up image illustrating the localized heating mechanism is shown in Figure 3.9(B). The temperature of the instrument was calibrated before PCR thermal cycling using a temperature sensor.

Figure 3.9 (A) In-house PCR instrument Microgene with a PCR array chip and a copper block placed on top of the TEC. (B) Localized heating mechanism.

3.5 Bacterial samples and PCR protocol

Four DNA templates were used to perform PCR amplifications on the PCR array chip. *Aeromonas hydrophilia* (AH), *Klebsiella pneumonia* (KP), *Staphylococcus aureus* (SA) and *Pseudomonas aeruginosa* (PA) were purchased from American Tissue Culture Collection (Rockville, USA). Genomic DNA of the bacterial cells was extracted using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). For internal positive control, we used human genomic DNA and primer pair from a commercial kit (β -actin Control Reagents (P/N 401846); Applied Biosystems, USA). The β -actin gene of human genomic DNA was chosen because its annealing temperature of the primer pair for β -actin gene was

similar as the annealing temperature of the primer pairs for the above four DNA templates. The sequences of forward and reverse primers for the DNA templates are listed in Table 3.2 below.

Table 3.2 Sequences of forward and reverse primers for the desired target organisms.

Target organism	Primer	Sequence (5'-3')	PCR product size(bp)
<i>Aeromonas hydrophilia</i>	Ahyd-F	GCCTTCTGCTATGACACG	466
	Ahyd-R	GGCTATCAGGATCTCGGT	
<i>Klebsiella pneumoniae</i>	Kpn-F	GTGCGATGCGGTCTTTG	398
	Kpn-R	GGGCGAACTGAACTGATG	
<i>Staphylococcus aureus</i>	Sau-F	AAACAAC TACAATCCCTTCA	319
	Sau-R	TACGGTTAGACTGCTTCG	
<i>Pseudomonas aeruginosa</i>	Pae-F	CCGACTGACGCCAACGA	211
	Pae-R	CGACCCTACCTCCCACGAAT	
Human genomic DNA (Internal positive control)	Beta-F	TCACCCACACTGTGCCCCATCTACGA	297
	Beta-R	CAGCGGAACCGCTCATTGCCAATGG	

Quantification of genomic DNA was done by using the PicoGreen DNA Quantitation Kit (Invitrogen) on TECAN GENios Plate Reader (Tecan, Mannedorf, Switzerland). Equal amounts (10ng/ μ l final concentration) of genomic DNA of the above bacterial cells were pooled together to be used as positive template for the PCR array chip. The positive control PCR mixture contained 10mM Tris-HCl (pH 9.0), 50mM KCl, 0.1% Triton X-100, 0.2mM each of dATP, dCTP, dTTP and dGTP, 3mM MgCl₂, 0.1U/ μ l of Platinum *Taq* DNA polymerase (Invitrogen, USA), 1 μ g/ μ l of BSA, 2X SYBR Green (Biotium, USA) and the pooled genomic DNA templates from the four pathogens (final concentration of each genomic DNA pathogens was 10ng/ μ l). In

negative control PCR mixture, equal amount of DI water was used to replace the DNA templates in the PCR mixture. The calibrated PCR temperature profile on the Microgene was illustrated in Figure 3.10 below.

Figure 3.10 Calibrated PCR temperature profile on in-house real-time PCR machine Microgene.

The PCR thermal cycling profile for the experiment consisted of initial denaturation at 95 °C for 10min, followed by 40 cycles of denaturation at 95 °C for 15 seconds, annealing at 55 °C for 15 seconds and extension at 72 °C for 15 seconds. The fluorescence of SYBR Green dye was measured at the 5th second into the extension step. Since the fluorescence intensity of the SYBR Green dye used in the experiments was always detected and measured in the extension stage of each PCR cycle at the same temperature of 72 °C, the possible change of fluorescence intensity due to temperature difference was eliminated. For melting curve analysis, the sample was heated from 50 °C to 99 °C and the SYBR Green fluorescence was measured every 1 °C.

3.6 PCR experiments on the PCR array chip

Amplifications of selected DNA templates were performed on the PCR array chip using the in-house real-time PCR instrument Microgene. Microreactor 1 and

7 (Figure 3.1) were loaded with forward and reverse primer pair liquor of PA. Microreactor 2 and 8 were loaded with forward and reverse primer pair liquor of SA. Microreactor 3 and 9 were loaded with forward and reverse primer pair liquor of KP. And Microreactor 4 and 10 were loaded with forward and reverse primer pair liquor of AH. Forward and reverse primer pair liquor of α -actin gene was loaded into the internal positive control (IPC) microreactor. The concentration of the loaded primer pair liquors was 10 μ M. The primer pair liquors were loaded into the microreactors through primer loading channel using syringe pumps. 1 μ l of primer pair liquors were dispensed into the respective microreactors through the primer loading ports which were connected to the syringe pumps by silicone tubing. Positive control (PC) PCR mixture containing the pooled DNA templates of PA, SA, AH, KP was loaded into the positive control loading channel. Due to capillary action, the PC mixture then filled Microreactor 1, 2, 3, 4 and mixed with the pre-loaded primer pair liquors in the microreactors. No-template control (NTC) PCR mixture without DNA templates was loaded into the no-template control loading channel. Due to capillary action, the NTC mixture then filled the Microreactor 7, 8, 9, 10. The internal positive control (IPC) PCR mixture containing α -actin gene template was loaded into the IPC microreactor. The PCR mixture stopped at the exit valves after filling the microreactors. Excessive mixture in the loading channels was removed through sample removal ports by syringe pumps and thus the microreactors were isolated. In order to evaluate the regeneratable PCR array chip, PCR amplifications were performed both in microfuge tubes on the commercial real-time PCR instrument Rotogene 3000 (Corbett Research, Sydney, Australia) and in the PCR array chip on in-house real-time PCR instrument Microgene. The desired PCR products from the Rotogene 3000 PCR instrument and the PCR array chip were confirmed by melting curve analysis and also on a capillary electrophoresis chip (DNA

Labchip 500) using Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The PCR amplifications were repeated three times on both in-house real-time PCR system Microgene and commercial PCR system Rotogene. And an average result (Ct value) was obtained.

3.7 Detection limit in the PCR array chip on Microgene

In order to determine the detection limit of the real-time PCR system Microgene by using the PCR array chip, decreasing concentrations of *Staphylococcus aureus* (SA) DNA was used as a sample DNA to be amplified on the PCR array chip. The SA DNA was added to the PCR mixture which contained 10mM Tris-HCl (pH 9.0), 50mM KCl, 0.1% Triton X-100, 0.2mM each of dATP, dCTP, dTTP and dGTP, 3mM MgCl₂, 0.1U/μl of Platinum *Taq* DNA polymerase, 1μg/μl of BSA and 2X SYBR Green such that the final sample DNA concentrations in the PCR mixture ranged from 100ng/μl to 0.1pg/μl. Specifically, the final SA DNA concentrations that were acquired from serial dilution were 100ng/μl, 10ng/μl, 1ng/μl, 100pg/μl, 10pg/μl, 1pg/μl and 0.1pg/μl, respectively. These different DNA concentrations were achieved by a 10 fold serial dilution and were confirmed by using PicoGreen DNA Quantification Kit from Invitrogen. The PCR amplifications of each specific DNA concentration was repeated in triplicate on the PCR array chip using Microgene and the average Ct value of the three measurements was taken. A negative control sample which had no DNA template of SA was run in parallel. In order to compare the detection sensitivity of the PCR amplification in the PCR array chip on the in-house real-time PCR system Microgene and conventional real-time PCR, amplifications were also performed in microfuge tubes on the commercial real-time PCR instrument Rotogene 3000. Each DNA concentration as mentioned above was run in triplicate as well on Rotogene.

The same PCR thermal cycling profile (as in Section 3.5) was used for both Microgene and Rotogene. The desired PCR products from the Rotogene 3000 PCR instrument and the PCR array chip were confirmed on a capillary electrophoresis chip using Agilent 2100 bioanalyzer. The PCR amplifications to determine the detection limit were performed three times on both in-house real-time PCR system Microgene and commercial PCR system Rotogene. And an average result (Ct value) was obtained.

Chapter 4 Regeneration of PCR array chip

The regeneration of PCR microfluidic devices is crucial in order to achieve continuous online detection of target samples and reduce the fabrication cost. However, carry-over contamination inhibits the reusability of PCR microfluidic devices. Subsequent PCR amplifications in the microreactors are contaminated by the residual PCR products from previous amplifications, generating a false positive or a false negative result. In this thesis, PCR array chip regeneration is realized by using washing buffers to decontaminate the microreactors between amplifications. The microfluidic operation of chip regeneration is solely achieved by capillary action and thus the complexity of the PCR array chip is greatly reduced.

4.1 Microfluidic operation of regeneratable PCR array chip

A PCR array chip of similar structure with two microreactors was used to illustrate the microfluidic principle of chip cleansing and regeneration. A schematic drawing of the PCR array chip with connected syringe pumps which were used for liquid loading and removal is shown in Figure 4.1. The A-A cross section view of the chip is illustrated in Figure 4.2.

The hydrophobic nature of PDMS layers prevents flow of pure water into the microreactors by capillary action. There are two methods to enable the capillary action in the hydrophobic PDMS channels and reactors. One of the methods is to use oxygen plasma to treat the PCR array chip. However the oxygen plasma treated PDMS surface will gradually change with time and the

surface will recover the hydrophobicity after a short while. The other method is to include Triton X-100 in the flow-in mixture. In the experiments illustrating the microfluidic operation of chip regeneration, 0.1% Triton X-100 detergent was added to DI water and it significantly reduced the contact angle, allowing the mixture to flow into the reactors and channels by capillary action. In order to visualize the flow of the liquid, blue dye, red dye and yellow dye were added to DI water representing preloaded primer pair liquor, PCR mixture and washing buffer, respectively. Syringe pumps (New Era Pump Systems Inc. USA) were connected to the primer loading channels for primer dispensing. Liquid (PCR mixture or washing buffer) was loaded into the loading channel or outlet channel by pipetting through the loading port at one end. A syringe pump which was used to remove the liquid was connected to the loading channel or outlet channel at the other end. The liquid pumping and withdrawing speed was set to 50 μ L/min.

Figure 4.1 The schematic drawing of the regeneration system including the PCR array chip with two microreactors and syringe pumps. Syringe pumps were connected for primer dispensing and mixture removal. The PCR array chip was used to illustrate the microfluidic operation of chip regeneration.

Figure 4.2 The A-A cross section view of the PCR array chip with two microreactors.

The PCR mixture loading and removal steps are shown in Figure 4.3. In Step A, primer pair liquors (represented by blue liquid) was dispensed into microreactors through primer loading channels by syringe pump A and syringe pump B. In Step B and Step C, PCR mixture (represented by red liquid) was introduced into the loading channel through the loading port by a pipette, and filled the microreactors in a sequential manner by capillary action. After filling the microreactors, the PCR mixture entered the outlet bridges and stopped at the exit valves due to the passive valve effect. Step D illustrates the removal of excess PCR mixture in the loading channel by syringe pump C. Following this, the microreactors were isolated from each other as illustrated in Step E. After the completion of PCR thermal cycling, PCR mixture was pipetted into the outlet channel through loading port as illustrated in Step F.

Upon filling the outlet channel, the PCR mixture was removed at one end of the outlet channel by syringe pump D. As shown in Step G and Step H, the microreactors, inlet and outlet bridges were emptied due to capillary action which was determined by the geometric design of the chip when the PCR mixture was removed from the outlet channel. After the microreactors and outlet channel were emptied, there was liquid remaining at the exit valves, as shown in Step I. A hair dryer was used to blow hot air at the exit valves for 1 min to evaporate the remaining liquid, as illustrated in Step J.

Figure 4.3 Microfluidic operations of PCR mixture loading and removal. (A) Primer pair liquor loading. (B) PCR mixture loading. (C) Filling of

microreactors with PCR mixture. (D) Excess PCR mixture removal. (E) Microreactor isolation. (F) Filling of PCR mixture in outlet channel. (G) Removal of PCR mixture (H) Microreactors emptied. (I) Liquid remaining at exit valves. (J) Liquid removed from exit valves.

After the chip was emptied, washing steps were introduced to remove the DNA residuals and eliminate contaminants in the channels and microreactors, as shown in Figure 4.4. In Step A, contaminated primer pair liquor (blue dye) was dispensed into the microreactors by syringe pump A and syringe pump B. Then washing buffer (represented by yellow liquid) was loaded into the loading channel through loading port. Due to capillary action, the washing buffer filled the microreactors and stopped at the exit valves, as illustrated in Step B and Step C. In Step D, excess washing buffer in the loading channel was removed by syringe pump C. Then the washing buffer was incubated inside the microreactors for a certain time. Next, washing buffer was loaded into the outlet channel through loading port in Step E. Upon filling the outlet channel, as illustrated in Step F and Step G, the washing buffer was removed at one end of the outlet channel by syringe pump D. The microreactors and outlet channel were emptied due to capillary. The remaining washing buffer at the exit valves was evaporated after a hair dryer was used to blow hot air at the valves, as shown in Step H.

Figure 4.4 Washing buffer loading and removal steps. (A) Dispensing of contaminated primer pair liquor into the microreactors. (B) Washing buffer loading. (C) Filling of microreactors with washing buffer. (D) Excess washing buffer removal. (E) Filling of washing buffer in outlet channel. (F) Removal of washing buffer. (G) Microreactors emptied. (H) Washing buffer removed from exit valves.

After the washing steps, a second round of amplification can be performed on the regeneratable PCR array chip. Figure 4.5 illustrates the microfluidic operation of loading primer pair liquor and PCR mixture into the regeneratable PCR array chip. In Step A, primer pair liquor was again dispensed into the microreactors through primer loading channels before the

loading of the PCR mixture. The primer pair liquor dispensed was not contaminated with DNA templates of previous amplification because contaminated primer pair liquor had been removed by washing buffer in the washing steps. In Step B and C, the PCR mixture was loaded into the loading channel for another round of PCR amplification.

Figure 4.5 Second round of PCR amplification performed on the regeneratable PCR reactor array. (A) Loading of primer pair liquor. (B) Loading of PCR mixture. (C) Filling of microreactors with PCR mixture.

4.2 Selection of washing buffers

In order to allow the chip regeneration and enable automated online detection, two methods were investigated to eliminate the carry-over contamination between serial amplifications. One method used DNA Away as cleaning agent and other method used propidium monoazide (PMA) as cleaning agent. In both methods, Triton X-100 was added to DI water as rinse solution.

DNA Away (Molecular Bioproducts Inc., San Diego, CA) is a widely used

surface decontaminant which is proved efficient in removing unwanted DNA contamination from laboratory instrument, glassware, pipettor barrels to bench-tops. This decontaminant can degrade DNA without having a significant residual effect on subsequent DNA samples. Some physical and chemical properties of DNA Away are listed in Table 4.1 below. DNA Away is stored in room temperature and used directly in the regeneration experiments.

Table 4.1 Some physical and chemical properties of DNA Away [39]

Appearance	Clear colorless liquid
Density	1 g/ml
Melting point	Around 0 °C
Boiling point	Around 100 °C
Solubility in water	Soluble
pH	11-12
Evaporation rate (water =1)	Around 1
Odor	Slight fragrant

PMA (Biotium, Inc. Hayward, CA) is a high affinity photoactive DNA binding dye. The dye is weakly fluorescent by itself but becomes more fluorescent after binding to nucleic acids. Upon photolysis, the photoreactive azido group on the dye is converted to a highly reactive nitrene radical, which readily reacts with any hydrocarbon moiety at the binding site to form a stable covalent nitrogen-carbon bond, thus resulting in permanent DNA modification. The dye is almost completely impermeable to cell membrane, and thus can be selectively used to modify exposed DNA from dead cells while leaving DNA from viable cells intact. This feature makes the dye highly useful in the selective detection of viable pathogenic cells by quantitative real-time PCR in

the presence dead cells whose DNA has been PMA-modified and thus could not be amplified. Due to its ability to interact with DNA to prevent it from being amplified in PCR reactions, PMA is used as the cleaning reagent in this regeneration experiment. For the convenience of storage and use, PMA was dissolved in 2% dimethyl sulfoxide (DMSO) (Sigma-Adrich, St. Louis, MO), distributed into 5 μ l aliquots into brown microfuge tubes, and then held at $-20\text{ }^{\circ}\text{C}$ until needed. To produce working solutions of different concentrations, different amount of sterile PBS was added to these microfuge tubes.

Triton X-100 is a nonionic surfactant which has a hydrophilic polyethylene oxide group and a hydrocarbon lipophilic or hydrophobic group. The hydrophobic group of the surfactant is absorbed onto the PDMS surface while the hydrophilic group sticks out into the buffer. Therefore the PDMS surface properties are changed and non-specific adsorption is minimized. It is commonly used as a detergent in biochemistry laboratories. Some properties of Triton are listed in Table 4.2. 0.1% Triton X-100 solution was prepared and used in the experiments as rinse solution.

Table 4.2 Some physical and chemical properties of Triton X-100 [40]

Molecular formula	$\text{C}_{14}\text{H}_{22}\text{O}(\text{C}_2\text{H}_4\text{O})_n$ ($n = 9-10$)
Appearance	viscous colorless liquid
Density	1.07 g/cm^3
Melting point	$6\text{ }^{\circ}\text{C}$
Boiling point	$> 200\text{ }^{\circ}\text{C}$
Solubility in water	Soluble
Vapor pressure	$< 1\text{ mmHg}$ (130 Pa) at $20\text{ }^{\circ}\text{C}$
Flash point	$251\text{ }^{\circ}\text{C}$

4.3 Regeneration experiment methods

In order to illustrate the feasibility of using DNA Away and PMA to remove residual DNA from the microreactor, 10 μ L glass capillaries coated with PDMS were used to mimic the microreactors. The PDMS-coated glass capillaries were prepared in the following manner: Firstly, PDMS prepolymer was loaded into the glass capillaries. Next, after the glass capillaries were filled, they were spun at 4000 rpm for 1min using a spin coater. Thus a thin layer of PDMS was coated on the inner surface of glass capillaries to simulate the microreactors on the PCR array chip. Finally, glass capillaries coated with PDMS were cured at 80 °C for 1 hour. For each PCR amplification performed in a PDMS-coated capillary on Microgene, a control test was run on commercial PCR instrument Rotogene. The 0.2ml PCR tubes used for Rotogene were also spin coated with PDMS prepolymer at a speed of 4000 rpm for 1 min. The PCR tubes were then cured at 80 °C for 1 hour. The PCR products of each round of amplification were validated by electrophoresis. *Pseudomonas aeruginosa* (PA) was used as DNA template in the regeneration experiments. The positive control PCR mixture contained the 10mM Tris-HCl (pH 9.0), 50mM KCl, 0.1% Triton X-100, 0.2mM each of dATP, dCTP, dTTP and dGTP, 3mM MgCl₂, 0.1U/ μ l of Platinum *Taq* DNA polymerase (Invitrogen, USA), 1 μ g/ μ l of BSA, 2X SYBR Green (Biotium, USA), 10 μ M forward and reverse primer pair liquor of PA and DNA template of PA (final concentration was 10ng/ μ l). In the case of negative control PCR mixture, equal amount of molecular biology water was used to replace the PA templates in the positive PCR mixture.

4.3.1 Method using DNA Away

To demonstrate the feasibility of chip regeneration, positive control and negative control PCR reactions were performed repeatedly in PDMS-coated glass capillaries. Three PDMS coated glass capillaries were used and positive control PCR mixture containing DNA sample (10ng/ μ l) was loaded into two capillaries (labeled Capillary A and Capillary C). Negative control PCR mixture without DNA sample was loaded into the third capillary (labeled Capillary B). Capillary A, B and C were then placed on the TEC of Microgene with copper block used for localized heating. Oil was used to facilitate heat conduction. After PCR thermal cycling, the mixture in capillaries was removed. Capillaries A and Capillary B were then washed with 0.1% Triton X-100 three times. Each time a pipette was used to perform the back and forth washing movement for 30 seconds. Then DNA Away was introduced into the capillaries and different lengths of incubation time were allowed. During incubation, Blu Tack, a reusable adhesive, was used to seal the capillaries with DNA Away inside to prevent evaporation of DNA Away solution. Decontamination was evaluated based on the lengths of incubation time. Next, the DNA Away was removed and capillaries were rinsed again with 0.1% Triton X-100 solution three times. Capillary C was only washed with 0.1% Triton X-100 solution for six times without using DNA Away. Then the next round of PCR amplification was performed using the capillaries. Negative control PCR mixture was loaded into Capillary A, B and C. In addition, two more fresh capillaries (without PCR amplification and washing) were used as internal controls. One fresh capillary was loaded with positive control PCR mixture while the other one was loaded with negative control PCR mixture. These control capillaries were used to confirm that the PCR mixture was free of possible contamination. In the following serial amplifications, two fresh capillaries were always used as

internal positive and negative controls. After the PCR thermal cycling, liquid in Capillary A, B and C were again removed and the abovementioned washing steps were performed. A total of 8 rounds of amplifications were carried out in Capillary A, B and C to demonstrate the robustness of reusability. Capillary A was used to perform alternating positive control and negative control PCR amplifications while Capillary B was used as a negative control to perform only negative amplifications. Capillary C was used to perform alternating positive and negative amplifications as Capillary A, but rinsed with only 0.1% Triton X-100 solution. PCR tubes coated with PDMS were used in Rotogene in accordance to the above serial amplifications. A table suggesting the serial amplifications is shown in the following (Table 4.3).

Table 4.3 Serial amplifications in the capillaries which were washed with Triton and DNA Away. ‘+’ indicates positive control PCR performed in the capillaries and ‘-’ indicates negative control PCR performed in the capillaries. One internal positive control and one internal negative control were performed in fresh capillaries.

Serial Amplification	Capillary A	Capillary B	Capillary C	Internal Control Capillaries
1	+	-	+	
	Washing steps using DNA Away and 0.1% Triton X-100		Washing steps using 0.1% Triton X-100	
2	-	-	-	+ and -
	Washing steps using DNA Away and 0.1% Triton X-100		Washing steps using 0.1% Triton X-100	
3	+	-	+	+ and -
	Washing steps using DNA		Washing steps	

	Away and 0.1% Triton X-100		using 0.1% Triton X-100	
4	-	-	-	+ and -
	Washing steps using DNA Away and 0.1% Triton X-100		Washing steps using 0.1% Triton X-100	
5	+	-	+	+ and -
	Washing steps using DNA Away and 0.1% Triton X-100		Washing steps using 0.1% Triton X-100	
6	-	-	-	+ and -
	Washing steps using DNA Away and 0.1% Triton X-100		Washing steps using 0.1% Triton X-100	
7	+	-	+	+ and -
	Washing steps using DNA Away and 0.1% Triton X-100		Washing steps using 0.1% Triton X-100	
8	-	-	-	+ and -

4.3.2 Method using PMA

The procedures of experiment using PMA was similar to the procedures using DNA Away. However, different concentrations of PMA solutions were used to investigate the cleaning effect. Three fresh capillaries were used and labeled A, B and C, respectively. Capillary A and Capillary C were filled with positive control PCR mixture containing DNA sample while Capillary B was filled with negative control PCR mixture without DNA template. Then Capillary A, B and C were placed on the TEC of the in-house real-time instrument Microgene. Copper block was used between capillaries and TEC to allow localized heating. After PCR thermal cycling, liquid in the capillaries were removed. Capillary A

and Capillary B were then rinsed with 0.1% Triton X-100 solution three times. Each time a back-and-forth movement was implemented for 30 seconds by using a pipette. PMA solution of a specific concentration was loaded into Capillary A and Capillary B. Then the capillaries were transferred into a light-tight black box (humidified with containers of water) and incubated for 10min. After incubation in the dark, the capillaries were placed on ice pad and exposed to an array of blue LED (480nm, 100W, Marl International Ltd, Cumbria, UK) for another 20min. The capillaries were placed at a distance of 3cm from the LED. Ice pad was used to reduce the temperature of the capillaries and thus prevent the evaporation of PMA solution inside the capillaries. Then PMA solution was removed and the capillaries were rinsed using 0.1% Triton X-100 solution three times. Capillary C was only washed with 0.1% Triton X-100 solution six times without using PMA. Then the next round of amplification was performed using the capillaries. Negative control PCR mixture was loaded into Capillary A, B and C. In addition, two more fresh capillaries (without amplification and washing) were used as internal controls. One capillary was loaded with positive control PCR mixture while the other capillary was loaded with negative control PCR mixture. These control capillaries were used to confirm that the PCR mixture was free of possible contamination. In the following serial amplifications, two fresh capillaries were always used as internal positive and negative controls. After the PCR thermal cycling, liquid in Capillary A, B and C were again removed and the abovementioned washing steps were performed. A total of 8 rounds of amplifications were carried out in Capillary A, B and C. Capillary A was used to perform alternating positive control and negative control PCR amplifications while Capillary B was used as a negative control to perform only negative amplifications. Capillary C was used to perform alternating positive and

negative amplifications as Capillary A, but rinsed with only 0.1% Triton X-100 solution. PCR amplifications in PDMS-coated tubes were performed in Rotogene in accordance to the above serial amplifications. A table suggesting the serial amplifications is shown below (Table 4.4)

Table 4.4 Serial amplifications in the capillaries which were washed with Triton and PMA. ‘+’ indicates positive control PCR performed in the capillaries and ‘-’ indicates negative control PCR performed in the capillaries. One internal positive control and one internal negative control were performed in fresh capillaries.

Serial Amplification	Capillary A	Capillary B	Capillary C	Internal Control Capillaries
1	+	-	+	
	Washing steps using PMA and 0.1% Triton X-100		Washing steps using 0.1% Triton X-100	
2	-	-	-	+ and -
	Washing steps using PMA and 0.1% Triton X-100		Washing steps using 0.1% Triton X-100	
3	+	-	+	+ and -
	Washing steps using PMA and 0.1% Triton X-100		Washing steps using 0.1% Triton X-100	
4	-	-	-	+ and -
	Washing steps using PMA and 0.1% Triton X-100		Washing steps using 0.1% Triton X-100	
5	+	-	+	+ and -
	Washing steps using PMA and 0.1% Triton X-100		Washing steps using 0.1% Triton X-100	
6	-	-	-	+ and -
	Washing steps using PMA and 0.1% Triton X-100		Washing steps using 0.1% Triton X-100	

Chapter 4 Regeneration of PCR array chip

7	+	-	+	+ and -
	Washing steps using PMA and 0.1% Triton X-100		Washing steps using 0.1% Triton X-100	
8	-	-	-	+ and -

Chapter 5 Results and discussions

5.1 PCR amplification results on the PCR array chip

The performance of the PCR array chip was demonstrated by detecting a pool of genomic DNA containing *Aeromonas hydrophilia*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. Positive PCR mixture containing the above DNA templates and no-template control (NTC) PCR mixture were loaded into the microreactors of the PCR array chip. Internal positive control (IPC) PCR mixture containing β -actin gene template was loaded into the internal positive control microreactor on the PCR array chip. PCR thermal cycling was performed on the in-house real-time quantitative PCR instrument Microgene. The amplification plot from the microreactors on the PCR array chip is presented in Figure 5.1.

Figure 5.1 PCR amplification plot on Microgene to detect Aeromonas hydrophilia, Klebsiella pneumoniae, Staphylococcus aureus and Pseudomonas aeruginos. β -actin gene was used as a internal positive control to detect any PCR failure caused by the system. NTC: no-template control; PC: positive control.

It is seen in Figure 5.1 that the PCR array chip can be successfully used for the simultaneous detections of multiple samples on the in-house real-time PCR instrument Microgene. Three PCR amplifications of the above four DNA templates were performed on Microgene. The average Ct values of the four DNA samples *Aeromonas hydrophilia*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Pseudomonas aeruginos* are 25.5, 25.8, 24.3 and 24.4 with a standard deviation of 0.6, 0.5, 0.5 and 0.6 respectively. The internal positive control β -actin gene has a average Ct of 17.1 with a standard deviation of 0.4. The repeated PCR amplifications on the PCR array chip suggested some consistent amplification results with a standard deviation of Ct values less than 1. There were no amplifications in no-template control microreactors, suggesting there was no cross contamination between microreactors on the PCR array chip. Melting curve analysis was performed to evaluate the purity of PCR product on Microgene (Figure 5.2). The melting curves of the four DNA samples and positive control template verified that the desired DNA templates were amplified on the PCR array chip using in-house real-time PCR machine Microgene. To evaluate the PCR on the PCR array chip, PCR amplification and melting curve analysis were also performed on commercial real time PCR instrument Rotogene. The Ct values of the sample DNAs were comparable to the results obtained using Microgene.

Figure 5.2 Microgene on-chip melting curve analyses to test the purity of amplified products. NTC: no-template control; PC: positive control.

The desired PCR products from the Rotogene 3000 real-time PCR instrument and the PCR array chip were confirmed on a commercial capillary electrophoresis chip (DNA Labchip 500) using Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The expected PCR product size was confirmed by comparing the size against an internal DNA ladder with lower marker (15 bp) and upper marker (1500 bp) (Figure 5.3). The results suggested that the regeneratable PCR array chip can be used to perform simultaneous detections of multiple DNA targets. Desired DNA targets were amplified in the respective microreactors while no cross contamination between microreactors was observed during the PCR amplifications. The on-chip no-template reactions can be used to monitor possible reagent contamination. And the on-chip internal positive control reaction can identify possible PCR failure caused by failure of thermal cycling instrument or optics.

Figure 5.3 Gel-like image of PCR product from the PCR array chip run on a DNA Labchip 500 using Agilent 2100 bioanalyzer. Lane L is the ladder. Lane 2,4,6,8 are no-template control reactions of *Aeromonas hydrophilia*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, respectively. Lane 1,3,5,7,9 are positive control reactions of *Aeromonas hydrophilia*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and internal positive control α -actin gene of human genomic DNA respectively.

5.2 PCR evaporative loss analysis in open microreactors during thermal cycling

Evaporative loss of PCR mixture is an important concern associate with unsealed microreactors. Evaporation loss incurred during PCR thermal cycling may lead to PCR failure. The evaporative loss during the thermal cycling stage is determined by a combination of factors such as the exposed surface area, temperature, the vapor pressure of the liquid, the type of the liquid (chemical nature of the liquid), etc. The eventual evaporative loss is the overall result of

all the above-mentioned factors. Increased surface area of the liquid can increase the liquid area from which liquid molecules can escape into the gas phase. Elevated temperature will increase the evaporation because liquid molecules have higher average energy at higher temperature which helps them to escape the liquid surface. The vapor pressure of the liquid, or the amount of the liquid molecules which are present in the gas phase near the liquid surface, affects the evaporation as well. In the case of water, the more water molecules in the air (higher humidity), the slower the evaporation. The strength of the intermolecular force between liquid molecules also determines the evaporation rate. Depending on the type of liquid, the stronger the force, the slower the evaporation. The PCR array chip described in this thesis is not sealed during PCR thermal cycling. Therefore in order to minimize the evaporation, the liquid surface temperature is lowered and the liquid surface area is reduced. The width of inlet bridge and outlet bridge on the two ends of the microreactor is reduced to 0.75mm in order to reduce the liquid surface area (0.6 mm^2) and therefore the liquid evaporation.

In addition to reducing liquid surface area, the localized heating mechanism can significantly reduce evaporation by lowering the liquid surface temperature. On the PCR array chip described in this thesis, the PCR mixture inside the microreactors was locally thermal cycled through a 0.1mm thick glass substrate on the copper block which was placed on the TEC surface of Microgene. Only the microreactors of the PCR array chip were in contact with copper block while the rest parts of the chip including inlet bridges and outlet bridges were not in direct contact with the TEC. Thus the inlet bridges and outlet bridges did not experience thermal cycling temperatures. Oil was used in between surfaces to facilitate heat conduction. It was found that during the denaturation step of the

PCR thermal cycling, when the microreactor was heated to a temperature of 95 °C, the temperature at both ends of inlet bridge and outlet bridge (liquid surface) which were 12mm away from the microreactor was measured at a maximum of 56 °C (room temperature is 22 °C) during the entire denaturation stage. The evaporation can therefore be reduced by increasing bridge channel length and lowering the liquid surface temperature.

By the combination of suitable chip substrate, optimized geometric design and localized heating method, the evaporative loss was reduced to less than 10% as shown in the following experiments, without implementing other thermal isolation and liquid replenishment methods which lead to complicated chip design, higher fabrication cost and prolonged fabrication time. With the use of PDMS and glass substrate in the fabrication of the PCR array chip, evaporative loss was greatly reduced. The evaporative loss in silicon based PCR microfluidic devices is much higher due to the higher thermal conductivity of silicon ($150 \text{ W m}^{-1}\text{K}^{-1}$) compared to glass ($1.4 \text{ W m}^{-1}\text{K}^{-1}$) and PDMS ($0.17 \text{ W m}^{-1}\text{K}^{-1}$). Thin bridge channels also helped to lessen the evaporation in the microreactors. The extent of evaporative loss in the microreactors is related to the length of the bridge channels (L).

In order to investigate the effect of bridge channel length on evaporative loss of PCR mixture in the microreactors, various lengths of bridge channels (0mm, 2mm, 5mm, 9mm, 12mm) were used during PCR thermal cycling. PCR array chips with each specific bridge channel length (0mm, 2mm, 5mm, 9mm or 12mm) was heated using the same thermal cycling profile as mentioned in Section 3.5. And for every bridge channel length, the evaporative tests were repeated six times. It was found that after 40 cycles, the average evaporative loss was 100% for microreactors with bridge lengths $L = 0, 2$ and 5mm, while the average

evaporative loss for microreactors with bridge lengths $L=9$ and 12mm was $35\% \pm 5\%$ and $10\% \pm 2\%$, respectively (Figure 5.4). The results in Figure 5.4 show that, in order to contain the evaporative loss to around 10%, the bridge channel length should be equal or greater than 12mm . Based on the results of Figure 5.4, the PCR array chip described in this thesis was designed with bridge channel length of 12mm . In theory, longer bridge channels are ideal, as they can better contain the evaporation. However, the upper limit of bridge channel length depends on the availability of space on the chip. For the PCR array chip harboring microreactors with bridge channel length $L=12\text{mm}$ in this thesis, the average evaporation loss was well contained to around 10%, even after 40 cycles, due to the chip design and localized heating mechanism.

Figure 5.4 Average evaporative loss in the PCR array chip with different bridge channel lengths. For reactors with bridge channel length $L > 12\text{mm}$, the average evaporative loss was less than 10% of the total volume of the PCR mixture.

5.3 PCR detection limit analysis on the PCR array chip

The detection limit of the in-house real-time PCR system Microgene was analyzed by amplifying a wide range of DNA concentrations on the PCR array chip. The final DNA concentrations used in the experiments were 100ng/ μ l, 10ng/ μ l, 1ng/ μ l, 100pg/ μ l, 10pg/ μ l, 1pg/ μ l and 0.1pg/ μ l, respectively. Three repeated PCR amplifications on each DNA concentration were performed and the averaged result was used. Figure 5.5 below shows the PCR amplifications of various DNA concentrations in the PCR array chip on Microgene.

Figure 5.5 PCR amplification results of various *Staphylococcus aureus* DNA concentrations on Microgene. The DNA concentrations had a range of 100ng/ μ l to 0.1pg/ μ l. A negative control which has no DNA template was run in parallel.

The amplification plot as shown in Figure 5.5 suggests that DNA samples with a concentration of 1pg/ μ l can be detected using Microgene. The average Ct values for the PCR mixtures with DNA concentrations of 100ng/ μ l, 10ng/ μ l, 1ng/ μ l, 100pg/ μ l, 10pg/ μ l and 1pg/ μ l are 14.9, 18.4, 22.5, 26.1, 30.3 and 34.4, with a standard deviation of 0.4, 0.5, 0.5, 0.6, 0.7 and 0.7, respectively. The Microgene amplification of DNA sample with a concentration of 0.1pg/ μ l was not detected by Microgene before 40 PCR cycles.

In order to compare the detecting sensitivity between Microgene and commercial real-time PCR instrument Rotogene, DNA samples of the same range of concentrations were amplified on Rotogene. And the amplification of each DNA concentration was repeated three times as well. The average Ct values for the Rotogene amplifications of the same DNA with concentrations from 100ng/ μ l to 1pg/ μ l fell into the range of 16.0-35.5. And no amplification was shown for DNA of concentration 0.1pg/ μ l before 40 cycles. The results suggested that the detection limit for Rotogene was also 1pg/ μ l, and the sample with a DNA concentration below 1pg/ μ l cannot be detected after PCR amplification. This result indicates that the detection limit on the in-house real-time PCR system Microgene using PCR array chip is comparable to the detection limit on commercial real-time PCR system Rotogene.

The PCR product of each amplification on Microgene was verified on a capillary electrophoresis chip using Agilent 2100 bioanalyzer. Figure 5.6 below shows the gel-image of PCR products after amplification of sample DNAs with various concentrations. It is seen in Figure 5.6 that the product bands (Line 1 to Line 6) for PCR amplifications of DNAs with concentrations

from 100ng/ μ l to 1pg/ μ l are shown clearly while the band (Line 7) for PCR product from amplification of 0.1pg/ μ l DNA is not shown. Thus it confirms the PCR detection limit of sample DNA concentrations.

*Figure 5.6 Gel-like image of PCR product from the PCR array chip run on a DNA Labchip 500 using Agilent 2100 bioanalyzer. Lane m is the ladder. Lane 1 to Lane 7 are the products of PCR amplifications on *Staphylococcus aureus* DNA samples with concentrations of 100ng/ μ l, 10ng/ μ l, 1ng/ μ l, 100pg/ μ l, 10pg/ μ l, 1pg/ μ l and 0.1pg/ μ l, respectively. Lane n is the product of negative control PCR reaction.*

5.4 Regeneration using DNA Away

5.4.1 Evaluation of regeneration using DNA Away of various incubation periods

DNA Away was used to clean the glass capillaries after PCR amplification. The capillaries were rinsed with Triton and washed with DNA Away as described in Section 4.3. Different lengths of incubation time were allowed

and decontamination results were analyzed and compared. Four different incubation periods of DNA Away in glass capillaries were investigated: 0.5 hour, 1 hour, 2 hours and 4 hours. A capillary which was only washed using 0.1% Triton X-100 was used in the experiment as a control. Positive control PCR mixture contained *Pseudomonas aeruginosa* template of concentration 10ng/μl while negative control PCR mixture was free of DNA template. After amplification of the positive control PCR mixture in the capillaries, DNA Away washing steps were performed with different incubation periods on different capillaries. Then the capillaries were used for amplification of the negative control PCR mixture. If DNA Away was not able to thoroughly decontaminate the capillaries and residual DNA from previous positive control amplification was present, the negative control PCR mixture would be contaminated and generated a false positive result. For each incubation time, the PCR amplification was repeated three times and the averaged Ct value was taken. The following figure (Figure 5.7) shows the Microgene results of negative control PCR amplification in the capillaries in which positive PCR amplification was performed followed by washing steps using DNA Away.

Figure 5.7 Negative control PCR amplification plot to evaluate the decontamination effect of various DNA Away incubation time. Negative PCR amplification was performed in the capillaries in which positive control amplification were performed followed by Triton rinsing and DNA Away washing steps. During the washing step, four different lengths of incubation time of DNA Away including 0.5h, 1h, 2h and 4h were allowed. A capillary which was only rinsed by Triton was used as control.

The average Ct values of negative control PCR amplifications in the capillaries in which DNA Away was incubated for 0.5 hour, 1 hour and 2 hours are 18.1, 26.3 and 34.1, with a standard deviation of 0.4, 0.4 and 0.6 respectively while there was no amplification of the negative control PCR mixture in the capillary in which DNA Away was incubated for 4 hours. The average Ct value of the PCR amplification in the capillary which was washed only using 0.1% Triton X-100 is 13.5, with a deviation of 0.4. Thus it was clear that DNA Away had the capability to eliminate residual DNA. However, the above results suggested that the capillaries in which the DNA Away was

incubated for 0.5 hour, 1 hour and 2 hours were not completely decontaminated and DNA residual from previous positive amplification was still present inside the capillaries. When DNA Away was incubated in the capillary for 4 hours or longer, the capillary was free of DNA carry-over contamination from previous run. Melting curve analysis was performed to ensure the amplification of the desired template in the capillaries. The PCR curve and melting curve on Rotogene were used to confirm the results. The PCR product of each amplification was verified on a capillary electrophoresis chip using Agilent 2100 bioanalyzer.

5.4.2 Robustness of regeneration using DNA Away method

In order to establish the robustness of the regeneration method using DNA Away, alternating positive control and negative control PCR experiments were repeatedly performed in the same capillary as suggested in Table 4.3. DNA Away was incubated in both Capillary A and Capillary B for 4 hours during the washing steps. The PCR amplification curves of 8 serial amplifications in Capillary A on Microgene are illustrated in Figure 5.8. Ct values of PCR amplifications in Capillary A, Capillary B and internal control capillaries are listed in Table 5.1 for comparison.

It can be seen that DNA was absent in the negative control PCR experiments (serial amplification 2, 4, 6 and 8) in Capillary A. Thus the washing steps using DNA Away which was incubated for 4 hours in the capillaries, were able to eliminate carry-over contamination caused by residual DNA from previous amplification thoroughly, and the capillaries can be washed with DNA Away and Triton X-100 repeatedly without generating any false positive

results.

Figure 5.8 Microgene plot of alternating positive and negative control amplifications on the same capillary (Capillary A) to evaluate the robustness of the DNA Away regeneration method. Washing steps using DNA Away were performed between PCR amplifications. Two fresh capillaries acting as internal positive and negative controls were included.

The Ct values of serial PCR amplification 1, 3, 5 and 7 are 17.6, 18.1, 18.3 and 17.9, respectively, as suggested in Table 5.1. When compared with Ct values of the PCR amplifications in internal positive control capillaries which were fresh and not washed with DNA Away, there was no obvious difference observed. The above results suggested there was no significant PCR inhibition caused by repeated washing steps using DNA Away and 0.1% Triton X-100 solution. Thus the capillaries can be repeatedly washed with DNA Away and

0.1% Triton X-100 without generating any false negative results due to possible PCR inhibition. The melting curve analysis on Microgene ensured the amplification of desired template in the capillaries. The PCR curve and melting analysis on Rotogene were used to confirm the results. The PCR product of each amplification was verified on a capillary electrophoresis chip using Agilent 2100 bioanalyzer.

Table 5.1 PCR amplification results of Capillary A, Capillary B and internal control capillaries. Alternating positive and negative amplifications were performed in Capillary A. Negative amplifications were performed in Capillary B acting as negative controls. Triton rinsing steps and DNA Away washing steps were applied between amplifications in Capillary A and B. Two fresh capillaries were used in each amplification as internal positive control and internal negative control to monitor any possible contamination of reagents and PCR mixture.

	Capillary A	Capillary B	Internal positive control capillary	Internal negative control capillary
Serial Amplification 1	Ct=17.6	No amplification	Ct=17.3	No amplification
Serial Amplification 2	No amplification	No amplification	Ct=18.4	No amplification
Serial Amplification 3	Ct=18.1	No amplification	Ct=17.9	No amplification
Serial Amplification 4	No amplification	No amplification	Ct=18.1	No amplification
Serial Amplification 5	Ct=18.3	No amplification	Ct=18.2	No amplification
Serial Amplification 6	No amplification	No amplification	Ct=18.0	No amplification

Serial Amplification 7	Ct=17.9	No amplification	Ct=17.7	No amplification
Serial Amplification 8	No amplification	No amplification	Ct=17.6	No amplification

5.5 Regeneration using PMA

5.5.1 Evaluation of regeneration using PMA of various concentrations

In the method using PMA to eliminate carry-over contamination, PMA of various concentrations was used to investigate the cleaning effect. There were six different concentrations of PMA used in the experiments: 25 μ M, 50 μ M, 100 μ M, 200 μ M, 400 μ M and 800 μ M. An additional capillary which was only rinsed with 0.1% Triton X-100 was used as a control. Positive control PCR mixture was firstly amplified in the capillaries, and the capillaries were rinsed three times with 0.1% Triton X-100 solution. Then PMA of different concentrations was used to wash the capillaries followed by another three times of Triton rinsing steps. Then negative control amplification was performed in the capillaries. The dark incubation time of PMA was 10min and the light exposure time of PMA was 20min under the 100W blue LED. If PMA and Triton washing steps were not able to eliminate the residual DNA from the previous positive amplification, the following negative control template would be contaminated and a false positive result would occur. For each PMA concentration, the PCR amplification was repeated three times and the averaged Ct value was obtained. Figure 5.9 shows the results of negative control amplification on Microgene in different capillaries which were washed using different concentrations of PMA.

Figure 5.9 Negative control PCR amplification plot to evaluate the decontamination effect using PMA of different concentrations. Negative control PCR amplification was performed in the capillaries in which positive control amplification were performed followed by Triton rinsing and PMA washing steps. During the washing step, six different concentrations of PMA were used to evaluate the decontaminating effect. An additional capillary which was only rinsed by Triton was used as a control.

The average Ct values of the PCR amplifications performed in capillaries which were washed with 25 μM , 50 μM , 100 μM and 200 μM PMA were 17.2, 26.6, 33.3 and 36.2, with a standard deviation of 0.4, 0.5, 0.5 and 0.7, respectively. There was no amplification in the capillaries which were washed with 400 μM and 800 μM PMA. The average Ct value of the PCR amplification in the control capillary which was only rinsed using 0.1% Triton X-100 was 12.4, with a standard deviation of 0.5. The above results suggested that low concentrations of PMA (25 μM , 50 μM , 100 μM and 200 μM) were not able to clean the capillaries thoroughly. DNA residual from positive PCR amplification remained in the capillaries despite the Triton rinsing and PMA

washing steps. And thus DNA carry-over contaminations occurred in the capillaries which were washed using the above PMA of low concentrations. However, when higher concentrations of PMA were used (400 μ M and 800 μ M), there was no amplification of the negative control PCR mixture in the capillaries, suggesting that the capillaries were free of residual DNA from previous positive control PCR amplification. The residual DNA was eliminated thoroughly by Triton rinsing and PMA washing steps and no carry-over contamination was observed. The melting curve analysis ensured the amplification of the desired DNA template. The PCR curve and melting curve on Rotogene were used to confirm the results. The PCR product of each amplification was verified on a capillary electrophoresis chip using Agilent 2100 bioanalyzer.

5.5.2 Evaluation of PCR inhibition caused by PMA

In order to investigate the inhibition effect on PCR, PMA of six concentrations (100 μ M, 200 μ M, 400 μ M, 800 μ M, 1600 μ M and 3200 μ M) was used as washing buffer to clean the capillaries. Six fresh capillaries were washed with the abovementioned PMA respectively. After washing with PMA, the capillaries were rinsed with 0.1% Triton X-100 three times. Then positive control PCR mixture was amplified in the capillaries and an additional fresh capillary. For each PMA concentration, the amplification was repeated three times and the averaged Ct value was acquired. The fresh capillary which was not washed with PMA was used as a control. Figure 5.10 shows the PCR amplifications in the six PMA-washed capillaries and the control capillary on Microgene. The melting curve analysis on Microgene ensured the correct PCR amplification. Again, control tests were run on Rotogene to verify the results.

The PCR product of each amplification was verified on a capillary electrophoresis chip using Agilent 2100 bioanalyzer.

Figure 5.10 PCR positive control amplification plot in PMA-washed capillaries on Microgene to evaluate PCR inhibition by PMA. Six different concentrations of PMA including 100 μ M, 200 μ M, 400 μ M, 800 μ M, 1600 μ M and 3200 μ M were used to wash the capillaries before positive PCR amplification. Capillaries were rinsed three times by Triton after PMA washing. A fresh capillary which was not washed with PMA was used as a control.

The average Ct value of PCR amplification in the control capillary is 17.6, with a standard deviation of 0.5, while the average Ct values of PCR amplifications in the capillaries which were washed with 100 μ M, 200 μ M, 400 μ M, 800 μ M, 1600 μ M and 3200 μ M PMA solution are 17.4, 18.2, 18.4, 17.9, 25.4 and 29.2, with a standard deviation of 0.4, 0.4, 0.6, 0.5, 0.5 and 0.6, respectively. Ct values increase significantly in the PCR amplifications

performed in the capillaries washed with 1600 μ M and 3200 μ M PMA. The results indicate possible inhibition of PCR when high concentrations of PMA (1600 μ M and 3200 μ M) were used while there was no obvious PCR inhibition when lower concentrations of PMA (100 μ M, 200 μ M, 400 μ M, 800 μ M) were used. The PCR inhibition was caused by the residual PMA in the capillaries. The rinsing step using 0.1% Triton X-100 was not able to remove the residual PMA in the capillaries thoroughly and considerable PMA remained in the capillaries when high concentrations of PMA were used.

In order to investigate the effect of multiple rinsing steps of Triton on the PCR inhibition, PMA of two high concentrations (1600 μ M and 3200 μ M) was used to wash the capillaries respectively. Then the capillaries were rinsed with 0.1% Triton X-100 solution once, three times, six times and nine times, respectively. Positive control PCR amplifications were then performed in the washed capillaries. A fresh capillary which was not washed with PMA was used as a control. Figure 5.11 shows the positive control amplification plot of the PCR reactions in the capillaries which were washed with 1600 μ M PMA and rinsed with Triton solution.

Figure 5.11 Positive control PCR amplification plot suggesting the effect of rinsing steps using 0.1% Triton X-100 on PCR inhibition caused by 1600 μM PMA washing steps. Capillaries were washed with 1600 μM PMA and then rinsed with Triton once, three times, six times and nine times, respectively. Then positive control PCR reactions were performed in the washed capillaries. A fresh capillary which was not washed with PMA was used as a control.

Figure 5.12 below shows the same amplification plot of positive PCR reactions in the capillaries which were washed with 3200 μM PMA and rinsed with Triton solution. In the above two experiments, the capillaries were rinsed with Triton once, three times, six times and nine times, respectively.

Figure 5.12 Positive control PCR amplification plot suggesting the effect of rinsing steps using 0.1% Triton X-100 on PCR inhibition caused by 3200 μ M PMA washing step. Capillaries were washed with 3200 μ M PMA and then rinsed with Triton once, three times, six times and nine times, respectively. Then positive control PCR reactions were performed in the washed capillaries. A fresh capillary which was not washed with PMA was used as a control.

Table 5.2 and Table 5.3 below summarize the Ct values of the amplification results as in Figure 5.11 and Figure 5.12, respectively.

Table 5.2 Ct values of positive PCR amplifications performed in the capillaries washed with 1600 μ M PMA and then rinsed with Triton once, three times, six times and nine times.

	Rinsed once	Rinsed three times	Rinsed six times	Rinsed nine times
Capillaries washed with 1600 μ M PMA	Ct=29.4	Ct=25.2	Ct=18.3	Ct=17.9
Control capillaries without washing with PMA	Ct=17.5	Ct=17.8	Ct=18.2	Ct=17.6

Table 5.3 Ct values of positive PCR amplifications performed in the capillaries washed with 3200 μ M PMA and rinsed with Triton once, three times, six times and nine times.

	Rinsed once	Rinsed three times	Rinsed six times	Rinsed nine times
Capillaries washed with 3200 μ M PMA	Ct=34.7	Ct=28.7	Ct=24.9	Ct=17.4
Control capillaries without washing with PMA	Ct=17.7	Ct=18.2	Ct=17.9	Ct=18.0

From the experiments above, it was shown that when more rinsing steps using Triton were applied, there was less residual PMA in the capillaries and less inhibition was observed. When the capillaries were rinsed six times using 0.1% Triton X-100 after washing with 1600 μ M PMA solution, no inhibition was observed any more. Nine times of rinsing were required to eliminate PCR inhibition for capillaries washed with 3200 μ M PMA solution.

5.5.3 Effect of light exposure time of PMA

The length of light exposure time of PMA solution using the blue LED (480nm, 100W, Marl International Ltd, Cumbria, UK) was investigated. Positive control PCR amplification was performed in capillaries. Then the capillaries were washed with 400 μ M/800 μ M PMA solution and rinsed with 0.1% Triton X-100. Different lengths of light exposure time (1min, 2min, 5min, 10min, 20min and 30min) were applied during the washing step following 10min dark incubation of PMA. A capillary washed with PMA that was not exposed to light was used as a control. Negative control PCR amplification was then performed in the capillaries. The effect of light exposure time on negative PCR amplification is shown in Table 5.4. The result shows increasing Ct values along with prolonged exposure time, while no amplification was observed in the capillaries in which PMA was light-exposed for 20min and longer. The increase in Ct values suggests that less DNA template from the previous positive PCR amplification was present in the capillaries with the prolonged light exposure times. No template remained in the capillaries which were subjected to 20min or longer light exposure time during PMA washing. Thus 20min light exposure time of 400 μ M and 800 μ M PMA solution was long enough to allow the sufficient cross-linking of DNA molecules and PMA molecules. Melting curve analysis and gel electrophoresis were performed to verify the results.

Table 5.4 PCR results suggesting the effect of light exposure time of PMA on PCR amplification. Ct values of negative PCR amplifications performed in the capillaries in which PMA was subjected to different lengths of light exposure time during washing steps were summarized.

	400 μ M PMA	800 μ M PMA
Control capillaries (no light exposure)	Ct=14.1	Ct=14.5
1min light exposure of PMA	Ct=24.3	Ct=24.0
2min light exposure of PMA	Ct=31.1	Ct=30.8
5min light exposure of PMA	Ct=33.4	Ct=33.6
10min light exposure of PMA	Ct=36.8	Ct=37.4
20min light exposure of PMA	No Amplification	No Amplification
30min light exposure of PMA	No Amplification	No Amplification

5.5.4 Effect of dark incubation time of PMA

The length of dark incubation time of PMA solution was also evaluated. Positive control PCR amplification was performed in capillaries. Then the capillaries were rinsed with Triton, washed with 400 μ M/800 μ M PMA solution and rinsed with Triton solution again. The light exposure time of PMA was 20min, and different lengths of dark incubation time (30s, 1min, 2min, 5min, 10min) were applied during the washing steps. A capillary washed with PMA that was not incubated in dark was used as a control. Negative control PCR amplification was then performed in the capillaries. The effect of dark incubation time on PCR amplification is shown in Table 5.5.

Table 5.5 PCR results suggesting the effect of PMA dark incubation time on the PCR amplification. Ct values of negative PCR amplifications performed in the capillaries in which PMA was subjected to different lengths of dark incubation time during washing steps were listed.

	400 μ M PMA	800 μ M PMA
Control capillaries (no dark incubation)	Ct=13.8	Ct=14.4
30s dark incubation of PMA	Ct=29.2	Ct=29.7
1min dark incubation of PMA	Ct=35.3	Ct=34.7
2min dark incubation of PMA	Ct=38.6	Ct=38.1
5min dark incubation of PMA	No amplification	No amplification
10min dark incubation of PMA	No amplification	No amplification

With the increase in dark incubation time of PMA solution, the Ct values of the negative control amplification increase accordingly, suggesting less residual DNA from the previous positive PCR amplification remained in the capillaries. For the incubation time longer than 5min, no amplification was observed and the capillaries were free of DNA template after PMA washing. From the table it was obvious that the Ct values increased rapidly when dark incubation time was increased from 30s to 5min, and the change flattened with longer incubation time. Identical tendencies were observed for PMA concentrations of both 400 μ M and 800 μ M. In the experiments performed in this thesis, a sufficiently long dark incubation time (10min) of PMA was allowed before light exposure.

5.5.5 Robustness of regeneration using PMA method

In order to establish the robustness of the regeneration method using PMA, alternating positive control and negative control PCR experiments were repeated as in Table 4.4. 400 μ M PMA solution and 0.1% Triton X-100 was used to wash Capillary A and Capillary B during the washing steps. PMA solution was dark incubated for 10 min and then light exposed for 20min. The PCR amplification curves of 8 serial amplifications in Capillary A on Microgene are illustrated in Figure 5.13.

Figure 5.13 Microgene plot of alternating positive and negative control amplifications on the same capillary (Capillary A) to evaluate the robustness of PMA regeneration method. Washing steps using 400 μ M PMA and Triton were performed between the PCR amplifications. Two fresh capillaries acting as internal positive and negative controls were included.

The Ct values of PCR amplifications in Capillary A, Capillary B and internal control capillaries are listed in Table 5.6 in the following for comparison.

Table 5.6 PCR amplification results of Capillary A, Capillary B and internal control capillaries. Alternating positive and negative amplifications were performed in Capillary A. Negative amplifications were performed in Capillary B acting as negative controls. Washing steps using 400 μ M PMA and Triton were applied between amplifications in Capillary A and B. Two fresh capillaries were used in each amplification as internal positive control and internal negative control to monitor any possible contamination of reagents and PCR mixture.

	Capillary A	Capillary B	Internal positive control capillary	Internal negative control capillary
Serial Amplification 1	Ct=17.4	No amplification	Ct=17.8	No amplification
Serial Amplification 2	No amplification	No amplification	Ct=17.9	No amplification
Serial Amplification 3	Ct=17.9	No amplification	Ct=17.4	No amplification
Serial Amplification 4	No amplification	No amplification	Ct=17.5	No amplification
Serial Amplification 5	Ct=18.1	No amplification	Ct=18.3	No amplification
Serial Amplification 6	No amplification	No amplification	Ct=18.5	No amplification
Serial Amplification 7	Ct=18.0	No amplification	Ct=17.7	No amplification
Serial Amplification 8	No amplification	No amplification	Ct=18.4	No amplification

As shown in the above figure and table, DNA was absent in the negative control PCR experiments (serial amplification 2, 4, 6 and 8) in Capillary A. Thus 400 μ M PMA and Triton solution was able to eliminate residual DNA template from previous run thoroughly and carry-over contamination was

eliminated. The capillaries can be washed with PMA and 0.1% Triton X-100 repeatedly without generating any false positive results. The Ct values of serial PCR amplification 1, 3, 5 and 7 are 17.4, 17.9, 18.1 and 18.0, respectively, as suggested in Table 5.6. When the above Ct values of positive PCR amplifications in the PMA-washed capillary were compared with Ct values of the PCR positive amplifications in internal control capillaries which were fresh and not washed with PMA, there was no obvious difference observed. The result confirmed there was no significant PCR inhibition caused by repeated washing steps using 400 μ M PMA and 0.1% Triton X-100. Thus the capillaries can be repeatedly washed with PMA solution and Triton solution without generating any false negative results due to possible PCR inhibition. The melting curve analysis on Microgene ensured the amplification of desired template in the capillaries. The PCR curve and melting curve on Rotogene were used to confirm the results. The PCR product of each amplification was verified on a capillary electrophoresis chip using Agilent 2100 bioanalyzer.

Chapter 6 Conclusions and future work

6.1 Conclusions

In the present research work, a regeneratable PCR array chip which enabled online continuous and repeated detection of target samples was designed and fabricated on PDMS substrate. The regeneratable PCR array chip comprised of an array of microreactors for simultaneous PCR analysis of multiple templates. The microreactors on the chip were unsealed and thus sophisticated microvalves were not required. Localized heating was used to contain the evaporative loss during PCR thermal cycling. The microfluidic operation of the PCR array chip was based on capillary action without the need of liquid handling systems for liquid loading and microreactor isolation. Key technical issues related to unsealed reactors such as evaporative loss has been addressed. The smooth internal surface of the microreactor, localized heating, optimized geometric design of the microreactor, chip substrate and reaction volume are critical parameters for successful PCR on the regeneratable PCR array chip. Simultaneous amplifications of four DNA templates were demonstrated using the PCR array chip and verified by commercial real-time PCR instrument as well as gel electrophoresis. The detection limit of the in-house real-time PCR system was determined and was demonstrated to be comparable to commercial real-time PCR system. The capillary-action based microfluidic operation of chip regeneration was demonstrated using a PCR array chip with a similar structure. Two washing buffers including DNA Away and PMA were used to eliminate the carry-over contamination which was one of the most important issues hindering chip regeneration. PDMS-coated glass capillaries which simulated the microreactors on the PCR array chip were used to

demonstrate the methods of regeneration using washing buffers. The effects of DNA Away incubation time and PMA concentration on the carry-over contamination were evaluated. Alternating positive and negative amplifications were performed in the capillaries to demonstrate the robustness of the regeneration methods. Other factors such as PMA light exposure time and dark incubation time were discussed to achieve optimized experimental conditions of regeneration. When comparing the cleaning methods of using DNA Away and PMA, it is suggested that PMA method is less time consuming. However, the method using DNA Away costs less and is relatively easy to perform.

Base on the work done, the following conclusions can be drawn:

- Successful parallel PCR amplifications of different DNA templates were performed on the regeneratable PCR array chip without any cross contamination. The results were confirmed by melting curve analysis and gel electrophoresis.
- Localized heating of the unsealed microreactors could be used to contain the evaporative loss during the PCR thermal cycling. Evaporation is reduced to less than 10% when bridge channels are equal or longer than 12mm.
- When using the PCR array chip, the detection limit of the in-house real-time PCR system Microgene is comparable to the commercial real-time PCR system Rotogene. DNA samples with a concentration of 1pg/ μ l or higher can be amplified and detected by Microgene.
- Due to proper geometric design of the PCR array chip, capillary action can be used in the microfluidic operations including liquid loading and chip regeneration using cleaning buffers. Thus sophisticated liquid

handling systems are not required and continuous online detection is made possible.

- DNA Away and 0.1% Triton X-100 can be used as washing buffers to clean the capillaries between serial PCR amplifications. Residual DNA from previous amplification can be thoroughly eliminated when DNA Away is incubated for 4 hours or above and no carry-over contamination occurs. PCR inhibition was not observed in using DNA Away as cleaning agent. Repeated PCR amplifications were performed to demonstrate the robustness of regeneration mechanism.
- Higher concentrations of PMA solution (400 μ M and above) and 0.1% Triton X-100 can be used as washing buffers to eliminate residual DNA templates thoroughly and prevent carry-over contamination.
- Excessive high PMA concentration (1600 μ M or higher) can produce inhibition on the subsequent PCR amplification. However, inhibition can be eliminated when more rinsing steps using 0.1% Triton X-100 are applied.
- When using PMA in the washing steps, less residual DNA remained in the capillaries with the prolonged dark incubation time. And a minimum of 5min of dark incubation time is needed in order to eliminate carry-over contamination thoroughly.
- When using 400 μ M and 800 μ M PMA in the washing steps, less DNA residual remained in the capillaries with the prolonged PMA light exposure time. For the above PMA concentrations used in the washing steps, 20min of light exposure time using 100W blue LED is sufficient to the allow cross linking of PMA and DNA molecules, and thus eliminate the carry-over contamination thoroughly.
- Repeated amplifications were performed when PMA and Triton were used

as washing buffers and it was demonstrated that the regeneration mechanism using PMA was robust.

6.2 Future work

6.2.1 PCR array chip regeneration

In this thesis, the microfluidic operation of chip regeneration was demonstrated using a PCR array chip with two microreactors. Thus in order to fully assess the microfluidic operation of chip regeneration, the designed PCR array chip with thirteen microreactors will be used in the future. In this thesis, glass capillaries coated with PDMS were used to simulate the microreactors on the PCR array chip. Repeated PCR amplifications and washing steps were performed in the PDMS-coated glass capillaries instead of the microreactors on the actual PCR array chip. In the future work, the designed PCR array chip will be used in the experiments of serial amplifications and washing steps to better evaluate the chip regeneration capability.

6.2.2 Integrated setup for automated sample loading and chip regeneration

In this thesis, manual operation was needed for sample loading and chip regeneration. For example, sample was pipetted into the loading ports on the chip manually. Besides, during the chip regeneration step, a hair dryer was manually held to blow hot air at the exit valves on the chip in order to evaporate the remaining liquid. Thus, in order to achieve automated operation of sample/primer loading and chip regeneration, an integrated setup including syringe pumps, tubing, valves, connectors and a device which can accelerate

evaporation was needed. The PCR array chip can be positioned on the setup, and the various loading and removal ports on the chip can be connected to the syringe pumps via valves, tubing and connectors. Thus, the entire process of sample loading and chip regeneration will be performed automatically on the integrated setup.

6.2.3 Vertical bridge channels on PCR array chip

Bridge channels were used to enable localized heating of microreactors and thus prevented the evaporation of PCR mixture inside the microreactors. It was demonstrated in this thesis that bridge channel of length larger than 10mm was required to contain the evaporation. Thus a large part of the PCR array chip was occupied by the bridge channels. And the overall number of microreactors which can be incorporated onto the chip was limited by the size of the TEC. In order to achieve high throughput, a PCR array chip which had vertical bridge channels was proposed (Figure 6.1). In the new PCR array chip, the horizontal bridge length was reduced and vertical bridge channels were imposed to contain the evaporation. A 10mm thick cover PDMS layer was bonded to the median PDMS layer which had similar structures as current PCR array chip including microreactors, loading channels and bridge channels of reduced length. Vertical bridge channels were fabricated on the cover PDMS layer to allow sample loading and contain evaporation of PCR mixture (The PCR mixture was represented by grey shading in Figure 6.1).

Figure 6.1 The schematic drawing of the top view of the PCR array chip with vertical bridge channels. Some short inlet bridges and outlet bridges were used to connect the microreactors to the vertical bridge channels. Thus the size of the chip was reduced and more microreactors can be incorporated onto the chip to increase the throughput. PCR mixture in the PCR array chip was represented by the grey shading.

Thus only the PCR mixture in the microreactors and the short inlet and outlet bridges which were in contact with TEC experienced thermal cycling temperatures, while the mixture in the vertical bridge channels did not experience the thermal cycling temperatures and evaporation was contained. By implementing the design of vertical bridge channels to contain the evaporation, the size of the PCR array chip was reduced. Thus, more microreactors can be incorporated onto the chip to increase the throughput of the PCR array chip. Figure 6.2 shows the A-A cross section view of the PCR array chip with vertical bridge channels mounting on the TEC.

Figure 6.2 The cross section view of the PCR array chip with vertical bridge channels mounting on TEC. A 10mm thick cover PDMS layer was bonded to the median PDMS layer. Vertical bridge channels connecting to the inlet/outlet bridges were fabricated on the cover PDMS layer. Mixture in the vertical bridge channels did not experience thermal cycling temperatures and the evaporation of the PCR mixture was contained.

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Appendix A DNA extraction protocol

Below is a protocol for DNA extraction using DNeasy Blood and Tissue Kit from Qiagen.

Important points before starting

- All centrifugation steps are carried out at room temperature (15–25 °C) in a microcentrifuge.
- Vortexing should be performed by pulse-vortexing for 5–10s.
- PBS is required for use in step 1. Buffer ATL is not required in this protocol.
- Optional: RNase A may be used to digest RNA during the procedure. RNase A is not provided in the DNeasy Blood & Tissue Kit.

Things to do before starting

- Buffer AL may form a precipitate upon storage. If necessary, warm to 56 °C until the precipitate has fully dissolved.
- Buffer AW1 and Buffer AW2 are supplied as concentrates. Before using for the first time, add the appropriate amount of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- Preheat a thermomixer, shaking water bath, or rocking platform to 56 °C for use in step 2.

Procedure

1. For blood with non-nucleated erythrocytes, follow step A; for blood with nucleated erythrocytes, follow step B; for cultured cells, follow step C. Blood from mammals contains non-nucleated erythrocytes.

Blood from animals such as birds, fish, or frogs contains nucleated erythrocytes.

- A. Non-nucleated: Pipette 20 μ l proteinase K into a 1.5ml or 2ml microcentrifuge tube (not provided). Add 50–100 μ l anti-coagulated blood. Adjust the volume to 220 μ l with PBS. Continue with step 2. Optional: If RNA-free genomic DNA is required, add 4 μ l RNase A (100mg/ml) and incubate for 2min at room temperature before continuing with step 2.
 - B. Nucleated: Pipette 20 μ l proteinase K into a 1.5ml or 2ml microcentrifuge tube (not provided). Add 5–10 μ l anti-coagulated blood. Adjust the volume to 220 μ l with PBS. Continue with step 2. Optional: If RNA-free genomic DNA is required, add 4 μ l RNase A (100mg/ml) and incubate for 2min at room temperature before continuing with step 2.
 - C. Cultured cells: Centrifuge the appropriate number of cells (maximum 5 x 10⁶) for 5min at 300 rpm. Re-suspend the pellet in 200 μ l PBS. Add 20 μ l proteinase K. Continue with step 2. When using a frozen cell pellet, allow cells to thaw before adding PBS until the pellet can be dislodged by gently flicking the tube. Ensure that an appropriate number of cells are used in the procedure. Optional: If RNA-free genomic DNA is required, add 4 μ l RNase A (100mg/ml), mix by vortexing, and incubate for 2min at room temperature before continuing with step 2.
2. Add 200 μ l Buffer AL (without added ethanol). Mix thoroughly by vortexing, and incubate at 56 °C for 10min. Ensure that ethanol has not been added to Buffer AL. Buffer AL can be purchased separately. It is essential that the sample and Buffer AL are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution.

3. Add 200µl ethanol (96–100%) to the sample, and mix thoroughly by vortexing. It is important that the sample and the ethanol are mixed thoroughly to yield a homogeneous solution.
4. Pipette the mixture from step 3 into the DNeasy Mini spin column placed in a 2ml collection tube (provided). Centrifuge at 8000rpm for 1min. Discard flow-through and collection tube.
5. Place the DNeasy Mini spin column in a new 2ml collection tube (provided), add 500µl Buffer AW1, and centrifuge for 1min at 8000 rpm. Discard flow-through and collection tube.
6. Place the DNeasy Mini spin column in a new 2ml collection tube (provided), add 500µl Buffer AW2, and centrifuge for 3min at 14,000 rpm to dry the DNeasy membrane. Discard flow-through and collection tube. It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during the following elution. Following the centrifugation step, remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-through, since this will result in carryover of ethanol. If carryover of ethanol occurs, empty the collection tube, and then reuse it in another centrifugation for 1 min at 14,000 rpm.
7. Place the DNeasy Mini spin column in a clean 1.5ml or 2ml microcentrifuge tube (not provided), and pipette 200µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at 8000 rpm to elute. Elution with 100µl (instead of 200µl) increases the final DNA concentration in the elute, but also decreases the overall DNA yield.

8. Recommended: For maximum DNA yield, repeat elution once as described in step 7. This step leads to increased overall DNA yield. A new microcentrifuge tube can be used for the second elution step to prevent dilution of the first elute. Alternatively, to combine the elutes, the microcentrifuge tube from step 7 can be reused for the second elution step. Note: Do not elute more than 200 μ l into a 1.5ml microcentrifuge tube because the DNeasy Mini spin column will come into contact with the elute.

Appendix B DNA quantification protocol

Below is a protocol for DNA quantification using RediPlate 96 PicoGreen DNA Quantitation Kit from Invitrogen.

1. RediPlate 96 Microplate Preparation

- 1.1. Allow the microplate to warm to room temperature. Remove the RediPlate 96 PicoGreen dsDNA quantitation microplate from the freezer and allow it to warm to room temperature. Do not open the foil packet until it is warm. The plate will typically take about 20 minutes to warm. Remove the plate from the foil packet by tearing or cutting above the resealable seal.
- 1.2 Remove any extra strips. Determine the number of strips required and carefully cut through the self-adhesive sealing film using a razor blade and remove any extra strips that are to be used at a later date. Store the extra strips at $\leq 20^{\circ}\text{C}$ in the foil packet with the included desiccators pack until future use. All of the strips contain equivalent amounts of the PicoGreen reagent. Empty strip holders from previously purchased RediPlate 96 kits are useful for storing extra as say strips.

2. RediPlate 96 PicoGreen dsDNA Quantitation Assay

The RediPlate PicoGreen dsDNA assay is easy to use with DNA samples from almost any source. Simply add buffer and the appropriate amount of the DNA sample to each well. The order of addition is not critical; it is only important that the final volume of solution in each well be 200 μL and that the amount of DNA added be within the linear range of the assay ($\sim 5\text{-}1000\text{ng/mL}$ or $\sim 1\text{-}200\text{ng}$

in a 200 μ L assay volume). The assay can thus be easily adapted for different types of liquid handlers and different concentrations of DNA samples, as described in the protocol below. Regardless of the method used, a DNA standard curve must be employed to convert the observed fluorescence into DNA concentration units.

2.1 Prepare DNA samples for a standard curve. Prepare a stock solution of DNA by diluting the λ DNA (Component C) 5-fold into TE buffer (Component B). Make serial dilutions in TE buffer to have concentrations ranging from 20 ng/mL to 20,000ng/mL (see Table 1). Volumes of only 10 μ L will be used for each assay; and in the 200 μ L assay, the final concentration will range from 1 ng/mL to 1000ng/mL. TE buffer alone will serve as the no-DNA control.

2.2 Add TE buffer. Add 190 μ L of TE buffer to as many assay wells as will be needed for all experimental samples and standard curve samples. Mix well. In order for very dilute DNA solutions to fall within the linear range of the assay, it may be necessary to decrease the amount of TE buffer added in this step to accommodate larger volumes of the DNA samples (see step 2.4). The PicoGreen reagent is not stable stored in aqueous solutions. Therefore, use wells containing the re-dissolved PicoGreen reagent within a few hours of preparation and do not save them for another day.

Table 1. Recommended DNA standards for use in the RediPlate 96 PicoGreen dsDNA quantitation assay.

Well	Concentration of the DNA standard (step 2.1)	Final concentration of DNA in the assay (step 2.3)
A	20,000 ng/mL	1000 ng/mL
B	10,000 ng/mL	500 ng/mL
C	3000 ng/mL	150 ng/mL
D	1000 ng/mL	50 ng/mL
E	300 ng/mL	15 ng/mL
F	100 ng/mL	5 ng/mL
G	20 ng/mL	1 ng/mL
H	0 ng/mL	0 ng/mL

2.3 Add the DNA standards. Pipette 10 μ L of DNA from each of the standard DNA solutions (prepared in step 2.1) into the assay wells. Mix well. Use one strip of wells for a single dilution series. If desired, use a second or third strip for duplicate or triplicate standard curves.

2.4 Add the experimental samples. Add 1.10 μ L of the experimental samples of DNA to the assay wells. Mix well. In order for very concentrated DNA samples to fall within the linear range of the assay, it may be necessary to dilute a portion of the DNA sample before adding it to the assay. In order for very dilute DNA solutions to fall within the linear range of the assay, it may be necessary to add a larger volume of the DNA sample solution in this step and decrease the amount of TE buffer added to the assay wells (in step 2.2) accordingly.

- 2.5 Incubate the samples. Incubate the loaded microplate for 5 minutes at room temperature, protected from light.
- 2.6 Read the fluorescence. Use a fluorescence-based microplate reader with excitation light and filter settings set for standard fluorescein wavelengths (excitation ~480nm, emission ~520nm). The PicoGreen reagent bound to DNA has excitation/emission maxima of approximately 502/523nm.
- 2.7 Correct for background fluorescence. For each value of sample fluorescence, subtract the value derived from the no-DNA control.
- 2.8 Determine the amounts of DNA. Using the data from the DNA standards, plot the amount of DNA versus the fluorescence intensity and fit a line to the data points. Use the standard curve to determine the amount of DNA from the fluorescence intensity measured for each experimental sample.

Appendix C Gel electrophoresis protocol

Below is a protocol for using capillary electrophoresis chip (DNA Labchip 500) on Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

1. Preparing the Gel-Dye Mix

- 1.1 Allow the DNA dye concentrate and DNA gel matrix to equilibrate to room temperature for 30 minutes. It is important that all the reagents have room temperature before starting the next step. Protect the dye concentrate from light.
- 1.2 Vortex the DNA dye concentrate for 10 seconds and spin down. Make sure the DMSO is completely thawed.
- 1.3 Pipette 25µl of the dye concentrate into a DNA gel matrix vial. Store the dye concentrate at 4 °C in the dark again.
- 1.2 Cap the tube, vortex for 10 seconds. Visually inspect proper mixing of gel and dye.
- 1.5 Transfer the gel-dye mix to the top receptacle of a spin filter.
- 1.6 Place the spin filter in a microcentrifuge and spin for 15 minutes at room temperature at $2240g \pm 20\%$ (for Eppendorf microcentrifuge, this corresponds to 6000 rpm).
- 1.7 Discard the filter according to good laboratory practices. Label the tube and include the date of preparation. The prepared gel-dye mix is sufficient for 10 chips. Use the gel-dye within 4 weeks of preparation. Protect the gel-dye mix from light as the dye will degrade when exposed

to light and this reduces signal intensity. Store the gel-dye mix at 4 °C when not in use for more than 1 hour.

2. Loading the Gel-Dye Mix

- 2.1 Allow the gel-dye mix to equilibrate to room temperature for 30 minutes before use. Protect the gel-dye mix from light during this time.
- 2.2 Take a new DNA chip out of its sealed bag and place it on the Chip Priming Station.
- 2.3 Pipette 9.0µl of the gel-dye mix at the bottom of the well marked. Insert the tip of the pipette to the bottom of the well when dispensing. Placing the pipette at the edge of the well may lead to poor results.
- 2.4 Set the timer to 60 seconds. Make sure that the plunger is positioned at 1ml, and then close the Chip Priming Station. The lock of the latch will click when the Priming Station is closed correctly.
- 2.5 Press the plunger of the syringe down until it is held by the clip.
- 2.6 Wait for exactly 60 seconds and then release the plunger with the clip release mechanism.
- 2.7 Wait for 5 seconds, and then slowly pull back the plunger to the 1ml position.
- 2.8 Open the Chip Priming Station.
- 2.9 Pipette 9.0µl of the gel-dye mix in each of the wells marked.
- 2.10 Pipette 5.0µl of the gel-dye mix in the well marked with the ladder symbol.

3. Loading the Marker

3.1 Pipette 5µl of the DNA marker into each of the 12 sample wells. Do not leave any wells empty or the chip will not run properly. Add 6µl of DNA marker to each unused sample well.

3.2 Do not leave any wells empty or the chip will not run properly. Add 6µl of DNA marker to each unused sample well.

4. Loading the Ladder and the Samples

4.1 Pipette 1µl of the DNA ladder in the well marked with the ladder symbol.

4.2 Pipette 1µl of each sample into each of the 12 sample wells. For optimal results, samples should be of pH 6 to 9 and should not have an ionic content greater than twice that of a typical PCR buffer.

4.3 Set the timer to 60 seconds. Place the chip in the adapter of the IKA vortex mixer. Vortex for 60 seconds at the indicated setting (2400 rpm).

4.4 Refer to the next topic on how to insert the chip in the Agilent 2100 bioanalyzer. Make sure that the run is started within 5 minutes.

5. Inserting a Chip in the Agilent 2100 Bioanalyzer

5.1 Open the lid of the Agilent 2100 bioanalyzer. Check that the electrode cartridge is inserted properly and the chip selector is in position.

5.2 Place the chip into the receptacle. The chip fits only one way. Do not use force.

5.3 Carefully close the lid. The electrodes in the cartridge fit into the wells of the chip.