Microfluidic array chip for highly parallel genomic and transcriptomic applications

Naveen Ramalingam

2011

Naveen, R. (2011). Microfluidic array chip for highly parallel genomic and transcriptomic applications. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/49499>

<https://doi.org/10.32657/10356/49499>

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**MICROFLUIDIC ARRAY CHIP FOR HIGHLY
PARALLEL GENOMIC AND TRANSCRIPTOMIC
APPLICATIONS.**

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SCHOOL OF MECHANICAL AND AEROSPACE
ENGINEERING**

2011

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PARALLEL GENOMIC AND TRANSCRIPTOMIC
APPLICATIONS.**

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A thesis submitted to the Nanyang Technological University
in partial fulfillment of the requirement for the degree of
Doctor of Philosophy

2011

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*To **Kulanthaiammal, Dr. Ramalingam & Anju.***

*With blessings from **KAKKAN***

ACKNOWLEDGEMENTS

I am deeply grateful to my Ph.D thesis advisor, **Professor Thomas Gong HAI-QING**, Micromachines lab 2 (BioMEMS), School of Mechanical and Aerospace Engineering, Nanyang Technological University, for his warm encouragement and thoughtful guidance through the duration of this work. Prof. Thomas introduced me to the exciting world of Bio-Micro-Electro-Mechanical Systems (BioMEMS) and shared with me his invaluable experiences and knowledge in this research field.

I thank my co-supervisor, **Professor Thomas LUFKIN**, Senior Group Leader, Stem Cell & Developmental Biology, Genome Institute of Singapore, Singapore, for his insightful discussions, and invaluable guidance for the biological areas of this research work. Special thanks to my technical mentor **Professor Ram RAJASEKHARAN**, Indian Institute of Science, Bangalore, India for inciting a spark to do the doctoral degree. I would also like to express my thanks and gratitude to all the research staffs in BioMEMS lab, especially Dr. Liu Hao-Bing, Robert Jiang, Chang-Chun Dai, Rajni Anup, Ratna Bandi, Serene Neo Chiew Hoon, Dr. Chen Longqing, and Charles. Their helps and valuable contributions are crucial to this project. Special thanks to Dr. Liu Hao-Bing for his help in development of 2-D PCR array chip, and design optimization of 1-D PCR array chip, Chan-Chun Dai in developing software to suit my project, and Robert Jiang for optics design and construction of real-time PCR instrument used in this project. I wish to express my warm and sincere thanks to Tong Chee San and Teo Jin Kai, students at Ngee Ann Polytechnic for their help during the development of microfluidic devices harboring unsealed reactors for real-time isothermal helicase-dependent amplification.

Finally, I owe my most sincere gratitude to my mentor and father Dr. K. Ramalingam, who constantly motivated without getting tired. His guidance helped me to improve the quality of this work. This work is a product of affection extended by my family members which include brother Neevan, sister Sumathi Sivakumar, and brother-in-law Sivakumar. Special thanks to my wife Anjana Naveen for her endless support and weekend sacrifices. My heart-felt thanks also goes to my friends Anup, Praveen, Bhupendra, Ranga, Sriram, Prasanna, John, Vaiyapuri, Rajesh and Karthikeyan. Without their support, this work would not have been possible.

EXECUTIVE SUMMARY

The Polymerase Chain Reaction (PCR) is an enzyme-based technique to amplify a single or few copies of Deoxyribose Nucleic Acid (DNA) in an exponential process, generating millions of copies of a known DNA sequence. The technique relies on thermal cycling; consisting of repeated cycles of heating and cooling of a reaction for DNA melting, target identification and enzymatic replication of the target DNA. The PCR technique is widely used for diagnosis, prognosis of diseases and enumeration of pathogen loads. Conventional PCR instruments are bulky, limiting its use in the field, but rapid development of miniaturized total analysis systems (μ TAS) enables commercialization of point-of-care devices. In resource-limited settings, it is impractical to get access to a diagnostic laboratory having sophisticated instruments and in such settings, it is desirable to use disposable point-of-care high-throughput diagnostic chips that do not require liquid handling or pumping instruments for sample distribution among an array of reactors. In addition to the pump-less sample loading method, the challenge to seal an array of reactors without the use of microvalves or mechanical parts persists. Implementation of microvalve array adds complexity to the chip fabrication and operation processes, and reduces the space on the microchip. The current point-of-care, PCR-based biochips are limited by its capability to test multiple samples or different gene targets.

In this doctoral work, a high-throughput, primer pair pre-loaded quantitative PCR chip platform for parallel analyses of multiple gene targets is developed. The PCR mixture distribution among an array of microreactors and subsequent isolation of the reactors were solely realized by a two-step surface tension-based microfluidic scheme, which eliminates the use of pumps, valves and liquid handling instruments. Sample loading and reactor sealing is achieved by implementing an unbalanced Young's force achieved by fabricating poly(dimethylsiloxane) (PDMS) and glass surface's with different wettability. The reactor-to-reactor diffusion of the pre-loaded primer pairs is investigated to eliminate the possibility of primer cross-contamination. Key technical issues such as primer pair degradation due to chip bonding conditions and bubble generation due to different PDMS-glass bonding methods are investigated. The capability of the developed PCR array chip is demonstrated by simultaneous (parallel) amplification of twelve different gene targets against cDNA template for

risk prediction of human hepatocellular carcinoma and simultaneous detection of multiple water-borne pathogens. In addition, a microfluidic device harboring an array of open/unsealed reactors was developed for isothermal amplification and detection of SARS cDNA template. Another version of high-throughput PCR array chip was developed as PDMS matrix chip. Sample loading and microwell sealing in this chip was achieved by vacuum and positive-pressure based loading of liquid PDMS prepolymer. Finally, the PDMS matrix chip was applied to solve an important clinical application. A microfluidic digital PCR was developed on the PDMS matrix chip for absolute quantification of live Methicillin-Resistant *Staphylococcus aureus* (MRSA) in mixed bacterial samples.

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INTRODUCTION

The RNA World referred to an hypothetical stage in the origin of life on Earth.

- **Sidney Altman** 1989 Nobel Laureate

for his work on the catalytic properties of RNA (Ribozyme).

Chapter 1 Introduction

The Polymerase Chain Reaction (PCR) is widely used for numerous biological applications such as disease diagnosis and prognosis, enumeration of pathogen loads, gene expression, genotyping, sample preparation for next-generation sequencing, DNA methylation analysis, copy number variation analyses. Conventional real-time/end-point PCR instrument are bulky and rapid development of miniaturized total analysis systems (μ TAS) enables commercialization of point-of-care devices. In resource-limited settings, it is highly desirable to use cost-effective disposable chips with minimal instrument for parallel detection of multitude pathogens [1, 2]. Considerable efforts have been made to develop microfluidic devices for performing multiple real-time qPCR [3, 4]. Microfluidic operation processes in these devices require external pump/instrument for sample loading among an array of reactors. In addition to this, an array of valves is used to reactor sealing. Implementation of mechanical/hydraulic valve array adds complexity to the chip fabrication and operation process and reduces the space on the microchip.

Three commercial high-throughput real-time PCR platforms are sold by Applied Biosystems (TaqMan low density array-TLDA; Applied Biosystems, Foster City, USA; www.appliedbiosystems.com), BioTrove (OpenArray® DLP Real-Time qPCR System; BioTrove, Woburn, USA; www.biotrove.com), SABiosciences (Qiagen acquired SABiosciences in November 2009) (Quantitative RT-PCR and PCR Array; SABiosciences, Frederick, USA; www.sabiosciences.com). The workflow of the three high-throughput platforms is shown in [Figure 1-1](#). These commercial, high-throughput platforms either requires bulky sample loading and reactor sealing instrument or requires arduous manual loading using eight-channel pipettor and sealing using optical adhesive film. During the course of this doctoral work, several new companies such as Stokes Bio (Limerick, Ireland), WaferGen (www.wafergen.com; last accessed on 16 January 2011), Raindance Technologies (www.raindancetechnologies.com; Lexington, USA; website last accessed on 6 January 2011), and QuantaLife Inc. (www.quantalife.com; last accessed on 16 January 2011) emerged with high-throughput PCR in their portfolio. More details about these emerging platforms will be presented in [Chapter 2](#).

Figure 1-1: Workflow of Applied Biosystem's (ABI) 7900HT Micro Fluidic card, BioTrove's OpenArray plate, and SABioscience's PCR array plate. (a) The ABI 384-well microfluidic card was developed by 3M technologies (Minnesota, USA) and can accept eight samples. Figure is compiled using images from www.appliedbiosystems.com and www.thermo.com. (b) BioTrove's stainless steel platen surface and reactor surface are differentially modified with hydrophobic and hydrophilic coating respectively to load sample under capillary action using OpenArray Autoloader. Figure is compiled using images from www.biotrove.com. (c) SABioscience's (earlier super array) PCR array plates are preloaded with assays and user manually loads sample and seals the PCR array plate.

1.1 Objective and significance.

The primary objective of this doctoral work is to develop a capillary-based microfluidics for loading PCR mixture among an array of microreactors without using micropumps/instruments and sequentially sealing these reactors without the use of microvalves. This work is a significant contribution towards development of high-throughput point-of-care diagnostic devices with minimal instrument for resource-limited settings.

The contribution of this report is four fold:

- Development of a surface-tension-based loading method without the use of micropumps for distribution of sample (PCR) mixture among an array of reactors.
- Development of a microfluidic sealing method without the use of microvalves or mechanical parts for achieving an array of closed reactors for high-throughput real-time PCR.
- Development of an array of solution-phase open/unsealed reactors for real-time isothermal amplification.
- Development of a vacuum aided microfluidics for rapid distribution of sample (PCR) mixture among an array of reactors.

1.2 Method of Approach

A novel, sequential capillary-driven microfluidics was developed for loading PCR mixture's containing nucleic-acid template among an array of solution-phase microreactors and sequentially sealing these reactors without the use of microvalves. During the chip fabrication process, different primer pairs were preloaded and dried inside different microreactors. Sample loading and reactor sealing is achieved by implementing an unbalanced Young's force achieved by fabricating surfaces with different wet ability. The surface tension generated pressure, which accounts for passive filling of PCR mixture and liquid sealant in a precise and reproducible manner. Isolation of microreactors is essential to prevent: (1) primer cross-contamination among fluidically connected reactors, and (2) evaporation of PCR

mixture. In order to isolate all the microreactors, PDMS prepolymer sealant was loaded in the inlet and air-venting channel.

In this work, two types of chip architecture for high-throughput PCR applications were developed: 1. PDMS/glass array chip, and 2. PDMS matrix chip. The PDMS/glass array chip was further developed with two types of chip architecture: 1. Sealed reactors, and 2. Unsealed or open reactor. The primary objective of developing microfluidic devices with unsealed reactor is to avoid the use to mechanical or moving parts for microvalves, which are required to seal an array of reactors. Different applications were demonstrated for these chip types. An overview of the chip architecture is shown in Figure 1-2.

Chip Format	Chip Image	Feature/Type	Applications
1. PDMS/glass Array Chip		Sealed Reactors	Gene Expression Analysis for Human Hepatocellular Carcinoma
		Unsealed Reactors: 1. Isothermal 2. Thermal cycling (PCR)	1. Detection of SARS cDNA 2. Detection of Waterborne pathogens
2. PDMS Matrix Chip		Sealed Nano-wells	1. Gene Expression Analysis for Human Hepatocellular Carcinoma 2. Quantification of methicillin resistant <i>Staphylococcus aureus</i> (MRSA)

Figure 1-2: An overview of the chip architecture developed in this thesis.

1.3 Organization of the thesis

In this doctoral thesis, chapter 3, 4, and 5 deals with the development of the PCR array chip, and chapter 6 and 7 discusses high-throughput applications. In chapter 6, data from two applications namely (1) parallel detection of multiple water-borne pathogens (genomic level), and (2) parallel profiling of gene expression (transcriptomic level) in human hepatocellular carcinoma (HCC) (liver cancer), are presented. Chapter 7 discusses a special and commercially relevant application of digital PCR for rapid and specific detection of methicillin resistant *Staphylococcus aureus* (MRSA).

Chapter 2 reviews the literature on current platforms available for massively parallel genomic and transcriptomic applications. In addition, this chapter also profiles the current state-of-the-art of PCR array chip used for parallel genomic applications. Commercially available qPCR array chips are also reviewed in this section.

Chapter 3 explains the specific chip design considerations for achieving surface-tension based microfluidics without air trapping (bubbles). Microfabrication methods used in the manufacturing of the reported devices are explained.

Chapter 4 explains the basics of surface-tension based microfluidics. Unbalanced Young's force achieved by differential surface wettability ensures precise and reproducible loading of sample mixture among an array of reactors.

Design and construction of a prototype real-time PCR instrument, which can accept array chip in a glass slide format, are explained in Chapter 5. Design of optics for detection of multiple fluorochromes from a single reactor is described in this section. This chapter presents the results of high-throughput, quantitative, nanoliter PCR using PDMS/Glass hybrid and matrix chip. This chapter also describes the development and validation of microfluidic devices harboring an array of unsealed reactors for high-throughput nucleic-acid amplification using isothermal helicase dependent amplification method.

Chapter 6 discusses the application of parallel detection and enumeration of multiple waterborne pathogens based on detection of genomic DNA (genomic level). In addition to this application, this chapter presents data for detection of transcripts in human liver cancer cells (HCC).

Chapter 7 discusses the development of multiplex TaqMan assay for rapid detection of MRSA and present results from the developed microfluidic digital PCR.

LITERATURE REVIEW

Science consistently produces a new crop of miraculous truths and dazzling devices every year.

- **Kary Banks Mullis** 1993 Nobel Laureate

for his invention of the polymerase chain reaction (PCR) method.

QUANTALIFE DROPLET PCR

Chapter 2 Literature Review

2.1 Highly parallel genomic and transcriptomic applications

Microarrays, next-generation sequencing/massively parallel sequencing, and digital single molecule analyses are three major platforms, which are used for parallel genomic, transcriptomic and epigenomic applications. In this section, a brief overview of the three technologies will be presented.

2.1.1 Microarrays

Microarrays were primary developed for high-throughput genomic and transcriptomic applications. This technology empowers users to screen a large number of genes. Their high-throughput capability for global, parallel gene expression monitoring makes them an ideal starting point for target discovery (Figure 2-1). Microarrays are typically used to screen ~10,000 gene targets.

Figure 2-1: Seamless workflow of microarrays for target discovery and target validation using qPCR.

However, microarrays require large amount of 5–20 μg of total RNA [5], which is at least 10^6 cells. Hence, historically scientists were using a pool of cells and looking at the average gene expression. Recent studies show that single cell gene expression is quite different from average expression from a pool of cells [6]. Single cell gene expression is of great interest (Figure 2-2) [7, 8]. Based on the re-association studies, three different class of mRNA *viz* highly, intermediate, and lowly expressed genes are

described [9]. In addition to large amount of input RNA, another drawback of microarray is that the dynamic range is relatively low ($<10^3$ logs).

Researchers generally validate the microarray results for two reasons:

- (1) To verify that the observed changes are reproducible in a large number of samples.
- (2) To verify that array results are not the result of inherent problems due to array technology.

In order to validate the array results, scientist use different methods such as TaqMan PCR, RNA-FISH; RNA-seq.

Figure 2-2: Comparison of heterogeneous population of cells and single-cell.

In the past two decades, immense efforts have been invested in the development of microarray technologies and methods for highly parallel genomic and transcriptomic applications [10]. In the late 1980's, Stephen P.A. Fodor came up with an idea to use semiconductor-manufacturing techniques to build vast amounts of biological data on a small glass chip for genomic applications [11]. The advent of this idea paved way for rapid development of highly parallel applications. This technology became the basis of a new company, Affymetrix, which began operating independently in 1992. On similar lines, another microarray based company Illumina was founded by David Walt

(inventor of BeadArray technology), of Tufts University. Illumina currently offers microarray-based products and services for an expanding range of genetic analysis sequencing, including SNP genotyping, gene expression, and protein analysis. [Figure 2-3](#) illustrates major applications at genomic and transcriptomic level, which are undergoing transformation to achieve a high-throughput format. Microarrays are majorly classified as five types ([Figure 2-9](#)): (1) printed microarray, (2) *In situ* synthesized, (3) high-density bead arrays, (4) electronic, and (5) liquid-bead suspension arrays.

[Figure 2-3](#): Major applications at genomic and transcriptomic levels.

Detailed analyses of different types of microarrays are described below:

- (1) **Printed microarrays**: Printed microarrays are primary used in research laboratories. Due to relatively large size of the features, printed microarrays are of lower density (~10,000 to 30,000)
- (2) ***In situ* microarrays** (high density- 15,000 - >10⁶): Oligonucleotides are synthesized directly on the surface of a quartz wafer using photochemistry. Multiple probe sets – one for perfect-match probe and one mismatch probe – are included per target. Prominent commercial companies such as Affymetrix, Roche-Nimblegen, and Agilent currently sells microarrays for numerous and ever-increasing applications ([Table 2-1](#)).

Table 2-1: Applications on commercial microarrays.

Company	Gene Expression	Genotyping	Arrays for capture (Sequencing)	MicroRNA Profiling	Mutation Profiling	Epigenetics	Copy number variation	Chromatin Immunoprecipitation
Illumina	*	*	N.A	*	*	*	*	*
Affymetrix	*	*	N.A	*	*	*	*	*
Agilent	*	*	Sure select DNA capture arrays	*	*	*	*	*
Roche Nimblegen	*	N.A	Sequence capture arrays	N.A	*	*	*	*
*Available. Information correct as on 10 th June 2011.								

(a) Working principle of DNA microarray:

A DNA microarray is a collection of synthetic DNA probes attached to designated location on a solid surface like glass (Figure 2.4). The resulting "grid" of probes can hybridize to complementary "target" sequences derived from experimental samples to determine the expression level of specific mRNAs in a sample.

Figure 2-4: Schematic of a DNA microarray. Each colored spot contains multiple copies of the probe. The target cDNA is fluorescently labeled (◆), and detected after hybridization at a specifically spot. Image taken from <http://bitesizebio.com/articles/introduction-to-dna-microarrays/>

Basic steps in a microarray experimental workflow are described below:

1. **Isolate and purify mRNA from samples of interest:** In-order to quantify the mRNA/transcript level, the cells are lysed and mRNA is purified.
2. **Reverse transcribe and label the mRNA (Figure 2.5 (a)).** In order to detect the transcripts by hybridization, they are fluorescently labeled, In the case of limited

sample amount; an amplification step is also included. Labeling usually involves performing a reverse transcription (RT) reaction to produce a complementary DNA strand (cDNA) and incorporating a fluorescent dye that has been linked to a DNA nucleotide, producing a fluorescent cDNA strand. Some protocols do not label the cDNA but use a second step of amplification, where the cDNA from RT step serves as a template to produce a labeled cRNA strand.

3. **Hybridize the labeled target to the microarray (Figure 2.5 (b)).** This step involves placing labeled cDNAs onto a DNA microarray where it will hybridize to their synthetic complementary DNA probes attached on the microarray. A series of washes are used to remove non-bound sequences.

4. **Scan the microarray and quantitate the signal (Figure 2.5 (c)).** The fluorescent tags on bound cDNA are excited by a laser and the fluorescently labeled target sequences that bind to a probe generate a signal. The total strength of the signal depends upon the amount of target sample binding to the probes present on that spot. Thus, the amount of target sequence bound to each probe correlates to the expression level of various genes expressed in the sample. The signals are detected, quantified, and used to create a digital image of the array.

Figure 2-5: Workflow for a DNA microarray. (a) Manufacturing of microarray: Synthesized oligonucleotides/PCR amplified probes are spotted on a glass substrate. (b) Total RNA from sample 1 (test sample) and sample 2 (control) are extracted and differentially labeled using Cy3 and Cy5. The labeled RNA is hybridized to the spotted probes. (c) After several washes, the chip is scanned for two different fluorophore. The raw images are analyzed to quantify the signal/mRNA level. Image from [12].

- (3) **High-density bead arrays** (high density- 50,000 - $>10^6$): Sequence-tagged beads are randomly assorted onto a fiber optic bundle or etched silicon wells. Illumina sells products based on high-density bead arrays.

(a) Illumina BeadArray technology background

Illumina's BeadArray technology is based on 3-micron silica beads that self assemble in microwells on either of two substrates: fiber optic bundles or planar silica slides (Figure 2.6). When randomly assembled on one of these two substrates, the beads have a uniform spacing of ~ 5.7 microns. Each bead is covered with hundreds of thousands of copies of a specific oligonucleotide that act as the capture sequences in one of Illumina's assays (Figure 2.7). BeadArray technology is utilized in Illumina's iScan system for a broad range of DNA and RNA analysis applications.

Figure 2-6: Illumina BeadArray. The Sentry Array Matrix (SAM) contains 96 1.4-mm fiber-optic bundles. Each bundle is an individual array consisting of 50,000 5- μm light-conducting fibers, each of which is chemically etched to create a microwell for a single silica beads. The Sentry BeadChips contains microwells on a silicon slide Image from [12].

Figure 2-7: Architecture of silica bead used in Illumina gene expression assay. The bead contains a unique 29nt address and a probe sequence to capture the target through hybridization.

(b) Illumina BeadArray technology background

Illumina gene expression protocols uses a first- and second-strand reverse transcription step, followed by a single *in-vitro* transcription (IVT) amplification that incorporates biotin-labeled nucleotides. Subsequent steps include array hybridization, washing, blocking, and streptavidin-Cy3 staining (Figure 2.8).

Figure 2-8: Workflow for production of biotin-aRNA sample for analysis with Illumina's Direct Hybridization assay.

Basic steps in a labeling kit for Illumina expression BeadChip are described below:

1. First-strand cDNA Synthesis: The Poly(A) RNA contained in a total RNA sample is reverse transcribed into first-strand cDNA. The reaction is primed from an oligo(dT)-primer containing a phage T7 RNA Polymerase promoter sequence at its 5' end. First strand cDNA synthesis is catalyzed by SuperScript III Reverse Transcriptase, and performed at an elevated temperature to reduce RNA secondary structure.

2. **Second-strand cDNA Synthesis:** The cDNA:RNA hybrid produced in Step 1 is converted to double stranded cDNA containing a T7 transcription promoter in an orientation that that will generate antisense RNA (aRNA; also called cRNA) during the subsequent in vitro transcription reaction. The cDNA produced can be used in the in vitro transcription reaction without the need for purification.
3. **In Vitro Transcription of Biotin-aRNA:** High yields of Biotin-aRNA (Biotin-cRNA) are produced in a rapid in vitro transcription reaction that utilizes the double-stranded cDNA produced in Step 2 as template.
4. **Biotin-aRNA Purification:** A purification column such as Qiagen RNeasy Mini Kit. (Qiagen cat. no. 74014) is used to remove unincorporated NTPs, salts, and other residuals to prepare for analysis with Illumina's Direct Hybridization assay.
5. **Hybridize BeadChip:** The cRNA is dispensed into BeadChips, and placed into the Hyb Chambers. Subsequently, the Hyb Chambers is placed in the Illumina hybridization oven for 14–20 hours at 58°C. Subsequently wash the BeadChip to remove unbound samples.
6. **Measure signal:** Cy3-Streptavidin is introduced to bind to the analytical probes that have been hybridized to the BeadChip. This allows for differential detection of signals when the BeadChips are scanned using iScan system.

(4) **Electronic arrays** (Low density- 400): Electric field are used to promote active hybridization of nucleic acids on a microelectronic device; streptavidin-biotin bonds immobilize the probes on the array surface. NanoChip 400 was originally developed by Nanogen Inc. It is currently sold by Savyon diagnostics.

(5) **Liquid-bead suspension** (Low density- 100): Spectrally unique microspheres provide solid support for application of probes or universal sequence tags; bead hybridization with fluorescently labeled target DNA is measured using flow cytometry.

Figure 2-9: Major types of microarrays. Images were taken from following websites: Affymetrix, Roche, Agilent, Illumina, Savyon and Luminex.

The heavy sample (~20 µg of total RNA) requirement leads most microarray users to “preamplify” their RNA. The sample is generally amplified after the reverse transcription (RNA to cDNA) step, and the cDNA is converted to labeled cRNA or aRNA (amplified RNA). Often, the labeling step is incorporated with the preamplification step. Amplification can be achieved by an enzyme mediated exponential process known as polymerase chain reaction (PCR) or T7 mediated linear amplification (Figure 2-10).

Figure 2-10: Importance of nucleic-acid amplification as backbone of various technologies.

2.1.2 Massively parallel sequencing.

In addition to microarray technology, masively parallel sequencing also known as “next-generation sequencing (NGS)” are widely used for high throughput genomic and transcriptomic applications. The evolution of masively parallel sequencing is shown in [Figure 2-11](#). As on June 2011, masively parallel sequencing are available from Roche [13], Illumina/Solexa [14-16], Life Technologies SoLiD and Ion Torrent. Single molecule sequencing is available through Helicos [17-22] and Pacific Biosciences [23-25]. For more information on these cutting-edge technologies, the reader is directed to these excellent reviews [26-28].

Figure 2-11: Evolution of massively parallel sequencing/”next-generation” sequencing. Images were taken Roche, Illumina, Life Technologies, Helicos Biosciences, Ion Torrent and Pacific Biosciences.

A major advantage with the high-throughput sequencing based approach is that a prior knowledge of the intended target sequence is not required. This basically open a large window for novel discovery in the areas of genomics, transcriptomics and epigenomics. A major drawback with masively parallel sequencing is high cost. Although the cost of sequencing, has tumbled down in the last decade (Figure 2-12), the cost per data point for parallel sequencing based approach is still very expensive. This high cost hinders the wide acceptance of this technology. With rapid development in the technology, the cost for such method is expected to drop to \$1000 [29] and further to \$30 dollars (GnuBIO Inc, Cambridge, www.gnubio.com last accessed on 15 June 11).

Figure 2-12: Cost of massively parallel sequencing methods. The numbers in the X-axis in the above graph refers to the following publication 1 (Sanger ABI)- [30], 2 (Sanger ABI)- [31],3 (Sanger ABI)- [32],4 (Roche 454)- [13],5 (Illumina)- [15],6 (Illumina) - [16],7- (Illumina) [14], and 8 (Helicos Biosciences)- [22]. The above graph is adapted from information presented in the reference [22].

(a) Working principle of massively parallel sequencing

Massively parallel sequencing can determine the sequence of huge numbers of different nucleic-acid strands at one time. These systems allow millions of reads to be gathered in a single experiment.

Except for single molecule sequencing methods such as Heliscope (Helicos Biosciences) and Pacific Biosciences, all the systems sequence populations of amplified template-DNA molecules (Figure 2-13). Sequencing technologies differs from each other in-terms of template preparation, sequencing chemistry and imaging/data analyses. A combination of different features makes a sequencing technology unique.

Figure 2-13: High-throughput sequencing technologies. a. Roche 454 GS FLX pyrosequencing, b. SOLiD system from Life Technologies, c. Illumina/Solexa GA sequencing, d. Heliscope from Helicos Biosciences, and e. Pacific Biosciences. Image from [33].

Template preparation

Since most of the imaging system cannot detect single fluorescent events, it is mandatory to amplify the templates. Two most common methods are emulsion PCR (emPCR) and solid phase amplification. In emPCR approach, a library of fragments is tagged to adaptors containing universal priming sites, which allows amplification of complex genomes with common PCR primers. After adaptor ligation, the DNA templates is denatured into single strands and captured on beads with a constrain that one DNA molecule per bead. Post amplification and enrichment of emPCR beads, they are either chemically cross linked to amino coated glass surface (SOLiD Life) or deposited into individual PicoTiterPlate (PTP) wells (Roche 454) to perform corresponding massively parallel sequencing chemistry (Figure 2-14).

Figure 2-14: Template immobilization strategies. a. Emulsion PCR (Roche 454.SOLiD Life Technologies) b. Solid-phase amplification (Illumina/Solexa). Image from [34].

In solid-phase amplification, randomly distributed, clonally amplified cluster or mate-pair templates are achieved on a glass slide. In this format, high-density forward and reverse primers are covalently attached to the slide, and the ratio of primers to template on the support defines the surface density of the amplified clusters. This approach can produce 100-200 million spatially separated template clusters with free ends on which a universal sequencing primer can be hybridized to achieve massively parallel sequencing chemistry (Figure 2-14).

Massively parallel sequencing chemistry

Pyrosequencing uses chemiluminescence for detection of each released pyrophosphate that occurs after incorporation of a nucleotide by the DNA polymerase. The four nucleotide are added to the sequencing reaction one at a time,

and the addition of correct nucleotide is accompanied by the release of light through a cascade of enzyme system. The amount of light produced is proportional to the number of incorporated nucleotides, allowing for detection of homopolymers. In Roche 454 system, about 1.6 million pyrosequencing reaction occur in parallel (Figure 2-15a).

Morozova O, et al. 2009.
Annu. Rev. Genomics Hum. Genet. 10:135–51

Figure 2-15: Massively parallel sequencing strategies. a. Pyrosequencing (Roche 454), b. Sequencing-by-synthesis for clonally amplified template (Illumina/Solexa), c. Sequencing-by-synthesis for single molecule (Helicos Biosciences), and d. Sequencing-by-ligation (SOLiD Life Technologies). Image from [35].

Sequencing-by-synthesis for clonally amplified template method is used by Illumina platform. In this approach, four fluorescently labeled nucleotide analogs that serve as reversible sequencing terminators, and special DNA polymerase that are capable of incorporating these analogs into the growing oligonucleotide chain is used. At each step, the correct nucleotide is identified by its color. The 3'-OH group is blocked to prevent further incorporation. Post imaging, the label is washed off and the chemical blockage is reversed, thereby allowing the synthesis to proceed (Figure 2-15b).

Sequencing-by-synthesis for single molecule method is used by Helicos biosciences. In this approach, single-molecule DNA template is captured on the flow cell surface; Cy-3 labels attached at both ends of each DNA molecule are used to

identify the location of each template bound to immobilized primers on the surface of the flow cell. The Cy-5 labeled nucleotide are added to the reaction one at a time, and the incorporated nucleotides are achieved by TIRF (total internal reflection fluorescence) (Figure 2-15c).

Sequencing-by-ligation method is used by SOLiD (Supported Oligonucleotide Ligation and Detection) system from Life Technologies. In this approach, successive rounds of hybridization and ligation events determine the sequence. The method uses 16 dinucleotide, each carrying a fluorescent label. Four fluorescent dyes are used in the system such that one dye labels four different dinucleotides. The identity of each base is determined from the fluorescent readout of two successive ligation reactions. The advantage of 2-base coding scheme is that each position is probed twice leading to accurate identification of the base with low sequencing error (Figure 2-15d).

(b) Working principle of single-molecule sequencing

In this section, the working principle of single-molecule sequencing from Helicos Biosciences and Pacific Biosciences is described.

Helicos Biosciences uses one color cyclic reversible termination method. The Helicos Virtual Terminator are labeled with the same dye and dispensed individually in a predetermined order. Following imaging using total internal reflection fluorescence method, a cleavage step removes the fluorescent dye and inhibitory group to permit the addition of the next Cy-5-2'-dNTP analogue (Figure 2-16). Since single-molecule gene expression is a relevant method for this thesis, the sample preparation protocol is illustrated in Figure 2-17.

Figure 2-16: One-color cyclic reversible termination method used by Helicos Biosciences. a. Sequence workflow of nucleotide incorporation, washing, dye cleaving and imaging, b. One-color images highlight the sequence data from two single-molecule template. Image from [34].

Sample preparation of single molecule digital gene expression is minimal, requiring neither PCR amplification nor ligation. Single-stranded cDNA is made directly from total RNA or poly-A+RNA using poly-U primer reverse transcription. The RNA is then digested away using RNase, and a poly-A tail is added to the cDNA at the 3' end using terminal transferase (Figure 2-17). The sample is then hybridized to Heliscope flow-cell surface and sequenced (Figure 2-18).

Figure 2-17: Overview of method utilized for single molecule sequencing digital gene expression performed on Helicos Biosciences system.

Figure 2-18: Schematic of the two-flow cell detection layout of the HeliScope single-molecule DNA sequencer. One flow cell is being imaged, and the other is simultaneously undergoing sequencing by synthesis chemistry.

Pacific Biosciences single molecule real-time sequencing is truly uninterrupted sequencing (Figure 2-19). This method is based on natural DNA synthesis by a DNA polymerase with phospholink nucleotides as it occurs in a continuous manner. Each SMRT chip contains thousands of zero-mode waveguides (ZMWs) [24]. The ZMW provides a platform to monitor DNA polymerase as it performs sequencing by synthesis. In contrast to other sequencing-by-synthesis methods using nucleotides with fluorophores, the phospholinked nucleotides of SMRT sequencing carry their fluorescent label on the terminal phosphate rather than the base. As a natural step in the synthesis process, the phosphate chain is cleaved when the nucleotide is incorporated into the DNA strand. Thus, the dye molecule is naturally cleaved. The phosphate-chain-dye complex quickly diffuses out of the detection volume, so that the background fluorescence is at low level. Subsequently, the polymerase advances to next base and the above process repeats.

Figure 2-19: Working principle of single-molecule, real-time DNA sequencing (SMRT Pacific Biosciences) A. Nanophotonic visualization chamber. A single polymerase molecule is attached to bottom of each nanowells. B. Schematic event sequence of phospholinked dNTP incorporation cycle. Image from [24].

2.1.3 Digital Single Molecule Analyses.

Digital single molecule analyses method is an upcoming technology with great promises [36]. As discussed in previous section 2.1.2, two massively parallel sequencing companies viz Helicos Biosciences and Pacific Biosciences. In this section, a non-sequencing based method known as nCounter system from Nanostring technology will be discussed (Figure 2-10). NanoString Technologies provides a complete solution for detecting and counting large sets of target molecules in biological samples. The company's products are based on a novel digital molecular barcoding technology invented at the Institute for Systems Biology (ISB) in Seattle under the direction of Dr. Leroy Hood. NanoString was founded in 2003 with an exclusive license to develop and market the technology. NanoString developed the

technology into the nCounter® Analysis System and began international sales operations in 2008.

Nanostring serves to bridge the gap between genome-wide (microarrays) and targeted (real-time quantitative PCR) expression profiling by virtue of its ability to multiplex up to 800 gene transcripts within a single reaction, with high sensitivity and linearity across a broad range of expression levels. The nCounter assay is based on direct imaging of mRNA molecules of interest that are detected using target-specific, color-coded probe pairs [37]. A key feature of Nanostring technology is that it does not require the conversion of mRNA to cDNA via reverse transcription or the amplification of the resulting cDNA via PCR. The large dynamic range of the assay and the lack of any enzymatic reactions allows direct analysis of a wide variety of sample types, including purified total RNA, crude cell and tissue lysates, RNA extracted from formalin-fixed paraffin-embedded (FFPE) tissues, and blood collected without globin mitigation.

Overview of the nCounter Gene Expression Assay

For each gene target of interest, two sequence specific probes (capture and reporter probe) are used (Figure 2-20). The capture probe contains a 35-50 bp sequence complementary to intended mRNA target, a short sequence containing two repeats of a 15-base sequence common to all capture probes, and a biotin affinity tag. The reported probe also contains a 35-50 bp sequence complementary to intended mRNA target, a color-coded molecular barcode, and a short sequence containing four repeats of a 15-base sequence. In total 16,384 different probes or “CodeSet” can be synthesized. The excitation and emission spectra for these four fluorescent dye in shown in Figure 2-20.

Figure 2-20: Probes architecture for Nanostring nCounter system. Image compiled from Nanostring technologies website.

Four fluorescent dyes (Alexa fluor 488, 594, 647 and Cy 3) are permuted at seven positions. In brief, the nCounter gene expression profiling workflow includes three steps: 1. Hybridization, 2. Post-hybridization processing, and 3. Digital data acquisition. During the hybridization step, the target mRNA is added to capture and reporter probe library (Figure 2-21). The reaction is incubated at 65C overnight. Following this, the post-hybridization processing is performed on an automated preparation station. During this step, the excess capture and reporter probe are captured using magnetic particle. Subsequently, the purified sample is loaded onto a laser cut acrylic sample cartridge using hydrostatic pressure. The biotin tagged tripartite complex is bound to the 30 μ m deep channel by streptavidin-biotin binding. An electric field is applied to extend and align the bound molecules. Finally, the cartridge is scanned using a CCD camera with 60X magnification and 600 field of view. A cartridge with 12 samples takes 4 hours for scanning.

Figure 2-21: Nanostring nCounter system workflow. Information compiled from Nanostring website.

As on June 2001, Nanostring offers probes sets for gene expression, microRNA gene expression, small RNA, and copy number variation (CNV) applications [38-40]. Nanostring technology uses small amount of total RNA (100 ng). The initial investment cost for the instrument ~ 230,000 USD, which leads to 1 USD price per data point. Although Nanostring nCounter system is an excellent technology in terms of input mRNA requirement, unbiased results due to amplification free sample preparation method, and simple workflow, they do not offer flexibility to the customers, as nCounter system is a closed platform. For any new studies or applications, the user needs to depend on the Nanostring technology for Codeset probe design and synthesis. Another point to note is that the limitation in the kinetics of solution-based hybridization puts a limit on the number of probes, which can be used in the multiplex hybridization reaction [41]. The current limit is at 800 probes. Although the company claims that theoretically, they can use 16,384 probes, achieving this might require multiple stages of optimization.

Except for single molecule analyses methods, most of the current methods for genomic, transcriptomic, and epigenomic applications require a nucleic-acid amplification step for further analyses in microarrays or NGS platforms. In addition to this, other nucleic-acid amplification methods such as real-time polymerase chain reaction (PCR) are used as a technology for all major application listed in [Figure 2-3](#), except sequencing method. There are numerous strategies for nucleic-acid amplification. The next section profiles a detailed review of the current methodologies for nucleic-acid amplification.

2.2 Nucleic-acid amplification methods

Nucleic-acid amplification is an indispensable step in genetic analyses. Nucleic-acid amplification methods may be categorized either based on the means of amplification strategy (target-, probe- and signal-based) or based on temperature requirement, which is further categorized as: (1) methods that require temperature cycling to separate double-stranded DNA, followed by primer annealing and extension. Polymerase chain reaction (PCR) and Ligase chain reaction (LCR) are examples of methods under this category; and (2) methods that involve co-ordinated catalytic activity of multiple enzymes with/without accessory proteins, at a single temperature (isothermal) [42]. Widely used isothermal DNA amplification methods include Strand displacement amplification (SDA), Q β replicase system, nucleic acid sequence-based amplification (NASBA), transcription-mediation amplification (TMA), signal mediated amplification of RNA technology (SMART), isothermal multiple displacement amplification (IMDA), single primer isothermal amplification (SPIA), Rolling circle amplification (RCA), Loop-mediated isothermal amplification (LAMP), SMart-Amplification Process (SMAP), Helicase dependent amplification (HDA) and circular helicase dependent amplification (cHDA). PCR was the first reported in vitro DNA amplification method [43].

In this doctoral work, the functionality of the developed microfluidic array chip is validated for the widely used PCR-based amplification and the isothermal helicase dependent amplification method.

2.2.1 Polymerase Chain Reaction (PCR)

The PCR is an enzyme-based technique to amplify a single or few copies of Deoxyribose Nucleic Acid (DNA) in an exponential process, generating millions of

copies of a known DNA sequence. The technique relies on thermal cycling; consisting of repeated cycles of heating and cooling of a reaction for DNA melting, target identification and enzymatic replication of the target DNA. During PCR, specific sequences are amplified (Figure 2-22). In the first step, the double helix of DNA is denatured (separates into two strands) at 95°C.

Figure 2-22: Principle of polymerase chain reaction (PCR).

In the second step, the temperature is lowered to 55-65°C for the two primers to specifically bind to the single stranded DNA. Finally, a heat stable DNA polymerase, *Taq* polymerase extends (copies) the two primers to generate a double strand from initially one single strand. The PCR is widely used as a nucleic-acid amplification method in areas such as expression profiling of genes and non-coding RNAs, genotyping, next-generation sequencing, epigenetics, clinical diagnostics, and forensic science. Real-time PCR has the ability to amplify, detect and quantify nucleic-acid in a single closed-tube reaction (homogenous method), thereby reduce the risk of cross-contamination and analyses time. The real-time PCR technology offer many advantages including high specificity, exquisite sensitivity (10 copies per cell), wide dynamic range of quantification (~8 log orders) and a high precision (< 2% SD) [44]. The thermal cycling profile of a typical real-time PCR is shown in Figure 2-23.

Figure 2-23: Thermal cycling profile for real-time polymerase chain reaction (PCR) and melting curve analyses.

The PCR process was first reported by Kary Banks Mullis in 1985 [45], for which he was awarded the Nobel prize in 1993. Based on the chemistry configuration, there are different types of real-time PCR (**Figure 2-24**).

Figure 2-24: Types of real-time PCR based on target sequence dependent, and sequence in-dependent.

Intercalating dye such as SYBR Green I/EvaGreen based real-time PCR assay are widely used due to economic reasons. These assays randomly intercalate into any double-stranded DNA. One of the major disadvantage of intercalating dye based assay is that false positive fluorescence signal may arise, if the primer design is not optimal

and it is subject to formation of primer-dimer. In such cases, it is essential to evaluate the purity of amplicon post-PCR. The purity and specificity of the PCR product is determined by melting curve analyses, in which temperature is raised by 1° each and the fluorescence is measured. This problem is overcome by implementation of sequence dependent assays such as TaqMan or Molecular Beacon based assay.

(a) Working principle of hydrolysis probe/TaqMan based assay.

In this method, a probe (short fragment of oligonucleotide) specific to the target is used. The probe/TaqMan probe is chemically synthesized and a fluorescent dye (FAM/VIC) is attached to the 5' end. The 3' end of this probe is chemically blocked so that they do not function as a primer. The TaqMan probe is designed in such a way that they hybridized between the forward and reverse primers. The probe is designed with a $T_m \sim 10^\circ\text{C}$ higher than the primers. This constrain will ensure that the probe hybridizes the target before the primers during the temperature transition from denaturation to annealing phase (Figure 2-25).

Figure 2-25: Working principle of TaqMan based real-time PCR.

An important feature of the TaqMan method is that the Taq DNA polymerase has a 5' nuclease activity. During the extension phase, the polymerase cleaves the TaqMan,

thereby separating the fluorophore from the close proximity of the quencher. Due to this separation, the fluorescence of the reaction increases.

(b) Working principle of molecular beacon based real-time PCR.

A molecular beacon is a single-stranded oligonucleotide 25 to 35 bases-long in which the last 5 to 7 bases on the 3' and 5' ends are complementary. Thus, a hairpin structure at ambient temperatures is formed. Molecular beacons have a fluorophore attached to their 5' end and a quencher attached to their 3' end, but they do not fluoresce at room temperature because the hairpin conformation brings these two moieties into close proximity. When the probe sequence in the hairpin loop hybridizes to its target, forming a rigid double helix, a conformational change occurs that removes the quencher from the vicinity of the fluorophore, thereby restoring fluorescence (Figure 2-26).

Figure 2-26: Principle of operation of molecular beacons.

The foundation for real-time PCR based microfluidic devices were laid in a span of 15 years (1985-2000; refer to Figure 2-27). The first concept of miniaturization, also known as miniaturized total analysis system (μ TAS), was introduced by a Swiss professor Andreas Manz in the year of 1990 [46].

Figure 2-27: Evolution of real-time PCR based microfluidic devices.

The main motivation for the development of miniaturized total system was to enhance the analytical performance rather than reduction in the device size. As the volumes of the micro total analysis system are reduced, the volume of reagents may not be reduced in proportion since the initial volume of test sample depends on the concentration of the analyte. The minimum sample volumes required for accurately determining DNA assays is $\geq 100 \mu\text{L}$ [47]. For each possible sample volume, there is an analyte concentration below which there is less than one molecule per sample, rendering the analyte statistically undetectable. A modified graph from the seminal paper by Manz [47] is shown in Figure 2-28. Biological analytes associated with clinical assays (10^{14} - 10^{20} copies/mL) can be detected in the sample volume ranging between picoliter to microliter range.

Figure 2-28: Detection limits as a function of the sample volume. Image adapted from publication [46].

The various steps of genetic analyses are illustrated in Figure 2-29. The major steps are cell culturing, cell isolation, cell lysis, nucleic-acid (RNA/DNA) purification, nucleic-acid amplification and detection. Most of cases, the concentration of the nucleic is lower than the detection limit of analytical systems. Hence, it is necessary to amplify or make more copies of nucleic acid by molecular biology methods such as polymerase chain reaction (PCR) and other isothermal nucleic acid amplification methods.

Figure 2-29: Different stages of genetic analyses system.

Parallel analyses of multiple gene targets or SNPs require a high-throughput PCR platform. BioMEMS played a crucial role in development of such a platform. Miniaturization led to reactions with reduced volumes, cost-effective assays, rapid analyses time, and efficient heat transfer due to reduced thermal mass. The first PCR chip was reported in 1993 by Northrup et al [48]. In following year, Peter Wilding and Larry J. Krick reported PCR in a microfabricated single chamber silicon microchip [49]. The PCR-based microfluidic devices can be divided into three major categories: (a) Single chamber devices, (b) Multi-chamber devices (high-throughput), a variant of this system is the droplet (emulsion) device, and (c) microfluidic flow-through devices. The first continuous-flow PCR chip was reported by Andreaz Manz in 1998 [50]. These PCR chips [48-50] provided the basis for subsequent development of numerous single chamber stationary PCR microfluidic based chips. Although, these devices exhibited the advantages of miniaturization such as reduced reaction volumes, faster PCR thermal cycling and cost-effective assay, they were not suitable for high throughput PCR applications. In order to address this concern, numerous research group developed multichamber stationary PCR microfluidics on a single chip [3, 4, 51-67].

Most of these devices require tedious manual PCR sample loading into individual reaction wells in a PCR chip, similar to that used in a well-plate operation, or required a costly liquid dispensing robot. The realization of high-throughput PCR-based biochips using microfabrication technology will significantly reduce the cost of genetic analysis by reducing the volumes required for PCR and also reduce the analysis time by precise thermal control due to reduced thermal mass. The salient features of a high-throughput PCR chip are low fabrication cost, disposable, uniform temperature across a large reaction array. In addition, a simple and cheap sample loading and reaction well sealing method is desired to reduce the operating cost. A few researchers have developed a microchamber array for PCR on silicon substrate [55, 60], on silicon-glass hybrid chip [61], and on poly-(dimethylsiloxane) (PDMS) substrate [4]. The fabrication and thermal characterization of multi-chamber for PCR applications were reported by a few other researchers [51, 53, 56, 64]. Successful amplification of nucleic-acid was not demonstrated in these devices. Baechi *et al.* reported a device with 300 valves/cm² using a thin layer of 3µm PDMS [51]. Chaudhari *et al.* developed a microfabricated 18-vessel array for PCR by sealing the microchambers on the silicon to a glass cover using a PCR-compatible acrylic-based

adhesive tape. The temperature fields in this microfabricated PCR vessel array were measured by thermochromic liquid crystals (TLC's) [53]. In another work, Iordanov *et al.* reported an array of nanoliter PCR chambers implemented in silicon with a glass cover, comprising integrated thermal and integrated optical detectors. The integrated heater and sensor increases the cost of the chip, significantly affects the temperature distribution on the chip, and hinders the development of a disposable PCR chip [56].

Park *et al.* reported a device with 16 wells with each well at 20 μl [64]. The integrated heater and sensor adds cost to the devices and limits its use as a disposable device. During the chip fabrication process, the PCR mixture is loaded and then bonded to another surface to achieve closed reactors. Issues related to effect of bonding on possible degradation of the PCR mixture was not addressed in this study [64]. Daniel *et al.* reported a silicon based device, in which a 260 bp fragment was amplified in 1.5 μl sample well covered with 1 μl silicone oil to prevent evaporation and cross-contamination during PCR process [55]. A unique feature of this device is that each micromachined chamber is suspended on four beams for thermal isolation. Although multiple chambers for nucleic-acid amplification was reported, the device still required cumbersome manual pipetting [55]. Like Daniel *et al.* device, Zou *et al.* amplified DNA on a 16-chamber plastic chip followed by off-chip amplicon analyses (end-point PCR) [67]. The microchip was sealed with a PCR-compatible adhesive tape from USA Scientific. Thermal multiplexing was demonstrated by fabrication of thermal cycler on a PCB card [67]. In another work, Yu *et al.* fabricated a PDMS-PDMS chip using a negative silicon master using SU-8 [66]. The chip contained 1064 chamber with each vertex holding a volume of 25 nL. The PCR was completed on a modified GeneAmp 2400 system from Perkin Elmer. The amplicon from this device was analyzed by Raman spectroscopy [66].

In 2002, Matsubara *et al.* reported a micromachined silicon chip (Figure 2-30) with 1248 chamber and each chamber at 40 nL [59]. Expensive nanoliter liquid dispensing system from Cartesian Technologies was implemented to load PCR mixture into the chambers. The functionality of this chip was demonstrated by amplifying 10,000 *Vibrio* template was amplified using SYBR Green I based assay on a conventional thermal cycling instrument from Takara Bio Inc. (Japan).

Figure 2-30: Microchamber array chip for nanoliter PCR developed by Yasutaka Matsubara and Eiichi Tamiya at the Japan Advanced Institute of Science and Technology. (A) Photograph of the microchamber array chip, which is placed on the peltiers of the miniaturized thermal cycler unit. (B) The miniaturized thermal cycler unit with six peltiers. (Image reproduced from ref. 55)

The fluorescence during PCR process was monitored using a microscope equipped with a CCD camera. Although, this study addressed numerous challenges associated with the development of high-throughput PCR device, but it failed to detect multiple target DNA or multiple assays (gene). In 2004, the same group used a similar device with 60 reactors to demonstrate digital PCR with 0.4 copies/reaction volume [58]. Subsequently, the same research group demonstrated detection of five different target genes (assays) against three different *E.coli* serotypes using TaqMan PCR. Again, the DNA sample was loaded by using a nanoliter dispensing robotic system followed by mineral oil for sealing the reaction wells [60].

Figure 2-31: Microchamber array chip for picoliter PCR developed by Hedenori Nagai and Eiichi Tamiya at the Japan Advanced Institute of Science and Technology. SEM images of the array chip: expanded view of 80 x 80 μm microchamber (Image reproduced from 55).

While, Nagai *et al.* developed a microchamber array with 10,000 chambers on silicon substrate for picoliter PCR (Figure 2-31) using manual sample loading step, and the amplification product was characterized by comparing the fluorescence intensity at the beginning and at the end of the PCR process [57]. The arrays micromachined in silicon substrate, were coated with SiO₂ using wet thermal oxidation techniques and resulted in chambers with volume ranging from 1.3 pL to 32 μ L, but PCR was successfully only in chamber with 86 pL volume.

Despite demonstration of high-throughput functional arrays, issues relating to cost effective sample delivery to individual chambers were not addressed. Like the device fabricated by Matsubara *et al.* [59], the device by Nagai *et al.* cannot manipulate multiple samples, but this device is suitable for parallel analysis of single-cell PCR or single-molecule PCR (digital PCR) [61]. In the same year, Nagai *et al.* used a similar device with 16 wells at 85 pL PCR volume to demonstrate rapid PCR to amplify 100 bp fragment for 40 cycles in 18 minutes [62]. In the past, few researchers have used microfluidics for performing PCR on-chip [3, 4], among them Mathies *et al.* demonstrated multiple PCR on chip with a microfluidic PCR mixture distribution (Figure 2-32), using a mechanical valve array for sample loading and well sealing [3].

Figure 2-32: Microfluidic PCR array chip developed by Mathies and Lagally at the University of California, Berkeley. The sample is pneumatically loaded past microfluidic valve from left to right direction. Finally, the valve and vent are pressure-sealed through an O-ring controlled by aluminum manifold.

In another breakthrough study, Liu *et al.* demonstrated the microfluidics for distribution of 2 μl PCR mixture among 400 independent reactors in a PDMS chip, using 2860 integrated hydraulic valves and pneumatic pumps [4]. In this work, the sample was loaded using a peristaltic pump and the microchambers were isolated from each other by a special hydraulic control channels (Figure 2-33). The high-throughput chip developed by Liu *et al.* is the basis of Dynamic and Digital array chip commercially sold by Fluidigm (www.fluidigm.com). Another group reported a similar high-throughput PCR, Leamon *et al.* reported a novel PicoTiterPlate™ platform in which three hundred thousand solid-phase asymmetric PCR with a reaction volume of 39.5 pL was simultaneously performed [56].

Figure 2-33: PDMS based high-throughput PCR array chip developed by Stephen R. Quake at the California Institute of Technology. Schematic diagram of the $N=20$ matrix chip layout, showing various input, output, and control ports. Inset: A single reactor color coded to indicate control lines (green), template sample (blue), DNA polymerase (yellow), primers (red), and rotary pump (white). $V_{\text{template}}:V_{\text{polymerase}}:V_{\text{primers}} = 3:3:4$. Scale bar. 6.4 mm (Image reproduced from ref. 4)

In another seminal work, Brenan *et al.* reported a rectilinear array of three thousand, seventy two 33 nL through-holes in a stainless steel platen, in which the primer pairs were immobilized in a matrix and the array of through-holes were sealed using UV curable epoxy [52]. Initially, Biotrove (www.biotrove.com) commercially sold the chips (OpenArray) developed in work by Brenan *et al.* Life Technologies (Earlier Applied Biosystems / Invitrogen) acquired Biotrove in November 2009.

Another elegant method of achieving high-throughput PCR is through emulsion microdroplets (water-in-oil droplets). The emulsions can be classified under two categories: (1) polydisperse, and (2) Monodisperse. Polydisperse droplets are manufactured using bulk emulsion using homogenization speed in an “eppendorf/microfuge” tube. In order to produce smaller droplets with narrow size distribution high speed of homogenization is required. In contrast, microfluidics provide a platform for precise tuning of reactor size as monodisperse droplets with consistent reaction rates. The seminal intellectual property (IP) rights for PCR based emulsion droplet microfluidics are owned by Lawrence Livermore National Laboratory, California, USA and described in two breakthrough papers in *Analytical Chemistry* [68, 69]. Vaccinia Western Reserve genomic DNA was amplified in 10 pL using TaqMan assay in a channel fabricated using silicon and bonded to a Pyrex glass cover [68]. This chip demonstrated a throughput of 1000 droplets/sec in a static chip format. A drawback, which was reported in this paper, is that droplet coalescence was observed with three doublets, two triplet and two quadruplets. This deleterious effect was taken into consideration during Poisson analyses for estimated copy number. Given that the number of doublets, triplets and other coalesced droplets may vary from experiment to experiment, it is difficult to implement the standard Poisson analyses for such chips. The same group report reverse-transcriptase PCR for MS2 virions [69].

The IP surrounding this work is licensed to two companies: (1) Raindance Technologies (www.raindancetechnologies.com; Lexington, USA; website last accessed on 6 January 2011), and QuantaLife Inc. (www.quantalife.com; last accessed on 16 January 2011). Raindance Technologies’ core RainStorm™ microfluidics technology can produce 10 million picoliter-volume droplets per hour. The throughput achieved by technology is far ahead of the throughput reached by the existing chamber based chips. Raindance Technologies uses this droplet microfluidics for two important applications: targeted sequencing (sequence enrichment) [70], single-cell

applications [71], genomics [72] and whole-genome amplification [73]. The typical workflow for sequence enrichment for next-generation sequencing (NGS) using droplet microfluidics developed by Raindance Technologies is illustrated in [Figure 2-34](#). The effect of mitomycin C drug effect on U937 single cells was studied on a hybrid PDMS-glass (tin oxide coated) chip using different additives such as 1% BSA, 1% Pluronic F-68 and Zonyl-FSO (fluorosurfactant) [71]. In a continuous flow-based PCR format, a 245 bp fragment of adenovirus genome was amplified on a PDMS-glass hybrid chip in a 65 pL volume [72]. Whole-genome amplification using isothermal hyperbranched rolling circle amplification (HRCA) was demonstrated on PDMS-glass chip using HFE-7500 fluorinated oil as encapsulant [73]. QuantaLife is yet to launch similar product in the market.

Figure 2-34: Raindance Technologies microdroplet PCR workflow for next-generation sequencing. (a) Primer library generation (1-4): Design primer and amplify specific segment, (b) Genomic DNA template preparation (5,6), (c) Droplet merging and PCR. (Image reproduced from ref. [70])

Although droplet PCR is an elegant method for high-throughput PCR, it is still in its infancy. There is still a limit for PCR product length, and issues related to leaking of small molecules persist. [Table 2-2](#) summarizes different droplet microfluidics work published in the literature. Efforts are ongoing by different research groups improve emulsion PCR based method [74, 75].

Table 2-2: Comparison of droplet microfluidics published in the literature.

Publication	PCR product size	Real-time/End point	Number of reactors	Comments
1. Schærli, Y., Wootton R. C., Robinson T., Stein V., Dunsby C., Neil M. A., et al. (2009) Continuous-flow polymerase chain reaction of single-copy DNA in microfluidic microdroplets. <i>Anal Chem.</i> 81,302-306.	85 bp	End-point	10 ⁶ droplets per day	34 cycles of PCR in only 17 mins.
2. Wang, F., Burns M. A. (2009) Performance of nanoliter-sized droplet-based microfluidic PCR. <i>Biomed Microdevices.</i>	56 bp	End-point	150 nL (20 droplets)	
3. Zeng, Y., Novak R., Shuga J., Smith M. T., Mathies R. A (2010). High-Performance Single Cell Genetic Analysis Using Microfluidic Emulsion Generator Arrays. <i>Anal Chem.</i>	624 bp	End-point	(3 nL) 6.4 × 10 ⁵ droplets per hour	
4. Kiss, M. M., Ortoleva-Donnelly L., Beer N. R., Warner J., Bailey C. G., Colston B. W., et al. (2008) High-throughput quantitative polymerase chain reaction in picoliter droplets. <i>Anal Chem.</i> 80,8975-8981.	245-bp	End-point	(65 pL) 1.8 million/hr	34 cycles in 35 mins
5. Kumaresan P, Yang CJ, Cronier SA, Blazej RG, Mathies RA. (2008). High-throughput single copy DNA amplification and cell analysis in engineered nanoliter droplets. <i>Anal Chem</i> ,80:3522-3529.	1139bp	End-point	(2-5 nL) 5000/hr	

Another company, which is yet to launch a high-throughput real-time PCR platform is WaferGen (www.wafergen.com; last accessed on 16 January 2011). It will be interesting to see the performance of these future systems. As mentioned before, during the development of this doctoral work, several companies emerged with high-throughput PCR as their portfolio. A comparison of their workflow is shown in [Figure 2-35](#). Life Technologies acquired Biotrove and Stoke Bio limited in November 2009 and April 2010 respectively. Raindance technology was founded in 2004, and is based on droplet PCR. The company demonstrated proof-of-principle for next-generation sample preparation [70]. QuantaLife and WaferGen are yet to launch a commercial product.

Figure 2-35: Commercial companies with high-throughput PCR. Images compiled from Life Technologies, Stokes Bio, Raindance Technologies, QuantaLife, and WaferGen.

In addition to the emulsion based PCR methods, high-throughput PCR can be achieved by continuous flow-through method as reported by Andreaz Manz in 1998 [50]. A layout of the chip developed by Manz is shown in Figure 2-36.

Figure 2-36: Chip layout of first microfluidic flow-through PCR chip developed by Andreas Manz at the Imperial College of Science, London. Chip layout. (A) Schematic of a chip for flow-through PCR. Three well-defined zones are kept at 95°, 77°, and 60°C by means of thermostatic copper blocks. The sample is hydrostatically pumped through a single channel etched into the glass chip. (B) Layout of the device. The device has three inlets on the left side of the device and one outlet to the right. Only two inlets are used: one carrying the sample, the other bringing a constant buffer flow (Image reproduced from ref. [50]).

Although, the PCR-based biochips described so far have the advantages of miniaturization such as reduction of nucleic-acid template and reagent consumption, reduced PCR thermal cycling time and precise control of thermocycling parameters, they still lack a simple, passive microfluidic PCR loading method and sealing of a array of reactors.

In this work, a manually operated sequential dual-fluid capillary microfluidics for PCR mixture loading and microreactor sealing is developed. The developed microfluidic approach for the PCR mixture loading and the microreactor sealing can be applied to a large number of microreactors. With the current chip design, the limits for the number of PCR microreactors on the PCR array chip depends on micromachining techniques to make small microreactors and improvement in microfluidics to control primer diffusion when microreactor density become very high. The advantages of the developed chip include reduced reagent consumption, parallel analysis of multiple genes on the same DNA/cDNA template, and a potential to integrate this PCR array chip with a nucleic-acid sample preparation system. In addition to the passive capillary-based microfluidic method, a vacuum based sample loading method was also developed to achieve high-throughput real-time PCR platform for highly parallel genomic applications.

2.2.2 Isothermal nucleic-acid amplification method: helicase dependent amplification (HDA)

Although PCR is a well established and widely used method for DNA amplification, the need for a specialized high-powered thermal cycling instrument limits the use of PCR in low-powered, hand-held DNA diagnostic devices. Alternatively, isothermal nucleic-acid amplification methods are being developed, as they are ideal for low-powered DNA diagnostic devices, since these methods do not require thermal cycling [76].

The various isothermal nucleic-acid amplification methods have their own advantages and limitations. SDA is not a true isothermal amplification method, because prior to addition of enzymes, the SDA reaction mixture is incubated at 95°C to denature the double-stranded DNA followed by 37°C to anneal primers [77]. Becton Dickinson (Sparks, USA) uses SDA technology for their commercial clinical diagnostic assays for detection of *Chlamydia* and *Mycobacteria*. In transcription-based amplification system (TAS), three enzymes: reverse transcriptase, RNase H, and T7 RNA polymerase, produces multiple copies of RNA using a double-stranded DNA intermediate [42]. The TAS can either produce sense or antisense amplified RNA product from a RNA sense strand [78]. Variations of TAS [also known as self-sustained sequence replication (3SR) system] include nucleic acid sequence-based amplification (NASBA) [79] and transcription-mediation amplification (TMA) [80]. Addition of dimethyl sulphoxide (DMSO) and optimization of reaction conditions to elevate the reaction temperature to 41°C improved the specificity of NASBA, when compared to the original 3SR system [81]. Biome'rieux (Durham, USA) uses NASBA for detection of HIV-1 and CMV. Transcription mediated amplification uses two enzymes: RNA polymerase and reverse transcriptase to amplify RNA or DNA with the help of two primers. Due to enhanced RNase activity of avian myeloblastosis virus reverse transcriptase (AMV-RT), a separate RNase H enzyme is not required. Gen-Probe (San Diego, USA) supplies a commercial system based on TMA for detection of *M. tuberculosis*.

Recently, three new isothermal DNA amplification methods: LAMP [82], SMAP [83] and HDA [84] have been described. LAMP technology uses four primers that recognize six distinct sequences on the target DNA to generate 10^9 copies in less than 60 min [82]. Although LAMP is highly sensitive, primer designing is arduous and requires dedicated software (PrimerExplorer V4). Another isothermal DNA

amplification method, SMAP, which is similar to LAMP was reported [83]. In this method, a single tube DNA amplification and SNP characterization was demonstrated by Mitani et al. [83], by developing a new mismatch-suppression technology. In 2004, mesophilic HDA (mHDA), a true isothermal DNA amplification technology, which does not require initial denaturation of double-stranded DNA was reported [84]. Subsequently, a thermophilic HAD (tHDA) platform was reported [85]. Although SDA, NASBA, TMA, LAMP, tHDA have been successfully commercialized, use of these methods in conventional instruments are limited by their capability to analyze multitude of genes in parallel. Hence, it is desirable to develop microfluidic devices as a high-throughput platform for isothermal nucleic-acid amplification methods.

An ideal isothermal amplification method for microchip devices should have: (1) high operation (incubation) temperature, as it reduces the incidence of non-specific target amplification; and (2) low number of enzymes, since multiple enzymes in the reaction will increase the probability of adsorption of these enzymes on the surface of the microchip and, disrupt the desired co-ordinated catalytic activity of the enzymes. Among the isothermal methods, LAMP, tHDA and SMAP are performed at 65, 64 and 60°C, respectively, while SDA, Q β replicase system, RCA, NASBA, TMA, SPIA are performed at 37, 37, 31, 41, 42, and 48°C, respectively. tHDA is advantageous when compared to other isothermal methods, since it requires two target specific oligos, similar to PCR, and uses two enzymes: DNA helicase and polymerase. Real-time isothermal DNA amplification methods are desired for the same applications as that of real-time PCR. In the literature, the real-time amplification of SMAP [83], mHDA [84], SDA [86], NASBA [87], and LAMP [88] has been reported.

Homogeneous real-time detection of SDA products utilized fluorescence resonance energy transfer (FRET) based “BDprobeTecET” probe. Real-time NASBA was demonstrated using molecular beacons, while “LUXTM” primer was used to develop real-time mHDA technology [84]. Recently, EvaGreen based real-time reverse transcriptase (RT)-HDA was reported [89]. Most of the reported high-throughput microfluidic devices employed the polymerase chain reaction for nucleic-acid analyses [3, 4, 52, 61], and utilized microvalves for reactor sealing [90, 91]. Additionally, these devices require micropumps/external pumping mechanism to distribute PCR mixture into an array of reactors. Implementation of microvalve arrays and micropumps increase the device cost, and add complexity to the chip fabrication

and operation process. By developing a microfluidic device harboring unsealed reactors with a capillary-based sample loading scheme, we eliminated the requirement of microvalve arrays and micropumps. Additionally, we implemented tHDA isothermal nucleic-acid amplification, so as to reduce the loss of reaction mixture due to the evaporation in the unsealed reactors. A few research groups have fabricated microchips to demonstrate the working of isothermal nucleic-acid amplification methods [92, 93]. Burns et al. demonstrated SDA reaction in a silicon-glass microchip followed by on-chip gel electrophoresis [92], while Gulliksen et al. used real-time molecular beacon based NASBA technology in 10 nL silicon-glass reaction chamber [93].

In this work a microfluidic device with open/unsealed reactors, which are preloaded with primer pairs for isothermal amplification of nucleic-acid using Helicase Dependent Amplification (HDA) technology is reported. The microfluidic operation includes a single-step manual pipetting step, which can be performed in a point-of-care or resource limited setting, without the need for micropumps or sophisticated liquid handling instruments. The capability of this microfluidic device is demonstrated by amplifying the BNI-1 fragment of SARS cDNA as a model test sample system.

CHAPTER

3

MICROFLUIDIC ARRAY CHIP DESIGN AND FABRICATION

Science produces knowledge and technology implements that knowledge.

- **Leland Harrison (Lee) Hartwell** 2001 Nobel Laureate

*for his contributions to the understanding of the cell cycle through years of
studying yeast.*

Chapter 3 Microfluidic Array Chip Design and Fabrication

Two different formats of PCR array chip was developed: (1) PDMS/glass hybrid chip with surface-tension based sample loading and reactor sealing, and (2) Micro-well PCR array chip with vacuum aided microfluidic sample loading. Henceforth, the first and latter format of the PCR array chip will be known as PDMS/glass array chip and PCR matrix chip respectively. The PDMS/glass array chip is fabricated by two different methods namely, (1) DRIE die, and (2) laser microfabrication. The PCR matrix chip is fabricated using an Electric Discharge Machining (EDM) based die.

3.1 PDMS/glass array chip

In this section, the design and fabrication of the PDMS/glass array chip by two different methods namely, (1) DRIE die, and (2) laser microfabrication will be presented.

3.1.1 Design of PDMS/glass array chip fabricated using DRIE die.

The schematic layout of the PCR microreactors and channel networks on the chip for the PDMS/glass array chip is shown in [Figure 3-1](#). During the fabrication of the PDMS-glass hybrid chip, different primer pairs are preloaded and dried inside different microreactors. The sample PCR mixture contains all the components of polymerase chain reaction, except for primer pairs. A primary requirement for the capillary-driven (unbalanced Young's force) microfluidics on the developed PDMS-glass hybrid chip is that the master mix should contain a PCR-compatible surfactant like Triton X-100 or Tween 20. The PDMS-glass hybrid chips in general consist of an inlet channel, which distributes the PCR mixture inside different microreactors. The inlet channel connects an array of orthogonal microreactors through inlet-bridge channels. When a sample PCR mixture is dispensed inside the inlet-port of the inlet channel, the PCR mixture will flow inside the microchannel conduit by capillary action. As the PCR mixture front in the inlet channel progresses, the orthogonal microreactors are sequentially filled with PCR mixture.

The PCR mixture enters the microreactors through inlet-bridge channels, then fills the microreactor, and finally fills the outlet bridge channel before its capillary force is restricted at the end of the outlet-bridge channel due to passive valve effect

arising due to abrupt change in the dimension of the two sides (PDMS walls) of the rectangular outlet microchannel. During the PCR mixture loading process the air inside the microreactors are purged through the outlet bridge channels, which are connected to a common outlet/air-venting channel. After complete loading of all the microreactors, it is essential to remove excess PCR mixture in the inlet channel, since fluidically connected reactors will provide a medium for diffusion of primer pairs from one reactor to another, ultimately leading to PCR with cross-contamination.

Figure 3-1: Layout of the PCR microreactors and the liquid loading network for PDMS/glass array chip. (a) The layout of the PCR microreactors and the liquid loading network. The inlet channel is used for distributing PCR mixture and subsequently used for loading liquid sealant. The air venting channel is also used for loading liquid sealant to fluidically isolate microreactors from each other. Different microreactors are pre-loaded with different primer pairs to analyze multiple gene targets. (b) Schematic representation of the microreactor shape parameters. (c) Photograph of the PDMS-glass chip comprising an array of twelve microreactors for PCR with open-top inlet and outlet channel.

The excess PCR mixture (dead-volume) inside the inlet channels is removed through an absorbent pad at the end of the inlet channel. During the process of excess PCR mixture removal from the inlet channel, the microreactors are isolated from each other. During PCR thermal cycling, it is required that the microreactors are sealed tightly at the ends of inlet and outlet bridge channels, so as to prevent PCR failures

due to sample evaporation. In order to achieve complete sealing of the microreactors, a flow able sealant was introduced both in the inlet and outlet channel. Different sealants were tried and finally PDMS prepolymer was selected as a suitable liquid sealant. Experiment results related to different sealants will be presented in [section 4.2.7](#).

The PCR array chip incorporating microreactors, inlet and outlet bridge channels was fabricated on a PDMS substrate by soft-lithography techniques [94], and then adhesively bonded to a glass substrate (0.1 mm) [95]. Different PDMS to glass bonding methods such as oxygen plasma [96], corona discharge treatment [97], and adhesive bonding methods [95] were evaluated and finally adhesive bonding treatment method was selected based on bubble-free microfluidics, threshold cycle concordance between conventional PCR and developed microchips. Detailed evaluation of different PDMS-glass bonding methods is described in [section 5.2.4](#). A hybrid (PDMS-glass) design circumvents the expensive glass micro-fabrication processes but still utilizes the superior properties of glass such as rigidity, flatness, and higher thermal conductivity [98, 99]. The dimension of microchannels and shape of PCR reactor on the PDMS-glass array chip were optimized to achieve capillary microfluidics without air/bubble trapping. Design consideration experiments are discussed in [Chapter 5](#). Post-optimization, the optimized parameters were implemented for the final version of the PDMS-glass hybrid chip. The inlet and air-venting channel of the chip illustrated in [Figure 3-1\(c\)](#) are 1.5mm wide, 1mm high with open-top. The microreactor, inlet bridge and outlet bridge have the same height of 180 μm . The widths of the inlet bridge channel and outlet bridge channel are 300 μm and 40 μm respectively. The distance between the adjacent bridge channels is 2 mm. The microreactor in [Figure 3-1\(b\)](#) has a hexangular shape with $\alpha = 30^\circ$ and $\beta = 45^\circ$ to facilitate the capillary-based microfluidics for PCR mixture loading and to avoid air-trapping during PCR mixture loading and reactor sealing step. The PCR microreactor is 1mm wide and 4 mm long, and holds a volume of $\sim 800\text{nl}$.

3.1.2 Fabrication of PDMS/glass array chip fabricated using DRIE die.

The silicon-based die for replicating the PDMS layer was dry etched by an ICP Deep Reactive Ion Etching system (DRIE) Si etching system with Bosch technology [100] with an etching depth of 180 μm . A thin layer of CF_2 polymer was coated on the

microfabricated Si surface using C_4F_8 plasma for easy release of the cured PDMS layer. The PDMS Sylgard Silicone Elastomer 184 and curing agent (Dow Corning Corporation Midland, MI, USA) were mixed at 10:1 weight ratio. It was degassed in a vacuum chamber for 20 minutes and cured on the silicon die at 80°C for 60 minutes to form a PDMS layer of 700 μm in thickness. The DRIE based silicon die with cured PDMS is shown in [Figure 3-2](#). After peeling off the PDMS layer from the silicon die ([Figure 3-3a](#)), the layer was diced to a suitable size and adhesively bonded to an acid-washed (piranha solution) [101] borosilicate glass substrate (Herenz Medizinalbedarf, Hamburg, Germany) pre-loaded with dried primer pairs.

At this stage of development, two problems were encountered:

1. ***Preloading of liquor primer without cross-contamination*** on an acid-washed glass substrate was a challenge since the contact angle of primer liquor on acid-washed glass substrate is 30°, which was measured using FTA 200 dynamic contact angle analyzer (First Ten Angstroms, Portsmouth, USA). Since the primer liquor wets the glass substrate, it was difficult to contain different prime liquor in a width of 1 mm, which is the width of a microreactor. In order to address this challenge, a hydrophobic pattern was developed and implemented ([Figure 3-3b](#)).
2. Another challenge was to develop a PDMS-glass bonding method, which can achieve ***bubble free loading*** and can survive 40 cycles of PCR thermal cycling. Initially plasma-bonding method was tried, but encountered issues related to PCR mixture loading with air-bubbles. The reason for bubble nucleation in plasma-bonded chip was found by extensive experimentation. In order to fix the source of this problem a bonding interface cladding technique was developed. Comprehensive experimental results from different bonding methods, which were tested will be presented in [section 5.2.4](#).

PCR has been successfully demonstrated on devices fabricated with glass surfaces coated with silanes [50, 102]. For the array chip, untreated glass substrate (acid-washed) was used, since coating with silanes like Sigmacote will render the glass surface hydrophobic and impede the developed capillary-based microfluidics. Instead of coating the glass substrate, dynamic coating of BSA is implemented at a

concentration of 1 $\mu\text{g}/\mu\text{l}$ and did not notice any deleterious effect of the bare glass surface on the yield of the PCR performed in microreactors with SVR of $\sim 13 \text{ mm}^{-1}$. In addition to my results, there is a report in which PCR was successfully performed without BSA in hybrid silicon-oxide chamber (SVR: 17.5 mm^{-1}) anodically bonded to an untreated pyrex (borosilicate) glass cover [103].

Figure 3-2: Photograph of DRIE etched silicon die with cured PDMS. Various length and width of outlet bridge channel was fabricated to optimize the parameters for efficient capillary loading of sample and sealant without trapping air/bubble.

To guide the primer liquor (mixture of forward and reverse primer) loading in the microreactors, a hydrophobic pattern of the liquid PDMS prepolymer was fabricated on an acid-washed glass substrate using a PDMS stamp with structure similar to the PDMS layer of the array chip [95]. The primer liquor was then loaded onto the acid-washed glass substrate and dried at 80°C for 10 minutes (Figure 3-3c). The hydrophobic liquid prepolymer pattern confined the primer liquor at desired spot inside the microreactor base. The PDMS layer and the glass substrate were bonded with GE RTV 615 as adhesive to form the chip. The liquid pre-polymer RTV 615 was spin-coated onto the surface of a 1 mm thick glass plate at 10,000 rpm for 1 minute to achieve a homogenous RTV 615 adhesive layer of thickness $\sim 6 \mu\text{m}$ (Figure 3-3d).

Figure 3-3: Schematic of the PDMS-glass chip fabrication process.

In order to produce a consistent fabrication method for 6 μm thin spin coated RTV 615 prepolymer, a spin curve was generated to have a better control on the spin speed, spin time and temperature with a power to predict the expected spin thickness. A typical spin curve for PDMS prepolymer at 25°C and 50°C is shown in [Figure 3-4](#).

[Figure 3-4](#): Spin curve for PDMS prepolymer at 25 and 50°C.

The PDMS replica was placed-on and subsequently peeled-off from the glass plate so as to “ink” the bonding surface of the PDMS layer with adhesive [95]. Following this, the PDMS layer was manually aligned to the glass substrate ([Figure 3-3e](#)). The PDMS-glass hybrid chip was then baked at 150°C for 20 minutes to cure the RTV 615 adhesive layer to achieve an irreversible bonding. Concerns about thermal degradation of the primer pairs due to exposure of the pre-dried oligonucleotides (primer pairs) to high-temperature (150°C for 20 minutes) during adhesive layer curing step have been addressed in [section 5.2.2](#). A photograph of the final PDMS-glass array chip is shown in [Figure 3-5](#).

Figure 3-5: Photograph of the PDMS-glass chip comprising an array of twelve microreactors.

3.1.3 Design of PDMS/glass hybrid microfluidic devices harboring unsealed reactors.

In order to ease the microfluidic operation process, the inlet and outlet bridge channel were elongated in length and nucleic-acid was amplified using an isothermal (single temperature) amplification process, to avoid the sealing step for an array of reactor. The initial thought was that by the time, sample evaporates in the inlet and outlet bridge channels, the nucleic-acid amplification process might have been completed. This concept was tested by fabricating PDMS-glass array chip with DRIE die. The functionality of the fabricated chip was successfully demonstrated by detection of SARS cDNA fragment cloned in a plasmid vector. The design and fabrication of the microfluidic devices harboring unsealed reactor will be presented in this and next section.

The microchip with incorporated reactors, inlet and outlet bridge channels, loading and waste channels was fabricated on a poly(dimethylsiloxane) (PDMS) material by soft-lithography techniques [94], and then adhesively bonded to a glass substrate (0.1 mm thick). The latter was pre-loaded with different primer pairs for simultaneous amplification at multiple loci of a nucleic-acid test sample. The use of glass substrate facilitates rapid heat transfer from the thermoelectric cooler (TEC) surface to the amplification mixture inside the microreactors [glass has a higher thermal conductivity than PDMS [104], and provides favorable hydrophilic surface conditions for capillary based microfluidics. The loading channel is used for

delivering the amplification mixture into an array of microreactors. During the loading step, the amplification mixture flows into the reactors and re-suspends the dried primer pair, and in the process the air inside the microreactor is purged out through the air venting port. In order to prevent primer cross-contamination, all the microreactors were isolated from each other by autonomous removal of excess amplification mixture from the loading channel by an absorbent pad through a waste channel. The microreactors on the chip are not sealed during the amplification process, and the amplification mixture evaporative loss in these unsealed reactors was well controlled by reactor design, smooth internal surfaces and a localized heating region of the reactor.

Figure 3-6: Schematic of the chip layout for microfluidic device with open/unsealed reactors.

The amplification mixture inside the reactors was locally heated on a TEC surface, while a part of the amplification mixture inside the bridge channels does not experience reaction temperature provided by the TEC surface. The microreactor, inlet and outlet bridge channels, loading and waste channels are 350 μm high. The inlet and outlet bridge channels are 200 μm wide and 10mm long, and the microreactor is 1 mm wide and 10 mm long with a surface-to-volume ratio (SVR) of 9.7 mm^{-1} , and holds a volume of $\sim 5\mu\text{l}$. A schematic for the microfluidic devices harboring unsealed reactor is shown in Figure 3-6.

3.1.4 Fabrication of PDMS/glass hybrid microfluidic devices harboring unsealed reactors.

To replicate the PDMS layer of the array chip, a silicon wafer was patterned with photo resist (AZ9260) by UV photolithography using a Karl Suss mask aligner. The wafer was then dry etched by an ICP DRIE (Deep Reactive Ion Etching) Si etching system with Bosch technology (STS) [100]. After the DRIE process, the residual photo resist on the wafer was cleaned with acetone, followed by isopropanol and water. The wafer was then treated with O₂ plasma for 5 minutes to remove any organic contaminants. Finally, a thin layer of CF₂ polymer was coated on the Si surface using C₄F₈ plasma, for ease in release of the cured PDMS layer.

The fabrication process of the PDMS-glass hybrid chip is very similar to [Figure 3-3](#) with minor modification such a punch holes (air-venting port) and location of the absorbent pad. Firstly, PDMS prepolymer (Sylgard Silicone Elastomer 184; Dow Corning Corporation Midland, MI, USA) was mixed homogenously on a magnetic stirrer. The prepolymer PDMS mixture was poured onto the fabricated silicon master, degassed in a vacuum chamber for 20 minutes and then cured at 150°C for 30 minutes. After curing, the PDMS replica containing the microchannels and reactors was peeled off from the silicon master ([Figure 3-3a](#)), and a smooth internal reactor surface was achieved by using PDMS prepolymer. For successful amplification of nucleic-acid in the PCR array chip, a smooth inner surface of the microreactors is imperative, so as to prevent bubble generation, which may cause purging of amplification mixture from the reactors, during the reaction incubation temperature. Secondly, the 0.1 mm thick glass substrate (Herenz Medizinalbedarf, Hamburg, Germany) was cleaned (acid-washed) using hot piranha solution (30% H₂O₂ in H₂SO₄) at 120°C for 60 minutes, and then rinsed with deionized water, and dried in nitrogen gas [101]. For the PCR array chip, the primer pairs were preloaded on the glass substrate in the desired position. To guide the primer liquor (mixture of forward and reverse primer) loading in the microreactors, a hydrophobic pattern of PDMS precursor (uncured) was fabricated on the acid-washed glass substrate using a PDMS stamp with structure similar to the PDMS layer of the array chip ([Figure 3-3b](#)). Thirdly, the primer liquor was then loaded onto the acid-washed glass substrate and dried at 80°C for 10 minutes ([Figure 3-3c](#)).

To fabricate the adhesive layer for bonding the PDMS layer to the glass substrate, the PDMS prepolymer was spun onto the surface of a 1 mm thick glass

plate (Herenz Medizinalbedarf, Hamburg, Germany) at 10,000 rpm for 1 minute to achieve a homogenous layer of thickness $\sim 6 \mu\text{m}$. The PDMS layer patterned with microreactors and channels was placed onto the PDMS coated glass plate. The embossed surface of the PDMS was in contact with the thin layer of adhesive. The recessed areas were free from the adhesive (Figure 3-3d). The PDMS layer was then peeled off from the PDMS coated glass plate and aligned on the glass substrate loaded with dried primer pairs, so that the dried primer pair is inside the microreactor (Figure 3-3e). The PDMS-glass hybrid chip was baked at 150°C for 20 minutes to cure the adhesive layer to form an irreversible bonding between the PDMS layer and glass substrate [95]. Finally, the air venting ports were punched as through hole to open it to the atmospheric air.

3.1.5 Design of PDMS/glass array chip fabricated using laser cutting techniques for detection of multiple water-borne pathogens.

In addition to isothermal amplification in unsealed reactors, we experimentally tested the open reactors for their suitability for PCR process. Again, the lengths of the inlet and outlet bridge channels were optimized to achieve successful PCR in unsealed reactors. This chip was fabricated using a laser cutting techniques as an alternate method of fabrication of PDMS-glass hybrid chip using DRIE die. The functionality of the fabricated chip was demonstrated by simultaneous detection of multiple water-borne pathogens.

The PCR array chip, incorporating microreactors, sample loading and waste channels, inlet and outlet bridge channels was fabricated on poly (dimethylsiloxane) (PDMS) substrate by a laser cutting technique, and then bonded to a 0.1 mm thick glass substrate. Such a hybrid design circumvents the expensive glass micro-fabrication processes, but still utilizes the superior properties of glass such as rigidity, flatness, and higher thermal conductivity compared to PDMS. The schematic layout of the PCR microreactor and channel networks on the chip is shown in Figure 3-7. During the chip fabrication process, different microreactors are deposited with different primer pair (dry form) for detection of multiple waterborne pathogens. The loading channel is used for distributing PCR mixture (without primer pair) containing a pool of DNA templates, into an array of microreactors. During PCR mixture loading step, the liquid flows into the microreactor and re-suspends the dried primer pair,

while the air inside the microreactors is purged out through the air venting port. After complete filling of the microreactors, the liquid samples inside the microreactors are isolated from each other by continuous and automatic removal of the excess PCR mixture in the loading channel by an absorbent pad.

Figure 3-7: Schematic of the chip layout for microfluidic device with open/unsealed reactors. (A) The loading channel is used for delivering PCR mixture containing multiple DNA templates into the microreactors. During PCR mixture loading step, the air inside the microreactors is purged through the air venting port to facilitate the microfluidics based on capillary action. Primer pair's 1, 2, 3 and 4 are in dried form and used to detect *Aeromonas hydrophilia*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* respectively. (B) The cross-sectional view of the PCR array chip with a localized thermal electric cooler (TEC) and heat-sink. (C) Isolation of microreactors by removal of excess PCR mixture from the loading channel by capillary suction flow using the absorbent pad.

The microreactors on the developed array chip are not sealed during the PCR thermal cycling, and the sample evaporative loss in these open/unsealed reactors was well controlled by reactor design, reactor surface and heating region of the PCR array chip. Reduction in sample evaporative loss in the open/unsealed reactors was achieved by:

(1). Localized heating of the microreactors with a peltier device, while the long narrow bridge channels connected to the microreactor is automatically maintained at the room temperature.

(2). Smooth internal surface of the microreactors, to prevent generation of air bubbles inside the reactors during PCR thermal cycling. Fabrication of a smooth internal surface of the reactor is critical for success of PCR in the developed array chip, since presence of air bubbles inside the microreactors will purge the liquid out of the reactors due to bubble expansion.

A photograph of the prototype PDMS-glass chip, comprising an array of nine microreactors for PCR is shown in [Figure 3-8](#). The chip contains four microreactors connected to a common loading channel for target sample (positive control reaction), four microreactors connected to another common loading channel for no-template control reaction (NTC), and a single microreactor for internal positive control (IPC) reaction. In the prototype PCR array chip, the bridge channels are 10 mm long and 300 μm wide; microreactors are 10 mm long and 1mm wide. All the channels and microreactors are 500 μm high. Reactors 1,2,3,4 ([Figure 3-8](#)) are pre-loaded with dried primer pairs, which are specific for *Aeromonas hydrophilia*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* respectively. When PCR mixture containing a pool of genomic DNA template from four waterborne pathogens (*Aeromonas hydrophilia*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*) was loaded in the sample loading port, the PCR mixture filled the microreactors and the dried primer on the glass substrate re-suspended in the PCR mixture.

During annealing step of the PCR process, the primer pairs specifically hybridized to the desired target. An internal positive control (IPC) reaction was included to identify PCR failures caused by failure of on-chip thermal cycling instrument and optics. In IPC reaction, the β -actin gene of human genomic DNA was amplified by PCR. The model template system for IPC reaction was selected based on two criteria, 1. It is unrelated to genomic DNA from waterborne pathogens, and 2. The annealing temperature of the primer pair for β -actin gene was same as the annealing temperature of primer pairs for detecting waterborne pathogens. Reactor 5 contained dried primer pairs specific for β -actin gene of human genomic DNA. The internal positive control PCR mixture contained human genomic DNA. Reactors 6,7,8,9 ([Figure 3-8](#)) are pre-loaded with dried primer pairs (similar to reactors 1,2,3,4), which are specific for *Aeromonas hydrophilia*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* respectively, for performing the

no-template control reaction for corresponding reactions (reactor 1,2,3 and 4). For no-template control reaction (NTC), PCR mixture without genomic DNA template was loaded into the NTC loading port.

Figure 3-8: Photograph of the PDMS-glass chip fabricated by laser cutting technique. PDMS-glass chip comprising an array of nine microreactors for PCR detection of four waterborne pathogens. PCR mixture loading and isolation of an array of microreactors is manually achieved by capillary microfluidics by a single step micropipette operation. Reactors 1-4 are for positive control reactions (*Aeromonas hydrophilia*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* respectively); reactor 5 is for internal positive control reaction, and reactors 6-9 are for no-template control reactions.

3.1.6 Fabrication of PDMS/glass array chip fabricated using laser cutting techniques for detection of multiple water-borne pathogens.

The PCR array chip is composed of three layers. [Figure 3-9](#) schematically illustrates the fabrication process of the PDMS-glass hybrid array chip. Firstly, a 0.5 mm thick PDMS median layer was placed on an acrylic substrate and was patterned with a 30W CO₂ laser (Versa Laser VLS2.30; Universal Laser Systems Inc., Arizona, USA) according to the shape desired for the microreactors. The optimized laser cutting parameters for different materials is listed in [Table 3-1](#).

Table 3-1: Optimized VersaLaser parameters for fabrication of PDMS layer.

Substrate	Z – height (mm)	Laser Watt (W)	Power (%)	Speed (%)	PPI (pulse per inch)
PDMS	1.0	30	5	1	600
PDMS	0.5	30	2	1	600
Acrylic sheet	2.0	25	6	5	1000

A 1mm thick PDMS cover layer, which has been punched with holes for sample loading and air venting, is then bonded with the median layer using a layer of PDMS prepolymer (fresh 10:1 mixture of Dow Corning Sylgard 184 kit) as adhesive. After that, the PDMS structure is peeled off from the acrylic substrate (Figure 3-9A). A smooth inner side wall surface of the microreactor was achieved by using PDMS prepolymer. Secondly, a 0.1 mm thick acid-washed borosilicate glass substrate (Herenz Medizinalbedarf, Hamburg, Germany) was deposited with primer pairs (Figure 3-9B). To guide the primer liquors (mixture of forward and reverse primer) loading in the preferred positions on the glass substrate, a hydrophobic pattern of PDMS prepolymer was formed on the glass substrate using the PDMS structure as a stamp. The primer liquor was then loaded onto the glass substrate and dried at 80°C for 10 mins. Finally the PDMS structure was bonded to the glass substrate, which was pre-deposited with primer pairs using the PDMS prepolymer as adhesive (Figure 3-9C).

A requirement of the fabricated chip is that the inner surfaces of the microreactors should be smooth to prevent bubble generation in the liquid sample during PCR thermal cycling. Since, in the above fabrication process, both the bonding of the two PDMS layers, and the bonding of the PDMS structure to the glass substrate were accomplished with adhesive (PDMS prepolymer), the reflow of the adhesive was able to cover the round corners and smoothen the relatively rough sidewalls, which were generated as a result of laser cutting fabrication process. The extent of the smoothening was controlled by the thickness of the adhesive layer, which in-turn was controlled with spin coating speed and duration. In this particular case, a 20 μm thick

PDMS prepolymer was used as adhesive, and no bubble generation inside the microreactors was observed during the PCR test.

Figure 3-9: Fabrication of the PDMS-glass chip by laser techniques. (A) Fabrication of the patterned PDMS structure. (B) Fabrication of hydrophobic pattern on a glass substrate for primer pair loading. Primer liquor (mixture of forward and reverse primer) preloading and drying. (C) Aligned adhesive bonding of the PDMS structures to the glass substrate.

3.2 PCR matrix chip.

In addition to the capillary-based sample loading for PDMS-glass hybrid chip, another version of high-throughput real-time PCR chip was developed with vacuum-based sample loading. The application of this chip was further expanded to demonstrate the feasibility of digital PCR. The chip is essentially a miniaturized 96-/384- well plate. The salient feature of this chip is rapid loading using vacuum. The vacuum pump is portable and hence can be used for field applications. Different density of matrix chip (100-, 400-, 1024- & 80,000 wells) were successfully fabricated.

3.2.1 Design of PCR matrix chip.

The matrix chip consists of three layers: a glass substrate/silicon layer, a transparent glass cover layer for fluorescent measurement from the top of the chip, and a PDMS

structure layer sandwiched in between (Figure 3-10). During initial development of the 100-well matrix chip, a 0.1 mm thick glass substrate was used. Successful amplification was achieved with a glass substrate. Subsequently a silicon layer was used as a substrate, due to its high thermal conductivity.

Figure 3-10: Schematic diagram showing three layers of the matrix chip.

Figure 3-11: Three dimensional view of the matrix chip.

The 100-well matrix chip has a total dimension of $22 \times 22 \times 1.2$ mm, and each well has a dimension of $0.5 \times 0.5 \times 0.5$ mm that hold sample liquid of about 120nl. The chip also contains one inlet channel, three outlet/air-venting channels and a headspace on top of the wells for microfluidic operation (Figure 3-11).

3.2.2 Fabrication of PCR matrix chip.

The PDMS layer is replicated from a stainless steel die, which was fabricated by an electrical discharge machining (EDM) process by a commercial vendor (Bytech Precision Engineering, Singapore). A photograph of the EDM machined die is shown in [Figure 3-12](#).

[Figure 3-12](#): Photograph of the stainless steel metal EDM die.

To replicate the PDMS layer, 2ml of liquid containing 10:1 PDMS Sylgard Silicone Elastomer 184 and Sylgard Curing Agent 184 (Dow Corning Corporation Midland, MI, USA) was mixed homogenously in a beaker using magnetic stirrer at 150 rpm for 1 hour. The PDMS prepolymer was poured onto the surface of the metal die and subjected to vacuum for 20 minutes to degas. Following this, a square glass block (160 g) with a smooth surface was placed on top of the PDMS prepolymer and the entire assembly was placed at 80°C for 2 hours for PDMS curing. The PDMS layer was peeled off and cut to a desired size. It was permanently bonded to a 0.1 mm thick acid-washed borosilicate glass substrate (Herenz Medizinalbedarf, Hamburg, Germany) using a 4 μ m thick spin-coated layer of PDMS prepolymer as adhesive. Following this, liquors containing different primer pairs were manually loaded under as stereomicroscope into wells using micro-tubing and dried by incubating the chip at 80°C for 10 mins. Finally, a 0.1 mm thick acid-washed borosilicate cover glass was adhesive bonded to the top of the chip to form enclosed microchannels and a headspace for microfluidic handling of PCR mixture and PDMS prepolymer. An optical micrograph of the PCR matrix chip is shown in [Figure 3-13](#).

In addition to the 100-well matrix chip, a 400-well chip (dimension of $0.4 \times 0.4 \times 0.4$ mm) that holds sample liquid of about 64nl was fabricated using another EDM die and successfully tested for its functionality.

Figure 3-13: Optical micrograph of the PDMS matrix chip. PDMS matrix chips comprising 100 wells of 120nl volume. The channels and wells are fabricated on a PDMS layer, and bonded to a glass substrate and a glass cover to form an enclosed headspace above the wells for microfluidic handling of PCR mixture and sealant.

The 400-well chip also contains one inlet channel, three outlet/air-venting channels and a headspace with height of 0.4mm on top of the wells for microfluidic operation. The chip has a total dimension of $24 \times 26 \times 1$ mm and consists of three layers: a glass substrate layer, a glass cover layer, and a PDMS structure layer sandwiched in between. A very high throughput chip with 80,000 wells with well dimension of $40 \times 40 \times 40 \mu\text{m}$ was also fabricated using a silicon die, which was micro fabricated by deep reactive ion etching (DRIE) (Figure 3-14).

Figure 3-14: Photograph of the 400 and 80,000 well chip. (A) Photograph of the 400-well matrix chip, (B) Photograph of the 80,000-well matrix chip, the inset shows 64 pL picowells.

SURFACE TENSION AND VACUUM BASED MICROFLUIDICS

*Progress in science depends on new techniques, new discoveries and new ideas,
probably in that order.*

- **Sydney Brenner** 2002 Nobel Laureate

*for his contributions to genetic regulation of
organ development and programmed cell death.*

Chapter 4 Surface Tension and Vacuum Based Microfluidics^{1,2}

In this chapter the basic principles of capillary-based microfluidics and a one-dimensional mathematical model for capillary microfluidics in a rectangular channel with two different surfaces is presented. In addition, the development of surface tension based and vacuum-based micro fluidics are reported in this section.

4.1 Principle of capillary microfluidics.

Capillary forces result from the interaction of liquid, gas and solid surfaces, at the interface between them. When a liquid drop is placed on a surface, the angle formed between the contact lines at the solid-liquid and liquid vapor interfaces is known as static contact angle (θ) (Figure 4-1). The contact angle is characterized by an interfacial, free-energy minimization. The surface energy is also referred to interfacial tension, and it cannot be measured, since solid do not deform like liquids. Surface energy/interfacial tension in liquids is known as surface tension with SI unit's mN/m or mJ/m². By taking balance of forces tangential to surface of the solid will yield the Young equation.

$$\gamma^{SV} = \gamma^{SL} + \gamma^{LV} \times \cos \theta \quad (4.1)$$

Where γ^{SV} , γ^{SL} , and γ^{LV} are the interfacial tensions between the solid and vapor, solid and liquid, and liquid and vapor, respectively, θ is the contact angle between γ^{LV} and γ^{SL} . When the contact angle is greater than 90°, the liquid will be “non-wetting” and surface is known as hydrophobic. When the contact angle is less than 90°, then the liquid will “wet” the surface, which is known as hydrophilic.

¹ Gong H, **Naveen Ramalingam**, Chen L, Che J, Wang Q, Wang Y, et al. Microfluidic handling of PCR solution and DNA amplification on a reaction chamber array biochip. **Biomed Microdevices** 2006,8:167-176.

² Liu H-B, Gong H-Q, **Naveen Ramalingam**, Jiang Y, Dai C-C, Hui KM. Micro air bubble formation and its control during polymerase chain reaction (PCR) in polydimethylsiloxane (PDMS) microreactors. **Journal of Micromechanics and Microengineering** 2007,17:2055-2064.

Figure 4-1: Schematic diagram illustrating the interfacial tension due to interaction of liquid, gas and solid surfaces.

If the surface tension of the fluid is below the surface energy of the solid, the liquid will spread on the surface. In the developed PCR array chip, the rectangular microchannel is composed of two substrates, glass substrum (one face) and PDMS (three faces) (Figure 4-2). The contact angles of liquid/PCR mixture on glass substrate and PDMS are denoted by θ_g and θ_p respectively. The pressure drop at liquid-air interface inside a rectangular microchannel with two different surfaces is expressed by Young-Laplace, one dimensional mathematical model Equation 4.2 [105].

$$p_c = \sigma_{PCR} \left(\frac{\cos\theta_g + \cos\theta_p}{d} + \frac{2\cos\theta_p}{w} \right) \quad (4.2)$$

Where P_c (P_c or $\Delta p = p_l - p_v$; p_l is pressure at liquid phase, p_v is pressure at vapor phase) is capillary pressure, σ_{PCR} is surface tension of PCR solution, d and w are depth and width of rectangular channel respectively, θ_p and θ_g are contact angle of PCR mixture on PDMS, and acid-washed glass surface respectively. The equivalent force F experienced by the capillary fluid column along the x -direction is given by Equation 4.3

$$F = P_c \times w \times d \quad (4.3)$$

When, $\Delta p = p_l - p_v > 0$ the channel is hydrophilic, and $\Delta p = p_l - p_v < 0$ the channel is hydrophobic.

Figure 4-2: Schematic diagram illustrating the principle of capillary pump. An unbalanced Young's force at position $L(t)$ drives the PCR mixture in the positive direction of x-axis inside the rectangular microchannel. w , $L(t)$, σ_{PCR} , τ , θ_g are width of the channel, displacement length of the PCR mix-air interface, surface tension of PCR mixture, shear stress on the channel wall and contact angle of the PCR mixture on a acid-washed glass substrate respectively.

In a capillary formed by the surfaces of two different materials (glass and PDMS), the liquid will spontaneously fill the rectangular capillary provided the contact angle of the liquid is less than 90° on both the surfaces. If the contact angle of liquid is greater than 90° , there are two ways of manipulating capillary microfluidics for passive filling. One way is to reduce the contact angle of the liquid below 90° by introducing surfactants. It is important to note that such addition of surfactants should not affect the performance of the downstream chemical or biological assays. The second way is to change the geometry of the channel. Different methods to manipulate capillary microfluidics are shown in [Figure 4-3](#).

Capillary pumps were developed by Prof. George Whitesides group at Harvard, for fabrication of polymer microstructures on substrates by a procedure known as MIMIC (micro molding in capillaries) [106], and subsequently used in variety of microdevices for various applications. Prof. Whitesides group studied the flow of different polymer in a rectangular PDMS microchannel with self-assembled monolayers for controlling the hydrophilicity of the channel surfaces. In 2001, Zhao *et al.* reported a device in which the surface was selectively controlled by forming a UV photocleavable SAM on glass surface to direct liquid flow inside microchannel [107]. Capillary pumps are ideal for point-of-care microdevices. Such an implementation was demonstrated by Dr. Emmanuel Delamarche group at IBM Zurich for autonomous point-of-care immunodiagnostics biochips [108-113].

Figure 4-3: Various methods published in literature to manipulate capillary microfluidics.

In this study, passive capillary pump based on liquid-liquid surface tension manipulations was developed to load PCR solution among an array of microreactors in a reproducible and economical manner.

4.2 Development of surface-tension based microfluidics.

Two microfluidic schemes based on surface-tension and vacuum are described in this section. The surface-tension-based microfluidic scheme is ideal for point-of-care application, since it does not require liquid handling or pumping instruments for sample distribution among an array of reactors.

4.2.1 Capillary pump for sample loading in a chip for point-of-care settings.

In the PCR array chip, sample loading and reactor sealing is achieved by unbalanced Young's force. The surface tension generated pressure, which accounts for passive filling of PCR mixture and liquid sealant. The hydrophobic nature of the PDMS layer in the PCR array chip prevents the flow of pure water or PCR mixture (without detergent) into the microreactors by capillary action. In order to load sample and PDMS prepolymers into the PDMS channel, two different strategies are possible:

- (1) **Dynamic manipulation method:** Commercial PCR master-mixes containing surfactant was implemented to reduce the contact angle of PCR mixture on the PDMS. This reduction in contact angle enabled capillary filling of PCR mixture.
- (2) **Static manipulation method:** The hydrophobic PDMS is treated with PCR-compatible chemicals, to modify it to a hydrophilic channel. Briefly, the PDMS layer was coated with n-dodecyl-beta-D-maltoside (DDM) by following the protocol by Richard N Zare [114]. The n-dodecyl-beta-D-maltoside coated PDMS was found to be PCR-compatible. Since this modification method is cumbersome as compared to the dynamic manipulation method, further characterization of the DDM coated PDMS was not done. Another excellent method, which can be used to modify PDMS using polyethylene glycol (PEG) was published by Emmanuel Delamarche from IBM Zurich [115].

Dynamic manipulation method was widely used for experiments in this doctoral work, due to its ease in use. Chemical surface treatment takes one day for chip preparation. In this section, the dynamic manipulation method using surfactant will be discussed in detail. The contact angle of the PCR solution (without detergent) on the PDMS layer was found to be 97° using FTA 200 dynamic contact angle analyzer (First Ten Angstroms, Portsmouth, USA). Experimental results show that PCR solution without any detergent failed to flow through the microchannels by capillary action ([Figure 4-4](#)).

PCR buffer/master mix from some vendors contains Triton X-100. Triton X-100 is a non-ionic detergent present in some commercial PCR buffers to enhance the *Taq* polymerase activity. It is also thought to suppress the formation of secondary structure [116]. Nonionic detergents, such as Tween®-20, NP-40, and Triton® X-100, have the added benefit of overcoming the inhibitory effects of trace amounts of strong ionic detergents, such as 0.01% SDS. Many enzymes remains active in the presence of Triton X-100; for example Proteinase K, remains active in 1% Triton X-100.

Figure 4-4: Contact angle of PCR mixture without detergent and PCR mixture with 0.1% Triton X-100 detergent (Promega) on cured PDMS layer and acid-washed glass substrate. (a) Contact angle of PCR mixture without detergent (97°) on cured PDMS. (b) Contact angle of PCR mixture with 0.1% Triton X-100 detergent (46°) on cured PDMS. (c) Contact angle of PCR mixture without detergent (30°) on acid-washed glass. (d) Contact angle of PCR mixture with 0.1% Triton X-100 detergent (14°) on acid-washed glass.

Experimental results from this doctoral work indicate that AmpliTaq Gold, the enzyme that copies DNA during PCR process is active in 0.1% Triton X-100. [Table 4-1](#) summarizes the components of PCR buffer from different vendors. Some vendors keep the composition of PCR buffer as proprietary information. The first five PCR buffer products (Promega, Funnymen, New England Bio labs, and Thermo Scientific) in [Table 4-1](#) contain Triton X-100. Hence, use of these buffer systems is recommended for the reported PCR array chip. In the work, *Taq* DNA polymerase buffer (M1902, Promega) containing 0.1% Triton X-100 is extensively used for experimentation. In addition, hot-start platinum *Taq* DNA polymerase from Invitrogen is used in conjunction with the Promega (M1902) buffer. The performance of the *Taq* DNA polymerase in Promega (M1902) buffer was evaluated, and efficiency was found to be above 90%.

Table 4-1: Composition of buffer systems with and without Triton X-100 from different vendors.

S.No	Vendor	Product name/part number	Composition (1X)
PCR Buffer/master mix with Triton X-100			
1	Promega	<i>Taq</i> DNA polymerase (M1902)	10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100
2	Promega	<i>Pfu</i> DNA Polymerase (M7741)	20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM (NH ₄) ₂ SO ₄ , 2 mM MgSO ₄ , 0.1% Triton® X-100 and 0.1 mg/ml nuclease-free BSA.
3	Finnzyme	DyNAzyme™ EXT Buffer (F-514S)	50 mM Tris-HCl (pH 9.0), 15 mM (NH ₄) ₂ SO ₄ and 0.1 % Triton® X-100
4	New England Biolabs	ThermoPol II (M0321S)	20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM (NH ₄) ₂ SO ₄ , 0.1% Triton X-100
5	Thermo Scientific	<i>Taq</i> DNA Polymerase Kits (AB0192)	75 mM Tris-HCl (pH 8.8), 20 mM (NH ₄) ₂ SO ₄ , 0.1% (v/v) Tween* 20
PCR Buffer/master mix without Triton X-100			
1	Finnzyme	DyNAzyme™ II Hot Start (F-522S)	15 mM Tris-HCl (pH 8.2), 2.5 mM MgCl ₂ , 30 mM KCl, 5 mM (NH ₄) ₂ SO ₄ and 0.02 % bovine serum albumin.
2	Invitrogen	Platinum <i>Taq</i> polymerase	20 mM Tris-HCl (pH 8.4), 50 mM KCl
3	Biorad	iTaq DNA polymerase	20 mM Tris-HCl (pH 8.4), 50 mM KCl
4	Roche	FastStart <i>Taq</i> DNA Polymerase (04 738 314 001)	50 mM Tris/HCl (pH 8.3), 10 mM KCl, 5 mM (NH ₄) ₂ SO ₄ , 2 mM MgCl ₂
5	Quanta Biosciences	AccuStart™ <i>Taq</i> DNA Polymerase (Cat# 95061-250)	20 mM Tris-HCl (pH 8.4), 50 mM KCl
6	Sigma Aldrich	JumpStart™ <i>Taq</i> DNA Polymerase. (D9307).	10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl ₂ , 0.001% (w/v) gelatin.

For PCR buffers without any detergents (refer to [Table 4-1](#)), Triton X-100 can be externally added at 0.1% to achieve capillary-based microfluidics on the PCR array chip. Addition of 0.1% Triton X-100 detergent to the PCR mixture significantly reduced the contact angle of the PCR solution on the PDMS layer to 46° ([Figure 4-4](#)).

Figure 4-5: Effect of Triton X-100 concentration on contact angle of PCR buffer on glass and PDMS surfaces. Error bars are standard deviation for $n = 5$ samples (n is number of technical replicates). PDMS prepolymer is used as liquid sealant in the developed PCR array chip.

The surface tension of PCR mixture and pure deionized water was found to be 30.8 ± 0.06 mN/m ($n = 4$; where n is number of technical replicates) and 71.4 ± 0.3 mN/m ($n = 4$) respectively using hanging drop method on FTA 200 dynamic contact angle analyzer (First Ten Angstroms, Portsmouth, USA). In order to validate the one-dimensional mathematical model (Equation 4-2), calculation were performed for outlet bridge channel in the developed PCR array chip, $d = 180$ μm , $w = 40$ μm , $\theta_p = 46^\circ$, $\theta_g = 14^\circ$. The capillary pressure (Equation 4-2) inside the outlet bridge channel is found to be about 1.35 kPa. Since $P_c > 0$, the PCR mixture flows inside the inlet and outlet bridge channel by capillary action.

Figure 4-6: Surface tension measurements of deionized water and PCR mixture containing 0.1% Triton X-100 by hanging drop method. (a) Surface-tension measurement of deionized water (R1-R4: Four replicate measurements). (b) Surface-tension measurement of PCR mixture containing 0.1% Triton X-100 (R1-R4: Four replicate measurements).

The microfluidic operation process of the PCR array chip consists of three steps: the PCR mixture loading, removal of the excess PCR mixture (dead volume), and sealing of the microreactors to prevent evaporation of sample and cross-contamination of primer pairs. In order to visualize the flow by capillary action, 0.1 % Xylene Cyanole FF (Sigma-Aldrich, Singapore) blue dye was added to the PCR mixture.

Figure 4-7(a) shows the PCR mixture loading step. The microreactors are filled with PCR mixture in a sequential manner by capillary action. After filling the microreactor, the PCR mixture enters the outlet bridge channel and stops at the exit to the air-venting channel due to the passive valve effect. Figure 4-7(b) shows the removal of the excess PCR mixture in the inlet channel by an absorbent pad. Following this, all the microreactors were isolated from each other by sealing the inlet and outlet bridge channels by using semi-cured liquid PDMS prepolymer (The liquid PDMS was precured at 72°C for 10 minutes in an oven) as a sealant (shown in Figure 4-7(c)). The sealant is loaded into the open-top inlet and air-venting channel through the loading port and the liquid PDMS prepolymer fluidically isolates the microreactors from each other by capillary action. Strong sealing of the microreactors is achieved by curing the sealant at 72°C for 10 minutes before thermal cycling and

the sealant totally cured during the PCR thermal cycling process. The curing temperature of the PDMS sealant (72°C for 10 minutes) did not produce any significant deleterious effect on the PCR process, since a hot-start *Taq* polymerase (activated at 95°C, which is higher than the sealant curing temperature) was used. The consistent threshold cycle (C_T) values and yield data from the PCR array chip, when compared to the PCR performed on a commercial PCR instrument, ascertain negligible effect of the sealant curing temperature on the PCR process.

Figure 4-7: Microfluidic operation process for chip fabricated using DRIE die (a) PCR mixture loading. For flow visualization 0.1%, blue dye was added. (b) Removal of the excess PCR mixture in the inlet channel by an absorbent pad to fluidically isolate all the microreactors to prevent primer cross contamination. (c) Microfluidic sealing of the microreactors by PDMS prepolymer to avoid sample evaporation during PCR thermo cycling.

4.2.2 Mathematical model for capillary meniscus movement in rectangular inlet channel.

Capillary filling in planar microchannels are modeled using the classic Washburn equation [117]. Large deviations from the Washburn model were noted for microchannels with dimensions in the nanometer range [118, 119]. Hibara *et al.* reported slowing in predicted capillary flow for nanochannels with heights of 330 nm

[118]. The deviation in this study was attributed to apparent increase in fluid viscosity in nm range. Tas *et al.* further developed this work and suggested electroviscous effect as a contributing factor for deviation from the predicted flow [119]. Subsequently, Nam-Trung Nguyen extensively modeled the effects of electroviscous on capillary flow [120, 121]. With the implementation of electroviscous effect on capillary flow, the authors demonstrate good agreement with Washburn's equation. Since the dimensions of microchannel used in this study were in micrometer range, electroviscous effect was not taken into account.

Geometry of microchannel.

In this experiment, the capillary flow in the 24 mm long rectangular inlet channel was studied. The dimensions of the inlet channel, microreactor and outlet channel used for this experiment is shown in [Figure 4-8](#). The values of various parameter used in the mathematical modeling is listed in [Table 4-2](#).

Figure 4-8: AutoCAD drawing showing the dimension of inlet channel, microreactor and outlet channel on a capillary microdevice used for mathematical modeling.

Table 4-2: Inlet channel dimensions and value of other parameters.

Parameter	Symbol	Values (Unit)	Reference
Length of the inlet channel	L_{in}	24 mm	Table 4-2
Width of the inlet channel	w	2.5 mm	Table 4-2
Depth of the inlet channel	d	0.18 mm	Table 4-2
Operation temperature (Room temp/Reference temperature)	T_{ref}	293 K	This study
Atmospheric/reference pressure	p_{ref}	1e5 Pa	
Density of air @293 K	ρ_{air}	$1.225 \frac{Kg}{m^3}$	
Dynamic viscosity of air @293 K	η_{air}	$1.789 \text{ e-}5 \text{ Pa} \cdot \text{sec}$	
Density of PCR mixture @293 K	ρ_{PCR}	$1000 \frac{Kg}{m^3}$	This study
Dynamic viscosity of PCR mixture @293 K	η_{PCR}	$1\text{e-}3 \text{ Pa} \cdot \text{sec}$	[122]
Kinematic viscosity of PCR mixture @293 K	ν_{PCR}	$1\text{e-}6 \frac{m^2}{s}$	This study
Surface tension of PCR mixture	σ_{PCR}	$30.8\text{e-}3 \frac{N}{m}$	This study
Static contact angle of PCR mixture on PDMS	θ_p	46° (0.8 rad)	This study
Static contact angle of PCR mixture on glass	θ_g	14° (0.24 rad)	This study

Governing equations.

The flow is considered laminar, incompressible, and Newtonian with isothermal condition. The governing equations are the Navier-Stokes and Continuity equation.

The volumetric flow rate ($\frac{dV}{dt}$), in a rectangular channel for a Newtonian fluid is given by Equation 4.4 [117].

$$\frac{dV}{dt} = \frac{d(wdL(t))}{dt} = \frac{w \times d \times \Delta p \times c_g}{\eta L} \quad (4.4)$$

where w and d are width and depth of the inlet channel, $L(t)$ is the length of the horizontal capillary column from the origin of x -axis (refer to Figure 4-8), η is the viscosity of PCR mixture, Δp is the pressure drop across the distance L , and c_g is the shape factor given by an infinite series described by Equation 4.5 [123].

$$c_g = \frac{d^2}{12} \left(1 - \sum_{i, odd} \frac{1}{i^5} \frac{192}{\pi^5} \frac{d}{w} \tanh \left(i\pi \frac{w}{2d} \right) \right) \quad (4.5)$$

The capillary pressure in the hybrid-PDMS glass microchannel is given in Equation 4.2. By integration of Equation 4.4, and use of approximation for the infinite series in

Equation 4.5 [123], the liquid meniscus position inside the rectangular microchannel is given by following Equation 4.6.

$$L^2(t) = \left[\frac{d^2 \times \sigma_{PCR}}{6 \times \eta_{PCR}} \times \left(\frac{\cos \theta_g + \cos \theta_p}{d} + \frac{2 \cos \theta_p}{w} \right) \left(1 - 0.63 \frac{d}{w} \right) \right] \times t \equiv a_t \times t \quad (4.6)$$

where a_t is the theoretically predicted constant of proportionality [124]. From the above equation, it can be noted that L^2 is directionally proportional to t .

In order to experimentally determine the position of meniscus inside the PDMS-glass hybrid channel with respect to time, a microchip device with dimensions mentioned in Table 4-2 was fabricated. The PCR mixture was loaded at the mouth the inlet channel and the movement of capillary meniscus in the PDMS-glass hybrid inlet channel with respect to time is monitored using a SONY camcorder (Model: DCR-HC 38, 40X optical zoom capability with Carl Zeiss® Vario-Tessar® Lens). A scale with 1mm pitch was included to measure of capillary displacement. The sequencing of images extracted from this video is shown in Figure 4-9.

The experiment data for $L(t)$ versus time (secs) is shown in Table 4-3.

Table 4-3: Experimental data for $L(t)$ vs Time (secs).

Time (secs)	Distance (mm)	Distance ² (mm ²)
2	4	16
4	8	64
6	11.5	132.25
8	15	225
10	17	289
12	20	400
14	23	529
Speed	1.64 mm/sec	Based on last data point

Figure 4-9: Displacement of capillary meniscus as a function of time.

The capillary front position vs time plots are shown in [Figure 4-10](#) and [4-11](#).

Figure 4-10: Plot of capillary front position ($L(t)$) versus time.

Figure 4-11: Plot of $L^2(t)$ versus time. $L(t)$ is the capillary front position.

From [Figure 4-11](#), $L^2(t)$ is approximately linear to time t . One of the reasons for small deviation between the theoretical prediction and experimental data is that, the [Equation 4.6](#) (theoretical prediction) provides the position of meniscus in a straight, horizontal rectangular microchannel. Whereas, for the capillary microfluidics, reported in this work, the inlet channel is orthogonally bifurcated into microreactors. Hence, there will be a difference in the measured and theoretical constant of proportionality (a_t). For simplicity, this mathematical model was used to understand the relation between L^2 and t .

4.2.3 Effect of surfactant (Triton X-100) on threshold cycle, PCR yield, PCR efficiency and amplicon melting temperature.

In order to achieve capillary microfluidics, it is essential that the PCR buffer contain surfactant such as Triton X-100 or Tween 20. Commercial PCR buffers, which do not contain Triton X-100/surfactant is spiked with 0.1% Triton X-100. Higher concentration of detergents is advantageous for our capillary-based microfluidics. However, studies show that Triton X-100 inhibits PCR at concentrations higher than 5% (v/v) [125]. In order to understand the effect of 0.1% Triton X-100 addition on threshold cycle, PCR yield, PCR efficiency and amplicon melting temperature, 189-bp amplicon were analyzed on RotorGene 3000. All reaction was tested in triplicates. For this experiment Invitrogen PCR buffer (pH 8.4; 20 mM Tris-HCl, 50 mM KCl) was spiked with 0.1% Triton X-100 and tested. The control PCR buffer reaction contained Invitrogen buffer without Triton X-100 and a commercial buffer from Promega, which contains 0.1% Triton X-100. The results are presented in [Figure 4-13](#). It can be inferred from [Figure 4-13](#), that addition of 0.1% did not affect the PCR performance metrics (Threshold cycle, PCR efficiency, melting temperature, and PCR yield). The use of Triton X-100 is advantageous for most of the primer-template systems including ours. However, it should be noted that this might not be a general case for other primer-template systems. It is advisable to optimize the concentration of Triton X-100 for each primer-template system.

Figure 4-13: Effect of Triton X-100 (0.1%) on PCR performance metrics. (a) threshold cycle of triplicate reactions. (b) PCR efficiency. (c) melting temperature (T_m) of amplicon. (d) PCR yield quantified on Agilent Bioanalyzer. Composition of Promega buffer: (pH 9.0;10 mM Tris-HCl,50 mM KCl, 0.1% Triton X-100). Composition of Invitrogen buffer: (pH 8.4;20 mM Tris-HCl,50 mM KCl). Composition of modified Invitrogen buffer: (pH 8.4;20 mM Tris-HCl,50 mM KCl,0.1% Triton X-100). Error bars are SDs for triplicate reactions.

4.2.4 Surface-tension-based microfluidic devices harboring open/unsealed reactors.

The above microfluidic scheme (4.2.1) was adapted for PDMS/glass hybrid chip fabricated using laser cutting techniques. A picture of the chip is shown in Figure 3-8. After the single step manual dispensing of PCR mixture into the loading port using a pipette, the capillary microfluidic process completes the fluidic operation process, which comprises two steps: 1. PCR mixture loading, and 2. Removal of excess PCR mixture (dead volume) to isolate reactors from each other, as illustrated in Figure 4-14. In order to visualize the PCR mixture flow, food coloring agents with 0.1% Triton X-100 was used. The microfluidic operation process for positive control reaction

(blue color), no-template control reaction (green color), and internal positive control reaction (red color) is shown in Figure 4-14. In the loading step, the PCR mixture flowed in the loading channel, and filled the microreactors in a sequential manner. After filling the microreactor, the PCR mixture entered the outlet bridge channel and stopped at the air venting port. In order to isolate the microreactor, the excess PCR mixture in the loading channel was automatically removed by an absorbent pad through a waste channel.

Figure 4-14: Microfluidic operation process for chip fabricated using laser technique (a) Fabricated PCR array chip showing on-chip heating zone and absorbent pad. Reactors 1-4 are connected to a common loading port and it is used for detection of target organism (positive control:PC). Reactor 5 is used for internal positive control (IPC) reaction. Reactors 6-9 are connected to a common loading port and it is used for no-template control (NTC) reaction. (b) The microfluidic operation process for PC is demonstrated by using a blue dye. (c) The microfluidic operation process for IPC is demonstrated by using a red coloured dye. Isolation of PC reactions (1-4) by removal of excess PCR mixture by the absorbent pad. (d) The microfluidic operation process for NTC is demonstrated by using a green coloured dye. (e) Isolation of NTC microreactors by removal of excess PCR mixture from loading channels. (f) Chip after completion of microfluidic operation process.

4.2.5 Chip design parameters consideration for capillary microfluidics.

In order to achieve capillary microfluidics on the array chip for PCR mixture loading and reactor sealing without air trapping, array chip with different dimensions were designed, fabricated and tested.

Effect of three different design parameters on microfluidic operation was studied ³ :

- (1) Shape of the microreactor,
- (2) Inlet bridge channel width and length, and
- (3) Outlet bridge channel with different width and length.

For easy flow visualization PCR mixture (containing 0.1% Triton X-100) was stained with 0.1% of Xylene Cyanole FF (Sigma Aldrich, Singapore) blue dye.

Shape of the microreactor

The shape of the microreactor is defined by four parameters: the width W , the length L , the inlet angle α , and the outlet angle β (Figure 4-15(A)). Microreactors with rectangle, diamond and hexagon shapes with different dimension ratios (length to width) were tested for microfluidics without air trapping.

Figure 4-15: Effect of microreactor shape on microfluidic operation process. (A) Parameters that defines the size and shape of a microreactor. (B) The hexangular microreactor (left) shows better sample loading property than the rectangular microreactor (right). (C) Effect of reactor shape in trapping of air bubble.

The shape of the microreactor defines the value of inlet (α) and outlet (β) angle, which influences the loading of the sample. For a rectangular chamber ($\alpha = 90^\circ$), the inlet bridge channel with a width of $100\mu\text{m}$ functions as a passive valve, and hence the

³ Design consideration experiments were performed with Dr. Liu Hao-Bing.

liquid sample cannot flow inside the microreactor, whereas liquid sample flows under capillary action in microchambers with hexagonal shape ($\alpha = 45^\circ$) with inlet bridge channel with $40\mu\text{m}$ width (Figure 4-15(B)). Smaller values of α will lead to faster loading of the PCR mixture. However, for a fixed value of W and L , smaller α reduces the liquid sample volume of the chamber. For the device reported in this work, α was selected as 30° . The shape of the microchamber also influences air trapping during sample loading (Figure 4-15(C)). During the initial phase of design considerations, etched glass, based chips were used for capillary microfluidics. Detailed design of the chip and fabrication can be found in the paper [126]. Eight different shape of microreactor was fabricated on the glass chip (Figure 4-16).

Figure 4-16: Eight different microreactor shapes. An illustration of a microfluidic biochip showing inlet channel (1) for PCR solution loading, outlet channel (2) for air venting, PCR microchambers of various shapes (3), inlet bridge channel (4) connecting a microchamber with inlet channel and outlet bridge channel (5) connecting a microchamber with outlet channel. The biochip cover is not shown.

Microfluidic operation process results shows that reactors with circle and diamond shape successfully got loaded with PCR solution without any bubble trapping (Figure 4-17). It is imperative to note that the surface hydrophilicity play a key role in sample loading without air trapping [126].

Figure 4-17: Sample loading failure modes based on reactor shapes. Effect of the shape of the microchamber on the loading of the PCR solution, where the black portion indicates the area filled with PCR solution. The sample loading failed for certain chamber geometries. The microchambers with shapes like square, hexagon, vertically standing rectangle, and horizontally standing rectangle were only filled in the area near the chamber sidewall. The off-sidewall area were not filled because of the air trapping caused by the blockage of the microchamber air venting exit by the fast moving liquid along the sidewall.

The air bubbles are detrimental to the PCR process, as they expand during PCR thermal cycling and cause failure of the PCR by purging the sample mixture out of the microreactor. In addition to this failure mode, bubbles that sustain inside the reactor through the 30-40 cycles of PCR, cause inefficient PCR in that microreactor, since, air bubble is a thermal insulator and thereby hinder uniform thermal distribution across the microreactor.

For the PDMS-glass hybrid chip, both rectangular and diamond shaped microreactors trapped air bubbles during sample loading, while PCR mixture was completely loaded in the hexangular shaped reactor without trapping air-bubbles (Figure 4-15(C)). Repeated experiments confirmed this observation. Based on this result, microreactor with hexangular shape with $\alpha = 30^\circ$ and $\beta = 45^\circ$ was fixed.

The width (W) of the microreactor affects the formation of vapors. Vapors always formed in microreactors with width and depth of 5 mm and 0.1 mm respectively. Even with a vapor barrier (0.1 mm thick glass cover) above the PDMS layer, PCR always failed in such a dimension of microreactor due to purging of PCR mixture from the microreactors by the vapor bubbles. PCR was successful in most of the microreactors with width (0.5 mm, 3 mm) and depth of 0.1 mm.

In some cases, PCR failed due to the following reasons

1. Air trapped between the PDMS layer and glass substrate during chip fabrication process.
2. Air trapped during improper microfluidic operation process,
3. Air trapped in defects introduced in the PDMS layer from the silicon die, and
4. Improper mixing of liquid PDMS sealant (weak sealant).

Inlet bridge channel width and length.

The width of the inlet bridge channel influences the loading of PCR mixture and the cross-contamination of primer pairs to the adjacent microreactors. A wider and shorter inlet bridge leads to faster sample loading, but it presents difficulty in sealing the reactors. Hence, a wider inlet bridge channel poses a high risk of primer cross-contamination due to easier diffusion of primer pairs in these channels. Therefore, it is ideal to have a longer and narrower inlet bridge channel. However, a longer inlet bridge consumes more space on the chip and leads to longer sample loading time. A narrower inlet bridge also leads to slower loading speed, and more seriously, prohibits the sample from flowing into the microreactor for some shapes of the reactor. Microfluidic operation process was tested in different PCR array chips with inlet bridges width of 40 μ m, 60 μ m, 80 μ m, 100 μ m, 140 μ m, 200 μ m, and 300 μ m with a rectangular microreactor. It was observed that microreactors with inlet bridge < 140 μ m failed to load PCR mixture inside the microreactor, due to activation of capillary valve at the end of the inlet bridge channel (Figure 4-18(A)). Another drawback of using a wider inlet bridge is that, during excess PCR mixture removal (dead-volume) from the inlet channel, more PCR mixture is lost from the inlet bridge channel (Figure 4-18(B)). Considering the above factors, an inlet bridge channel with 300 μ m width and 600 μ m length was fixed.

Figure 4-18: Effect of inlet bridge channel parameters on microfluidic operation process. (A) Effect of inlet bridge width. The narrower inlet bridge (100µm width) prevented sample loading into a rectangular microreactor. (B) More liquid in the wider inlet bridge is removed during the microfluidic operation process for removal of excess sample from the inlet channel.

Outlet bridge channel width and length.

The width of the outlet bridge is a critical parameter for microfluidic operation process, since it functionally acts as a passive capillary valve to confine the PCR mixture inside the microreactor. This geometric confinement is based on an abrupt change of outlet dimensions, where capillarity breaks down. Different widths of 40µm, 60µm, 80µm, 100µm and 200µm with length of 500µm and 1000µm were fabricated to study the effectiveness of the capillary valve. An outlet bridge channel with 1000 µm length and 80 µm width successfully confined the PCR mixture, while the same channel with longer width of 100 µm failed to confine the PCR mixture (Figure 4-19(A)). Similarly, for an outlet bridge channel with 500 µm length and 60 µm width successfully confined the PCR mixture, while the same channel with longer width of 80 µm failed to confine the PCR mixture (Figure 4-19(B)). Based on this result, the dimensions of length and width of the outlet bridge channel was fixed to 800µm and 40µm respectively.

Figure 4-19: Effect of outlet bridge channel parameters on microfluidic operation process. (A) An outlet bridge channel with 1000 μm length. 80 μm wide channel successfully confined the PCR mixture, while 100 μm wide channel showed functional failure (B) An outlet bridge channel with 500 μm length. 60 μm wide channel successfully confined the PCR mixture, while 80 μm wide channel showed functional failure.

4.2.6 PCR mixture confinement by passive capillary stop valves.

Confinement of PCR mixture inside the microchambers is a challenging problem. We employed a passive valve for confinement of PCR mixture. The passive valve at the exit of the outlet bridge channel was achieved by abrupt change in channel dimension on the three sides (PDMS wall) of the outlet bridge channel. Successful confinement of liquid depends on the contact angle of PCR mixture on the cured PDMS layer (θ_p) and on acid-washed glass substrate (θ_g) and outlet bridge channel expansion angle (δ) [127]. We measured the contact angle of PCR mixture on PDMS layer and on acid-washed glass substrate to be about $46^\circ(\theta_p)$ and $14^\circ(\theta_g)$ respectively using FTA 200 dynamic contact angle analyzer. The critical expansion angle [127] $\delta_c (= \pi/2 - \theta_p)$, for the developed chip was calculated to be about 44° . For our chip, the passive valve will not fail to confine the PCR mixture, if the expansion angle of the PDMS walls (three sides) of the outlet bridge channel is higher than 44° . We designed and fabricated our chip with outlet bridge channel expansion angle $\delta = 90^\circ$ for three sides of PDMS walls, and $\delta = 0^\circ$ for glass substrate. Although higher concentration of Triton X-100 aids in the capillary microfluidics, it is important to note that PCR mixture with concentration higher than 0.1% Triton X-100 might lead to failure of the passive capillary valve.

4.2.7 Capillary microfluidic reactor sealing.

Sealing of array of microreactors on the chip is required to prevent evaporation during thermal cycling and prevent cross-contamination of preloaded PCR primers due to diffusion in fluidically connected microreactors. A proper sealing of the microreactors is critical to prevent any PCR mixture evaporation and bubble generation, which may lead to various problems at the denaturation step of a PCR thermocycling process. In the literature different microreactor sealing methods such as mineral oil [126, 128], pneumatic or mechanically activated diaphragms [3, 129, 130], thermally activated phase change pistons [131, 132].

Ideally, it is desirable to use a curable liquid sealant that becomes rigid upon curing. In addition to its mechanical property, a microreactor sealant is desired with the following salient features:

- (1) It must be immiscible with the PCR mixture to avoid interference of sealant in the microfluidic operation and the PCR amplification process.
- (2) It should cause no inhibition to the PCR in both the uncured and the cured states of the sealant, since the sealant in the both states is in contact with the PCR mixture during the chip operation.
- (3) It should cause minimal absorption of real-time PCR mixtures including Taq polymerase, DNA template, and fluorescent dye.
- (4) The sealant should be able to generate a surface tension at the sealant-PCR mixture interface in the same direction as the purging flow.
- (5) The above positive surface tension at the sealant-PCR mixture interface should not be too large to cause the sealant to flow into the microreactor by either breaking the flow restriction force at the exit of the outlet bridge channel or displacing the PCR mixture out of the microreactors from its inlet bridge channel.
- (6) The sealant should have a low viscosity to assist the flow, especially when the inlet and outlet channels are narrow.
- (7) The sealant should be able to gain a good adhesive strength at a partially cured state at a low temperature and within a short time period.

Five different liquid adhesives were considered as a potential sealant:

1. Epoxy resin EPO-TEK 301 (Epoxy Technology, Billerica, USA),
2. PDMS prepolymer (Sylgard 184, Dow Corning), and RTV-615 prepolymer (GE Silicones, Wilton, USA).
3. Mineral oil of PCR grade (Sigma, Singapore, M-8662),
4. Acrylic based UV-curing resin (PN 3046, Three Bond, Tokyo)
5. Acrylic based visible light/UV-curing resin (Loctite PN 3311, Henkel technologies)

EPO-TEK 301, Loctite #3311 showed auto-fluorescence in the spectrum of SYBR Green 1/Evagreen/FAM fluorophore (Emission = 520 nm). Auto-fluorescence is not desirable as it may interfere with fluorescent signal from the microreactor. In addition to this, the UV-curing resin from Three Bond was also dropped, since implementation of UV-curing might induce mutation in the target nucleic-acid template.

PDMS prepolymer from Dow Corning and GE silicone, and PCR grade mineral oil fulfilled almost all the above criteria. Both these sealants were successfully implemented in the developed PCR array chip. Due to high viscosity of PDMS prepolymer (~4000 cps), the flow rate inside the inlet and outlet channel is low. Typical flow rate is 0.75 mm/mins. Since it takes a long time ~20 mins to fill a 15 mm long channel. An alternate strategy was developed for faster loading of PDMS prepolymer. Sylgard 184 was first mixed with the 184 curing reagent at 10:1 ratio, followed by 200 Fluid 50 cs (Dow corning) at 1:1 ratio. The curing profile of the PDMS prepolymer + 200 Fluid 50 cs was comparable to the Sylgard 184 prepolymer. Mineral oil loading is shown in [Figure 4-20](#).

[Figure 4-20](#): Mineral oil as a sealant for microfluidic PCR array chip.

4.2.8 PCR failure modes due to air bubble.

There are different types of bubble-caused PCR failure modes, which depend on the original amount and size of the air bubbles, chip bonding strength, and sealing strength of the liquid loading orifices. A common PCR failure mode is the leakage of PCR mixture through inlet or outlet bridge channel by disruption of sealant due to pressure generated by expanding bubbles inside the microreactor during thermocycling. Such a PCR failure mechanism is illustrated in [Figure 4-21](#). Micro-bubbles start to generate and expand to a certain size at 72°C ([Figure 4-21 A-C](#)). When the chip was heated to 95°C, the bubbles expanded rapidly due to thermal expansion, and disrupted the sealant, leading to purging of PCR mixture out of the reactor ([Figure 4-21 D-E](#)). When the chip was cooled to the primer annealing temperature (~ 60°C), the size of the bubble reduced due to thermal contraction ([Figure 4-21F](#)). However, during subsequent heating of the chip to 95°C, the PCR mixture completely purged out of the microreactor ([Figure 4-21G](#)). Once the visible-bubbles were generated inside the reactor, it was highly likely that the PCR mixture purging might happen, except in some cases, when the bonding and sealing were strong enough to limit the expansion of the bubbles, and withstood the pressure generated by the bubbles. However, such an exceptional condition cannot be considered stable because the chip is under stress during thermocycling. The expansion and contraction of the bubbles generates a dynamic pressure that is more devastating than a static one, and at the same time the material of the chip (PDMS/glass chip) swell or shrink (probably at different rates) due to the different ramping rates. The best way to avoid PCR failure due to bubbles is to prevent formation of bubbles.

Figure 4-21: Purging of PCR mixture due to bubble expansion. A: PCR mixture sealed inside the micro reactor with sealant at both ends. B: After incubating at 72°C for few minutes, bubbles started to generate. C: The bubbles increased in size during incubation at 72°C for a longer time. D~E: At 95°C, the expansion of bubbles disrupted the sealant and pushed the liquid out of the reactor. F: When the chip was cooled from 95°C to 60°C, the bubbles contracted. G: When the chip was heated again to 95°C, the remaining liquid was completely purged out of the reactor.

Bubble source can be divided into three categories: the macro bubbles, the bubbles that are formed by air trapping, and the micro bubbles.

Macro bubble

The macro bubbles are visible with bare eye inside the liquid PCR sample, even before thermocycling. These bubbles are air trapped in the chamber because of sub-optimal chip design, e.g. some chamber shapes that trap macro bubbles [Figure 4-15](#), defected chip fabrication, or improper loading or sealing operation, e.g. bubble trapped between sealant and liquid. The macro bubbles can be eliminated with proper design, fabrication and operation of the chip.

Bubbles formed by air trapping

The fluidic microreactors are formed by bonding of two layers, in this case, PDMS and glass. Because of the defects of the bonding surfaces or dusts, small amount of air can trap between the bonding surfaces. When the chip is heated, the trapped air expands and in some cases, splits the relatively weak bonding and finally finds its way into the chamber, as shown in [Figure 4-22](#). This problem is solved with a stronger

interface bonding, which can withstand the air pressure and with cleaner surfaces for bonding.

Figure 4-22: Bubbles formed by air trapping. A: A visible air pocket existed between the bonding surfaces. B: The air pocket expanded after heating at 72°C for 35secs. C: The expanded air pocket delaminated the bonding after heating at 72°C for 2mins 15secs. D: A bubble formed by the air tunneling into the chamber (72°C for 2mins 35secs). E: More air tunneled into the chamber and the bubble expanded (72°C for 3mins 35secs).

Microbubbles

Micro bubbles are very small air bubbles trapped on or near the inner surface of the chamber. They are so small that only after heating and expansion that they visible to naked eyes. [Figure 4-23](#) are microscope photos of top and side view of the micro bubbles after they expanded to certain sizes. The micro bubbles are located at the corner formed by the PDMS sidewall and glass bottom.

Figure 4-23: Nucleation site for micro bubbles. The microscope photos of the side and top views of the microreactor indicate that the micro bubbles are mostly generated at the corner formed by the PDMS sidewall and the glass.

4.3 Development of vacuum based microfluidics.

The design and fabrication of the PCR matrix chip is discussed in [section 3.2](#). The sized PDMS chip is adhesively bonded to the bottom substrate (glass/silicon) by spin coating and curing the adhesive layer at 100C for 20 mins. In order to amplify different gene targets against cDNA template, different primer pair were preloaded and physically dried inside the nanowells.

4.3.1 Primer pair preloading on matrix chip.

Different primer liquor pairs (forward and reverse primer) were prepared at a final concentration of 0.3 μ M each. In order to aspirate the primer liquor, an ART GEL, gel loading pipette tip (Molecular BioProducts, San Diego, USA) was sized and made to fit 2.5 μ l pipettor, which was set to 0.150 μ l (~150 nL). The long and narrow pipette from Molecular Bioproducts renders the aspiration and dispensing of primer liquor pair process step easy. The primer liquor pairs were then manually loaded into the nanowells using under a stereo microscope. The primer loading operation workflow is shown in [Figure 4-24](#).

Since the PDMS layer is naturally hydrophobic, two different primer pair preloading process was tested:

(1) primer liquor (forward and reverse primer at 0.3 μM each) with Triton X-100 detergent, and

(2) primer liquor without Triton X-100.

Figure 4-24: Primer pair liquor loading operation workflow.

The presence of Triton X-100 did not aid the primer loading process, and the Ct values for PCR with and without Triton X-100 in the primer liquor were comparable. The primer pairs inside the nanowells were dried by baking the chip at 80°C for 10 mins.

Although the manual primer preloading process with an aid of microscope is easy and the process of loading of 12 different gene targets in triplicate well can be achieved in ~5 mins, this method is not feasible and economical when the scale of gene targets/primer pairs increases. In such cases, an automated liquid dispensing system can be used pre-load primer pairs. Such primer pairs can be loaded inside nanowells using well established techniques such as Inkjet and microjet deposition. During the course of this thesis work, such systems were developed by commercial

companies, such as BioTrove/Open Array (Life Technologies) and WaferGen. Currently, Life Technologies (Open Array) and WaferGen use such in-house system to pre-load primer pairs. Such a system can be developed or adapted to work with the developed PCR matrix chip, which is reported in this thesis. Recently, precise nanoliter dispensing and liquid handling systems are commercially available. One such system is available from BioTek (NanoQuot Microplate Dispenser). This system can precisely dispense 100nl in a 384-well plate. In addition to this, microarray spotter can be used to bind primer pairs to the bottom of the surface to achieve solid-phase PCR. Li *et al.*, used such a system:- SmartArray-48 Microarray Spotter (CapitalBio, China) to deposit primer pairs on a glass microwell array chip [133]. PCR efficiency is not good in such solid-phase PCR devices, which is in contrast to liquid-phase PCR array device developed in this work. A novel chip-to-chip microfluidic dispenser was reported [134]. In this device, microchannels are fabricated on PDMS layer and 115 nl volume was accurately dispensed on glass microwell array. The working principle of this device is shown in Figure 4-25. Such a device can be integrated with the developed PCR matrix chip for primer preloading.

Figure 4-25: Working principle of chip-to-chip nanoliter dispenser published by Wang *et al.* Image from [134].

4.3.2 Microfluidic operation for sample loading in PCR matrix chip.

Finally, a 0.1 mm thick acid-washed borosilicate top-cover glass (Herenz Medizinalbedarf, Hamburg, Germany) was bonded to the qPCR matrix chip using liquid PDMS to form closed microchannels and head-space above the nanowells for microfluidic handling of PCR mixture and liquid sealant. In some cases, the matrix

chip was sandwiched between two 1 mm thick diced glass, to achieve tight sealing as required for vacuum loading. [Figure 4-26](#) shows the sandwich setup with the tubing connections.

The vacuum system for PCR mixture loading and isolation of the nanoliter wells consists of a vacuum enclosure made of acrylic plastic plates, a pipe connected to the vacuum pump, a valve to control the vacuum, and a pinch valve to control the sample liquid flow ([Figure 4-27](#)). Prior to PCR mixture loading into the wells, the chamber is cleaned with DNA AWAY (Molecular Bioproducts) to avoid any aerosol cross-contamination of nucleic-acid template from the previous run.

[Figure 4-26](#): Sandwich setup to achieve tight sealing of matrix chip layers.

For the loading of the PCR mixture into the wells, a 2 cm long Teflon tubing (PTFE 30 TW, Cole Parmer, USA) was inserted into the inlet channel of the chip. A pipette tip, which acts as a reservoir for holding PCR mixture was connected with the Teflon tubing through a 6.5 cm long silicone tubing (I.D. 0.020" and O.D. 0.083", Cole Parmer, USA) ([Figure 4-26](#)). The microfluidic operation procedure includes few steps. Briefly, the pinch valve is first closed, and the vacuum inside the acrylic enclosure was reached to a value of 2.0 Torr by a rotary vane vacuum pump (PN 79203-20; Cole Parmer, Illinois, USA). The pressure inside the enclosure is monitored through a digital vacuum gauge (VG64; Supco Sealed Unit Parts Co., Inc, New Jersey, USA) ([Figure 4-28](#)).

Figure 4-27: Vacuum enclosure for the matrix chip. The vacuum system set-up for PCR mixture loading into the PDMS matrix chip. The parts shown in the inset are disposable.

Following this, 70 μ l of PCR mixture was loaded into the reservoir using a pipette. Subsequently, the pinch valve is manually opened to allow the PCR mixture loading into the headspace and nanowells under vacuum. The atmospheric air following the PCR mixture purged the excess PCR mixture inside the headspace and isolated the nanowells from each other. Manual loading using syringe is not effective, as it traps air during the loading process.

Figure 4-28: Vacuum system set-up for the matrix chip.

Hence, vacuum is essential for efficient loading of PCR mixture inside the microwells without trapping air. The vacuum valve was then closed, and the matrix chip was removed out of the vacuum enclosure. In order to seal the nanowells, liquid sealant (PDMS prepolymer) was injected into the headspace of the chip through the reservoir by a syringe pump (NE-1000, New Era Pump Systems Inc., New York, USA). The

sealed qPCR matrix chip was thermocycled on a peltier based real-time PCR instrument.

The primer re-suspension process has been discussed in [section 5.3.1](#). In order to simulate and visualize primer re-suspension and PCR mixture loading into the wells, a blue dye (0.1% of xylene cyanol) was selectively loaded into some wells (to form “NTU” characters) and dried. The microfluidic operation procedure is shown in [Figure 4-29](#), and the results for fluidic sample loading and sealing are shown in [Figure 4-30](#). The above microfluidic operation process is described in detail below:

Step 1: The liquid control pinch valve was closed. The vacuum pump was switched ON and subsequently the vacuum control valve was opened to achieve a vacuum level of 2 Torr. Following this PCR mixture (70 μ l) was loaded into the reservoir using pipette ([Figure 4-29\(A\)](#), [Figure 4-30\(1\)](#)).

Step 2: The pinch valve was opened. The sample liquid was jetted into the headspace of the chip due to vacuum. The presence of walls surrounding the well matrix helped the liquid column to redistribute to cover the entire area of the well matrix ([Figure 4-29\(B, C\)](#), [Figure 4-30\(2~3\)](#)). Air followed the liquid column into the headspace, and purged the remaining sample (dead-volume) in the headspace. After this step, all the wells that have been loaded with liquid were isolated ([Figure 4-29\(D\)](#), [Figure 4-30 4~5](#))). The entire sample loading and well isolation process can be completed in a fraction of a second, e.g. in 0.44 second as shown in [Figure 4-29](#)

Figure 4-29: Fluidic operation process for PCR matrix chip (A) A column of PCR mixture is loaded into the reservoir, and vacuum is established in the headspace and wells. (B,C) The pinch valve is opened; the sample column is filled in to the headspace. (D) Under vacuum, air followed the liquid into the headspace purging out the liquid in the headspace and isolated the wells. (E) Vacuum closed, and the sealant is injected into the headspace.

Step3: The vacuum valve was closed. The dried blue dye resuspended into the PCR mixture. Only the wells preloaded with dye were rendered blue color (Figure 4-30 6)). This shows that the rapid sample loading and well isolation method is free of primer cross-contamination.

Step 4: Following this, liquid sealant (a PDMS prepolymer) was injected through the reservoir into the headspace of the chip, in order to seal all the wells (Figure 4-29(E), Figure 4-30 7~9)).

Step 5: The vacuum enclosure was opened and the chip was retrieved for PCR thermocycling.

The microfluidic operation process snapshot images for a chip with 400 nanowells are shown in Figure 4-31.

Figure 4-30: Visualization of the sample loading in 100-well matrix chip. Visualization of the sample loading, well isolation and sealing process on the chip recorded at 25 fps. 1: The chip was placed in the vacuum enclosure before PCR mixture loading. Some of the wells were preloaded with blue dye dried on the well surface. 2~3: Under vacuum, the liquid is injected into the headspace. 4-5: Air followed the sample liquid immediately and purged the excess liquid out of the headspace through the venting channels, leaving all the wells isolated from each other. Subsequently, the vacuum is shut OFF. 6: Resuspension of the dried dye rendered the wells blue color. 7~9: The sealant (PDMS prepolymer) was injected into the headspace from the tubing to seal the wells.

Figure 4-31: Visualization of the sample loading in 400-well matrix chip. Visualization of the liquid sample loading, well isolation and sealing process on the chip recorded at 25 fps. A: The chip was placed in the vacuum enclosure before sample loading. Some of the wells were preloaded with blue dye dried on the well surface. B~D: liquid filling. Driven by vacuum established in the headspace and wells, the sample liquid was injected into the headspace at high-speed and redistributed to fill the headspace and the wells in a fraction of a second after opening the pinch valve. E~G: Sample isolation. Air followed the sample liquid immediately and purged the extra liquid out of the headspace through the outlet channels, leaving all the wells isolated from each other. Afterwards, the vacuum was shut off. H: Resuspension of the dried dye gave the wells a blue color, showing six blue characters, “NTU NCC”, in the wells matrix, while the rest of the wells remained clear. This shows that there is no visible cross-contamination among the wells. I~K: Optional sealing process. The sealant was injected into the headspace from the tubing and all the wells were sealed.

CHARACTERIZATION OF MICROFLUIDIC ARRAY CHIP

CHAPTER

5

*Take the time to talk to each other. New ideas will be born in those
conversations. That's the key to progress.*

- **Craig Cameron Mello** 2006 Nobel Laureate

for the discovery of RNA interference.

Chapter 5 Characterization of Micro fluidic Array Chip^{4 5 6}

In this chapter, the performance characterization of the PDMS/glass array and matrix chip is described. A prototype real-time PCR instrument, which can accept chips in a glass-slide format, was developed. This prototype instrument was developed by Robert Jiang and Chang Chun Dai. I was involved in the design of filter optics for detection of fluorophores used for nucleic-acid detection.

5.1 Real-time PCR amplification instrument.

A real-time PCR instrument for the developed PDMS-glass array chips was constructed and tested (Figure 5-1). The temperature of the array chip was cycled using a TEC (Melcor Corp., Trenton, NJ, USA). A RTD (Resistive Temperature Detector) was mounted on the TEC to measure the temperature and it was used as a feedback control [135]. The optics of the real-time PCR instrument was designed to measure the fluorescence of SYBR Green I, a widely used DNA intercalating dye. The SYBR Green I fluorophore was excited using an array of blue LED (Marl International Ltd, Cumbria, UK) at a fixed angle of 45° to the plane of the PCR-array chip surface to prevent the interference of the excitation light on the light path of the detection unit. Both the excitation light (intensity peak at 480 nm) from blue LED array and the emission light from the chip were filtered using a band pass filter of 465-495nm and 515-555nm (Chroma Technologies Corp, Brattleboro, USA), respectively. The fluorescence image of the entire chip is captured by a cooled CCD camera (DTA, Pisa, Italy; 14-bit monochrome camera; 768 x 512 resolution). The real-time PCR system is fully automated and integrated with multiple functions including thermocycling control, real-time fluorescence imaging, on-line image processing and data analysis.

⁴ Naveen Ramalingam, Liu H-B, Dai C-C, Jiang Y, Wang H, Wang Q, et al. Real-time PCR array chip with capillary-driven sample loading and reactor sealing for point-of-care applications. **Biomedical Microdevices** 2009,11:1007-1020.

⁵ Naveen Ramalingam, San T, Kai T, Mak M, Gong H-Q. Microfluidic devices harboring unsealed reactors for real-time isothermal helicase-dependent amplification. **Microfluidics and Nanofluidics** 2009,7:325-336.

⁶ Liu H-B, Naveen Ramalingam, Jiang Y, Dai C-C, Hui KM, Gong H-Q. Rapid distribution of a liquid column into a matrix of nanoliter wells for parallel real-time quantitative PCR. **Sensors and Actuators B: Chemical** 2009,135:671-677.

5.1.1 Temperature control and heat transfer.

PCR is a temperature-controlled enzymatic reaction, which amplifies the target nucleic-acid by periodical repetitions of three different temperatures (denaturation, primer annealing, and extension). Because of its dependence on temperature, minor temperature difference may significantly affect the efficiency of PCR. The developed PCR instrument used peltier-based Thermo-Electric device (Melcor Corp., Trenton, NJ, USA).

Figure 5-1: Schematic diagram and photo of the real-time PCR instrument.

For efficient heat transfer, few issues were taken into consideration. A TE device consists of an array of parallel p-n junctions, and for a given voltage, each p-n junction have its own temperature differential. In order to overcome this limitation, a thick copper wafer was attached to the TEC through thermal grease. The uniformity of the temperature across the TEC was calibrated using a RTD sensor, which was calibrated using boiling water (100°C). In order to ensure good thermal contact between the TE surface and glass substrate, thermally conductive PCR grade mineral oil was added between the two surfaces.

Active proportional-integral-derivative (PID) control regulated the chip's temperature. The PID temperature controller is connected to host computer via RS232 communication. A thermocouple (type T, 0.6mm diameter) is installed on the surface of the TEC. The measured temperature is used a feedback control to heat the TE device. However, for low thermal mass of PCR in biochips and the fast ramp rates, the integral term of simple PID/PI control will tend to cause overshoots that can be detrimental to PCR particularly at denaturing temperatures. However, the PID values were optimized to achieve PCR thermal cycling without any overshoots, as seen in Figure 5-2.

Figure 5-2: Temperature profile on the TEC surface used for thermal cycling PCR microreactor. Denaturation at 95°C for 15 seconds, annealing at 60°C for 15 seconds and extension at 72°C for 15 seconds. The average ramping rates are 3°C/s and 6.5°C/s for heating and cooling respectively.

5.2 Real-time quantitative PCR in PDMS/glass array chip.

For PCR experiments using SARS cDNA, plasmid pGEM-3Z containing BNI-1 fragment of SARS cDNA was used as a DNA template for the PCR process. A pair of primers was used to amplify a 189 bp gene fragment with forward primer sequence: 5'-ATG AAT TAC CAA GTC AAT GGT TAC-3' and reverse primer sequence: 5'-CAT AAC CAG TCG GTA CAG CTA-3'. The PCR mixture (10 μ l) contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100 (Promega M1902), 0.2 mM each of dATP, dCTP, dTTP and dGTP, 3 mM MgCl₂, 0.3 μ M each of forward and reverse gene specific primer, 0.1 U/ μ l of hot-start Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, USA), 1 μ g/ μ l of non-acetylated bovine serum albumin (BSA), 1X SYBR Green I (Cambrex Biosciences, Maine, USA), and varying concentration of plasmid DNA template. Note that non-acetylated BSA was added as a surface passivation reagent. The importance of BSA in PCR in high surface-to-volume microreactors is discussed in [section 5.2.6](#).

The performance of the array chip was tested by a sequence non-specific, SYBR Green I assay for the detection of SARS cDNA. A gradient-PCR was performed on PTC-200 cycler (MJ Research, Waltham, MA, U.S.A.) to determine the optimum annealing temperature of the primer pair. An additional experiment was performed on a commercial real-time PCR instrument (RotorGene 3000, Corbett Research, Sydney, Australia) to determine the optimum MgCl₂ and primer pair concentration. Optimization experiments showed that PCR with annealing temperature at 60°C, MgCl₂ at 3 mM and primer pair at 0.3 μ M concentration gave highest PCR yield.

Different concentrations of SARS cDNA template were amplified in the developed array chip (number of experiment: $n = 5$). The primer pairs were pre-loaded and dried in four different PDMS chip (for four varying concentrations of the template DNA). Each chip contained five microreactors: four microreactors for performing positive template control (PC) reactions in quadruplicates and one microreactor to perform no-template control (NTC) reaction. It is important to note that the chip was fabricated in such a way that the microreactor for NTC was not connected to the common inlet channel. The SARS cDNA fragment cloned in pGEM-3Z plasmid template was diluted (ten-fold) to varying concentrations of 10, 1, 0.1, and 0.01 pg/ μ l yielding 3×10^7 , 3×10^6 , 3×10^5 , 3×10^4 copies of SARS template cDNA per reaction mixture respectively. Each PDMS chip was loaded with PCR mixture containing a fixed concentration of cDNA template and sealed using PDMS prepolymer. PCR thermal cycling was performed on the in-house real-time quantitative PCR instrument. The NTC microreactor on each chip was manually loaded with PCR mixture without SARS cDNA. For each cDNA template concentration, the average amplification plot from a reactor is presented in [Figure 5-3\(a\)](#). The formation of primer-dimer (PD) in NTC and the threshold cycle (C_T) values for different template concentrations have been validated on RotorGene 3000 real-time PCR instrument ([Figure 5-3\(b\)](#)).

Melting curve analysis was performed to evaluate the purity of the PCR product from the developed PCR array chip ([Figure 5-3\(c\)](#)). The T_m (melting temperature) values for the desired PCR product and PD in the NTC reaction determined by the real-time PCR instrument are 86°C and 83.7°C respectively. The T_m values are comparable to the values determined by RotorGene 3000 (PC: 85.7°C and NTC: 84°C). The standard curve was plotted for the amplification of different template concentrations on the PDMS chip and on the polypropylene microfuge PCR tube used in RotorGene 3000 real-time PCR instrument. The efficiency of the PCR on the developed real-time quantitative PCR instrument is 0.96, which is very close to the efficiency of the PCR on RotorGene 3000 (0.97) ([Figure 5-3\(d\)](#)).

Figure 5-3: Amplification plot from PDMS-glass hybrid chip (a) Amplification plot for different template concentration on different PDMS-glass hybrid chip. The large increase in fluorescence signal for PCR with 10 pg/μl is due to freshly prepared SYBR Green I fluorescent dye (b) Amplification plot for different template concentration on RotorGene 3000. (c) Melting curve analysis performed in the PDMS chip. (d) The standard curve for amplification of different template concentration on PDMS chip and on polypropylene microfuge PCR tube used in RotorGene 3000 real-time PCR instrument.

The specificity of the PCR and the purity of the PCR products from the RotorGene 3000 real-time PCR instrument and the PDMS chip were confirmed on a commercial capillary electrophoresis chip (number of experiment: $n = 2$) (DNA Labchip 500) using Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) (Figure 5-4(a)). An incision was made on the PDMS layer at the entrance of the inlet bridge channel by using a sterile scalpel blade to extract the PCR product for post-PCR analyses. Subsequently, a gel loading pipette tip (Dia: 0.17mm, Molecular BioProducts Inc., CA, USA) connected to a pipetman was inserted into the microreactor with a negative pressure maintained inside the tip. Finally, the PCR product was extracted by the release of the negative pressure. The expected PCR product size was confirmed by comparing the size against an internal DNA ladder with lower marker (15 bp) and upper marker (600 bp). Besides the expected PCR product band (189 bp), there were no dominant additional bands. The PCR product

size determined by DNA Labchip 500 using Agilent 2100 bioanalyzer was 192 bp, which is comparable to the designed 189 bp PCR product. The yield of PCR from the developed PDMS chip is comparable to that from the commercial RotorGene 3000 real-time PCR instrument (Figure 5-4(b)).

Figure 5-4: Gel-like image of PCR product from PDMS-glass hybrid chip (a) Gel-like image of 189 bp PCR product run on a DNA Labchip 500 using Agilent 2100 bioanalyzer. Lane L is the ladder. Lane 1 and 2 are PC and NTC reaction run in RotorGene 3000 respectively. Lane 3 and 4 are PC and NTC reaction run in the PDMS chip respectively. (b) Concentration of PCR product (yield) from polypropylene (pp) tube and PDMS chip. The yield's of PCR product from polypropylene tube and PDMS chip are 12.45 ± 2.1 ng/μl (mean ± SD, $n=4$) and 14.31 ± 2.2 ng/μl (mean ± SD, $n=6$) respectively.

5.2.1 Primer cross-contamination tests.

During the PCR mixture loading step, all the microreactors are microfluidically connected with each other, while the pre-loaded primer pairs on the glass substrate re-suspends into the PCR mixture. In this case, there is a possibility of primer cross-contamination by diffusion and hence the timing of the PDMS prepolymer sealant introduction is a critical factor for performing primer cross-contamination free PCR. Alternate reactors were loaded with primer/s to test for any possible cross-contamination of primers. Seven microreactors were used for this experiment (Figure 5-5). Reactors 2, 4 and 6 contained both forward and reverse primer, reactor 3 lacked both the primers (No-primer control: NPC), reactor 5 contained only forward primer and reactor 7 contained only reverse primer. The experiment was designed to test the following possibilities 1. both primers diffuse together 2. only forward primer diffuse

and 3. only reverse primer diffuse. PCR mixture without primer was microfluidically loaded into the microreactors 2-7, sealed using PDMS prepolymer and PCR was performed. For no-template control reaction in microreactor 1, PCR mixture without template DNA was loaded individually. PCR was successful only in reactors 2, 4 and 6 and the C_T values from all these reactors were consistent (Figure 5-5). It can be concluded from Figure 5-5 that primer diffusion is negligible for the reported dimensions of microreactors in the developed chip. We repeated the experiment three times and got similar results.

Figure 5-5: Primer cross-contamination free PCR in PDMS-glass hybrid chip (a) Amplification plot for no-template reaction control (reactor 1), which was individually loaded; positive reaction control (reactor 2, 4 and 6) containing 3×10^7 copies of template DNA; no-primer reaction control (reactor 3); forward primer asymmetric PCR (AS1, reactor 5), and reverse primer asymmetric PCR (AS2, reactor 7). (b) Gel-like image of 189 bp PCR product run on a DNA Labchip 500 using Agilent 2100 bioanalyzer. (c) Fluorescent images of the PDMS chip captured by CCD camera at different cycles during amplification.

Another experiment was carried out to reinforce negligible primer cross-contamination in the developed chip. The diffusion of a HPLC purified 20 mer long oligonucleotide (primer) tagged with FAM fluorophore (5'-(6 FAM)-TCG TGC GTG GAT TGG CTT TG) was monitored in a microreactor with dimensions smaller than the microreactors used for performing PCR. Microreactors with smaller dimensions were experimented to gain knowledge about primer diffusion in lower length scale. For this study, microreactors with 3.2 mm length, 600 μm width and 180 μm depth was used. The oligonucleotide tagged with FAM was preloaded and dried inside a reactor (microreactor 2, refer to Figure 5-6). The microreactors flanking the reactor 2 on either side lacked preloaded oligonucleotides. For this study, the PDMS chip layer was not adhesively bonded to the glass substrate to prevent temperature (adhesive layer curing at 150°C for 20 minutes) effects on the FAM fluorophore. The PCR mixture without SYBR Green I dye was loaded in the inlet channel and the diffusion of the primer tagged with FAM was monitored for 30 mins on a fluorescence microscope setup (Olympus optical, Germany, Olympus BX 60). The fluorescent images were taken using a 4 $\times$ microscope objective lens. Figure 5-6 shows the images of FAM tagged primer diffusion in a single microreactor at different time points. Analyses of fluorescent data values, extracted from the images showed negligible primer cross-contamination in the developed PCR array chip.

Figure 5-6: Fluorescent images of primer diffusion in PDMS-glass hybrid chip. Fluorescent images of FAM tagged oligonucleotide diffusion at different time points. The 20 bp oligonucleotide tagged with FAM was preloaded in microreactor 2 (refer to image t = 0 min). The adjacent microreactors (1 and 3) contained PCR mixture during the experiment.

If we assume the diffusion constant of the primers to be $3\text{-}5 \times 10^{-6} \text{ cm}^2/\text{s}$, the distance diffused by primers in 30 mins is $\sim 1.3 \text{ mm}$ [136]. The diffusion distance is small when compared to the length of inlet bridge channel on the microfabricated PCR array chip. Moreover, the time taken to load the PCR mixture and seal the microreactors is relatively short (less than 5 minutes) when compared to 2 hrs required by the primers to diffuse 2-5 mm. One of the advantages of high surface-to-volume ratio microreactors is that the molecular diffusion coefficient of the primers decreases due to increased interaction between primer pairs and the reactor walls [137].

5.2.2 Effect of chip bonding conditions on primer pair degradation.

The chip bonding conditions includes adhesive layer curing temperature and incubation time. Typically, the adhesive layer can be cured at lower temperature (70°C) with longer incubation time, or at higher temperature (150°C) with shorter incubation time. Primer pairs were heat treated at 70 and 150°C for different incubation time of 30, 60, 90 and 120 minutes on a polypropylene (PP) PCR tube. Subsequently, PCR mixture without primer pair was added to suspend the heat-treated primer pair. The PCR mixture containing primer pair was aspirated and real-time PCR was performed in a new PP tube on the RotorGene 3000 PCR instrument. Freshly prepared PCR mixture with primer pair (0 minute) and primer pair incubated at room temperature (RT) for 120 minutes were used as controls to separate the effects of temperature from incubation time. For primer pairs incubated at 70°C for 120 minutes, no effect of the heat-treated primer pairs on the C_T values (Figure 5-7) was observed, when compared to the primers incubated at room temperature for 120 minutes. The small difference in C_T values between the incubated primer pairs (at room temperature, 70°C and 150°C) and freshly prepared PCR mixture (0 minute) may be due to increased interaction of the primer pairs with the PP surface during thermal incubation, which might lead to incomplete re-suspension of the primer pairs in the PCR mixture.

For primers treated at 150°C for 120 minutes, the C_T values were not consistent with the positive control reaction incubated at RT for 120 minutes. The increase in fluorescence level was lower for PCR with primer treated at 150°C for 120 minutes (number of experiment: $n = 2$). The yields (using Agilent 2100 bioanalyzer)

of PCR containing heat-treated primer pair (150°C for 30 minutes) and freshly prepared PCR (no heat-treatment of the primer pairs) were comparable (Figure 5-4(b)). To support this result, there is a report demonstrating the stability of high-temperature exposed oligonucleotides [138]. Okamoto and coworkers reported no damage to the oligonucleotides (10-300 bp), when subjected to high-temperature (~200°C) during covalent attachment of oligonucleotide to a glass surface using Bubble Jet technology for DNA microarray fabrication [138].

Figure 5-7: Effect of chip bonding conditions on primer pair degradation Effect of chip bonding conditions (temperature and incubation time) on primer pair degradation. The primer pairs were heat-treated at 70 and 150°C for different incubation time of 30, 60, 90 and 120 mins on a polypropylene (pp) PCR tube.

5.2.3 PCR mixture loss analysis during thermal cycling.

It is well-known that the PDMS layer is permeable to gas. Hence, sample loss due to evaporation through the top of microreactor is a concern. For the developed PDMS-glass chip with microreactor top thickness of 0.7 mm, nearly no loss of the PCR mixture during thermal cycling for the reported dimensions of the microreactors was observed, except in occasional cases (1:10 cases) when air bubble formatted and

expanded in the microreactors due to defects. I performed a crude method to approximately quantify the PCR mixture loss during thermal cycling. A known weight of the PCR mixture was loaded in four microreactors, sealed with PDMS prepolymer, and the chip was subjected to thermal cycling (40 cycles of 95, 60 and 72°C for 15 secs each). Using a calibrated micropipette, the density of the PCR mixture was found to be $\sim 1 \text{ g/cm}^3$. PCR product was extracted after thermal cycling by using a gel loading pipette tip and weighed again. The loss of the PCR mixture was found to be in the range of 1.4 - 3.6%.

5.2.4 PDMS/glass bonding methods and their influence on micro bubble formation during PCR.

A reliable and strong bonding of the PDMS chip layer to the glass substrate is important to prevent PCR mixture leakage due to the pressure generated in the reactor during the PCR thermal cycling. PDMS can be bonded with glass by O₂ plasma treatment [139], corona discharge treatment [97], and an adhesive layer of uncured PDMS [95]. All these methods resulted in strong permanent bonding in our tests, when the parameters of the processes were well controlled. However, PCR failure due to generation of bubbles in microreactors was usually observed for the chips bonded using O₂ plasma or corona discharge treatment. With further investigation it was found that the micro-bubbles mainly generated from the bonding corner area in the reactor. Some micro features in this area were responsible for the bubble generation, including surface roughness of the bonding surface of the PDMS replica and micro pinholes on this surface, which may form micro air bags in the bonding interface when bonded with flat glass, and micro cavities on the bonding corner (Figure 5-8(A)). For the chips bonded by using O₂ plasma or corona discharge treatment, the bonding corner has a wedge shape (Figure 5-8(B)) due to the round corner of the PDMS replica which is replicated from the round corner on the silicon die, which is an intrinsic result of the DRIE process [100]. The wedge shape forms a wetting barrier, and micro cavities in the wedge area tend to trap micro bubbles. During PCR thermal cycling, especially at the DNA denaturation temperature of 95°C, some of the micro bubble and micro air bags expand and form visible air bubbles in the reactor, which further leads to sample leakage and/or introduce uneven temperature distribution in the sample.

The methods that can be used to control the micro bubble formation includes surface treatment of the PCR reactor [99, 126, 140], pressurized sealing of the reactor [141, 142], degasification of the PCR sample [143], and locally changing the bubble generation features. It was found that the adhesive bonding had the potential to change the micro features in the wedge of the reactor, when the thickness of the adhesive layer was controlled. This led to a reliable and simple bonding interface cladding technique. With thickness controlled by spin coating speed and duration, the adhesive layer was able to cover the surface roughness and pinholes at the bonding interface, and partly filled the micro cavities and wedge at bonding corner, thus eliminated the bubble generation sites (Figure 5-8 (C)).

Figure 5-8: SEM photos of chip bonding corner using different bonding technique. (A) The surface of the PDMS replica before bonding. (B) When using O₂ plasma bonding of PDMS with glass, a wedge area forms and micro cavities and pinholes in this area tend to generate bubbles during PCR thermal cycling. (C) With the thickness controlled adhesive bonding, the adhesive fills the wedge, which is a common site for trapping air bubbles.

5.2.5 Characterization of single chamber PDMS-glass hybrid chip with high surface area-to-volume ratio microchambers.

Miniaturization of PCR-based biochips have the advantages of reduced consumption of nucleic-acid template and reagents, reduced PCR cycling time by efficient heat transfer to the PCR mixture, precise control of thermocycling parameters and potential towards a high-throughput platform. However, the effect of surface on PCR dominates in miniaturized PCR biochips and hence it is difficult to perform efficient PCR with uniformity across a high-throughput biochip. PCR in nanoliter range was performed in various surface-to-volume ratio microchambers and the reaction yield was studied. The adsorption of *Taq* polymerase enzyme, effect of BSA and SYBR

Green I concentration on PCR yield was investigated. This study provides opportunities to enhance the efficiency of PCR in nanoliter range to achieve a reproducible high-throughput PCR platform. Miniaturization of PCR-based analysis systems has necessitated studies on feasibility and efficiency of PCR in nanoliter range.

Miniaturization leads to increase in surface-to-volume ratio (SVR) of the chamber. At high SVR chambers, effects related to the non-specific adsorption of *Taq* DNA polymerase and template DNA to chip-surfaces becomes significant. To perform nanoliter PCR efficiently on the chips, the surface of the chips requires a PCR compatible coatings. Additionally, additives such as BSA, gelatin are added to the PCR mixture to prevent adsorption of *Taq* polymerase onto the biochip substrates. The PCR microchamber was fabricated by soft lithography techniques. The microchip consists of two parts, a PDMS layer containing the reaction chamber and channels and a glass substrate. The rhomboidal microchamber was connected to inlet and outlet (air venting) channel. Microchambers with different dimensions were fabricated to study the effects of surface area-to-volume ratio ($SVR = \frac{S}{V} \sim \text{size of chamber}$) of the microchambers on different parameters of the PCR. Table 5-1 lists the dimensions of the microchambers with different SVR.

Table 5-1: Dimensions of microchambers with different SVR.

Width of microchamber (mm)	Depth of microchamber (mm)	SVR (mm-1)	Length of microchamber (mm)	Volume of PCR (ul)
2	0.25	9	4	2
0.5	0.25	12	16	2
3	0.1	20.7	6.7	2.01
3	0.1	20.7	13	3.9
3	0.1	20.7	20	6
3	0.1	20.7	33	9.9
0.5	0.1	24	40	2

Effect of SVR on yield of PCR for a fixed PCR volume.

For fixed volume of PCR, the effect of SVR on the yield of PCR was studied. The effect of SVR on PCR yield was studied in the presence and absence of PCR additive BSA. At high SVR it is thought that, BSA prevents adsorption of *Taq* polymerase. At low SVR (9 and 12 mm⁻¹), the PCR was successful without BSA, and yield decreased for SVR 12 mm⁻¹, when compared to 9 mm⁻¹. The PCR failed for reaction without BSA at SVR 20.7 mm⁻¹ for 2 µl volume. At SVR 20.7 mm⁻¹, the PCR was successful in the presence of 1 µg/µl of non-acetylated BSA (Figure 5-9).

Figure 5-9: Effect of Surface-to-Volume Ratio (SVR) on PCR yield. For PCR without BSA additive, the PCR was successful in microchambers with SVR 9 mm⁻¹ and 12 mm⁻¹, but it failed for microchambers with SVR 20.7 mm⁻¹. At SVR 20.7 mm⁻¹, the PCR was successful in the presence of 1 µg/µl of non-acetylated BSA.

Effect of Volume on yield of PCR for a fixed SVR.

The effect of PCR volume on the yield of PCR for a fixed SVR was studied. Since a typical PCR microchamber have SVR $\sim 18\text{-}20\text{ mm}^{-1}$, the effect of PCR volume on PCR yield was studied at 20.7 mm^{-1} .

Figure 5-10: Effect of PCR volume on PCR yield. For PCR at 20.7 mm^{-1} with BSA additive, the PCR yield for chamber with $4\text{ }\mu\text{l}$ was low. The yield of PCR increased for chamber with $6\text{ }\mu\text{l}$, when compared with chamber with $4\text{ }\mu\text{l}$. There was no increase in PCR yield for PCR volume $10\text{ }\mu\text{l}$, when compared to chamber with $6\text{ }\mu\text{l}$.

The effect of volume at 4 , 6 and $10\text{ }\mu\text{l}$ was studied. The yield of PCR increased for chamber with $6\text{ }\mu\text{l}$, when compared with chamber with $4\text{ }\mu\text{l}$. There was no increase in PCR yield for PCR volume $10\text{ }\mu\text{l}$, when compared to chamber with $6\text{ }\mu\text{l}$ (Figure 5-10).

5.2.6 Effect of BSA concentration on PCR.

Bovine Serum Albumin has many uses as a carrier protein and as a stabilizing agent in enzymatic reactions and a blocking agent. The inclusion of BSA in the PCR mix is to reduce the problem of the abstraction of *Taq* polymerase and possible abstraction of PCR reagents on the PDMS and glass substrate. The addition of BSA reduces the non-specific binding of the enzyme to the internal surfaces of the PDMS-glass hybrid chip. When BSA was not included, the exponential amplification of PCR products is lower than when BSA was included.

Reagent abstraction can be easily reduced by the inclusion of BSA in the PCR mix to a certain extent. However, BSA will lead to sporadic precipitation in

some commercial PCR mixes during repeated cycles of denaturizing at 95°C. This results in the failure of PCR amplification, while acetylated BSA may kill polymerase. It is found out that dynamic coating of BSA to be very effective in preventing surface denaturation of polymerase on the internal glass surfaces. BSA can be categorized into three types: they are acetylated BSA, non-acetylated BSA and additive reagent BSA. The difference between acetylated and non-acetylated BSA is that acetylation process that changes the BSA's binding characteristics as a blocking agent and has been shown to inhibit PCR amplification and presume to kill polymerase. At high SVR, BSA is required for performing successful PCR (Figure 5-10). At SVR 20.7, increasing the concentration of BSA did not affect the yield of PCR, but PCR yield was higher when the concentration of Taq and SYBR Green I was increased to 2X (Figure 5-11).

Figure 5-11: Effect of BSA, Taq and SYBR Green I concentration on PCR yield. Effect of BSA, Taq and SYBR Green I concentration on PCR yield. PC is PCR control in the chip with 0.1 U/μl of Taq DNA polymerase, 1 μg/μl of BSA, 1X SYBR Green I.

5.2.7 Amplification of different copy number (sensitivity).

Different concentrations of SARS DNA template were amplified in the PDMS chip. The primer pairs were pre-loaded and dried in five different microreactors for different template concentrations, and a microreactor to perform no-template control (NTC) reaction. SARS DNA fragment cloned in pGEMT-3Z plasmid template was diluted (ten-fold) to varying concentrations of 100, 10, 1, 0.1 and 0.01 pg, yielding 3 x

10^7 , 3×10^6 , 3×10^5 , 3×10^4 , 3×10^3 copies of template DNA per reaction mixture respectively. Each microchamber was individually loaded with PCR mixture containing a fixed concentration of DNA template, sealed using liquid PDMS and PCR thermal cycling was performed on the in-house real-time quantitative PCR instrument. For each DNA template concentration, the amplification plot from the chip and the NTC reaction from the chip with highest copy number (3×10^7) are presented (Figure 5-12). The lowest tested DNA template copy number in the chip is 3000, though it is not the lowest template detection limit of the developed chip. The threshold cycle (Ct) for primer-dimer amplification in the NTC for the DNA template-primer pair is around 26 cycles, while the Ct for 3000 copies in the chip is around 24 cycles (Figure 5-12). The formation of primer-dimer in NTC and the Ct values for different template concentrations has been validated on RotorGene 3000 real-time PCR instrument.

Figure 5-12: Amplification plot for different template concentration. Amplification plot for different template concentration. The delta Ct for 3000 copies and primer-dimer amplification in NTC is 2 cycles.

5.2.8 Geometry of reaction chamber: Effect of top-coverglass on PCR.

It is thought that PCR failure in PDMS chip is due to porosity of PDMS, and exposure of PDMS layer to the atmosphere. Most of the PDMS-glass hybrid chip described in literature has a glass cover at the top of PDMS layer to prevent possible evaporation of PCR mixture, which may lead to formation of bubbles and failure of PCR. PCR in the chip with and without top cover glass was tested. The structure of the chip is shown in Figure 5-13. For PDMS layer thickness of 0.8 mm above the chamber, no

evaporation for chambers with and without top coverglass was observed. The PCR was successful in six wells with top coverglass and six wells without top cover glass. There was no evaporation in wells without top cover glass and Ct was consistent in all the wells (Figure 5-14).

Figure 5-13: Structure of single chamber PDMS glass hybrid chip. The structure of single chamber PDMS glass hybrid chip. The height of the microchamber for PCR is 200 μm , and height of PDMS layer above the microchamber is 0.8 mm. The effect of 0.1 mm thick top cover glass on evaporation of PCR was studied.

Figure 5-14: Effect of top-surface cover on PCR

5.2.9 Effect of primer concentration on PCR yield.

For primer cross contamination studies, it is desired to find the concentration of primers at which PCR fails. PCR was performed in the chip at a concentration of 300 nM of primer concentration. PCR was performed in a conventional real-time PCR instrument RotorGene 3000 instruments, and the PCR product yield was enumerated on a capillary electrophoresis chip (DNA Labchip 500, Agilent Technologies) using Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The PCR

failed for 5 nM concentration of primer. The PCR product yield increased with increasing concentration of primers (Figure 5-15)

To study the primer diffusion in the developed chip, the diffusion of a HPLC purified 20 mer long oligonucleotide (primer) tagged with FAM fluorophore (5'-(6 FAM)-TCG TGC GTG GAT TGG CTT TG) was monitored at two concentrations of FAM fluorophore (300 nM and 5 nM). The oligonucleotide tagged with FAM at two different concentrations was preloaded and dried inside a reactor (microreactor 1 contained primer tagged FAM at 300 nM and microreactor 3 contained primer tagged FAM at 5 nM, refer to Figure 5-16), and the microreactor 2 lacked preloaded oligonucleotides. The PDMS chip layer was not adhesively bonded to glass substrate, in order to prevent temperature (adhesive layer curing at 150°C for 20 mins) effects on the FAM fluorophore. The PCR mixture without SYBR Green I dye was loaded in the inlet channel, and the diffusion of the primer tagged with FAM was monitored for 30 mins on a fluorescence microscope setup (Olympus optical, Germany, Olympus BX 60).

Figure 5-15: Effect of primer concentration on PCR product yield. The PCR failed yield for 5 nM concentration of primer. The PCR product yield increased for increasing concentration of primers. The PCR was performed in RotorGene 3000.

The fluorescent images were taken using a 4X microscope objective lens. The images of FAM tagged primer diffusion in a single microreactor at different time points are shown in Figure 5-16. To track the diffusion of oligonucleotides, the fluorescent values were extracted from the images and plotted on a graph (Figure 5-17). Figure 5-17, indicate that the fluorescence values in the empty microreactor without any pre-loaded primer are comparable to microreactor with 5 nM primer concentration, the concentration of primer at which PCR failed (Figure 5-15).

Figure 5-16: Fluorescent images of FAM tagged oligonucleotide diffusion. Fluorescent images of FAM tagged oligonucleotide (300 nM and 5 nM) diffusion at different time points. The diffusion was monitored for 30 mins. The 20 bp oligonucleotide tagged with FAM was preloaded in microreactor 1 at 300 nM concentration and microreactor 3 at 5 nM concentration (refer to image $t = 0$ min). The sandwich microreactor 2 contained PCR mixture during the experiment.

Figure 5-17: Graphical illustration of FAM tagged oligonucleotide diffusion measured along the center line

5.2.10 Effect of primer preloading on glass substrate.

The primer was preloaded and dried on glass substrate. Experiment was done to study the effect of primer suspension on Ct. The PCR in microchambers with preloaded primer was compared to PCR in microchambers with primer pairs in the PCR

solution. The delta Ct for PCR with primer preloaded when compared to PCR with primer in PCR solution is 2 cycles. (Figure 5-18).

Figure 5-18: Effect of primer preloading on the glass substrate. Three wells were loaded with PCR mixture with primer pair and three wells were preloaded with primer pairs. The delta Ct for PCR with primer preloaded when compared to PCR with primer in PCR solution is 2 cycles.

5.2.11 Development of fast thermal cycling protocol.

In order to test if the developed chip and instrument can support fast thermal cycling protocol, SARS cDNA template (3×10^7 copies) was amplified for a 78bp amplicon of BNI-1 fragment. The two step fast thermal protocol: 95°C-60 sec; 40 cycles of 95°C-0sec; 58-1sec; 58-5 sec. The ramp time for 58-95°C is 15 sec, whereas for 95-58 it is 6 sec. The CCD based camera imaging takes 9 sec. In summary, one cycle with imaging took 33 secs. The PCR mixture contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 3 mM MgCl₂, 0.2 mM each of dATP, dTTP, dGTP, and dCTP, 1.0 µg/µl of BSA, 2X EvaGreen fluorescent dye (Biotium, USA), 0.3 µM each of gene specific primer, 0.2 U/µl of Platinum hot-start *Taq* DNA polymerase (Invitrogen, Carlsbad, USA).

Figure 5-19: Amplification plot for two-step fast thermal cycling.

5.3 Real-time quantitative PCR in matrix chip.

The PCR results from the matrix chip will be presented in Chapter 6. In this section, the characterization of the matrix chip is presented.

5.3.1 Primer cross-contamination tests in PCR matrix chip.

During the PCR mixture loading step, the headspace above all the wells is filled with liquid, Hence, all the PCR wells are fluidically connected with each other. There is a possibility of primer cross-contamination among the wells as the dried pre-loaded primer pairs inside the wells re-suspends into the PCR mixture. Hence, the timing of liquid removal in the headspace is critical factors in performing primer cross-contamination free PCR in the developed PDMS matrix chip. Based on my PCR experiments, it was found that primer cross-contamination occurred if the PCR wells were fluidically connected for a few minutes. When the incubation time of fluidically connected wells was reduced to 25 seconds, negligible primer cross-contamination from the PCR result was observed. In the sample loading process of the developed chip, the liquid in the wells connects for only 0.5 second, which is adequate to avoid primer cross contamination.

Further studies on the primer diffusion in the chip was performed by monitoring the fluorescence from HPLC purified 20 mer long oligonucleotides (primers) tagged with FAM fluorophore (5'-(6 FAM)-TCG TGC GTG GAT TGG CTT TG). The oligonucleotide tagged with FAM was preloaded and dried in selected number of PDMS wells (with reference to Figure 5-20) while the remaining wells were kept empty. The PCR mixture without EvaGreen dye was loaded and the wells

were isolated using the vacuum aided microfluidics. The fluorescence from the primers tagged with FAM was monitored for 5 mins on a fluorescence microscope setup (Olympus optical, Germany, Olympus BX 60). The images of FAM tagged primer movement in two wells at different time points are shown in Figure 5-20, indicating that there is no significant primer movement to the adjacent wells. Figure 5-20 also shows that even long after the microfluidic operation is completed; the primers still stay near the bottom of the wells.

Figure 5-20: Visualization of primer resuspension process during sample loading. Visualization of primer (oligonucleotide) resuspension process during sample loading. The fluorescence images show no observable cross-contamination of primers between wells after sample loading. A: the top view of the shape of the wells. B: fluorescence image of the wells before sample loading. The wells at the upper-left and the lower-left corners have been preloaded with HPLC purified 20 mer long FAM tagged oligonucleotides and they have been dried in the wells. B~C: the fluorescence images shots at selected time intervals after sample loading show the resuspension process of the oligonucleotides. D: enlarged view of the fluorescence image before sample loading. The dried FAM tagged oligonucleotide appears concentrated along the corners of the bottom of the well. E: Nine seconds after sample loading. F: 99 seconds after loading, further diffusion makes the fluorescence intensity even in the well. The boundary of the fluoresced region shows that the oligonucleotides remain near the bottom of the well. G: 279 seconds after loading, a larger fluorescence area compare to F indicates the oligonucleotides have diffused upward from bottom.

5.4 Microfluidic Devices Harboring Unsealed Reactors

A microfluidic device with open/unsealed reactors preloaded with primers for isothermal amplification of nucleic-acid using HDA technology is reported in this chapter. The microfluidic operation includes a single-step manual pipetting step, which can be performed in a point-of-care or resource limited setting, without the need for micropumps or sophisticated liquid handling instruments. The capability of

the developed microfluidic device was demonstrated by amplifying the BNI-1 fragment of SARS cDNA as a model test sample system. To my best knowledge, this is the first report of real-time HDA technology in a microchip system.

5.4.1 Real-time helicase-dependent amplification (rt-HDA).

For isothermal amplification, IsoAmp tHDA (thermophilic Helicase-Dependent Amplification) kit from New England Biolabs (Beverly, MA, USA) was used. Plasmid pGEM-3Z containing BNI-1 fragment of SARS cDNA was used as a DNA template. A pair of primers was used to amplify a 78 bp gene fragment with forward primer sequence: 5'-TTA TCA CCC GCG AAG AAG CT-3' and reverse primer sequence: 5'-CTC TAG TTG CAT GAC AGC CCT C-3'. The tHDA reaction contained 1X annealing buffer, 1X tHDA mix, 3 mM MgSO₄, 1 µg/µl of BSA and 0.1% Triton X-100, 0.2 µM each of forward and reverse primer, 0.1X EvaGreen fluorescent dye, and pGEM-3Z containing BNI-1 fragment of SARS cDNA (0.01 ng/µl). The manufactures of the tHDA kit suggest titration of MgSO₄ concentration from 3 to 4.5 mM. The MgSO₄ concentration (3, 4 and 5mM) was titrated to study the effect of MgSO₄ concentration on HDA yield. For the template-primer system, the optimal reaction condition was determined to be 3mM.

The IsoAmp tHDA kit did not contain BSA, Triton X-100 and fluorescent dye, and these components were externally added to the HDA reaction in the chip. I externally added BSA, Triton X-100 and fluorescent dye for surface passivation of the chip, capillary microfluidics and real-time amplification monitoring respectively. For real-time monitoring of DNA amplification product accumulation, the performance (in terms of inhibition) of two DNA-intercalating fluorescent dyes, SYBR Green I (Cambrex Biosciences, Maine, USA) and EvaGreen (Biotium Inc, Hayward, CA, USA) was tested. EvaGreen is a DNA-intercalating fluorescent dye, which shows much lower PCR inhibition than SYBR Green I [144]. The absorption and emission spectra of EvaGreen are similar to SYBR Green I. The tHDA (thermophilic Helicase Dependent Amplification) kit from New England Biolabs contains thermostable UvrD helicase (Tte-UvrD), and thermostable DNA polymerase (large fragment of *Bst* DNA polymerase). The concentrations of helicase and polymerase are proprietary. According to the manufacturer's protocol, the IsoAmp tHDA kit can amplify a short DNA sequence from 70 bp-120 bp and this limitation is attributed to the limited speed

and processivity of UvrD helicase [85]. For isothermal amplification, the HDA reaction mixture was prepared and loaded in the sample port. The distribution of the amplification mixture among the reactors and isolation of the reactors was autonomously achieved by capillary microfluidics. Following this, the PDMS-glass hybrid chip was incubated on the in-house real-time quantitative nucleic-acid amplification instrument at 62°C for 30 minutes. The fluorescence intensity (FI) values of the EvaGreen dye was measured every 1 minute.

5.4.2 Optimization of HDA method.

Experiments were performed to optimize the primer annealing temperature, MgSO₄, NaCl, and primer pair concentration in a commercial nucleic-acid amplification instrument (RotorGene 3000, Corbett Research, Sydney, Australia) to achieve higher amplicon yield, which was enumerated using a Agilent 2100 Bioanalyzer. Although, HDA was successful for reactions with circular plasmid DNA, linearized plasmid DNA using BamH1 (Promega, Madison, USA) restriction enzyme at 146 bp upstream of forward primer annealing region was used to study the effect of plasmid linearization on (1) ability to amplify regions with size outside the specified range (70-120bp), (2) ability to amplify low concentration of plasmid DNA template, and (3) amplicon yield. I found that the yields of HDA with circular and linearized primer were comparable. Even for reactions with linearized plasmid DNA, the HDA technology failed to amplify region with size of 189 bp (beyond the manufacturer's specified range), and reactions with low concentration of plasmid DNA. I performed all the HDA experiments with DNA template at a concentration of 0.01 ng/μl (3×10^7 copies/μl). For template-primer system, addition of NaCl partially inhibited the HDA reaction, which was evident from my yield data. The HDA product yield was highest for reactions with 3 mM MgSO₄, 0.2 μM each of forward and reverse primer, and 62°C HDA reaction temperature. For real-time HDA, 1X concentration of SYBR Green I and Eva Green showed partial inhibition of HDA reaction. HDA showed better performance in the presence of Eva Green, when compared to SYBR Green I. EvaGreen at a concentration of 0.1X was used. The yields of HDA with and without EvaGreen (0.1X) were comparable.

5.4.3 Isothermal amplification of cDNA in PDMS-glass hybrid chip

The performance of HDA was tested using the EvaGreen assay for the detection of SARS cDNA as a model template system. Each PDMS chip was loaded with HDA mixture, and incubated at 62°C for 30 minutes on the in-house real-time nucleic-acid amplification instrument. BNI-1 fragment of SARS cDNA was successfully amplified using HDA technology in real-time format. [Figure 5-21a](#) displays the sigmoidal curve for the HDA reaction (~5 µl) in the chip. The characteristic sigmoidal curves were base-line subtracted and smoothed using a three point moving average method [145]. In addition to the PDMS-glass hybrid chip (SVR: 9.7 mm⁻¹), HDA was successfully performed in a borosilicate glass capillary (Microcaps, Drummond Scientific Co., USA; ID: 0.63 mm; SVR: 6.3 mm⁻¹) in 5 µl reaction volumes. Since the SVR of the glass capillary was low (6.3 mm⁻¹), HDA was successfully performed in glass capillary without BSA. The surface effects of microstructures with low SVR on the yield of the amplification reaction are negligible [146].

Addition of BSA at concentrations 1 or 2 µg/µl did not increase the yield of HDA reaction in borosilicate glass capillaries. Results indicate that the coordinated catalytic activity of two enzymes (UvrD helicase, DNA polymerase I) still persist on PDMS-glass and borosilicate chip surfaces. To compare and evaluate the performance of HDA in the PDMS-chip, HDA in 5µl volumes was performed in a commercial real-time nucleic-acid amplification instrument. Since the presence of EvaGreen at 1X concentration partially inhibited HDA, HDA was performed without fluorescent dye and post-amplification analysis was performed by adding EvaGreen (1X), to determine the melting temperature (T_m) of amplicon. The T_m values of amplicon and primer-dimer determined by a commercial nucleic-acid amplification instrument are 83.7°C and 79.5°C respectively.

The T_m of amplicon product and primer-dimer of HDA performed in the chip are ~83°C and 79°C respectively ([Figure 5-21b](#)). HDA in nanoliter volumes was also performed using five PDMS-glass hybrid rectangular microreactor (length x breath x depth = 2 x 0.6 x 0.16 mm). The SVR of this microreactor is ~16.8 mm⁻¹, and it holds 192 nL reaction volume.

Figure 5-21: Amplification plot for HDA. (a) Amplification plot to test the reproducibility of HDA in five different microreactors (HDA reaction volume ~ 5.0 μl) on a PDMS-glass chip. The mean T_t (Threshold time based on manually set threshold fluorescence level) is 16.1 ± 0.11 minutes (mean $\pm$ S.D). (b) Melt curve analysis of amplicon generated by HDA in ~ 5.0 μl volume. The T_m (melting temperature) for 78 bp DNA amplicon and primer-dimer in the PDMS chip is $\sim 83^\circ\text{C}$ and $\sim 79^\circ\text{C}$ respectively. (c) Amplification plot of HDA in 192 nl reaction volume in five different microreactors on a PDMS chip. (d) Melt curve analysis of amplicon generated by HDA in 192 nl volume. (e) Gel-like image of 78 bp PCR product run on a DNA Labchip 500 using Agilent 2100 bioanalyzer. Lane L is the ladder. Lane 1 and 4 are no-template control of PCR in polypropylene (PP) tube, and HDA in PDMS chip respectively. Lane 2 and 3 (duplicates) are positive PCR control in PP tube. Lane 5 and 6 (duplicates) are positive HDA in PP tube. Lane 7 and 8 (duplicates) are positive HDA in the PDMS-glass array chip. (f) The yield of HDA in polypropylene (PP) tube (6.58 ± 0.36 ng/ μl ; mean $\pm$ SD); yield of HDA in PDMS chip (6.80 ± 0.15) and PCR (6.7 ± 0.15) in PDMS chip are comparable. NTC1-2: No-template control duplicates; PC1-5: Positive control reaction in five different microreactors on the same chip.

For nL HDA, the reactors were sealed using PDMS prepolymer, due to unavailability of 2mm wide TEC surface. Figure 5-21c shows the characteristic sigmoidal curve of real-time nL HDA format. The low increase in fluorescence and variability in threshold time (T_t), evident from Figure 5-21c, can be attributed to increased SVR of reactors. At high SVR, the effects of surface on HDA become dominant. The average threshold time for reactions in 192 nL volumes is in agreement with threshold time of HDA reaction in micro-scale (5 μ l) in the chip. Melt curve analyses of nanoliter HDA product showed that the desired product is pure (Fig. 5-21d). To further evaluate the purity of the amplicon generated by HDA in the chip, capillary electrophoresis using a DNA Labchip 500 (Figure 5-21e) was performed. The PCR product size determined by the DNA Labchip 500 was 83 bp, which is comparable to the designed 78bp PCR product.

5.4.4 Comparison of PCR and HDA on-chip.

The performance of HDA technology on-chip was compared with the widely used PCR on-chip. Each PDMS microreactor was loaded with PCR mixture and sealed using semi-cured PDMS prepolymer (precured at 72°C for 10 minutes in an oven). Strong sealing of the microreactors is achieved by curing the sealant at 72°C for 10 minutes before thermal cycling. The curing temperature of PDMS prepolymer (72°C for 10 minutes) did not produce any significant deleterious effect on PCR, since a hot-start Taq polymerase (activated at 95°C, which is higher than the sealant curing temperature) was used. The consistent yield data from the developed PCR chip, when compared to PCR from a commercial PCR instrument, demonstrate a negligible effect of sealant curing temperature on PCR [147]. The amplification plots for no-template control (NTC) reaction and positive control (PC) of PCR in five microreactors from a chip in microliter (5 μ l) and nanoliter (192 nL) volume range are presented in Figure 5-22a and Figure 5-22c respectively. HDA and PCR in nanoliter ranges was compared in the chip. The variability in C_t for PCR (11.12 ± 0.11 cycles; mean $\pm$ S.D) is comparable to variability in T_t for HDA reaction (16.8 ± 0.77 minutes; mean $\pm$ S.D). Melt curve analysis of amplicons generated by PCR (5 μ l volume) in the chip, indicated that the T_m of amplicon in the PDMS chip is $\sim 84^\circ\text{C}$ and for primer-dimer is 79°C (Figure 5-22b), The T_m values are in agreement with values determined by a commercial real-time nucleic-acid amplification instrument (amplicon: 84°C ; primer-dimer: 78.5°C). The performance of HDA/PCR in the chip was evaluated by yield of

nucleic-acid amplification using DNA Labchip 500. The yields of PCR in PDMS-glass chip, HDA in PP tube, and HDA in PDMS-glass chip are comparable (Figure 5-21f).

Figure 5-22: Comparison of HDA and real-time PCR. (a) Real-time PCR amplification plot for five microreactors in 5 µl reaction volume on a PDMS-glass hybrid chip. (b) Melt curve analysis of amplicon generated by PCR in 5 µl volume in PDMS chip. The T_m (melting temperature) for 78 bp DNA amplicon and primer-dimer in the PDMS chip are $\sim 85^\circ\text{C}$ and $\sim 79^\circ\text{C}$ respectively. (c) Amplification plot of PCR in 192 nl reaction volume in five different microreactors on a PDMS chip. NTC: No template control and PC1-5 is positive control reaction in five different microreactors on the same chip.

5.4.5 Evaporative loss studies in unsealed reactors.

Unlike sealed reactors, a concern associated with unsealed reactors is evaporative loss of amplification mixture, which may lead to failure of PCR or HDA depending on the extent of evaporation. Several research groups have reported different methods to reduce evaporative loss in open and closed reactors. Most of the reported microchip reactors for nucleic-acid amplification, utilized microvalves for sealing the reactors [3, 4]. Recently, Burns *et al.* reported an unsealed reactor for end-point PCR, in which optimized design geometry, thermal isolation and vapor replenishment were employed as methods to reduce evaporative loss [148], while Quake *et al.* implemented hydraulic control channels as replenishment method for sealed reactors

[149]. Burns *et al.* found that for reactors having bridge channel volumes of less than 10% of the total reaction volume, the overall PCR efficiency in unsealed reactors, was not significantly affected [148]. For the array chip, the evaporative loss of amplification mixture was controlled by implementing optimized reactor design geometry, smooth internal reactor surfaces, external heater, selective heating zone, suitable chip substrate based on thermal conductivity and reaction volume. The evaporative loss was evaluated in the developed unsealed reactor with different bridge channel lengths (L) with isothermal incubation for HDA, and compared the evaporative loss in unsealed reactors for PCR. For HDA, the evaporative loss was monitored at 62°C incubation for 40 minutes, while thermal cycling profile was followed for evaporative loss analysis due to PCR.

Figure 5-23: Evaporative loss in a locally heated unsealed microreactor. Evaporative loss in a locally heated unsealed microreactor with different bridge channel lengths. Evaporative loss in unsealed reactor for HDA was compared to reactors experiencing PCR thermal cycling.

The elimination of the use of thermal isolation and vapor replenishment methods is advantageous, since incorporation of these methods leads to complex design, higher fabrication cost and longer fabrication time. Evaporative loss is greatly reduced in the developed chip due the choice of chip substrate (glass) and external heater, when compared to silicon based devices integrated with heaters [148], since the thermal conductivity of silicon ($150 \text{ W m}^{-1} \text{ K}^{-1}$) is much higher than glass ($1.4 \text{ W m}^{-1} \text{ K}^{-1}$). The relatively low thermal conductivity of the glass substrate and PDMS material ($0.17 \text{ W m}^{-1} \text{ K}^{-1}$) together with the thin bridge channel that prevents thermal convection, assured lower temperature, thus the evaporation of the PCR mixture

inside the bridge channel is less, even when the reactors are heated to 95°C. To approximately quantify the evaporative loss during the amplification process a crude method was performed, in which reactors were loaded with amplification mixture and subjected to thermal cycling (PCR)/incubation (tHDA). The reactors were aligned against a length scale and evaporative losses of the amplification mixture at different cycles were enumerated by monitoring the receding front in the bridge channels against the length scale. For reactors with no bridge channel ($L=0$), both PCR and HDA failed, due to evaporative loss of 100% and 44% respectively (Figure 5-23). For reactors with $L=5$ mm, performance of HDA and PCR were comparable, since after 40 cycles/minutes, evaporative loss was ~10% for both HDA and PCR. A small difference in evaporative loss due to PCR and HDA method was observed for reactors with $L=10$ mm. This difference can be attributed to lower amplification mixture-air interfacial temperature, which is one of the driving forces for evaporation. Based on the result from Figure 5-23, reactors with bridge channel length of 10 mm were designed.

The principle of evaporative loss in valveless microreactor is based on the concept of diffusion-limited evaporation. Wang *et al.* presented an extensive theoretical analysis of this concept for a hybrid microchannel fabricated using silicon and glass [148]. For the microreactor design reported in this work, the inlet and outlet bridge channel to the reaction chamber provide a resistance that decreases the evaporation rate from the reaction fluid. Vapor that evaporates from the reaction mixture must diffuse through the microchannel before it can mingle with the ambient air present in the inlet channel. The evaporate rate of reaction mixture is limited by vapor diffusion rate, since there is no other convective flow of air inside the microchannels. The diffusion-limited evaporation can be defined by mass transfer equations. At a snapshot of time, there exist vapor-pressure gradients along the inlet and outlet bridge channel. As evaporation proceeds, the amplification mixture-interface recedes in the channel, resulting in increased diffusion length over time.

The position of amplification-mixture-interface/capillary front position (x_F) from the origin of the inlet bridge channel (refer to Figure Figure 5-24), as a function of time at a constant temperature is given in Equation 5.1. The derivation of Equation 5.1 can be found in Wang *et al.* [148].

$$x_F^2 = \frac{2 \times P \times M_w \times D_v \times t_F}{\rho_w \times R \times T_i} \times \ln\left(\frac{P - p_0}{P - p_v}\right) \quad (5.1)$$

where x_F is the position of capillary front at a given time, P is atmospheric pressure (Pa), D_v is vapor diffusivity of amplification mixture in air ($\frac{m^2}{s}$), ρ_w and M_w are the density and molecular weight of water, respectively, R is the ideal gas constant, T_i is the amplification mixture-air interface temperature, p_v and p_0 are the amplification mixture-air interface and ambient vapor pressure respectively, and t_F is the time.

The volume loss (V_{loss}) can be estimated by the Equation 5.2

$$V_{loss} = A_c \times (x_F) \quad (5.2)$$

where A_c is the cross-sectional area of the rectangular inlet microchannel.

In order to compare the experimental results with the theoretical prediction, a microreactor with 10 mm long inlet and outlet channel was used. The microreactor, inlet and outlet bridge channels, loading channels are 350 μm high. The inlet and outlet bridge channels are 200 μm , and the microreactor is 1 mm wide and 10 mm long to simulate the isothermal ($T_i = 335K$) amplification. The inlet opening angle is 16 degrees. The values used in the theoretical calculation are listed in Table 5.2.

Table 5-2: Values used for theoretical calculation of evaporation length scale.

	Symbol	Unit	Value
Atmospheric pressure	P	Pa	101000
Molecular weight of water	Mw	g/mol	18
Diffusivity of vapor in air@328K	Dv	m ² /s	2.97E-05
Density of water	ρ (w)	Kg/m ³	1
Ideal gas constant	R	J/K.mol	8.314
Amplification mixture interface temperature	Ti	K	335
Sample interfacial vapor pressure	pv	Pa	15737

The evaporation length in the inlet and outlet bridge channel was monitored for 40 minutes. The theoretical and experimental data is presented in Figure 5-24. It can be noted from Figure 5-24 that the experimental time is systematically lower than the theoretical value. One possible reason is that, the theoretical model uses an assumption that the amplification mixture interface temperature is not affected by the thermal dissipation from the chip material. In other words, the model assumes that the locally heated region is thermally isolated and the temperature at amplification mixture interface is same as the liquid inside the microreaction. This assumption is not true for evaporation in devices reported in this work. In the device reported in this work, there is no thermal isolation of microreactor heating and there is thermal dissipation through PDMS and glass substrate. Due to this, the temperature at the amplification mixture interface is expected to be lower than the temperature of the liquid inside the microreactor.

Figure 5-24: Comparison of theoretical and experimental data for evaporation of liquid meniscus during isothermal DNA amplification.

HIGHLY PARALLEL GENOMIC AND TRANSCRIPTOMIC APPLICATIONS

CHAPTER

6

What I worry about is that if we start messing around with eggs and sperm, at some point, since this is a human enterprise, we're going to make a mistake.

- **Mario Renato Capecchi** 2007 Nobel Laureate

for his discoveries of principles for introducing specific gene modifications in mice by the use of embryonic stem cells

Chapter 6 Highly Parallel Genomic and Transcriptomic Applications⁷

This chapter is divided into two sections: 1. Genomic applications, 2. Transcriptome applications (Gene expression analyses). For the genomic applications, results from parallel detection of multiple waterborne pathogens are presented. For the transcriptomic applications, gene expression profiles of liver cancer vs normal liver cells are presented.

6.1 Highly parallel genomic detection of multiple water borne pathogens.

A PDMS/glass hybrid chip comprising an array of reactors, which are pre-loaded with primer pairs for simultaneous (parallel) PCR analyses of multiple waterborne pathogens is reported. The microfluidic operation is simple, involves a single manual pipeting step, and can be performed in a point-of-care setup, without the need of sophisticated liquid handling systems for sample loading, and isolation of microreactors. The capability of the PCR array chip was demonstrated by simultaneously detecting four waterborne pathogens: *Pseudomonas aeruginosa*, *Aeromonas hydrophila*, *Klebsiella pneumoniae* and *Staphylococcus aureus*. However, the number of reactors on the chip can be expanded to analyze a panel of waterborne pathogens.

6.1.1 Bacterial samples and PCR experiment.

Aeromonas hydrophila (ATCC 35654), *Klebsiella pneumoniae* (ATCC 13883), *Staphylococcus aureus* (ATCC 25923), and *Pseudomonas aeruginosa* (ATCC 15692) were purchased from American Tissue Culture Collection (Rockville, USA). Bacterial cells were grown in LB broth for 18 hrs and genomic DNA was extracted using DNeasy blood and tissue kit (Qiagen, Hilden, Germany). Quantification of genomic DNA was done by using the PicoGreen ds DNA Quantitation Kit (Invitrogen) on TECAN GENios Plate Reader (Tecan, Mannedorf, Switzerland). Equal amounts (0.1 ng/μl final concentration) of genomic DNA were pooled together and used as a model template system for the PCR array chip. The sequences of forward and reverse

⁷ Naveen Ramalingam, Rui Z, Liu H-B, Dai C-C, Kaushik R, Ratnaharika B, et al. Real-time PCR-based microfluidic array chip for simultaneous detection of multiple waterborne pathogens. **Sensors and Actuators B: Chemical** 2010,145.

primers for the desired target organisms are listed in Table 6-1. The PCR mixture contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 0.2 mM each of dATP, dCTP, dTTP and dGTP, 3 mM MgCl₂, 0.2 µM each of forward and reverse primer, 0.1 U/µl of Platinum *Taq* DNA polymerase (Invitrogen, USA), 1 µg/µl of BSA, 2X EvaGreen (Biotium, USA) and pooled genomic DNA from four pathogens (final concentration of each genomic DNA pathogens is 0.1 ng/µl). For internal positive control, human genomic DNA and primer pair from a commercial kit (β-actin Control Reagents (P/N 401846); Applied Biosystems, USA) was used.

Table 6-1: Sequences of forward and reverse primers for the desired target organisms

Target organism	Primer	Sequence (5'-3')	PCR product size(bp)
<i>Aeromonas hydrophila</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	AAACAACCTACAATCCCTTCA	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	
<i>E.coli</i>	Eco-F	CTGCTGCTGTCGGCTTTA	205
	Eco-R	CCTTGCGGACGGGTAT	

To compare and evaluate the PCR on the PDMS-glass chip, the PCR was performed both in polypropylene (PP) microfuge tube on a commercial real-time PCR instrument (RotorGene 3000, Corbett Research, Sydney, Australia) and in the PDMS-glass array chip on the in-house real-time quantitative PCR instrument with the following thermal cycling profile: initial denaturation at 95°C for 60 seconds followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 15 seconds and extension at 72°C for 15 seconds. The fluorescence of EvaGreen dye was measured at the extension step of every PCR cycle. For melting curve analysis the sample was heated from 50°C to 99°C and the EvaGreen fluorescence was measured every 1°C. The desired PCR products from the RotorGene 3000 PCR instrument and the PDMS array chip were confirmed by melting curve analysis method and also on a capillary

electrophoresis chip (DNA Labchip 500) using Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA,USA).

6.1.2 Simultaneous detection of waterborne pathogens using PDMS/glass hybrid chip.

The performance of the PDMS/glass hybrid chip is demonstrated by detecting a pool of genomic DNA containing *Aeromonas hydrophilia*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. The PDMS chip was loaded with PCR mixture containing a fixed concentration of genomic DNA template. PCR thermal cycling was performed on the in-house real-time quantitative PCR instrument. The amplification plot from microreactors from a chip and the NTC reaction from the chip are presented in Figure 6-1. The formation of primer-dimer in NTC and the Threshold cycles (C_T) values for PCR with different primer pairs has been validated on RotorGene 3000 real-time PCR instrument. Melting curve analysis was performed to evaluate the purity of PCR product from RotorGene 3000 and the real-time PCR instrument (Figure 6-1(B)).

Figure 6-1: Amplification plot to detect water-borne pathogens. (A) Amplification plot to detect *Aeromonas hydrophilia* (19,000 copies/ μ l), *Klebsiella pneumoniae* (17,000 copies/ μ l), *Staphylococcus aureus* (32,000 copies/ μ l), and *Pseudomonas aeruginosa* (14,000 copies/ μ l). (B) On-chip melting curve analysis to test the purity of the amplified product. NTC: no-template control; PC: positive control.

The desired PCR products from the RotorGene 3000 real-time PCR instrument and the PDMS 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 6-2). The yield of PCR from the developed PDMS chip is comparable to that from the commercial RotorGene 3000 real-time PCR instrument.

Figure 6-2: Gel-like image of PCR product from water-borne pathogens. 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 1,3,5,7,9 are no-template control reaction of *Aeromonas hydrophilia*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and human genomic DNA(internal positive control) respectively. Lane 2,4,6,8,10 are positive control reaction of *Aeromonas hydrophilia*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and β -actin gene of human genomic DNA respectively.

In this section, a PCR array chip harboring unsealed reactors for simultaneous detection of four waterborne pathogens was reported, and successfully demonstrated the potential of capillary microfluidics in generating a large number of solution-phase real-time PCR. The advantages of the developed system are single step capillary flow based sample loading without the use of pumps and unsealed reactors, which does not require microvalves. With the design of microfluidic network, this new platform is able to perform three functions on the same microchip: (1). ability to detect and quantitate waterborne pathogens, (2). ability to monitor template contamination in reagents, through the use of on-chip, no-template control reaction, and (3). ability to identify PCR failure caused by failure of on-chip thermalcycling instrument or optics, by using internal positive control reaction. Key technical issues related to unsealed reactors such as evaporative loss and PCR yield have been addressed. It found that the smooth internal surface of the microreactor, localized heating, optimized design

geometry of the microreactor, chip substrate and reaction volume are critical parameters for successful PCR on the array chip. Although evaporative loss of PCR mixture during PCR thermalcycling has been successfully controlled in the developed chip, it can be further reduced by isothermal nucleic-acid amplification methods, which typically operates at a much lower constant temperature (~60°C). A pool of DNA template from multiple pathogens was used, as an example to demonstrate the capability of the developed array chip. However, the developed chip can be integrated with a sample preparation module, to directly detect waterborne pathogens for integrated genetic analyses of a panel of waterborne pathogens.

6.2. Highly parallel transcriptomic detection on the PDMS/glass array chip.

For PCR experiments using human hepatocellular carcinoma (HCC) cDNA, cancerous HCC surgical biopsies were obtained and immediately frozen in liquid nitrogen. All tissue samples employed in this study were approved and provided by the Tissue Repository of the National Cancer Centre, Singapore. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, USA) by following the manufacturer's protocol. cDNA was synthesized with random hexamers (Invitrogen, Carlsbad, USA) and oligo(dT)₁₂₋₁₈ primers using SuperScript II reverse transcriptase. All reverse transcription reaction was performed with a starting amount of 1-5 µg of DNase I-treated total RNA in a final volume of 20 µl.

The PCR mixture (10 µl) contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 0.2 mM each of dATP, dCTP, dTTP and dGTP, 2.5 mM MgCl₂, 0.3 µM each of forward and reverse gene specific primer, 0.1 U/µl of hot-start Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, USA), 1 µg/µl of BSA, 1X SYBR Green I (Cambrex Biosciences, Maine, USA), and 0.5 µl of HCC cDNA template. Primer pairs for HCC cDNA samples were designed with similar T_m (melting temperature) of ~ 55°C ([Table 6-2](#)).

Experiments were performed to optimize the annealing temperature, MgCl₂ concentration, and primer concentration for amplification of HCC cDNA on a commercial real-time PCR instrument (RotorGene 3000, Corbett Research, Sydney, Australia). To compare and evaluate the PCR on the PDMS-glass array chip, HCC cDNA templates were PCR amplified in a polypropylene (PP) microfuge tube on RotorGene 3000 (PCR volume: 10µl) and in the developed PDMS-glass array chip on the in-house real-time quantitative PCR instrument. The thermal cycling profile for

HCC cDNA: Initial denaturation at 95°C for 900 seconds; followed by 40 cycles of denaturation at 94°C for 15 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds. The fluorescence of the SYBR Green I dye was measured at the extension step of every PCR cycle.

Table 6-2: Primer sequence for twelve gene targets of liver cDNA

“f” and “r” denotes forward and reverse primer respectively.

Gene Symbol	Gene Name	Primer sequence
Actin-f	Actin	GGGTGAATACCACTGCCAAG
Actin-r		GTTTCATGCCCAACAAGCTCT
RAS-f	Ras GTPase activating protein	GCAGATGCAGTCTACGGACA
RAS-r		CCTCAGGAGCAAGGACAAAC
OSN-f	Cystein rich osteonectin	GAGAAGGTGTGCAGCAATGA
OSN-r		AGGACGTTCTTGAGCCAGTC
FBJ-f	FBJ murine osteosarcoma viral oncogene homolog B	AAACTCTGGCTCAGCCTGTC
FBJ-r		AAAATCTCATGTCCCCAACG
APC-f	Adapter related protein complex	AATCTGAGGCTGGCTTAGCA
APC-r		GATGACTCTTCCCCCTGGAT
PD2-f	PD2 domain	TGGGCTAGAGGATGAGGATG
PD2-r		AGGGGTGTCAAGTGGATCAG
OST-f	Osteopontin, bone sialoprotein I	ACTGATTTTCCACGGACCT
OST-r		CCATTCAACTCCTCGCTTTC
FOS-f	v-fos FBJ murine osteosarcoma viral oncogene homolog	CTAACCGCCACGATGATGTT
FOS-r		GTGGGAATGAAGTTGGCACT
PK-f	Protein kinase DNA activated	TGCAAGCAGATGTTCTCAC
PK-r		CAACACACCAGCTGCATTTT
HSP90-f	Heat shock protein 90 KDa alpha(cytosolic)	GGCCGACAAGAATGATAAGG
HSP90-r		GGGGGATCTCATCAGGAAGT
ANX-f	Annexin A2	ATGTTCCCAAGTGGATCAGC
ANX-r		CATACAGCCGATCAGCAAAA
ADE-f	Adenylyl cyclase-associated protein 2	TCAATGGTGTCATTGCAGGT
ADE-r		CTCGTGGGGTTGTTGGTACT

For melting curve analysis the sample was heated from 72°C to 99°C and the SYBR Green I fluorescence was measured at every 1°C. The desired PCR products from the RotorGene 3000 PCR instrument and the PDMS array chip were confirmed by melting curve analysis method and on a capillary electrophoresis chip (DNA Labchip 500) using Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA,USA).

A chip was preloaded with twelve primer pairs targeting twelve gene targets on cancerous liver cDNA sample (number of experiment: n=3) to demonstrate the capability of our PCR-array chip to simultaneously amplify multiple gene targets. Optimization experiments were performed for all the primer pairs (twelve sets) to determine the optimum annealing temperature, MgCl₂ and primer pair concentrations. The PCR mixture loading and reactor sealing were achieved by employing our capillary microfluidics. The array chip was subsequently thermocycled on our real-time PCR instrument. [Figure 6-3](#) shows the amplification plot for twelve gene targets. On-chip PCR product confirmation and evaluation of the PCR product purity were performed by melting curve analysis ([Figure 6-3\(b\)](#)).

Figure 6-3: Amplification plot for twelve transcripts. (a) Amplification plot for twelve different gene targets on PDMS-glass hybrid chip (b) Melting curve analysis of twelve amplified PCR product performed in our PDMS chip

The C_T values and T_m (PCR product) values are in accordance with the values obtained from a commercial real-time PCR instrument ([Table 6-3](#)).

Table 6-3: Comparison of threshold cycle (Ct) values and melting temperature (Tm) values from our PCR array chip with a conventional real-time PCR instrument.

S.No	Gene Name	Symbol	RotorGene 3000		PCR array chip	
			Ct	Tm (oC)	Ct	Tm (oC)
1	Actin	Actin	18.23	84.8	20.5	84.2
2	Ras GTPase activating protein	RAS	19.19	89	19.2	89.4
3	Cystein rich osteonectin	OSN	20.74	92.2	20	92.1
4	FBJ murine osteosarcoma viral oncogene homolog B	FBJ	17.93	91.2	16.5	91.8
5	Adapter related protein complex	APC	15.39	86	18.2	86.5
6	PD2 domain	PD2	17.27	86.8	16	87
7	Osteopontin, bone sialoprotein I	OST	20.98	89	21	89.5
8	v-fos FBJ murine osteosarcoma viral oncogene homolog	FOS	22.19	94.5	22	93.5
9	Protein kinase DNA activated	PK	21.54	89.5	20.5	90
10	Heat shock protein 90 KDa alpha(cytosolic)	HSP90	16.46	89	15.8	89.5
11	Annexin A2	ANX	18.94	87.5	18	87.2
12	Adenylyl cyclase-associated protein 2	ADE	19.16	90.3	18.5	90

6.3 Highly parallel transcriptomic detection on the matrix array chip.

In this section the PCR data from the 100- and 400-well matrix array chip are presented.

6.3.1 Highly parallel transcriptomic application on a 100-well chip.

Cancerous human hepatocellular carcinoma (HCC) surgical biopsies were obtained and were immediately snap frozen in liquid nitrogen. All tissue samples employed in this study were approved and provided by the Tissue Repository of the National Cancer Centre, Singapore. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, USA) by following the manufacturer's protocol. cDNA was synthesized with random hexamers (Invitrogen, Carlsbad, USA) and oligo(dT)12-18 primers using SuperScript II reverse transcriptase. All reverse transcription reaction was performed with a starting amount of 1-5 µg of DNase I-treated total RNA in a final volume of 20 µl. To validate the primer-cDNA template system, compare and evaluate the real-time PCR on the PDMS matrix chip, ten different gene fragment was PCR amplified both in polypropylene (PP) microfuge tube on a commercial real-time PCR instrument (RotorGene 3000, Corbett Research, Sydney, Australia) and in the PCR matrix chip on the prototype real-time quantitative PCR instrument with the

following thermal cycling profile: Initial denaturation at 95°C for 900 seconds; followed by 40 cycles of denaturation at 94°C for 15 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds. The fluorescence of EvaGreen dye was measured at the extension step of every PCR cycle.

Primer pairs were designed with similar T_m (melting temperature) of ~ 55°C (Table 6-4).

Table 6-4: Primer sequence for amplification of ten different gene targets of liver cDNA

Gene Symbol	Gene Name	Primer sequence
Ade-f	Adenylyl cyclase-associated protein 2	TCAATGGTGTTCATTGCAGGT
Ade-r		CTCGTGGGGTTGTTGGTACT
Aldo-f	Aldo-keto reductase family	ATCACCGTTACGGCCTACAG
Aldo-r		CGTGCTGGTGTTCACAGACTT
Anx-f	Annexin A2	ATGTTCCCAAGTGGATCAGC
Anx-r		CATACAGCCGATCAGCAAAA
HSP90-f	Heat shock protein 90 KDa alpha(cytosolic)	GGCCGACAAGAATGATAAGG
HSP90-r		GGGGGATCTCATCAGGAACT
PK-f	Protein kinase DNA activated	TGCAAGCAGATGTTCTCTCAC
PK-r		CAACACACCAGCTGCATTTT
PD2-f	PD2 domain	TGGGCTAGAGGATGAGGATG
PD2-r		AGGGGTGTCAAGTGGATCAG
APC-f	Adapter related protein complex	AATCTGAGGCTGGCTTAGCA
APC-r		GATGACTCTTCCCCCTGGAT
FBJ-f	FBJ murine osteosarcoma viral oncogene homolog B	AAACTCTGGCTCAGCCTGTC
FBJ-r		AAAATCTCATGTCCCAACG
Osn-f	Cystein rich osteonectin	GAGAAGGTGTGCAGCAATGA
Osn-r		AGGACGTTCTTGAGCCAGTC
Ras-f	Ras GTPase activating protein	GCAGATGCAGTCTACGGACA
Ras-r		CCTCAGGAGCAAGGACAAAC

The PCR mixture contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM MgCl₂, 0.2 mM each of dATP, dTTP, dGTP, and dCTP, 1.0 µg/µl of BSA, 2X EvaGreen fluorescent dye (Biotium, USA), 1.0 µM each of gene specific primer, 0.1 U/µl of Platinum hot-start Taq DNA polymerase (Invitrogen, Carlsbad, USA), and cancerous liver cDNA template. The desired PCR products from the RotorGene 3000 PCR instrument was confirmed on a capillary electrophoresis chip (DNA Labchip 500) using Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

To test the performance of the PDMS matrix chip and to investigate cross-contamination between wells, primer pairs specific for ten different target regions of cancerous liver cDNA was preloaded. The samples were run in duplicates. In addition to the PDMS matrix chip with wells containing positive template control reactions (PC), a different PDMS matrix chip with wells containing no-template control reactions (NTC) was also thermal cycled. Using the vacuum aided microfluidic scheme, the primer pre-loaded PDMS matrix chip containing positive template control reactions was loaded with PCR mixture containing a fixed concentration of cDNA template and was sealed using PDMS prepolymer. The chips were then thermal cycled on the in-house real-time quantitative PCR instrument. The threshold cycle (C_t) values for amplification of ten different regions of cDNA from cancerous human hepatocellular carcinoma PDMS matrix chip (Figure 6-4(A)), were comparable to corresponding reactions performed in a commercial RotorGene 3000 real-time PCR instrument. Melting curve analysis was performed to evaluate the purity of PCR product from the real-time PCR instrument (Figure 6-4(B)). The T_m (melting temperature) for the desired PCR products and primer-dimer in NTC reaction were comparable to the values determined by RotorGene 3000 commercial real-time PCR machine.

Figure 6-4: Amplification plot of ten different gene targets of cDNA from human hepatocellular carcinoma. (A) Amplification plot of ten different gene targets of cDNA from human hepatocellular carcinoma. (B) Melt curve analysis to test the purity of PCR product from the PDMS matrix chip. Information about abbreviations is given in [Table 6-4](#).

The well-to-well variability was studied by amplifying 18S rRNA gene in cDNA template from normal liver tissue. For $n=100$ wells, the mean Ct value is 12.78 and the standard deviation was 0.63 ([Figure 6-5](#)). In order to estimate the efficiency of PCR in our matrix chip, we serially diluted cDNA template by 10-fold, and for each dilution we preloaded and physically dried the cDNA template in three microwells (replicates). Following this, PCR mixture with GAPDH primer pairs and other PCR components (excluding template) was loaded into the matrix chip. The amplification plot for 10-fold serially diluted cDNA template is shown in [Figure 6-6](#). The efficiency of PCR as estimated from the standard curve is 1.07.

Figure 6-5: Amplification plot for 18S rRNA gene in 100 wells. The mean Ct value is 12.78. The chip image at 1st and 30th cycle is shown in the inset.

Figure 6-6: Amplification plot for GAPDH gene using 10-fold serially diluted cDNA template. The efficiency of PCR in our matrix chip is 1.07.

In order to test for any possible primer cross contamination, 22 wells were preloaded with dried primers pairs; the remaining 78 wells did not contain preloaded primer pairs. In addition to the PDMS matrix chip with wells containing positive template control reactions, a different PDMS matrix chip with wells containing no-

template control reactions was thermal cycled. Each PDMS matrix chip with pre-loaded primer pairs was loaded with PCR mixture containing a fixed concentration of DNA template, sealed using liquid PDMS and top cover glass. Following this, PCR thermal cycling was performed on the in-house real-time quantitative PCR instrument. The threshold cycle (C_t) for amplification of 3×10^7 copies of templates from 22 wells in our PDMS matrix chip is ~ 11 cycles (Figure 6-7), which is comparable to the commercial RotorGene 3000 real-time PCR instrument. The C_t values for amplification of 3×10^7 copies of template DNA in 22 wells were consistent within a chip (Figure 6-7).

Figure 6-7: Reproducibility and primer cross-contamination test in PDMS matrix chip. Well 1 to 10 contained positive reaction control (PC) with 3×10^7 copies of template DNA. NTC 1 and 2 are no-template control reactions.

6.3.2 Highly parallel genomic application on 400-well chip.

In order to validate the high-throughput functionality of the 400-well chip, PCR amplification of BNI fragment of SARS cDNA was performed in ten randomly selected wells. Plasmid pGEM-3Z containing BNI-1 fragment of SARS cDNA was used as a template. A pair of primers was used to amplify a 189 bp gene fragment with forward primer sequence: 5'-ATG AAT TAC CAA GTC AAT GGT TAC-3' and reverse primer sequence: 5'-CAT AAC CAG TCG GTA CAG CTA-3'. The PCR

mixture (200 μ l) contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 0.2 mM each of dATP, dCTP, dTTP and dGTP, 3 mM MgCl₂, 0.3 μ M each of forward and reverse primer, 0.1 U/ μ l of hot-start Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, USA), 1 μ g/ μ l of BSA, 2X EvaGreen fluorescent dye (Biotium, USA), and 0.01ng/ μ l of plasmid DNA template. Experiments were performed to optimize annealing temperature, MgCl₂ concentration, and primer concentration for amplification of SARS cDNA on a commercial real-time PCR instrument (RotorGene 3000, Corbett Research, Sydney, Australia). The 400 picowells chip was loaded by employing vacuum aided high-speed microfluidics. Following this, PDMS prepolymer was manually injected to cover all the wells using a syringe.

Figure 6-8: Amplification plot of a 400 nanowells chip. Detection of SARS cDNA in ten random wells using. NPC1 and 2 are replicates of no-primer control (total: 390 wells) on the chips. The average threshold cycle (C_T) is 11.22 ± 0.12 (Mean $\pm$ S.D).

Finally, the sealed chip was placed on our prototype real-time quantitative PCR instrument with the following thermal cycling profile: initial denaturation at 95°C for 60 seconds (hot-start of *Taq* polymerase), followed by 30 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 15 seconds and extension at 72°C for 15 seconds. The fluorescence of EvaGreen dye was measured at the extension step of every PCR cycle. The amplification plot is shown in Figure 6-8. The average threshold cycle (C_T) is 11.22 ± 0.12 (Mean $\pm$ S.D).

Other possible high-throughput applications for the 400-well include molecular diagnostics [150, 151], gene expression profiling of genes [152] and non-coding RNAs, genotyping [153], validation of microarray data [154], quantitative methylation analysis [155], copy number variance analysis [156], oncogene mutation profiling [153], and forensic science [157]. van't Veer et al. proposed a 70-gene prognosis signature for selection of lymph node-negative breast cancer patients for adjuvant systemic therapy [150]. Golub et al. reported an approach for cancer classification (distinction between acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL)) based on gene expression monitoring [151]. The 400-well chip can be used for point-of-care, molecular diagnostic applications similar to van't Veer and Golub's approaches. For high-throughput gene expression profiling applications, Caldana et al. reported analyses of 2500 lowly expressed transcription factor genes to determine their involvement in developmental or physiological processes [152]. In another seminal report for high-throughput genotyping applications, Thomas et al. reported profiling of 238 known oncogene mutation involving multiple cancer genes for simultaneous cancer classification and therapeutic interventions [153]. A multi-centre report used real-time PCR for validation and comparison of various microarray platforms [154]. A high-throughput quantitative methylation detection method named "Sensitive Melting Analysis after Real Time (SMART)-methylation specific PCR was reported [155]. For such an application, the 400-well PCR chip can be used for detection of tumour suppressor genes that are currently used as molecular biomarkers for early cancer detection. Gene copy number variations (CNV) are considered as risk factors for complex diseases serving as valuable and detectable biomarkers. Currently available methods are limited in throughput for applications involving higher range of copy number. For example CCL3L1 or the DEFB4 cluster, requires accurate ability of a method to distinguish five copies from six [156].

CLINICAL APPLICATIONS

CHAPTER

7

Letting the person do their own thinking is the whole key in science.

- **Elizabeth Blackburn** 2009 Nobel Laureate

Blackburn co-discovered telomerase, an enzyme that replenishes a protective structure at the end of chromosomes called the telomere.

An enzyme with exciting implications for the study of cancer and human longevity.

Chapter 7 Clinical Application: Absolute Quantification of Methicillin-resistant *Staphylococcus aureus* using Microfluidic Digital PCR.

Staphylococcus aureus is a Gram-positive coccoid bacterium. Its importance as a human pathogen is ever increasing due to emergence of antimicrobial resistance strains - particularly to penicillin, methicillin and recently vancomycin- which has made treatment and surveillance difficult and ultimately lead to increased mortality rate. In humans, *S. aureus* can cause a wide-range of illnesses from minor skin infections, to life-threatening diseases such as endocarditis, toxic shock syndrome (TSS), bacteremia, and sepsis. The methicillin-resistant *Staphylococcus aureus* (MRSA) has become a major cause of nosocomial and community & hospital-acquired infections worldwide. The Centers for Disease Control and Prevention (CDC, USA) estimates that each year in the United States there are about 1.7 million nosocomial infections in hospitals and 99,000 associated deaths with total direct costs estimates of nosocomial infections ~ \$17 billion [158]. A patient with *S. aureus* infection had, on average, three times the length of hospital stay, three times the total charges, and five times the risk of in-hospital death than patients without this infection [159].

The control and reduction of such infections through rapid point-of-care detection of MRSA at bedside form an important component of efforts to improve healthcare safety in the world. The primary habitat of *S. aureus* is the human nostril [160]. Comprehensive analyses of human nostril microbiota indicate presence of few other common bacterial residents [161-164]. Culture-independent methods such as next-generation sequencing method using Roche 454 platform [161, 162], and 16S rRNA gene microarray [164] reveal complex microbiota in nostrils, mostly comprising of *Firmicutes* family *Staphylococcaceae*, and the *Actinobacteria* families *Corynebacteriaceae* and *Propionibacteriaceae*. In nature, the target *S. aureus*, is present with another commensal bacteria *Staphylococcus epidermidis* (*Firmicutes*). Based on the effect of methicillin/oxacillin drug, the bacterial strains of *S. aureus* and *S. epidermidis* are majorly divided into two categories: methicillin-sensitive (MS) and methicillin-resistant (MR). In a recent study by Iwase *et al.*, 93% of *S. aureus* carrier subjects also carried *S. epidermidis*, while 97% of *S. aureus* non-carrier subjects

showed colonization of *S. epidermidis* [163]. The authors showed the existence of two forms of *S. epidermidis*: the inhibitory JK16, and the non-inhibitory JK11 strain, which restricts or allows the growth of commensal *S. aureus* species, respectively. Both the JK11 and JK16 strains are methicillin-sensitive (personal communication with Iwase). The simultaneous cocolonization of methicillin-resistant *S. aureus* (MRSA), methicillin-sensitive *S. aureus* (MSSA), methicillin-resistant *S. epidermidis* (MRSE), and methicillin-sensitive *S. epidermidis* (MSSE) in nasal carriage presents challenge to molecular methods for specific and rapid detection and quantification of MRSA in clinical samples.

Numerous polymerase chain reaction (PCR) based methods, implement prior selective enrichment of MRSA either using broth containing oxacillin drug [165-168] or immuno-qPCR [169]. Such culture based methods are time-consuming (24-48 hrs) and restricts development of rapid MRSA diagnostic methods, which can process clinical samples directly (Figure 7-1).

Figure 7-1: Steps involved in detection of MRSA using classic microbial cultures.

3M medical diagnostic company implemented a magnetic particle based detection of MRSA using selective enrichment broth and selective lysis using Lysostaphin. In this method the ubiquitous adenylate kinase (AK) enzyme is detected by addition of ADP, which is converted to ATP by AK. This ATP is detected by firefly luciferin and luciferase (Bioluminescence). This method sometimes non-

specifically binds MRSE and hence leads to low positive predictive value (Figure 7-2).

Figure 7-2: Magnetic particle based MRSA detection method from 3M medical diagnostic company.

Direct gDNA extraction of nasal clinical samples without culture-based enrichment methods will lead to a multitemplate (gDNA from various nasal microbiota) system [170]. Use of non-sterile specimen imposes a challenge, since the MRSA is present with other staphylococci species such as methicillin-sensitive *S. aureus* (MSSA), methicillin-resistant *S. epidermidis* (MRSE), and methicillin-sensitive *S. epidermidis* (MSSE) [171]. The *mecA* gene, which contributes to resistance, is present in both MRSA and MRSE (Figure 7-3). In such a situation, any probe signal that was directed to *mecA* cannot be linked to presence of MRSA alone. Thus, several multilocus PCR that simultaneously amplify *mecA* (resistance gene) and a *S. aureus* species specific gene such as *sa442*, *nuc*, *femA*, *femB*, *clfA* and *spa* were reported [166, 168, 172, 173]. Although multilocus PCR are capable of producing quick and accurate results for blood culture or culture medium enriched samples, they cannot be applied directly to clinical samples as ambiguous results might appear due to presence of *mecA*-positive CoNS in the mixed clinical samples. This presents an unmet specificity problem, which leads impaired results due to false-positive, when applied to mixed flora specimens/clinical samples. The specificity problem is clearly explained in an excellent journal article by Sturenburg [174].

Figure 7-3: Schematic representation of presence and absence of target genes (*mecA*, *nuc*) in MRSA, MSSA and MRSE. The relative position of *mecR1*, *mecI*, *mutS* and *mutL* is shown only for MRSA. Arrows indicate direction of gene.

In order to address the specificity problem as defined by Sturenburg. [174], a single-locus real-time PCR targeting the amplification of DNA sequence where the staphylococcal cassette chromosome *mec* (SCC*mec*) integrates with open reading frame *orfX* was reported in 2004 [170]. The staphylococcal cassette chromosome *mec* (SCC*mec*) harbors the *mecA* gene. The SCC*mec*-*orfX* junction is unique to MRSA, and hence a single locus PCR is sufficient to specifically detect MRSA in the presence of MRSE, MSSA, MSSE, and other commensal flora specimens. It is important to note that the method reported by Huletsky et al. (2004) did not directly target the *mecA* gene, and it was thought that the SCC*mec* mobile genetic element was stable and detection of SCC*mec*-*orfX* junction indirectly infers presence of *mecA* gene.

The single locus PCR targeting the SCC*mecA*-*orfX* junction is widely used, since application of this method does not require specific enrichment of MRSA. At that time, it was thought the specificity problem for rapid detection of MRSA in clinical sample was solved by the method of Huletsky et al. Subsequently numerous variant methods using SCC*mecA*-*orfX* junction were reported. The method reported by Huletsky et al. is the basis of most of the commercial available MRSA detection test including the three United States Food and Drug Administration (US-FDA) approved kits (LightCycler MRSA advanced Test (Roche Molecular Systems), GeneXpert MRSA (Cepheid), and GeneOhm MRSA assay (Becton Dickinson)). Although, satisfactory performance of the FDA approved kits such as LightCycler MRSA advanced Test [175], GeneXpert MRSA [176, 177], and GeneOhm MRSA [178, 179] were reported, there are numerous report of increased false-positive and false-negative results. A caveat with SCC*mecA*-*orfX* junction method is that the

SCCmecA, which is a mobile genetic element, is not stable, which is contradictory to earlier thoughts. In addition, this method does not directly detect the *mecA* gene which is the sole contributor to the resistance mechanism. In the original report by Huletsky et al., a relatively low and acceptable false positive rate of 4.6% was reported. Recent studies have shown that the reasons for false-negative PCR results are due to unstable nature of the SCCmecA cassette element, where the *orfX* fragment merges with the *mecA* gene, thereby eliminating the SCCmecA-*orfX* junction. The reasons for false-positives PCR results are due to different reasons, such as presence of an *orfX* gene in CoNS that is homologue in sequence to *Staphylococcus aureus* [180]; SCCmec cassette which lacks *mecA* gene [181]; and in some rare situation, *mecA* gene is replaced by a different gene [180].

To complicate further, the SCCmec are of different types. In the original protocol, Huletsky et al. developed primer and probes to detect SCCmec types I, II, III, IVa, IVc, and IVd. False negatives have been reported secondary to variations in this region, new variants of the SCCmec (type IV and V) and nontypeable variants [169]. This approach will always be vulnerable to epidemiological variations and emergence of new SCCmec types. In addition to above reports, documenting the pitfalls associated with the method reported by Huletsky et al., numerous report showed that the FDA-approved commercial kits are also prone to false-positive and negatives PCR results [182-185]. The issues pertaining to high rate of false negative PCR results due emergence of new SCCmec types affected the real-world as Cepheid requested a class I recall for their Xpert MRSA/SA Blood Culture Assay on April 5, 2010 (source FDA website, List of 2010 medical devices recall, last accessed on May 18, 2011). The only way to reliability detect MRSA in a universal setting of unknown SCCmec epidemiology, is to directly detect *mecA* gene and to make sure the *mecA* signal is due to *Staphylococcus aureus* and not due to coexisting MRSE. Based on the current knowledge, it seems like the SCCmec concept is not the best method and probably the multiple locus method may be a favorite choice again. This observation is corroborated by a research article by Thomas et al. [185]. Given the inherent limitations and issues associated with the SCCmec-*orfX* junction detection methods, it seems like the specificity problem as reported by Sturenburg. [174], still persist. The specificity problem related to rapid detection (without any enrichment method) of MRSA directly in clinical samples remains as an unmet clinical need.

Microfluidic digital PCR (mdPCR) offers such a platform. Digital PCR is based on amplification of single template molecule [136, 186]. Another unique advantage of digital PCR is that absolute quantification of target DNA copy number is possible without the requirement of a standard/calibration curve [187]. Specific detection of MRSA gDNA in a multitemplate PCR presents numerous challenges, which are generally associated with any multitemplate PCR, such as amplification bias due to primer mismatch, different primer binding energies, and reannealing of template [188-190]. Hence, it is desirable to develop a system, which can process clinical samples directly without amplification bias, which is inherent of multitemplate PCR. Most of the current MRSA assays are either quantitative with a paramount requirement to construct standard curves for every experimental run [191, 192] or they are just qualitative including the United States of America Food and Drug Administration (US-FDA) approved commercial kits LightCycler MRSA advanced Test (Roche Molecular Systems), Xpert MRSA/SA nasal assay (Cepheid), and GeneOhm MRSA assay (Becton Dickinson). Precise and absolute quantification of MRSA in the presence of other pathogens is relevant, as studies have shown that mean bacterial load is higher in persistent carriers [193], resulting in extensive dispersal of MRSA in the environment with an increased risk of infections and associated deaths [194]. Estimation of MRSA bacterial load will enable doctors to stratify patient's risk and treat them better.

In the past, digital PCR have been successfully demonstrated on a 96-well plate [195], microfluidic arrays [196], and droplet-microfluidics [68] for a variety of applications such as detection of rare KRAS mutations [195] and ABL tyrosine kinase in chronic myeloid leukemia [197], DNA methylation analysis (epigenetics) [198], copy-number variation [199], and prenatal diagnosis of fetal aneuploidy [200]. However operation of these chip require microvalves, which are controlled by external pressure source. Recently, there were two reports of microfluidic digital PCR on a spinning disk platform [201] and "SlipChip" [202]. The spinning disk platform implements serial dilution of target template along a microchannel, which may have inherent loading bias in some areas of well along the channel. The authors did report failure of template loading in first few wells [201]. Although the "SlipChip" has a simple sample loading method, which is ideal for point-of-care diagnostics, they require cumbersome chromium etch fabrication process on a glass substrate inside a clean room, which ultimately add cost to the chip. Following chromium etching the

authors cleaned the chip and coated the chip surface with a hydrophobic coating of dichlorodimethylsilane. This step is critical in the fabrication process, since any inefficiency at this step may lead to possible chromium residues which may inhibit the PCR, as observed in a study by another group [203].

In this work, a disposable microfluidic digital PCR matrix chip fabricated using polydimethylsiloxane (PDMS) with a rapid, vacuum-based sample loading method was developed. The vacuum-based sample loading can load 1024 wells in less than a second in a parallel dilution format instead of serial dilution, as practiced by the current platforms. The loading method utilizes a portable vacuum pump without the use of on-chip valves, centrifuge equipment's or manual slide operation action. The microfluidic chip has been specially designed for rapid detection of live MRSA in mixed bacterial samples using multiplex, hydrolysis probe-based (real-time) fast PCR protocol. The mixed bacterial sample is treated with propidium monoazide followed by genomic DNA extraction off-chip, and digital PCR on PDMS matrix chip to selectively quantify genomic DNA from viable MRSA. Although digital PCR primarily requires end-point PCR scan, implementation of real-time format for digital PCR provides additional power to the user to decide for any false-positive or false-negative using a sigmoidal amplification plot. The improvements of our report, when compared to the existing digital PCR platform are primarily classified as (1) chip design and operation, and (2) applications. Under the chip design and operation, the improvements are low-cost disposable digital PCR chip, easy fabrication using soft-lithography which is suitable for bulk manufacturing of digital PCR matrix chip, rapid sample loading method (parallel dilution), which can be easily implemented in a point-of-care set-up without the use of on-chip valves or centrifugation instruments. On the applications side, we implemented fast PCR to reduce sample-to-result time, detect and quantify only live MRSA in a complex background of sample without the need to specifically enrich MRSA, thereby reducing the sample processing time. In addition to these improvements, the matrix chip reaps the benefit of digital PCR such as high target to background template ratio, dilution of possible PCR contaminants and background templates in parallel dilution format, insensitive to high standard deviation (σ) in quantification cycle (C_q) due to stochastic amplification of single template molecule [204], elimination of PCR bias, high signal-to-noise ratio (SNR) in a single template molecule PCR well, and high primer to target ratio.

7.1 Bacterial Strains and growth conditions

Reference strains *Staphylococcus aureus* subsp. *aureus* ATCC 33591 (MRSA, strain 328), methicillin-sensitive *Staphylococcus aureus* subsp. *aureus* ATCC 25923(MSSA, strain Seattle 1945) were purchased from American Type Culture Collection (ATCC). Pure genomic DNA isolated from *Staphylococcus aureus* subsp. *aureus* ATCC 33591 and methicillin-resistant *Staphylococcus epidermidis* ATCC 35984 (MRSE, strain RP62A) were also obtained from ATCC. Bacterial cells were grown in Tryptic Soy Broth (TSB 43592 Fluka, Sigma Aldrich, Singapore) at 37°C overnight with aeration on a rotary shaker at 220 rpm to approximately reach an optical density of 1.0 at 600 nm.

7.2 Oligonucleotide and probe design and validation

The specificity of MRSA detection is based on the presence of two genes *mecA* and *nuc*. Gene *mecA* is present in methicillin-resistant *S. aureus* and methicillin-resistant *Staphylococcus epidermidis*, whereas *nuc* gene is *S. aureus* specific. A schematic representation of the presence and absence of the gene is illustrated in [Figure 7-3](#). A consensus sequence, which was obtained by multiple sequence alignment of X52593 (*mecA* gene), V01281 (*nuc* gene) and CP000029 (*S. epidermidis* RP62A, complete genome) using Clustal W, was used to design the primers and hydrolysis probes. Two pairs of oligonucleotides (1st Base, Singapore) were designed to amplify a 97-bp and 83-bp region of *mecA* and *nuc* genes respectively. The specificity of the primer pairs were checked by primer-BLAST using the NCBI (National Center for Biotechnology Information) BLAST server. The absence of amplicon secondary structures at the designed regions of *mecA* and *nuc* genes was confirmed using Zucker DNA folding program [205]. The probe for *mecA* gene was labeled with 6-FAM and for *nuc* gene was labeled with VIC. Both the probes had MGB quenchers (Life Technologies, USA).

7.3 Digital PCR chip design and fabrication

The initial development and extensive characterization of the PCR matrix chip was reported in our earlier paper [206]. The matrix chip presented in this paper is an improved version of our earlier chip. In contrast to our earlier version, we used a

black colored liquid PDMS for fabrication of the matrix chip to prevent possible fluorescence cross-talk (Figure 7-4).

Figure 7-4: Comparison of black PDMS and transparent PDMS. The peaks represent fluorescence from nine consecutive nanowells. The fluorescence from wells in transparent PDMS is relatively higher compared to black PDMS indicating that chips with transparent interface walls might be prone to fluorescence cross-talk.

To replicate the middle black PDMS layer, 2 ml of liquid PDMS containing 10:10:2 ratio of PDMS Sylgard silicone elastomer 184 (part A), Sylgard 567 primerless silicone (part A), and Sylgard curing agent 184 (part B) (Dow Corning Corporation, USA) respectively, were mixed to a homogenous state in a beaker using magnetic stirrer at 150 rpm for 1 hour. The black color is contributed by Sylgard 567 primerless silicone (part A, Dow Corning Corporation, USA). The middle PDMS layer is replicated using soft-lithography technique using a stainless steel die micromachined using electrical discharge machining (EDM) process by a commercial vendor (Bytech Precision Engineering, Singapore).

During initial phase of development, Sylgard 567 primerless silicone (part A & B, Dow Corning Corporation, USA) was used to fabricate black PDMS ([Table 7-1](#)) by curing Sylgard 567 at 100°C for 90 minutes. However, this method was not successful, since the cured Sylgard layer on the stainless steel EDM die was rigid and had issues with ability to peel it off. Hence, the temperature of curing was reduced to 80°C for 90 minutes. This again, was not a good solution since, 2 out of 3 chips fabrication failed due to tearing of PDMS layer during demolding. Hence, a mixed approach of combining Sylgard 184 and 567 (10:10:2 ratio of PDMS Sylgard silicone elastomer 184 (part A), Sylgard 567 primerless silicone (part A), and Sylgard curing agent 184 (part B) (Dow Corning Corporation, USA) respectively) was followed. Henceforth, this 10:10:2 ratio of PDMS will be known as mixed Sylgard 184 + 567. Fabrication method using mixed Sylgard 184 + 567 is robust. No issues related to PDMS tearing were encountered for at least nine different chip fabrications.

Table 7-1: Comparison of properties between Sylgard 184 (transparent) and Sylgard 567 (black).

Typical Properties	SYLGARD(R) 184 SILICONE ELASTOMER KIT	SYLGARD(R) 567 PRIMERLESS SILICONE ENCAPSULANT KIT
Color	Clear	Black
Dielectric Constant at 100 Hz	2.65	N/A
Dielectric Constant at 100 kHz	2.65	N/A
Dielectric Strength	= 540 volts per mil v/mil	N/A
Dissipation Factor at 100 Hz	0.0005	N/A
Dissipation Factor at 100 kHz	< 0.001	N/A
Durometer - Shore A	= 50 Shore A	= 45 Shore A
Dynamic Viscosity	= 3900 Centipoise	= 1500 Centipoise
Flowable	Yes	Yes
Heat Cure	45 Minutes @ 100 Deg C	90 Minutes @ 100 Deg C
Hydrophobic	Yes	Yes
Mix Ratio	10:1 Base to Catalyst 87-RC	1:1 Base to Catalyst
Room Temperature Cure - Hours	= 48 Hours	N/A
Self Leveling	Yes	Yes
Shear	N/A	= 140 psi
Shelf Life	= 720 Days	= 720 Days
Specific Gravity @ 25C	1.03	1.24
Temperature Range	-45 Deg C to 200 Deg C	-45 Deg C to 200 Deg C
Thermal Conductivity	= 0.16 Watts per meter K	= 0.3 Watts per meter K
UL 94 V-0 @ 8.4 mm thickness	Yes	N/A
UL 94 V-1 @ 6.0 mm thickness	Yes	N/A
Volume Resistivity	= 1.2e+014 ohm-centimeters	N/A
Water Resistant	Yes	Yes
Working Time	> 120 Minutes	= 4320 Minutes

In order to further characterize the black PDMS, the Shore A hardness and percentage shrinkage (post-curing) were enumerated. For this experiment, three chips were fabricated using Sylgard 184, Sylgard 567, and mixed Sylgard 184+567 (used in this study) by curing at 80°C for 90 minutes. The location of hardness measurement is shown in [Figure 7-5](#), and the measured data is shown in [Table 7-2](#). Based on [Table 7-2](#), it is clear that the PDMS tearing issues encountered for Sylgard 567 is due to very low Shore A hardness (18.63), when compared to the Shore A hardness of the widely using Sylgard 184 (44.38). The mixed Sylgard 184+567 had a Shore A harness value of 38.5, which is very close to Sylgard 184 (44.38). Another disadvantage of using Sylgard 567 is that the percentage shrinkage post-curing is high (1.39%), when compared to Sylgard 184 (0.76%).

Location of hardness measurement and shrinkage distance

Figure 7-5: Location of eight points for hardness measurement. The percentage shrinkage was estimated based on chip with initial length of 30 mm from the inlet channel to outlet channel on the opposite end.

Table 7-2: Shore A hardness and percentage shrinkage data for Sylgard 184, Sylgard 567 and Mixed Sylgard 184+567.

All the three chips were cured at 80 degree C for 90 minutes												
	Shore A hardness: location of measurement on chip									Shrinkage		
Sample	1	2	3	4	5	6	7	8	Average	Standard Deviation	Shrinkage (mm)	Shrinkage (%)
Sylgard 184	44	45	44	45	45	44	44	44	44.38	0.52	0.23	0.76
Sylgard 567	19	18	19	19	18	20	18	18	18.63	0.74	0.42	1.39
Mixed Sylgard 184 + 567	39	39	38	38	38	40	38	38	38.50	0.76	0.17	0.56

The chip is made of three materials, a silicon substrate (diced silicon wafer) for uniform thermal uniformity across the chip, poly-(dimethylsiloxane) (PDMS) harboring nanowells and microchannels, and a top glass cover (Herenz Medizinalbedarf, Germany; 33 x 33 mm) for optical imaging of the nanowells. A schematic of the digital PCR chip is shown in Figure 7-6. The digital PCR chip for MRSA detection can accept three samples. A top row of 32 nanowells for no-template control (NTC) reaction. A middle matrix of 1024 nanowells (32 x 32 matrix) for positive control or test reaction and a bottom row of 32 nanowells for internal positive control (IPC) reaction. The chip has a total dimension of 33 mm x 33 mm x 1.2 mm. Each nanowell has a dimension of 0.5 x 0.5 x 0.5 mm that can hold approximately 120 nl.

Figure 7-6: Schematic representation of the digital PCR chip showing the three layers. The bottom silicon substrate provides a uniform thermal heating to the chip, the centre PDMS layer harbors the channels and microwells, the top transparent glass cover allows fluorescence image capture by a CCD camera.

The PDMS prepolymer was poured onto the surface of the metal die and subjected to vacuum for 20 minutes to degas. Following this a square shaped crystal weight (160 grams) was placed on the top of the metal die, and the entire assembly was placed inside an oven at 80°C for 90 minutes to cure the liquid PDMS. Subsequently, the PDMS layer was peeled off the die and diced to desired size.

Figure 7-7: Photograph of the fabricated microfluidic digital PCR chip without top glass cover. (a) Top view of the chip. The red dashed line shows inlet ports. NTC: No-template control reaction; PC: Positive control reaction.

The diced PDMS layer was bonded to a silicon substrate and top cover glass, using a thin spin-coated layer of PDMS prepolymer as adhesive. The interface spin-coated PDMS adhesive was cured at 80°C for 30 minutes. The chip was cooled to room-temperature before use or stored in a sealed pack for future use (Figure 7-7).

7.4 Detection of MRSA in nasal swab

Nasal samples were collected using premoistened BBL™ CultureSwab™ Liquid Stuart, Single Swab (PN 220099, BD Diagnostics, USA) with swabs rubbed inside one nostril followed by other. The swab was broken off into a screw-top microfuge tube containing 100 µl of PBS (P5493, Sigma Aldrich, Singapore). Swabs were vortexed for 30 seconds. Following this, the swab was removed from the microfuge tube and propidium monoazide (PMA) (Biotium, USA) was added at a final concentration of 50 µM in a microfuge tube. The sample was briefly incubated on ice in a dark place for 5 mins before being exposed to blue wavelength light-emitting diodes (LED) for 2 mins. The cells were then pelleted and the PBS buffer was discarded by aspiration. The cells were then resuspended in 100 µl of 1U/µl of achromopeptidase (T8787, Sigma Aldrich, Singapore) in 1X TE (pH 8.0, Sigma Aldrich, Singapore). The samples were thoroughly vortexed and incubated at 30°C for 15 mins. Finally, the samples were incubated at 99°C for 5 mins. The 100ul sample was directly used for PCR without any purification step.

7.5 Extraction of bacterial DNA for PCR protocol development using a commercial kit

Bacteria were grown overnight in 10 ml of TSB, and 1.5 ml of this culture was centrifuged at 13,000rpm for 10 mins. The DNeasy blood and tissue kit (PN 69504, Qiagen, Singapore) was used with modification to the protocol for Gram-positive *Staphylococcus aureus*. The bacterial pellet was resuspended in 200 µl of modified enzymatic lysis buffer and incubated at 37°C for 30 mins. The 1X modified enzymatic lysis buffer contained 20 mM Tris-Hcl (pH 8.0, T2694, Sigma Aldrich, Singapore), 1.2% Triton X-100 (T8787, Sigma Aldrich, Singapore), 20 µg of Lysozyme (L6876, Sigma Aldrich, Singapore)/ml, and 200 µg of Lysostaphin (L7386, Sigma Aldrich, Singapore)/ml. Following this, 56 µl of RNase cocktail enzyme mix (AM2286, Ambion, Life Technologies, Singapore) was added with 200 µl of AL buffer (kit component) and incubated at 70°C for 10 mins. Subsequently, 25 µl of proteinase K (kit component) was added and incubated at 56°C for 30 mins. The mixture was then processed on the DNeasy mini spin column by following the manufacturer's instruction. The genomic DNA was finally eluted in 100µl of buffer AE (kit component). Quantification of genomic DNA was done by using the PicoGreen

dsDNA Quantitation Kit (Invitrogen, Singapore) on TECAN GENios Plate Reader (Tecan, Mannedorf, Switzerland) using black flat bottom 96-well assay plates (PN 353241, Becton Dickinson, USA).

7.6 Microfluidic digital PCR chip operation.

The chip uses a vacuum to load test sample, NTC reaction and IPC reaction. The basic operation process using a vacuum enclosure is extensively described in our earlier work [206]. Following sample loading, the nanowells were isolated using liquid sealant (PDMS prepolymer) by injecting it through the head-space of the chip.

In order to prevent cross-contamination among NTC, PC and internal PC during microfluidic operation process, the following steps were implemented.

1. All the three reactions NTC, PC and iPC (internal PC) used separate reservoir with attached Teflon and silicone tubing (Figure 4-23). In order to prevent subsequent contamination, this setup is a disposable component of the system.
2. The reactions were loaded in the following sequence: NTC, iPC, and PC.
3. Every reaction was sealed with liquid prepolymer before next reaction was loaded in the chip. NTC was first loaded and sealed, second iPC was loaded and sealed. Finally the PC was loaded and sealed.
4. In order to prevent contamination, after every vacuum loading step, the chamber (vacuum enclosure) is cleaned with DNA AWAY (Molecular Bioproducts) to avoid any aerosol cross-contamination of nucleic-acid template from the previous run.

Finally, the chip was thermocycled on a prototype real-time quantitative PCR instrument.

7.7 Real-time quantitative PCR instrument for multiplex TaqMan PCR.

The prototype real-time PCR instrument consists of a thermocycling and optical imaging module. A flat square shaped thermoelectric cooler (TEC) (RS components, Singapore) powered with 12 volts was used to thermocycle the digital PCR chip. A RTD (resistive temperature detector) was mounted on the top of TEC surface to monitor the temperature on the TEC. The measured temperature is used as an input (feedback) to the proportional–integral derivative (PID) algorithm, which controls the power output to the TEC surface. Rapid cooling of the TEC was achieved by forced

cooling using a fan under the TEC. The performance of the developed real-time PCR instrument was demonstrated in our earlier publication with PCR amplification efficiency of above 90% [207, 208]. The optics of the real-time PCR instrument was designed to measure the fluorescence of 6-carboxyl-X-rhodamine (ROX) ($\lambda_{\text{ex}} = 580$ nm; $\lambda_{\text{em}} = 605$ nm), 6-carboxyfluorescein (FAM) ($\lambda_{\text{ex}} = 497$ nm; $\lambda_{\text{em}} = 525$ nm), and proprietary VICTM fluorescent dye ($\lambda_{\text{ex}} = 538$ nm; $\lambda_{\text{em}} = 554$ nm). The ROX and VIC fluorescent dye was excited using high power LED with a circular excitation filter $D580 \pm 10\text{x}$ and $D530 \pm 5\text{x}$ (Chroma Technologies, USA) respectively. The FAM fluorophore was excited using an array of diffuse LED in rectangular format with a custom made rectangular (88 x 45 x 3 mm) filter $480 \pm 15\text{x}$ (Chroma Technologies, USA). The LED light casing was fixed at an angle of 45° to the plane of the digital PCR chip so as to prevent interference of emitted fluorescent light from the nanowells. The emitted light from the nanowells were filtered using another set of three filters on a filter wheel before being detected by a CCD camera (DTA, Pisa, Italy). The 25mm circular emission filters fixed on a filter wheel are $D520 \pm 10\text{m}$ (FAM), $HQ\ 567 \pm 7.5\text{m}$ (VIC), and $D620 \pm 20\text{m}$ (ROX) respectively. All the filters were purchased from Chroma Technologies, USA. Software was written to control, automate, and integrate multiple functions including thermal cycling control, filter wheel control, real-time fluorescence imaging, on-line image processing, and real-time PCR data analyses.

7.8 Real-time TaqMan-based digital PCR.

In order to demonstrate the digital PCR on the matrix chip we specifically amplified and detected genomic DNA of MRSA. The digital PCR mixture (20 μl) contains 1X LuminoCt qPCR ReadyMix (Sigma Aldrich, Singapore), 1X ROX internal reference dye (Sigma Aldrich, Singapore), 1 $\mu\text{g}/\mu\text{l}$ of non-acetylated bovine serum albumin (B8667, Sigma Aldrich, Singapore) and genomic DNA. Each primer-hydrolysis probe mixture for *mecA* and *nuc* gene, contains probe at 250 nM and forward and reverse primers at 900 nM each. The LuminoCt qPCR ReadyMix contains optimized concentrations of Tris-HCl (pH 8.3), KCl, dNTPs, stabilizers, MgCl_2 and Jumpstart Taq DNA polymerase. For internal positive control, *mecA* gene cloned in a plasmid, which is component of IsoAmp rapid *mecA* detection kit was obtained from BioHelix Corporation (USA). The internal positive control reaction

contains *mecA* control template pMECA201 at 0.1 ng/μl, 1X LuminoCt qPCR ReadyMix (Sigma Aldrich, Singapore), 1X ROX internal reference dye (Sigma Aldrich, Singapore), 1 μg/μl of non-acetylated bovine serum albumin (B8667, Sigma Aldrich, Singapore), 2X EvaGreen (Biotium, USA) and 300 nM of each primer (Forward: 5'-GAA AAA TGA TTA TGG CTC AGG TAC TGC and reverse: 5'-TGG ATA GAC GTC ATA TGA AGG TGT GCT; primer from reference [209]). After sample loading and nanowells sealing, the digital PCR was performed with following fast PCR thermal profile: Initial denaturation at 95°C for 120 minutes; followed by 40 cycles of denaturation at 95°C for 3 seconds, and annealing/extension at 55°C for 15 seconds. At every PCR cycle, automated images were collected at the extension phase for ROX, FAM and VIC fluorophore.

7.9 Optimization of PCR condition for *mecA* and *nuc* genes.

The optimum annealing temperature for *mecA* and *nuc* gene primer pairs is found to be 55°C by performing a gradient PCR at 55, 60 and 65°C on DNA Engine (PTC-200) from MJ Research (now Bio-Rad). The reaction with highest PCR yield, which is enumerated using a capillary electrophoresis chip (DNA Labchip 1000 Series II) using Agilent 2100 bioanalyzer (Agilent Technologies, Singapore), is chosen as optimum annealing temperature. The *mecA* and *nuc* gene PCR product is enumerated as 101 and 89 bp, which is comparable to the designed PCR product size of 97 and 83 bp respectively (Figure 7-8).

Figure 7-8: Gel-like image of *mecA* and *nuc* PCR product from RotorGene 3000, tested on a DNA Labchip 1000 using Agilent 2100 bioanalyzer. Lane L is the ladder. NTC is no-template control. R denotes replicate reactions.

The efficiency of the designed primer-probe set for *mecA* and *nuc* gene is tested using a 10-fold serially diluted MRSA genomic DNA template on a

commercial real-time PCR instrument (RotorGene 3000, Corbett Research, Australia; now Qiagen). The PCR efficiency is calculated using the equation $E = 10^{(-1/\text{slope})} - 1$. The efficiencies for *mecA* and *nuc* gene are 97 and 94% respectively for 5 logs of gDNA template concentration (Figure 7-9). The specificity of the single-plex reactions using MRSA, MSSA and MRSE gDNA is tested using *mecA* and *nuc* primer-probe set. The presence of *mecA* gene was confirmed in MRSA and MRSA, while *nuc* gene was confirmed in MRSA and MSSA.

Figure 7-9: Standard curve for 10-fold serially diluted genomic DNA from MRSA. Five logs of genomic DNA are used to generate the standard curve. Based on the slope the efficiency of the *mecA* and *nuc* gene is found to be 97 and 94% respectively. Error bars represent standard deviation for sample size $n = 2$.

7.10 Absolute quantification of MRSA gDNA.

Pure MRSA gDNA was diluted to ~39 copies in 60 μ l of total PCR volume. The 60 μ l was loaded in a 100-well chips fabricated using black PDMS. Post-PCR, the number of positive wells were counted and found to ~ 36 positive well. The distribution of biomolecule among array of reactors is governed by Poisson distributions. If λ is the mean number of template copies and x is the number of templates of interest present

for a given well, then the Poisson distribution of x is described by the following equation.

$$P(X=x) = \frac{[e^{-\lambda} \lambda^x]}{x!}$$

The amplification plot for the digital PCR chip is shown in [Figure 7-10](#).

[Figure 7-10](#): Amplification plot for single molecule PCR. Inset shows 40th cycle image for a 10x10 matrix.

The quantification cycle (Cq) value from the 36-positive wells is shown in [Table 7-3](#).

[Table 7-3](#): Quantification cycle values from positive wells from a digital PCR chip.

Well No.	Cq	Well No.	Cq	Well No.	Cq
Nanowell 1	27.90	Nanowell 13	28.94	Nanowell 25	27.77
Nanowell 2	29.10	Nanowell 14	28.60	Nanowell 26	29.22
Nanowell 3	27.86	Nanowell 15	27.08	Nanowell 27	28.55
Nanowell 4	28.65	Nanowell 16	29.93	Nanowell 28	28.86
Nanowell 5	28.24	Nanowell 17	29.06	Nanowell 29	27.15
Nanowell 6	28.42	Nanowell 18	28.81	Nanowell 30	30.00
Nanowell 7	27.62	Nanowell 19	27.46	Nanowell 31	29.66
Nanowell 8	27.38	Nanowell 20	28.54	Nanowell 32	28.27
Nanowell 9	30.15	Nanowell 21	31.29	Nanowell 33	29.92
Nanowell 10	28.33	Nanowell 22	30.95	Nanowell 34	28.00
Nanowell 11	30.00	Nanowell 23	29.34	Nanowell 35	28.84
Nanowell 12	29.50	Nanowell 24	29.66	Nanowell 36	28.33
				n	36
				Mean	28.82
				SD	1.02
				%CV	3.54

The Cq values from 36 positive wells fit a normal distribution with a P value of 0.67 (Figure 7-11). Based on the Poisson distribution the expected estimated range for 39 single molecule PCR is 32.8 to 45.2 copies. Based on the digital PCR run, the number of copies, which was experimentally enumerated, was 36 copies. Since this values lies in the Poisson range, the experimentally enumerated result from the developed digital PCR agrees with the Poisson distribution.

Figure 7-11: Normal distribution of quantification cycle values for single molecule PCR.

CONCLUSIONS

The potential of surface-tension based microfluidics in generating an array of solution-phase real-time PCR was successfully demonstrated. The surface-tension generates pressure in the inlet channel, which distributes the PCR mixture among an array of reactors. The PCR mixture is confined inside the reactor by abrupt enlargement of capillary at the exit of the outlet bridge channel causing drastic change in the contact angle between the PCR mixture and channel wall surface. The developed capillary microfluidics can be applied to any high-throughput chemical or biological analysis platforms for liquid sample distribution and reactor sealing. Since PDMS is inert to many chemicals and biologically compatible, the reported array chips can be used for other analytical processes. With the current chip design and dual-fluid microfluidics, the upper limit for the number of reactors on the developed chip depends on the improvement in microfluidics to control primer diffusion when reactor density on the chip is high, ability to perform efficient PCR in picoliter range. The salient features of the microfluidic method presented in this work are manual pipetting (non-robotic) with a simple two-step pipetting operation, cost-effective, ease in operation when compared to multiple-step manual pipetting operation or specialized centrifugation/vacuum instrument for loading sample liquids (TLDA card) and other liquid handling instruments. [Table 8-1](#) shows comparison of features of the developed PCR array chip with a commercially available microfluidic chip from Applied Biosystems.

Table 8-1: Comparison of features between Applied Biosystems TaqMan Low-Density Array chip and the developed PCR array chip

S.No	Feature	Applied Biosystems Microfluidic TLDA chip	Our PCR matrix chip
1	Microfluidic sample loading	Centrifugal force (1200rpm-2mins). Requires centrifuge instrument	Surface-tension force. Passive loading (No instrument)
2	Reactor sealing	Specialized sealing device	Surface-tension force. Passive loading(No instrument)
3	PCR volume	1500 nL	120 nL
4	Max. number of samples	8	1 (Scaleable)
5	Max. assays for each sample	48	100 (Scaleable)*
6	Max. number of reactors	384	100 (Scaleable)*

The advantages of the developed PCR array chip, when compared to other similar work's published in the literature are presented in [Table 8-2](#).

Table 8-2: Comparison of the developed PCR array chip with other similar work's [4, 53, 55, 56, 60, 61] published in the literature.

No	Features	Array chip developed in this study	Daniel et al.	Matsubara et al.	Nagai et al.	Liu et al.	Chaudhari et al.	Iordanov et al.
1	Sample loading	Passive loading	Manual pipetting: same reaction in all the wells	Nanoliter dispensing robotic system	Nanoliter dispensing robotic system	Peristaltic pump	Manual pipetting	N.A
2	Reaction array sealing	Passive sealing	Manual pipetting: mineral oil	Nanoliter dispensing robotic system: Mineral oil	Sealing using a glass cover with weight	Special pneumatic hydraulic control channel	Acrylic-based adhesive tape	N.A
3	PCR format	Real-time PCR	End-point PCR	Real-time PCR	End-point PCR	End-point PCR	Real-time PCR	N.A
4	Reaction volume	800 nL	1500 nL	40 nL	0.86 nL	3 nL	5000 nL	N.A
5	Total no. of reactors	12*	N.A	60	10000	400	18	N.A
6	Surface treatment	Dynamic coating	Yes, BSA	Yes, BSA and mineral oil	Yes, SiO ₂ layer	Dynamic coating	Yes, SiO ₂ layer	N.A
7	Position of the heater and sensor	Integrated in the PCR instrument	Integrated in the PCR array chip	Integrated in the PCR instrument	Integrated in the PCR instrument	Integrated in the PCR instrument	Integrated in the PCR instrument	Integrated in the PCR array chip
* can be expanded			N.A : Not-Available					
Daniel et al.(1998): Silicon microchambers for DNA amplification. Sensors and Actuators A: Physical 71(1-2): 81-88.								
Matsubara et al. (2005): Microchamber array based DNA quantification and specific sequence detection from a single copy via PCR in nanoliter volumes. Biosensors and Bioelectronics 20: 1482–1490.								
Nagai et al.(2001): Development of a microchamber array for picoliter PCR. Anal Chem 73(5): 1043-7.								
Liu et al.(2003): Solving the "world-to-chip" interface problem with a microfluidic matrix. Anal Chem 75(18): 4718-23.								
Chaudhari et al. (1998): Transient liquid crystal thermometry of microfabricated PCR vessel arrays. Microelectromechanical Systems, Journal of 7(4): 345-355.								
Iordanov et al.(2003): PCR array on chip - thermal characterization. Sensors. 2003. Proceedings of IEEE.								

FUTURE WORKS

A surface tension based sample loading and microreactor sealing method is successfully developed, and the functionality of the developed method is demonstrated by reliably performing PCR in a series of microreactors. In a different chip array format, a nanowell array or PDMS matrix chip is developed with vacuum-based microfluidic sample loading and reactor sealing. Subsequent works that can build on the work reported in this thesis are summarized below.

- 1. Miniaturization of real-time PCR instrument:** The developed capillary based microfluidic array chip is developed for point-of-care applications for use in the field, where access to sophisticated instrument is impossible. Although the real-time PCR system developed in this work is portable, development of hand-held real-time PCR instrument, which can accept the surface-tension driven device, will be useful, as it will help to realize the true potential of point-of-care application. Smiths detection (Watford, UK) currently sells such a hand-held real-time PCR instrument (Bio-Seeq PLUS). The weight of Bio-Seeq PLUS is 3 Kgs, and it can detect only 6 targets simultaneous, limiting its use for multiple sample or assays greater than six. Future work can be performed in the area of instrumentation to develop a lighter weight hand-held real-time PCR instrument, which can accept the developed capillary microfluidic devices, and test more targets per run.
- 2. Automated system for primer preloading for matrix chip:** In this work, primer preloading inside the nanowells was achieved by a manual pipetting step. Although, there are numerous sophisticated and precise liquid handling robots, which can go down to dispensing nanoliter volumes, there is still a missing interface, which can seamlessly integrate the existing liquid handling system with the developed PCR matrix chip. A cost-effective method of primer preloading on the matrix chip should be motivation for the development of an integrated method, which can serve the purpose. An alternative path is to explore a micronozzle array, which can be fabricated using standard lithography techniques, to dispense primer pair liquor into nanowells arrays.
- 3. Targeted extraction of PCR amplicons:** In order to perform downstream analyses of the amplicons, it is desired to extract the amplicons post-PCR. For

example, applications such as targeted sequencing or amplicon barcoding for the massively parallel sequencing/next-generation sequencing require extraction of amplicon. Since the PCR is performed in nanoliter range, it will be a great challenge to extract it without getting contaminated. In addition, not all the reactions, may be of interest to analyze further. In such a situation, it will be desired to barcode the nanowells, so that it can precisely targeted for extraction.

- 4. Fast PCR thermal cycling and multicolor optics:** A fast PCR thermal cycling can be optimized to achieve fast sample-to-results in the field. Such a need will require improvement in the thermal module of the real-time PCR instrument. The current optics of the real-time PCR instrument is designed to detect three fluorescent molecules (FAM/HEX/ROX). The optics may be upgraded to detect five fluorescent molecule to improve the productivity of the instruments in the field.

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PUBLICATION LIST

Journal paper

- I. **In preparation** – (2011) Calibration-curve-free Absolute Quantification of Live Methicillin-Resistant *Staphylococcus aureus* (MRSA) in Mixed Bacterial Samples Using Microfluidic Digital PCR.
- II. **Naveen Ramalingam**, Rui Z., Liu H.-B., Dai C.-C., Kaushik R., Ratnaharika B., et al. (2010) Real-time PCR-based microfluidic array chip for simultaneous detection of multiple waterborne pathogens. *Sensors and Actuators B: Chemical*. 145,543-552.
- III. **Naveen Ramalingam**, Liu H.-B., Dai C.-C., Jiang Y., Wang H., Wang Q., et al. (2009) Real-time PCR array chip with capillary-driven sample loading and reactor sealing for point-of-care applications. *Biomedical Microdevices*. 11,1007-1020.
- IV. **Naveen Ramalingam**, San T., Kai T., Mak M., Gong H.-Q. (2009) Microfluidic devices harboring unsealed reactors for real-time isothermal helicase-dependent amplification. *Microfluidics and Nanofluidics*. 7,325-336.
- V. Liu H.B, **Naveen Ramalingam**, Jiang Y, Dai C.C, and Kam M. Hui, and Gong H.Q (2009) Rapid distribution of a liquid column into a matrix of nanoliter wells for parallel real-time quantitative PCR. *Sensors and Actuators B: Chemical*, Volume 135, Issue 2, 671-677.
- VI. Liu H.B, Gong H.Q, **Naveen Ramalingam**, Jiang Y, Dai C.C, and Kam M. Hui (2007) Micro air bubble formation and its control during polymerase chain reaction (PCR) in polydimethylsiloxane (PDMS) microreactors. *Journal of Micromechanics and Microengineering*, 17 2055-2064.
- VII. Gong H.Q, **Naveen Ramalingam**, Chen L, Che J, Wang Q, Wang Y, Yang X, Yap PH, Neo CH (2006) Microfluidic handling of PCR solution and DNA amplification on a reaction chamber array biochip. *Biomed Microdevices* 8:167-76.

Book Chapter

- VIII. Gong H.Q, **Naveen Ramalingam**, Liu H.B, Chen L.Q, Wang Q.H, Yang X.H, Deng L.Q, Peng Huat E.Y., Kam M. H, Jiang Y, Dai C.C. (2009) Sequential microfluidic formation of PCR microreactor array for high throughput genetic testing. *Microfluidics for Nucleic Acid Analysis (Methods in Molecular Biology)* by Dennis J. OKane. Publisher: Humana Press; 1 edition (*Expected date of release*: December, 2011).

Conference Paper

- IX. **Naveen Ramalingam**, LongQing C., XinHao Y., Deng L., Wang Q., Eric Yap P.H., Neo C.H, and Gong H.Q (2009) A surface-directed microfluidic scheme for parallel nanoliter PCR array suitable for point-of-care testing. The Seventh International Conference on Nanochannels, Microchannels, and Minichannels Paper number: ICNMM2009-82052. June 22-24, 2009, Pohang, South Korea.

My contributions to the published journal papers:

Paper I. I developed the design, fabrication and fluidic operation process of the microfluidic digital PCR chip. Designed and developed the multiplex assay for detection and quantification of MRSA. I performed the experiments and wrote the paper.

Paper II. I developed the design, fabrication and fluidic operation process of the microfluidic chip for parallel detection of multiple waterborne pathogens. I also performed the biological experiment to demonstrate the detection of waterborne pathogens. I wrote the paper.

Paper III. I developed the fabrication process of the microfluidic device, designed the experimental setup and performed the biological experiments. I performed the characterization of the microfluidic device and the data analysis. I wrote the paper.

Paper IV. I developed the design, fabrication and fluidic operation process of the microfluidic device. I also performed the experimental validation of the device using isothermal helicase dependent nucleic-acid amplification method. I wrote the paper.

Paper V. I fabricated the chip, performed the fluidic operation process and validated the functionality of the chip by demonstrating parallel detection of ten gene targets involved in prediction of recurrence of human hepatocellular carcinoma. I performed the characterization experiments together with Liu Hao-Bing. I performed the data analysis for biological experiments, and I wrote the paper together with Liu Hao-Bing.

Paper VI. I fabricated the chip and performed the biological experiments. Analyzed the biological data and wrote the corresponding section with Liu Hao-Bing.

Paper VII. I performed the biological experiment and wrote the paper with Prof. Thomas Gong Hai-Qing.

Book chapter VIII. Related results from paper II and V are presented in this book chapter. I wrote the chapter with Associate Professor Thomas Gong Hai-Qing and Liu Hao-Bing.

Conference paper IX. Related results from paper II are presented in this conference paper. The chip was designed and fabricated by Chen Long-Qing. I performed the experiments related to surface characterization based on contact angle and biological validation of the PCR array chip. I wrote the conference paper.

Scientific publications, which are not included in this doctoral thesis:

- I. Fang T.H, **Naveen Ramalingam**, Xian-Dui D, Tan S.N, Xianting Z, Kuan A.T.L, Peng Huat E.Y, Gong H.Q. (2009) Real-time PCR microfluidic devices with concurrent electrochemical detection. *Biosensors and Bioelectronics*, Volume 24, Issue 7, 2131-2136.
- II. Teh H.F, Gong H.Q, **Naveen Ramalingam**, and Tan S.N (2008) A microfluidic flow-through reactor with electrochemical sensor array for real-time quantitative PCR. Invited talk, Proceedings of The Second International Symposium on Physics of Fluids. June 9-12, 2008, Nanjiang, China.
- III. Teh H.F, Gong H.Q, **Naveen Ramalingam**, and Tan S.N (2007) Electrochemical sensor array for a microfluidic flow-through real-time quantitative PCR. Proceedings of The First Shenyang International Colloquium on Microfluidics. Oct 21-24, 2007, Shengyang, China.
- IV. Kong J, Gong H.Q, Dong X.D, **Naveen Ramalingam**, NEO C.H (2007) Detection of SARS using electrochemiluminescence immunoassay chip. International Conference on Materials for Advanced Technologies 2007. 1-6 July 2007, Singapore.
- V. Dinish U.S, Seah L. K, Murukeshan V. M, Gong H. and **Naveen Ramalingam** (2006) Detection of DNA Sample Fluorescence Using Frequency Domain Imaging Technique. **IEEE**: The International Conference in Biomedical and Pharmaceutical Engineering. 11th-14th December 2006, page: 373-376 at Singapore.
- VI. Yin Tan, Haiqing Gong, **Naveen Ramalingam**, Qinghui Wang, Liquan Deng (2006) A Miniaturized Fluorescence Imaging System for Detecting Disease Genes on a Micro PCR Biochip. (MD6) International Optical Design Conference (IODC), June 4-8, 2006 at Vancouver, British Columbia, Canada.
- VII. Tan Yin, **Naveen Ramalingam**, Neo Chiew Hoon, Haiqing Gong (2005) A portable DNA detector based on a multiple polymerase chain reaction (PCR) chip. International Conference on Sensing Technology (ICST) 2005, Paper ID: ICST_168, November 21-23, 2005 at Palmerston North, New Zealand.