DEVELOPMENT OF MICRO TOTAL ANALYSIS SYSTEM FOR DETECTION OF WATER PATHOGENS

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SUMMARY

The objective of this project is to develop a micro total analysis system for water pathogen detection. This micro total analysis system will consist of a micro Polymerase Chain Reaction (μPCR) chip integrated with a continuous-flow based DNA microarray.

A silicon/glass hybrid μPCR chip had been developed. The μPCR chip was able to achieve fast heating/cooling with good temperature uniformity due to the side heating concept with etched through slot surrounding the reaction chamber for thermal isolation. The design was optimized using numerical simulation and was fabricated using Micro-Electro-Mechanical Systems (MEMs) technology. Successful amplification of fecal indicator *Escherichia coli's (E.coli)* had been demonstrated by the μPCR chip.

The silicon/glass hybrid DNA microarray was designed with a passive mixer to allow mixing of PCR amplicons and hybridization buffer. Pathogen specific capture probes for *E.coli* and *Shigella* were spotted on the DNA microarray. Continuous flow of DNA targets to the capture probes in the micro device allowed hybridization to be detected within 20 mins.

The μPCR chip and the DNA microarray were integrated by packaging the two chips on an acrylic housing. The pathogen sample has been successfully detected in our micro total analysis system through DNA amplification by the μPCR chip follow by direct transfer of the amplicons to the DNA microarray for detection within 3 hours.

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CHAPTER 1 INTRODUCTION

The microbiological quality of drinking water is a concern to consumers, water suppliers, regulators and public health authorities alike. The potential of drinking water to transport microbial pathogens to great number of people, causing subsequent illness, is well documented in countries at all levels of economic development. Waterborne pathogens continue to contaminate drinking water supplies and cause waterborne disease outbreaks despite current regulations that are designed to prevent and control their spread. Annually, it is estimated that pathogen infected drinking water results in about a million new cases of illness and about a thousand deaths [1].

In general, waterborne pathogens are disease-causing organism that live in water, and can be classified as bacteria, viruses, protozoa, or algae. There are hundreds of different pathogens that can be transmitted through exposure to contaminated water. Many of these pathogens are enteric in nature, meaning that their primary site of infection is the intestines. Exposure to enteric pathogens is typically through consumption of food or water that contains the pathogens. These pathogens can enter drinking water supplies or water resources through fecal contaminations (enteric pathogens) while some are indigenous to natural aquatic environments. They are environmentally stable, infectious in notably lower doses, and resistant to many conventional methods used to control bacterial pathogens.

Existing methods (EPA method 9131 and 9132) [2] that are used to assess the microbial water quality is based on culture based approaches which requires more than a day. This result in a long delay in obtaining results thereby causing a time lag between the

occurrence of the contamination event and its detection to be able to safe guard the consumers' health. Therefore, there is a demand for a faster analytical method for the above purpose.

1.1 Objective

The main objective of this project was to develop a micro total analysis system as a faster analytical method for the detection of water pathogen as compared to the classical method that uses cultivation. This micro total analysis system would be based on molecular techniques which consist of a μPCR chip integrated with a continuous flow DNA microarray. The expected total analysis time was targeted to be within 3 hours.

1.2 Scope

In this project, a micro total analysis system was developed. This report begins with a literature survey on microbial safety of water and molecular techniques for detection of water pathogen. This is followed by a chapter on design and numerical analysis of μPCR chip and DNA microarray. The experimental procedures, results and discussion of μPCR chip, DNA microarray and the integration of both chips to form a micro total analysis system will be covered in the next three chapters. Conclusions and recommendations are touched in the last chapter.

CHAPTER 2 LITERATURE REVIEW

2.1 Microbiological Safety of Water

Water is essential to sustain life, and a satisfactory (adequate, safe and accessible) supply must be available to all. One of the most important attributes of good quality water is to be free of disease-causing organisms-pathogenic bacteria, viruses, protozoa, or parasitic worms (microbiological quality). Water contaminated with sewage may contain such organisms because they can be excreted in the faeces of infected individuals. If contaminated water is consumed by others before it is properly treated, the cycle of disease can continue in epidemic proportions. However, it is difficult and time consuming to test for the presence of individual pathogens such as Salmonella typhose bacterium which causes typhoid fever in water. The concentrations of these organisms in a contaminated water sample may be small enough to elude detection, making it necessary to test large volumes of water. Further it would be necessary to test for a wide variety of different organism before the water could be considered safe. A more practical and reliable approach than testing for individual pathogens is to test for a single species that would signal the possible presence of sewage contaminations. If sewage is present in the water, it can be assumed that the water may also contain pathogenic organisms and is a threat to public health.

2.1.1 Indicator Organisms

The measured microbiological water quality is to monitor for indicator organisms. They are not harmful to health but their presence indicates that other fecal organisms (including harmful pathogens) may also be present in water. Members of the coliform

group of bacteria are used as indicators of water quality. This group contains many species of bacteria that grow in the environment, but a sub-group of coliform bacteria, called thermotolerant coliforms (coliforms preferring warmer temperatures), are found predominantly in the intestine and faeces of humans and other warm-blooded animals. One member of the thermotolerant coliform group, *Escherichia coli* (often referred to as *E. coli)* is recognized as the most specific indicator of recent fecal contamination in water supplies. This organism is now the preferred indicator for assessing the microbiological quality and safety of drinking water [1].

2.1.2 Testing for Coliform

According to EPA regulations, a system that operates at least 60 days per year, and serves 25 people or more or has 15 or more service connections, is regulated as a public water system under the Safe Drinking Water Act [2]. Under the Safe Drinking Water Act, EPA requires public water systems to monitor for coliform bacteria first because this test produces faster results. When a sample is tested positive, the same sample must be analyzed for fecal coliform or *E. coli*. which are both indicators of contamination with animal waste or human sewage.

Two EPA approved methods that are used for detecting and measuring coliforms in water are multiple tube fermentation method (Method 9131) and membrane filter method (Method 9132).

2.1.2.1 Multiple Tube Fermentation Method [3]

This technique is based on the fact that coliform organism can use lactose, the sugar occurring in milk as food and produce gas in the process. A measured volume of water sample is added to a tube that contains lactose broth nutrient medium. A small inverted vial in the lactose broth traps some of the gas that is produced as the coliform bacteria grow and reproduce. The gas bubble in the inverted vial along with a cloudy appearance of the broth provides visual evidence that coliforms may be present in the sample. But if gas is not produced within 48 hrs of incubation at 35 ºC, it can be concluded that coliforms were not present in the sample volume injected into the broth

The failure of gas formation after incubation is called a negative test. The appearance of gas and the accompanying cloudiness in the broth is called a positive presumptive test. As some bacteria other than coliforms occasionally produce gas in lactose, it is usually necessary to perform another test (confirmed test) to prove that it was the coliform bacteria that produced the gas in the positive presumptive tube.

The confirmed test involves transferring the nutrient medium from a positive presumptive tube to another fermentation tube that contains a different nutrient medium, called brilliant green bile. If the gas is again formed within 48 hrs of incubation at 35 ºC, the presence of coliforms is confirmed. In some cases, a third procedure called the complete test may have to be performed. The fermentation tube procedure can be used to test for fecal coliforms as well as total coliforms, but a higher temperature of 44.5 ºC is used for fecal indicators.

2.1.2.2 Membrane Filter Method [4]

In this procedure, a measured volume of sample is drawn through a special membrane filter by applying a partial vacuum. The filter, a flat, paper-like disk about the size of a silver dollar, has uniform microscopic pores small enough to retain the bacteria on its surface while allowing the water to pass through.

After the sample is drawn through, the filter is placed in a sterile container called a Petri dish which also contains a special culture medium that the bacteria use as a food source. This nutrient medium is usually available in small glass containers called ampules, from which is readily transferred into the Petri dish. Its composition is such that it promotes the growth of coliforms while inhibiting the growth of other bacteria caught on the filter.

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The Petri dish holding the filter and nutrient medium is usually placed in an incubator at 35 ºC for 24 hrs which appear as specks or dots, with a characteristic metallic sheen.

The coliform concentration is obtained by counting the number of colonies on the filter. A basic premise for the membrane filter test is that each colony started growing from one organism. From this it can be assumed that each colony counted represents only 1 coliform in the original sample.

Coliforms concentration is expressed in terms of the number of organism per 100ml of water. The basic procedures described here for the membrane filter test can be applied to tests for total coliforms or fecal coliforms, but different nutrient media are used and the fecal indicator test is conducted at 44.5 ºC or 35 ºC.

2.2 Molecular Method for Detection of Water Pathogen

Traditional methods of pathogen detection and identification include microbiological culturing techniques, where the pathogen is identified based on biochemical characteristics and immunological techniques to detect specific antigens of the pathogen [5]. However, these detection methods are very time consuming, as some microorganisms are difficult to culture and grow slowly. As well, immunological methods can result in false-positive results because of cross-reactivity of antibodies. In addition, routinely used biochemical and immunological tests do not provide information about the potential pathogenicity or virulence of identified microorganisms.

Molecular detection technologies offer several potential advantages over conventional microbiological techniques. Several nucleic acid-based methods have been developed for the rapid detection of pathogens in food, soil, and water with high degrees of sensitivity and specificity and without the need for complex cultivation [6]. In general, these methods allow detection within hours rather than days as is normally required by culture techniques.

Among the molecular detection technologies, PCR is the most commonly employed as it is highly sensitive and specific [7]. It has the ability to detect multiple pathogens in a single analysis, to make highly specific identifications, and to detect very low numbers of target organisms in a short time [8]. However a major limitation to this approach is the utilization of one specific primer pair per gene detection reaction. Although multiple primer sets may be successfully combined in one reaction, they rarely exceed more than six primer sets due to the generation of non-specific products or false negatives [208].

Another difficulty with multiplex PCR is that it requires additional post amplification analysis to discriminate the products. Size separation by electrophoresis is frequently used to discriminate multiplex PCR products, but this requires additional labour and that the amplicons of each reaction be significantly different in size, which can limit the primer pairs that can potentially be multiplexed. Consequently, general pathogen detection by PCR can be both labour-intensive and costly [208].

DNA microarray represents an important advance in molecular detection technology. It allows simultaneous detection of labeled DNAs from many different pathogenic organisms on a small glass slide containing thousands of surface-immobilized DNA probes. Both basic types of microarrays, i.e., immobilized oligonucleotides and PCR amplicons probes, have been used to successfully detect [188] and/or characterize [189] pathogens. As the sensitivity of microarrays hybridized with total genomic DNA from complex mixtures is usually inadequate to provide detection of low pathogen concentrations [190], the hybridized DNA (target) usually consists of PCR amplicons [191]. This mode of pathogen detection necessitates the combination of PCR prior to their hybridization on DNA microarrays.

2.2.1 PCR [11,12]

PCR is a biochemistry and molecular biology technique [13] for exponentially amplifying a fragment or sequence of interest of DNA, via enzymatic replication, without using a living organism (such as *E. coli* or yeast). PCR is a three step amplification process first introduced by Saiki and co workers in 1985 (Figure 2.1). These in vitro enzymes mediated method facilities the generation of nucleic acid sequence based on choice of

specific primers. During the first step (denaturation), the hydrogen bonding stabilizing the double strand DNA template is broken to form two complementary single strands. In order to provide the energy necessary to break the bonding, this step is commonly performed at temperatures between 94 ºC and 96 ºC. The temperature is then lowered for the annealing steps where primers specifically bind to the complementary sequences of the DNA template. Then the temperature is raised to allow extension where the template is typically replicated by a thermostable DNA polymerase at a temperature close to 72 ºC. The denaturation-annealing-extension cycle is repeated between 25 to 40 times.

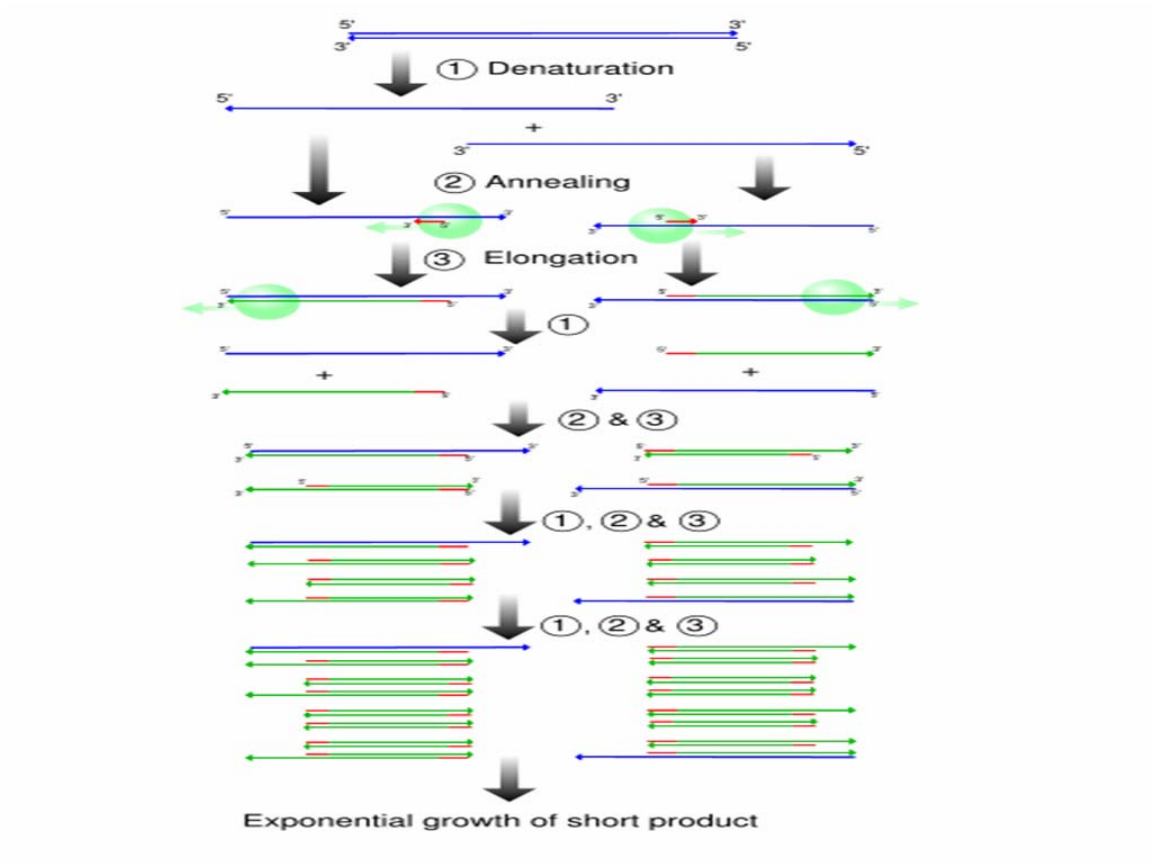


Figure 2.1: Schematic drawing of the PCR cycle. (1) Denaturing at 94-96°C. (2) Annealing at ~55°C (3) Elongation at 72°C. Four cycles are shown here [11].

Besides the basic PCR method described above, some other PCR variations for different applications are described briefly in Table 2.1.

Table 2.1: Variations of PCR [11, 12]

PCR type	Description
Allele-specific (AS) PCR	To determine the genotype of single nucleotide polymorphisms (SNP) by using primers whose ends overlap the SNP and differ by that single base
Assembly PCR	An artificial synthesis of long gene products by performing PCR on a pool of long oligonucleotides with short overlapping segments to selectively produce their final product
Asymmetric PCR	To preferentially amplify one strand of the original DNA more than the other
Colony PCR	To rapidly screen bacterial clones for correct DNA vector constructs
Helicase dependent amplification	Similar to traditional PCR but maintains a constant temperature rather than cycling
Hot start PCR	A technique that reduces non-specific amplification during the initial set up stages of the PCR
Intersequence specific (ISSR) PCR	A PCR method for DNA fingerprinting that amplifies regions between some simple sequence repeats to produce a unique fingerprint of amplified fragment lengths
Inverse PCR	A method used to allow PCR when only one internal sequence is known. This is especially useful in identifying flanking sequences to various genomic inserts.
Ligation-mediated PCR	Use to detect vector insertion sites into a genome

Multiplex Ligation-dependent Probe Amplification (MLPA)	This method permits multiple targets to be amplified with only a single primer pair, thus avoiding the resolution limitations of multiplex PCR
Multiplex-PCR	The use of multiple, unique primer sets within a single PCR reaction to produce amplicons of varying sizes specific to different DNA sequences
Nested PCR	This method increases the specificity of DNA amplification, by reducing background due to non-specific amplification of DNA.
Quantitative PCR	Use to measure the quantity of a PCR product (preferably real-time). It is the method of choice to quantitatively measure starting amounts of DNA, cDNA or RNA. Q-PCR is commonly used to determine whether a DNA sequence is present in a sample and the number of its copies in the sample.
RT-PCR	This is a method used to amplify, isolate or identify a known sequence from a cellular or tissue RNA
TAIL-PCR - thermal asymmetric interlaced PCR	This is used to isolate unknown sequence flanking a known sequence
Touchdown PCR	This is a variant of PCR that aims to reduce nonspecific background by gradually lowering the annealing temperature as PCR cycling progresses.
Strand displacement amplification (SDA)	Isothermal amplification reaction
Nucleic acid sequence –based amplification amplification (NASBA)	
Rolling circle amplification (RCA) and the Qβ replicase reaction	

2.2.1.1 µPCR Chip

MEMS are the integration of mechanical elements, sensors, actuators, and electronics on a common silicon substrate through microfabrication technology. These miniaturized devices are being developed in the semiconductor industry and the characteristic dimensions of those small structures are on the order of 1-1000 µm. These devices will represent a central technology in many systems used for biological, chemical and medical applications, whose advances promise to revolutionize many process of detection of pathogens or environmental pollutants [14, 15]. PCR devices are one of the many devices that have been manufactured in MEMs technology.

The traditional PCR machines are slow in PCR speed as these peltier effect or metal block based PCR system are characterised by high thermal mass, large reaction volume and thus slow heating/cooling rates. With advent of micro-electro-mechanical system (MEMS) technology, the development of µPCR chip becomes possible [10, 13] and this can help to improve PCR speed by increasing the heat transfer rate or decreasing the thermal mass. With miniaturizaton, its applications in the chosen fields will lead to many improvements such as decreased cost of fabrication and use, reduced reaction time, reduced consumption of reagent and increased potential of portability and integration of PCR device [16, 17, 18, 19, 20].

2.2.1.1.1 µPCR Chip Substrates

Currently, the three most popular materials for µPCR fabrication are silicon [21-25], glass [26-30] and plastic. Silicon is an excellent material for a thermal cycler chamber. It

has a high thermal conductivity and once it is thermally isolated, the chamber has good thermal uniformity [49-52]. Micromachining for silicon is also well established. However the drawback is that the silicon surface itself inhibits the PCR and its surface has to be covered with another material, such as silicon dioxide, SiO_2.

On the other hand, μPCR chip fabricated from glass has a thermal conductivity more than a hundred times lower than that of silicon. Due to its low thermal conductance, the systems made of glass are thermally isolated. But at the same time, it will also take longer to heat up and cool down as compared to silicon device. Moreover creating a device by glass machining is rather difficult as compared to either silicon or plastic processing [106].

The third material commonly used for PCR is plastic (such as polycarbonate [36-40] and polydimethylsiloxane (PDMS) [31-35]). All those materials have a cost advantage over both silicon as well as glass and they are simple to process. Polycarbonate can be shaped by a hot embossing technique, while PDMS polymerizes in a mold. The common drawback is the low thermal conductivity of the plastic.

Although no single substrate material can offer a preferable solution to the restrictions such as cost, ease of fabrication, biocompatibility, optical transparence, many researchers have taken full advantage of the respective properties of silicon, glass or plastic and have investigated μPCR chip based on hybrid substrate materials, for instance silicon/glass hybrids [70-72], plastic/silicon [73] and plastic/glass [74, 75]. With the presence of a wide range of substrates to choose from, the suitability of each substrate for μPCR chip will depend on the applications and technologies available.

2.2.1.1.2 Surface Treatment

The PCR efficiency is often limited by interactions between the chip surface and the biomolecules in the PCR solution, primarily due to the increase of the surface-to-volume ratio in a micro-scale environment. In general, the hydrophilic PCR solution is not easily introduced into the hydrophobic chip [146]. Therefore, a proper surface treatment is required to ensure the success of on-chip PCR. The treatment processes can be classified as static treatment and dynamic treatment.

In static surface passivation of this type, the inner surface of μPCR chip is pre-coated by using a PCR-friendly substance during the fabrication of PCR chip or before starting the PCR chemistry. In most silicon/glass hybrid PCR chip, a thin layer of silicon oxide surface coating is deposited to enhance PCR compatibility. Sometimes, this type of surface coating technique can also be used to deposit the inner surfaces of plastic substrates for PCR chip. An obvious advantage of the silicon oxide layer [85, 147, 148] method is that the passivation process is accomplished during chip fabrication and the subsequent sealing of the chips with glass wafer by an anodic bonding technique is not being intervened with. Furthermore, deposition of oxide surfaces is a standard industry procedure that is reproducible and inexpensive and can be accomplished in a batch production setting.

Another commonly used static passivation procedure is chemical silanization of inner surfaces which is performed by filling the reaction chamber/channel with a silanizing agent and incubating the filled chip for a period of time. This is followed by removal of the excess silanizing agent [108, 152,153, 154] and then the silanized chip is dried and washed. However, silanization is a time-consuming and complex labor-intensive process,

and the chips need to be stored in liquids to protect the silane film from damage, which could be a serious problem for practical applications.

The second type of passivation is dynamic passivation. This passivation procedure occurs during the practical operation of PCR chip and is realized by adding the passivation agent to PCR solution. For this passivation technique, the most frequently used passivation agents include a competing protein adjuvant-bovine serum albumin (BSA) [85, 131, 151], polymer solutions such as polyethylene glycol (PEG) [150, 151] and polyvinylpyrrolidone (PVP) [88], and the nonionic surfactant Tween 20 [148]. BSA is often included into the PCR solution to stabilize the polymerase enzymes and to reduce undesired adsorption of polymerase onto the inner surfaces of reaction chamber. PEGs with different molecular weights (e.g. PEG 400, PEG 1000, PEG 8000, etc.) had been included into the PCR solution respectively and the effect of their addition on the PCR had been tested. The best results were achieved by addition of PEG 8000 at a 0.75% (w/v) concentration. With respect to the PVP, the addition of only PVP may not have a significant effect on PCR in the μPCR chip, regardless of its concentration. It may need to be utilized in combination with some other passivation techniques. The PCR buffer is completed by using Tween 20 as an additive which is found to be effective for PCR on chip. Tween 20, which acts on the relaxation of the surface tension of solutions and is often utilized in fields of protein and nucleic acid handling, serves as a dispersant, emulsifier and solubilizer in protecting the enzyme.

2.2.1.1.3 Architectures for µPCR Chips

µPCR chips can offer an opportunity for automation of PCR amplification, shorter processing time, higher sample throughput and minimum human/world to PCR intervention and contamination. Two basic approaches have been developed comprising mainly of stationary µPCR chip and flow-through µPCR chip.

2.2.1.1.3.1 Stationary µPCR Chip

The stationary µPCR chips work in the same manner as conventional PCR thermal cycler, where the PCR solution is kept stationary and the temperature of the PCR reaction chamber is cycled between three different temperatures. After completion of PCR, the amplification products are recovered from the chamber for detection. The stationary chamber PCR chip can perform very well in terms of fluidical and thermal control, and present beneficial properties such as reduction of thermal and fluidic cross-talk between PCR reaction microchambers. [77-81].

2.2.1.1.3.2 Flow-Through µPCR Chip

The flow-through systems typically have zones at three constant temperatures. The sample will be moved between zones of different temperatures to go through different stages of PCR. This type of PCR system is faster than stationary µPCR chip but it requires an implementation of a mechanism to move the sample around during the reaction. In addition, this approach lacks the flexibility in control as the cycle number is fixed as it is dictated by the channel layout [106,199].

2.2.1.1.4 Heating Methods

μPCR devices can be also categorized based on the heating system, which is either direct or indirect/non contact. Direct heating PCR chips have heaters as well as the temperature sensors integrated with the device. A disadvantage associated with the contact heating is that a certain amount of thermal mass is added in the PCR chip assembly, which inevitably hinders fast thermal transitions. Moreover, when PCR and analytical function are integrated on a single chip, it is very difficult to confine the contact heating to the PCR chip itself and not analysis part of the chip [106].

In order to overcome the issues associated with direct heating, interest in indirect/non-contact heating continues to grow [104]. Recently, the use of non contact infrared red (IR)-mediated heating [105] and the use of an alternating-electric-current induced buffer Joule heating effect without an external heater component [106] have been implemented. The disadvantage of non contact heating approaches is the low heating rate and cooling rate.

2.2.1.1.5 Temperature Measurements

In μPCR chip, it is very important to select methods for temperature measurement to accurately control temperature during temperature cycling. Presently, the temperature measurement methods are usually divided into two categories which are contact and non-contact temperature measurement. The contact temperature measurement methods include thin-film-type temperature sensing and non-thin-film-type temperature sensing.

The thin film temperature sensors comprise platinum [107-110], aluminum [111-113], ITO [114, 115], polysilicon [116], and even copper temperature sensors [117]. The thin

film temperature sensors are usually made from some metallic, nonmetallic or oxide materials by using thin film deposition techniques, which can provide the μPCR chips with a higher degree of integration, small footprint and good biocompatibility.

The non-thin-film temperature sensors generally include thermocouples [118-121], Pt100 electrical-resistance thermometers [122-124], semiconductor electrical-resistance thermometers (thermistors) [125, 126], and diode thermometers [127, 128]. The utilization of non-thin-film temperature sensors to measure the temperatures may lead to adverse problems such as biocompatibility and/or integration. However, they are still widely used in μPCR chip because of their lower cost and convenience.

But whatever the contact temperature sensors' nature, they will add their own thermal mass to the PCR system, which ultimately adversely affect the thermal cycling performance of μPCR chips. Additionally, the contact temperature measurement techniques can yield temperature data only at a few discrete points or lines and only indirectly reflect the temperature of the PCR solution, and so the precision and accuracy of temperature measurement is limited. Although direct contact between the temperature sensors and the PCR solution may lead to a more accurate temperature measurement, the presence of the sensor may cause side effects on the PCR and increase the risk for sample cross-contamination and can inhibit the PCR by inactivating the Taq polymerase through irreversible adsorption [129].

In order to overcome these problems, some researchers have made attempts to develop non-contact temperature measurement techniques for μPCR chips, such as infrared red (IR) thermometry [130, 131]. The advantages of this type of temperature measurement

technique include rapid response, continuous temperature readings, higher spatial resolution, and no interference with the object observed. However, IR thermometry has also disadvantages such as a precision lower than that of contact measurements. Also, only information about the two-dimensional surface temperature of the IR-absorbing substrate is obtained, which can be easily affected by the intermediate medium such as vapor and carbon dioxide.

2.2.1.1.6 Temperature Control

PCR is a typical temperature-controlled reaction system, so temperature control of the PCR solution is a key issue. Within current μPCR chips, the most commonly used temperature control algorithm is accomplished through a proportional-integral-derivative (PID) module (within a certain software program) [86, 107, 132, 134]. PID control is one of the earliest developed control strategies, which has been widely used for the control of industrial processes. However, in order to meet the requirements of temperature control for μPCR chips, some researchers have to make some modifications to the PID control strategy and adopt alternative temperature control algorithms such as proportional control [129], PI control [109, 128] , PD control [142], and PD–PID control [116].

2.2.2 DNA Microarray

DNA microarray technology has been widely developed in many platforms since its introduction. In the microarray platform, complementary probes (either PCR products cDNA or oligonucleotides) are immobilized in a patterned array on a solid support to facilitate simultaneous hybridization of corresponding DNA/RNA targets. When the

targets hybridize to their complementary probes, they are detected using some types of reporter molecules to allow for rapid and high throughput analysis.

The cDNA and oligonucleotide probes can be deposited on any type of substrates, with modified glass being the most common followed by filter membranes and silicon surface [158-160]. There are a myriad of approaches to modify slides and to attach probes to the slide surfaces. Commonly used surface coatings for the attachment of nucleic acids to slide surfaces include aldehyde, silane, poly-L-lysine, polyacrylamide and various electrophilic chemicals with different functional groups [161-163].

The oligonucleotide or cDNA probes are immobilized generally through two formats. In the first format, oligonucleotide are synthesized directly onto a slide using the same solid-phase chemistry as used in conventional DNA systhesis through photolithography techniques [164] or ink jet printing technology [165,166]. The second format is to directly spot pre-systhesized oligonucleotide probes or single stranded cDNA onto microarray substrate through covalent or non covalent attachments to surfaces [167] usually through the use of contact or non contact robotic spotting arrayer system.

Once DNA microarrays have been printed, targets are prepared for hybridization. Depending on the objective, targets may be PCR products, genomic DNA, total RNA, cDNA, plasmid DNA, or oligonucleotides. In most cases, the targets incorporate either a fluorescent label (e.g. Cy-3) or some other moiety such as biotin that permits subsequent detection with a secondary label. Once post-hybridization steps are completed, the arrays are imaged using a high-resolution scanner. These are laser- or filter-based systems that

use specific light spectra to excite fluorescent molecules and collect the subsequent emission spectra using CCD cameras.

In general, the most mature applications of this technology have been in comparative genomics, single nucleotide polymorphism (SNP) assays, and gene expression [163]. It also has clear application as a multiplexed format for bacterial identification and clinical diagnostics. Among possible diagnostic targets, the gene encoding the 16S rRNA offers the most comprehensive database of sequences of both cultured and uncultured microbiota, and has received increasing attention as a target for probes immobilized on DNA microarrays [168-172].

The challenge of microbial diagnostics places additional demands on accurate interpretation of hybridization results, which often requires discriminating by a single nucleotide base-pair [170,172]. Although the influence of an unknown mismatch composition on duplex stability cannot be generally predicted, the specificity of an individual probe can be improved by optimizing critical hybridization parameters (i.e., temperature, ionic strength, and concentration of denaturant).

The other serious limitation on the reaction of biomolecules is the slow diffusion kinetics. [173]. DNA microarray hybridization is typically performed overnight to ensure the reactions run to completion. Accelerating the reaction by using flow through 3D micro channels [174], active mixing using pneumatically powered pumps [175], passive mixing using sample oscillations [176] or chaotic advection [177], low density array [178],

reduction of DNA microarray channel height [179] were some of the strategies adopted which greatly helped to reduce the hybridization time.

2.3 Integrated µPCR Chip with DNA Microarray

To take advantages of the superiority of µPCR chip and DNA microarrays, integrated microfluidic devices have been investigated. While there are many works directed at PCR chip and DNA microarrays separately, only a few µPCR chip and DNA microarray combined systems have been described. However none of these studies on integration of µPCR chip and DNA microarray have shown applications in water pathogen detection.

One of the most relevant and cited work is from HKUST [183] which demonstrates the integration of DNA microarray into PCR micro reactor. Their group had developed a novel micro-DNA amplification and analysis device consisting of multiple PCR microreactors with integrated DNA microarrays on a single silicon chip. In their device, there were four PCR microreactors with different samples of 3 µl internal volume allow to perform parallel analysis of DNA sample, and furthermore the oligonucleotide probes are printed on the bottom wall of each microreactor so that no buffer exchange or sample transfer is needed, thus leading to reduction of assay time and of contamination risk. The static hybridization following PCR will take at least 3 hours to run to completion.

CHAPTER 3 DESIGN AND NUMERICAL SIMULATION

3.1 µPCR Chip

3.1.1 Design of µPCR Chip

Several aspects and parameters of the µPCR Chip function and operation were defined and considered during the design stage. Parameters such as reagent volumes, analysis time, temperature efficiency, ease of control and biocompatibility of material were also considered. The µPCR chip designed in this study was a silicon/glass hybrid single chamber microchip (Figure 3.1). Silicon was used for its good thermal performance while glass was transparent and allowed viewing of the sample in the chamber. The chip consisted of a serpentine-like chamber etched on silicon for easy flow of sample. The chamber was supported by silicon beams and thermally isolated from the surrounding substrate by air gaps to reduce thermal cross talk. Aluminium was integrated on chip as side heaters and sensors to provide fast and accurate heat and control. The chip was packaged on a thermal conductive printed circuit board (PCB) for electrical connections for power and feedback. An acrylic housing was used for sample delivery through its embedded channels connected to the inlet and outlet of the chip. The volume of the µPCR chip was 10 µl sufficient for reactions downstream (e.g. detection) and measured at 25 mm by 11.6 mm. The µPCR chip's detail dimensions and mask design are included in Appendix A.

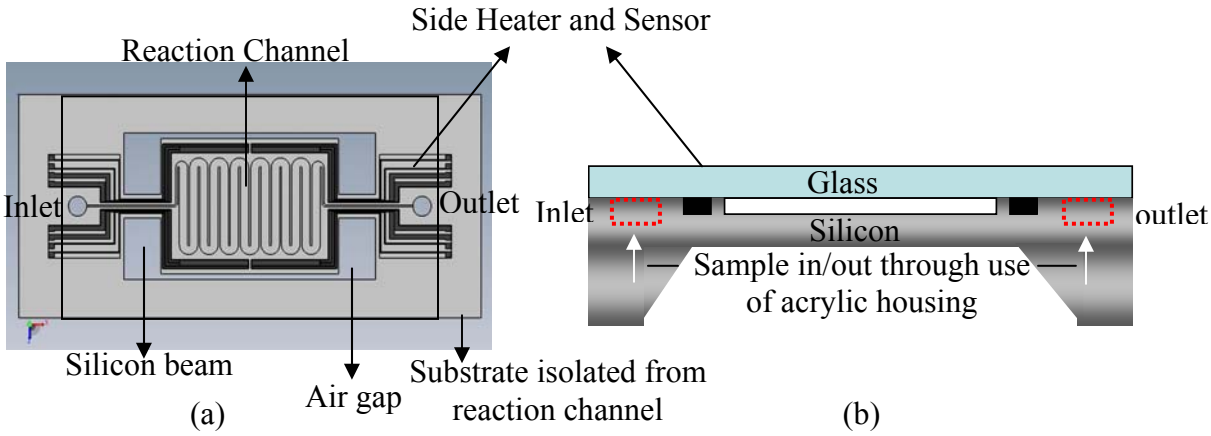


Figure 3.1: Schematic of μPCR chip. (a) Top view; (b) Side view

3.1.2 Aluminium Heater and Sensor Design

Heater and temperature sensors were carefully designed at proper locations. Heaters were located around the joints of the reaction chamber and the silicon beams for good heat distribution. The relationship between the locations and the achieved temperature uniformity had been obtained from numerical simulations in section 3.4. Multiple sensors were placed on the area of interest for monitoring the temperature distribution around the reaction chamber.

Metal (aluminium) was used for both heater and sensor because of the simple process and the high temperature coefficient of resistivity (TCR). The high TCR makes metal a good resistance temperature detector (RTD) that converts changes in voltage signals to temperature by the measurement of resistance. As aluminium was used as heating material, line width and thickness of the heater needed to be designed in such a way that the maximum current density was less than the critical limit where electromigration occurs (e.g. $1x10^6$ A/cm^2). Electromigration is the transport of material caused by the gradual movement of the ions in a conductor due to the momentum transfer between

conducting electrons. These diffusing metal atoms caused by electromigration must be avoided as it will damage and ultimately results in failure of the affected circuitries or interconnects.

3.1.3 Printed Circuit Board (PCB) Design

PCB (Figure 3.2) was made of thermal conductive material 96% Alumina with 0.3 µm thick gold being printed as circuitry and bond pads. Pitch size was 3.96 mm with 1.54 mm thickness based on vendor's fabrication requirements. The central area of the PCB was removed to reduce contact with silicon chip for thermal isolation as PCB itself was thermal conductive. The µPCR chip was bonded to PCB using a heat conductive epoxy which required 30 mins to cure at 150 °C before wire bonding. The purpose of PCB was to provide electrical connections between µPCR chip and external power supply and data acquisition units through wire bonding. The dimensions of PCB were 39mm by 34 mm. Material properties and detailed dimensions of PCB are provided in Appendix B.

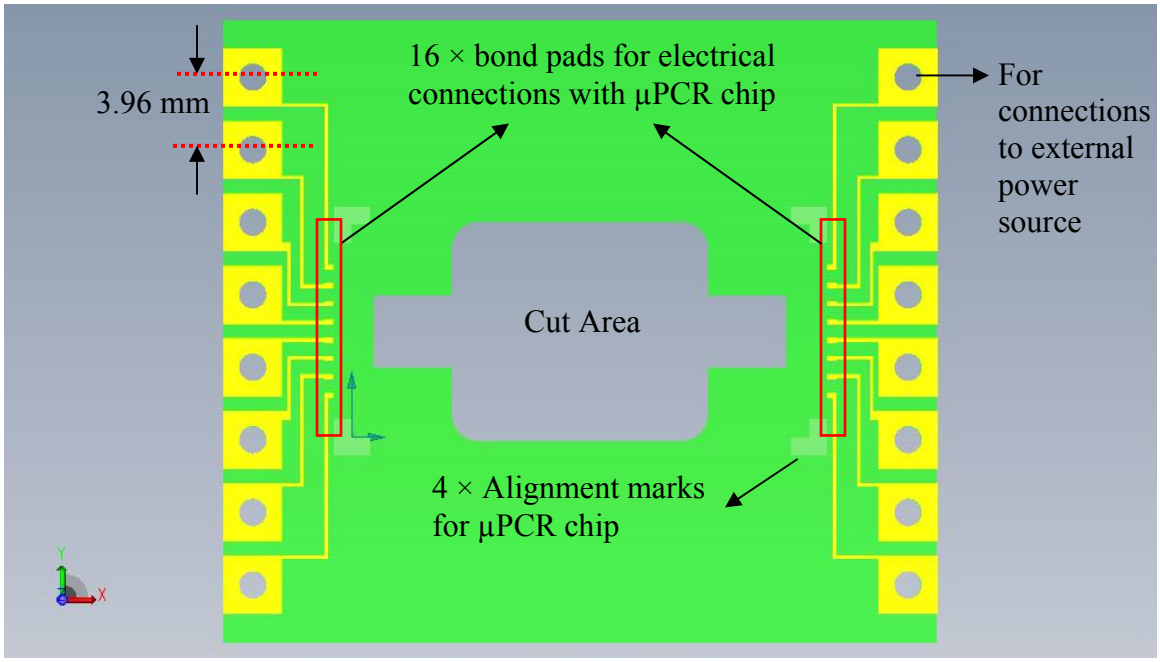


Figure 3.2: Top view of PCB

3.1.4 Acrylic Housing for µPCR Chip

µPCR chip was housed in a custom made acrylic housing (Figure 3.3) with embedded channels to facilitate transfer of PCR mixture in and out of the µPCR chip. "O"-rings were used as an interface between the connection point of chip and acrylic housing to prevent leakage. The dimensions of the housing were 50 mm by 26.6 mm with depth of 10 mm. Drawings of acrylic housing are attached in Appendix C.

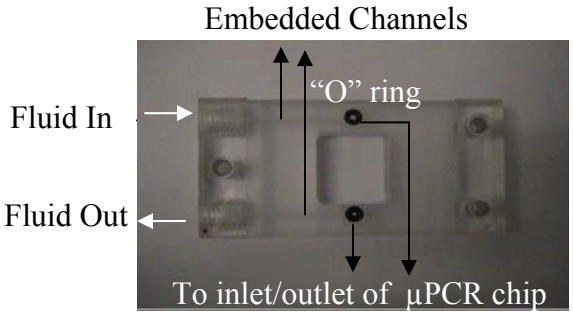


Figure 3.3: Acrylic housing for µPCR chip

3.2 DNA Microarray

3.2.1 Design of DNA Microarray

The DNA microarray design was based upon the concept of a continuous flow device where oligonucleotide/PCR amplicons and hybridization buffer would be continuously transfer to the DNA capture probes for hybridization. The DNA microarray consisted of a serpentine channel on which the DNA capture probes were spotted. It also consisted of a passive mixer incorporated into the device (Figure 3.4) to allow efficient mixing of hybridization buffer and oligonucleotide/PCR amplicons from µPCR chip. Detailed dimensions of the DNA microarray are attached in Appendix D.

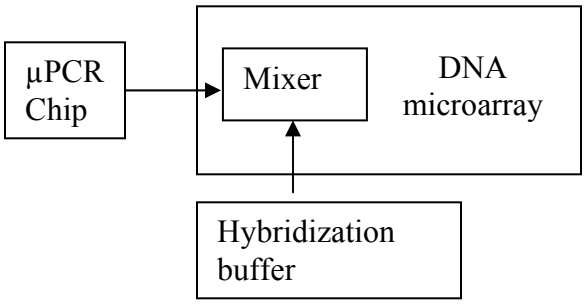


Figure 3.4: Design concept of DNA microarray

3.2.2 Acrylic Housing for DNA Microarray

The DNA microarray was housed in an acrylic housing (Figure 3.5) with embedded channels to allow transfer of PCR amplicons/oligonucleotides and hybridization buffer to the microarray. Similarly, "O" rings were used between the chip and housing at connections points for sealing. The dimensions of DNA microarray acrylic housing were 40 mm by 40 mm by 10 mm with drawings attached in Appendix E.

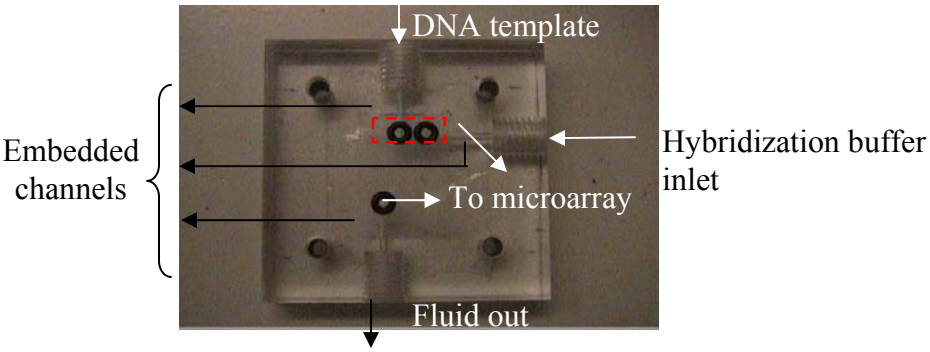


Figure 3.5: Acrylic housing for DNA microarray

3.3 Design of Micro Total Analysis System

The micro total analysis system was made up of a µPCR chip packaged with the DNA microarray using an acrylic housing (Figure 3.6). The transfer of fluid from one device to the other was done through the embedded channels in the acrylic housing. "O" rings were

used as described previously to prevent leakage. The device measured at 80 mm by 26.6 by 10 mm with detailed drawings provided in Appendix F.

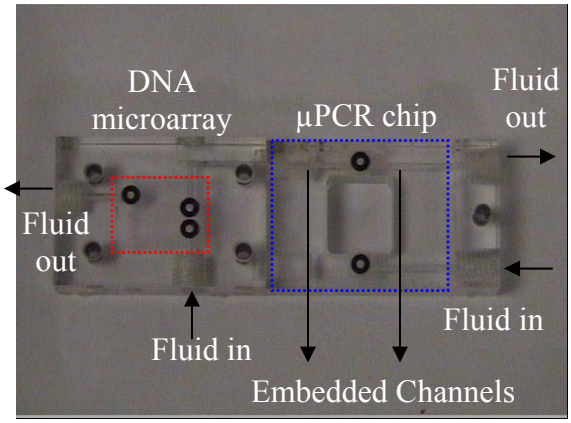


Figure 3.6: Acrylic housing for micro total analysis system

3.4 Numerical Simulation of µPCR Chip

3.4.1 Thermal Analysis of µPCR Chip

Finite element analysis (FEA) was used to optimize the thermal model, using ANSYS (Version 8.0). Figure 3.7a shows the parameters used in the simulations. Parameters studied included thermal mass, chamber geometry, heater position/configuration and air gap's size. A quarter three dimensional thermal model (Figure 3.7c) was used to study the parameters as described above and steady state (to predict the temperature distribution) and transient state analysis(to predict the thermal response speed of model) were utilized to optimize the parameters.. A quarter model was used due to symmetry to shorten analysis time. The thermal model included silicon chip (reaction chambers, beams, substrate), glass covering plate, sample filled in chamber (water) and PCB (Figure 3.7b). The material properties of the device are shown in Table 3.1.Thermal SOLID 90 element in ANSYS was selected for precise modeling. A steady state numerical analysis of a two

dimensional thermal model (Figure 3.7d) was also used to compare the thermal performance between bottom heater and side heater.

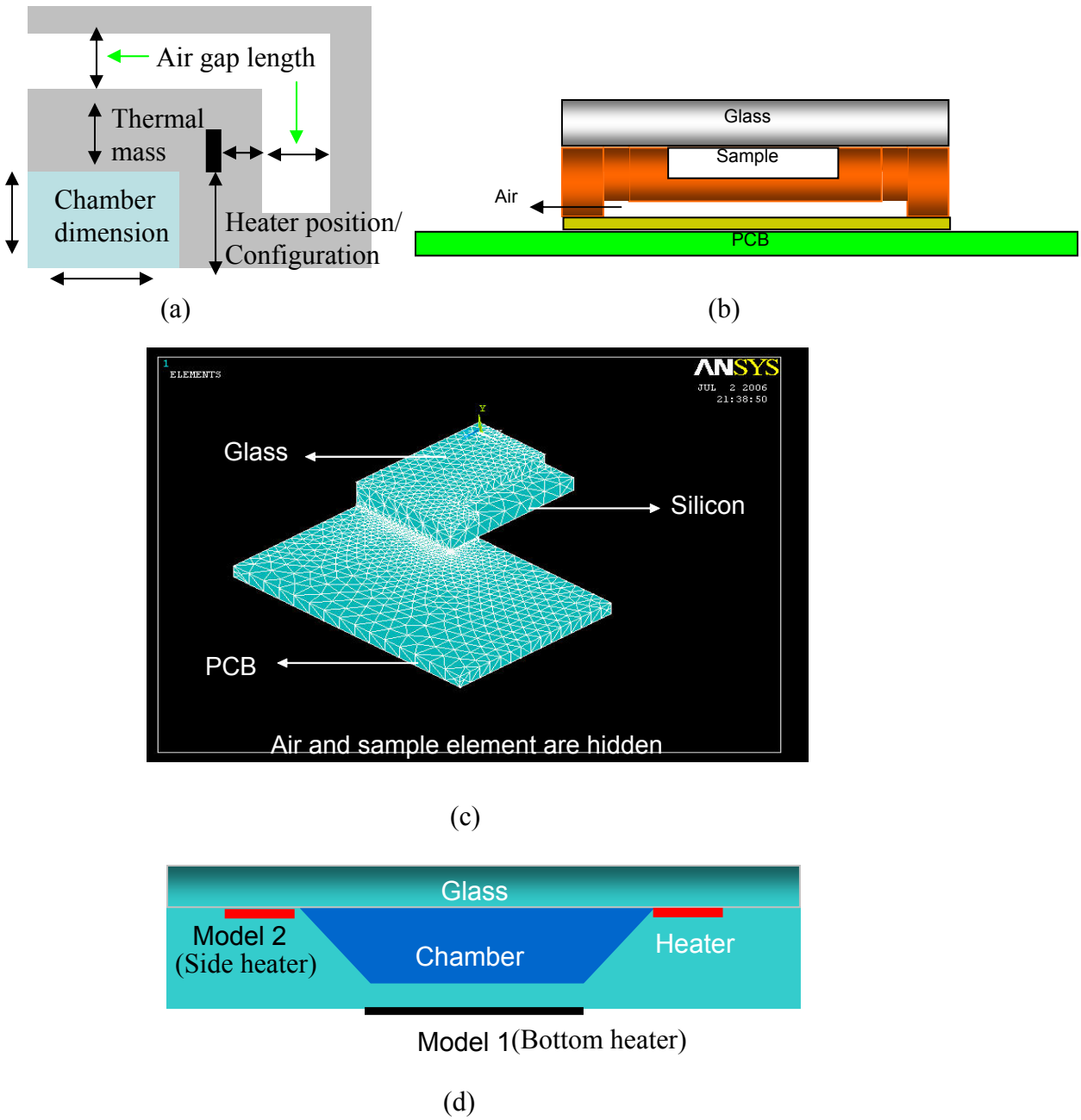


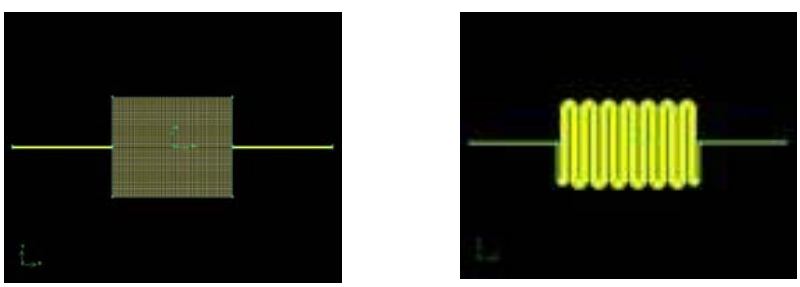
Figure 3.7: Thermal models for numerical analysis. (a) Quarter model of µPCR chip showing various parameters; (b) Side View of thermal model; (c) 3-D thermal model (Quarter model); (d) 2 D model to compare between bottom heater(model 1) and side heater(model 2)

Table 3.1: Material properties for numerical analysis

Material	Thermal Conductivity (W/m-K)@room temperature	Specific Heat capacity J/Kg-K	Density kg/m^3
Silicon	150	700	2329
Pyrex glass	1.143	750	2280
DNA sample/water	0.58	4.181	997.1
PCB	24	800	3780
Air	0.24	1.005	1.205

3.4.2 Channel Geometry of µPCR chip

The channel geometry was modelled using computational fluid dynamics software Fluent 6.1. The main objective of this analysis was to determine the best geometry that would allow maximum recovery of sample and minimum dead volume. This was especially important for integration work to ensure that sufficient amount of sample was available for reactions downstream. Two geometries which included the rectangular and serpentine like chamber (Figure 3.8a and b) were being considered.



(a) (b)

Figure 3.8: Geometries for µPCR chip chamber numerical analysis. (a) Chamber Reactor; (b) Serpentine channels

3.5 Numerical Analysis for DNA Microarray Mixer

Fluent (Version 6.1) was used to determine the best mixer design for effective mixing of oligonucleotides/PCR amplicons and hybridization buffer. The efficiency of the mixers were simulated and gauged on the mixing capability of two samples.

CHAPTER 4 METHODS AND MATERIALS

4.1 µPCR Chip

4.1.1 Fabrication of µPCR Chip

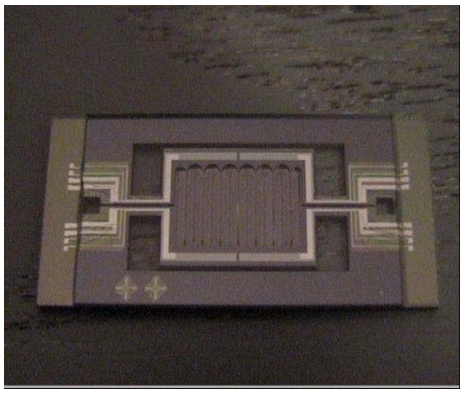
Two different types of µPCR chips were fabricated to facilitate different sets of experiments. Besides the complete version of the µPCR chip, a simplified version of µPCR chip known as 2-Mask PCR chip[1] with only a reaction chamber was fabricated. The 2-Mask PCR chip was used solely to evaluate the effect of silicon surface on PCR.

4.1.1.1 µPCR Chip (Complete Version)

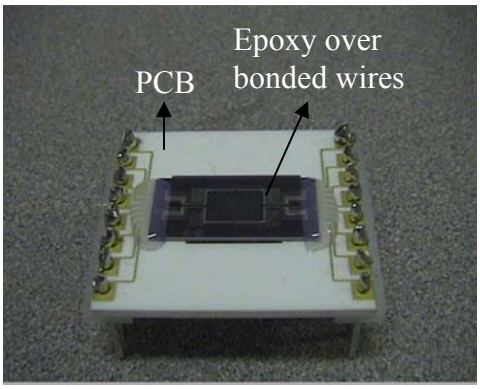
The substrate for device fabrication was an 8-inch silicon wafer. The device was fabricated using microfabrication techniques, which consisted of photolithography, dry/wet etching and anodic bonding[2]. A layer of aluminium that was suitable for use as a temperature sensor and resistive heater was deposited to a thickness of 0.8µm on silicon substrate. This layer was then patterned and etched to form temperature sensors and heater element. The reaction channels, air gaps and silicon membrane were fabricated to different depths respectively by etching. The inlet and outlet were etched through by laser drilling. Finally before encapsulation with glass, the bare silicon wafer was covered by a layer of SiO_2 through plasma enhanced chemical vapour deposition (PECVD) to prevent DNA from reacting with it. Pyrex Glass was then anodic bonded to the silicon wafer and diced to size of 25 mm by 11.6 mm.

[1] 2-Mask PCR chip: Chip was named 2 Mask PCR chip as it requires 2 mask patterns to fabricate
[2] Anodic bonding is the bonding done at elevated temperature clamped between 2 electrodes which will supply voltage across the glass to cause it to react chemically with silicon to form a strong permanent bond between them.

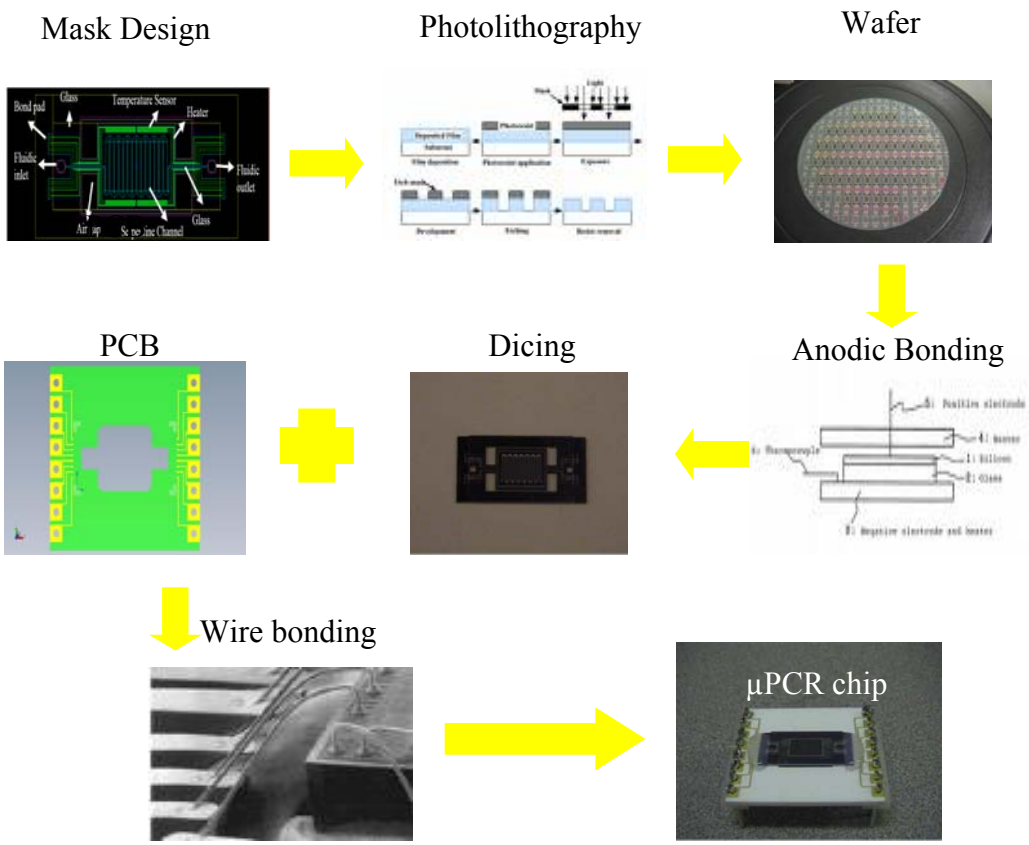
The diced μPCR chip (Figure 4.1a) was bonded onto the custom design printed circuit board (PCB) using standard quick cure epoxy (Hysol Loctite). The PCB served as the platform for electrical connections between the μPCR chip and external power source. These electrical connections were established when μPCR chip's metal heater and sensors were wire bonded (F& K wire bonder) to the gold pads of the PCB. A protective layer of non conductive epoxy (silicon rubber) was finally applied to protect the bonded wires. Figure 4.1b and Figure 4.1c illustrate the final device and process flow respectively. Operating procedures, design of experiment and parameters for wire bonding are attached in Appendix G.



(a)



(b)



(c)

Figure 4.1: µPCR chip process. (a) Diced µPCR chip; (b) µPCR chip bonded on PCB and wire bonded; (c) Process flow of µPCR chip

4.1.1.2 2-Mask PCR Chip

The 2-Mask PCR chip was a simplified version of the final µPCR chip and required less processing steps and time to fabricate. The 2-Mask PCR chip (Figure 4.2) had only reaction channels. Similar to the process as mentioned in the previous section, the substrate for device fabrication was an 8-inch silicon wafer. The fabrication processes consisted of photolithography and etching of the channels and air gap to 300 µm. The inlet and outlet were etched through as entry and exit points for sample flow into the channels. The bare silicon wafer was covered by a layer of SiO_2 through PECVD to

prevent DNA from reacting with it. Pyrex Glass was then anodic bonded to the silicon wafer and diced to size of 25 mm by 11.6 mm.

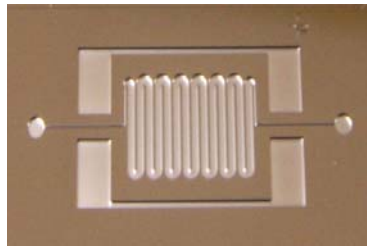


Figure 4.2: 2-Mask PCR chip

4.1.2 Device Characterization

The reliability and the performance of the aluminium sensors depended on its temperature coefficient of resistance (TCR). This was gauged by the comparison of the measured value with the theoretical value. The integrated aluminium sensors on the device were characterized by probing the device at the probe station (Cascade Microtech, Inc) at different temperatures. Resistance of the aluminium temperature sensors had a linear relationship with temperatures based on the following formula:

$$R = Ro[1+\alpha(T-To)] \quad (4.1)$$

where R = sensor resistance (Ω) at temperature T (ºC)

Ro = reference resistance (Ω) at reference temperature To (ºC)

α = temperature coefficient of resistance (TCR) of the sensor.

The TCR of the sensor was determined by equating the slope of the sensor resistance temperature plot with αRo.

4.1.3 System Setup for PCR Chip

4.1.3.1 System Setup for 2-Mask PCR Chip

2-Mask PCR chip was used to evaluate the effect of silicon chip surface on PCR and thus the need of surface passivation for the PCR chip. PCR mixture was pipetted into the 2-Mask PCR chip and placed on the loading plate of the Attocycler™ genetic analyzer for thermal cycling (Figure 4.3). PCR was also carried out concurrently on conventional thermal cycler (Applied Biosystem 2720).

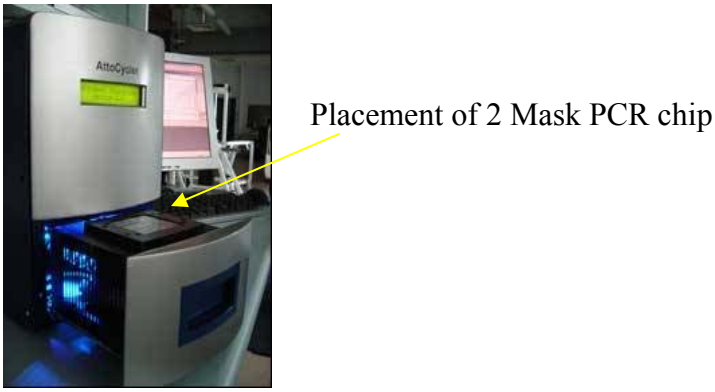


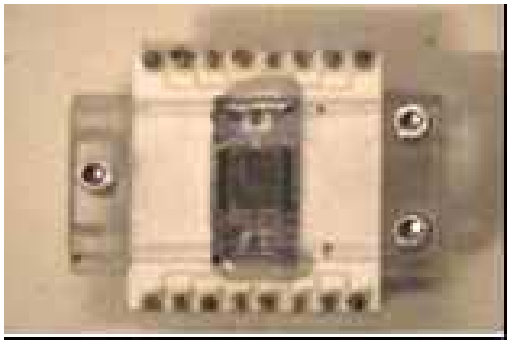
Figure 4.3: 2-Mask PCR experimental setup using Attocycler™ genetic analyser (Attogenix). Attocycler™ genetic analyzer is a peltier based thermocycler controlled externally by a laptop.

4.1.3.2 System Setup for µPCR Chip

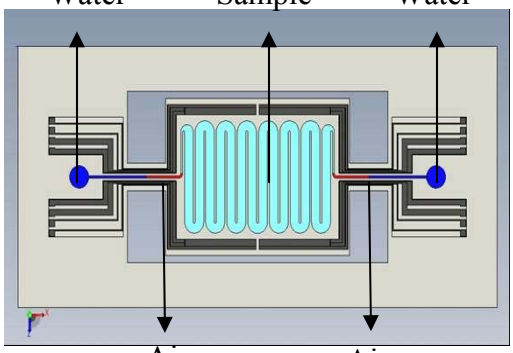
The µPCR chip was housed in an acrylic housing (Figure 4.4a) as described previously. A syringe pump (KDS100, KD Scientific, Boston, MA) was used to move and position the fluid through a manual three-way valve into the µPCR chip's reaction channels. The three-way valve was used to control the fluid flow sequence to create a fluidic zone with water/air/sample/air/water arrangement (Figure 4.4b). Stoppers (Figure 4.4c) were used to cap the inlet and outlet of the acrylic housing to further enclose the system during PCR to prevent evaporation.

The chip-based PCR control apparatus consisted of three main units including data acquisition system, power supply source and labview control unit. The µPCR chip's temperature sensors (RTD) were electrically connected to the data acquisition (DAQ) card (NI 4351 National Instruments) via a terminal block (TBX 68T National Instruments) which gave the temperature sensors an excitation current of 1 mA. The conversion from voltage reading to temperature was based on equation 4.1. The µPCR chip's integrated heater was connected to PXI Triple-Output Programmable DC power supply card (NI-4110 National Instruments) with three channels of ± 20 V and 1 channel of ± 6V providing current of 1 A from external power sources. Power was modulated through a custom Labview program (National Instruments, Austin, TX, USA) (Figure 4.4d) that allowed independent, flexible tuning of control parameters and easy setup of PCR protocol (e.g. PCR cycle number, pre denaturation temperature and time etc.). For precise temperature control and fast temperature transient without overshoots at the three temperature set points, the program used the application of gain scheduling[3] along with the digital feedback of PI [109,128]. A typical Labview program code, electrical connections, hardware and operating procedures are provided in Appendix H.

[3] Gain scheduling is an approach to control of non-linear systems that uses a family of linear controllers, each of which provides satisfactory control for a different operating point of the system.

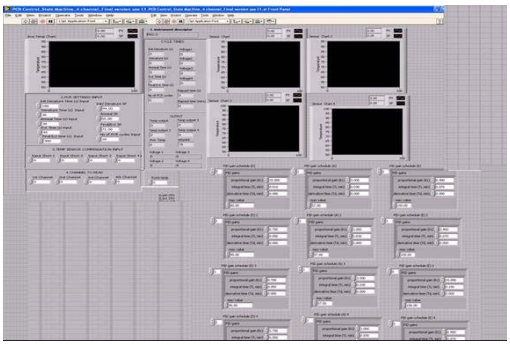


(a)

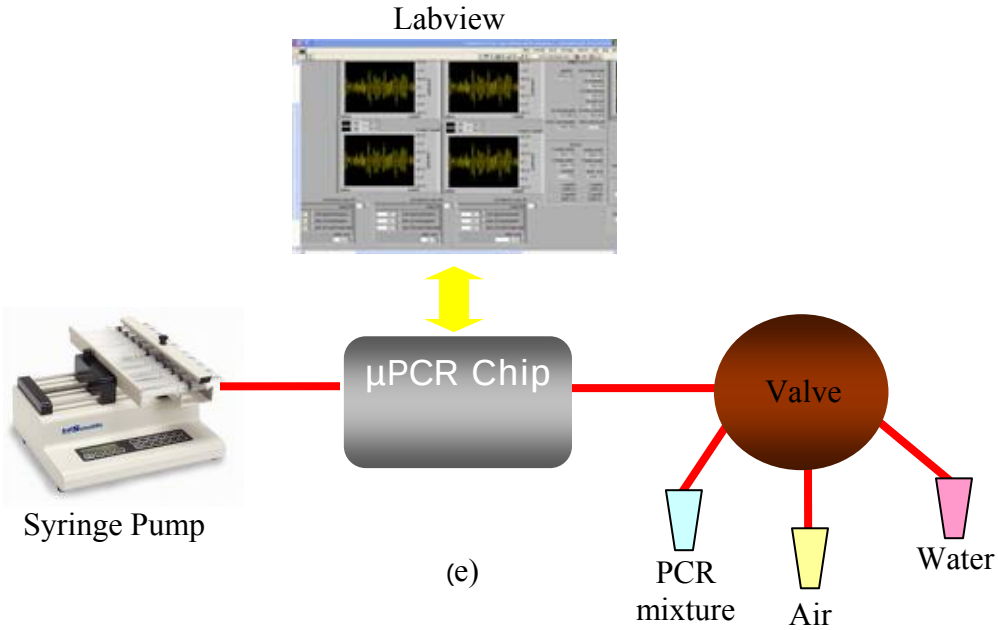


(b)

(c)



(d)



(e)

Figure 4.4: µPCR chip experimental setup. (a) µPCR chip in acrylic housing; (b) water/air/sample/air/water zone arrangement; (c) µPCR chip system setup (d) Labview program; (e) System setup

4.1.4 Cleansing

In order to carry out consecutive successful PCR using the μPCR chip, there was a need to prevent carry over from previous PCR runs and to remove PCR inhibitors. Cleansing protocol which included incubating the PCR chamber in 70% ethanol for 2 mins followed by 0.3% sodium hypochlorite for 2 mins thereafter flushed with 3 volumes of water was added in to eliminate possible contamination and inhibitors.

4.1.5 Symmetric PCR Protocol

DNA template for PCR was extracted from *Escherichia coli (E. coli)* ATCC15222 FDA strain PCI 1657 [K12; NCIB 10416]. *E. coli* was used as the target organisms as it is used in the EPA approved methods [1]. *E. coli* with concentrations ranging from 1 to 10^8 cfu/ml were extracted using Qiagen DNA min kit 51304 (Protocol attached in Appendix I). DNA extracted from *E. coli* with concentrations of 10^8 cfu/ml was used for all PCR experiments unless otherwise stated.

Three sets of universal primers: reverse primers R2 (5'-G(T/A)ATTACCGCGGC(T/G)G CTG-3') and forward primers F1(GAGTTTGATCCTGGCTCAG), reverse primers EUB338 (5'-GCT GCC TCC CGT AGG AGT-3') and forward primers F1, reverse primers R2 and forward primers EUB338 (5'-ACT CCT ACG GGA GGC AGC-3') were chosen to amplify three regions of 16S rRNA region.: bp 11-536(526 bp), bp 11-338(328), bp 338-536 (199 bp) respectively. All primers were purchased from Proligo. Although three sets of primers were designed so that they might be potentially used to identify other microbial targets, (such as *Cryptosporidium parvum*, *Giardia lambli*,

Salmonella sp., *Yersinia* sp., *Campylobacter* sp., *Enterococcus* sp. *Escherichia coli*, *Shigella* sp., *Vibrio* sp.) the focus in this study would be on the use of 199 bp product for proof of concept on the micro devices.

For each symmetric PCR experiment, 100 µl of master mix was prepared and aliquot to 10 tubes of 10 µl each. All PCR reagents were purchased from Qiagen (Taq PCR Core Kit 201223). The mixture contained 10µl of 10 × PCR buffer solution (Tris.HCl, KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$; pH 8.7 (20 ºC)), 4µl of 25 mM $MgCl_2$, 2µl of 10 mM dNTP mix (200 µM of each dNTP), 2 µl of 10 µM forward primer and 2µl of 10 µM reverse primer, 0.5µl of 2.5 units/reaction Taq DNA polymerase, 74.5µl of autoclaved double-deionized water (ddH_2O). Concentration of BSA was determined based on experiments conducted for surface passivation (next section). PCR amplification was carried out using Applied Biosystem 2720 thermal cycler under the following thermal program as recommended from Qiagen Taq PCR Core protocol: Initial denaturation at 95ºC for 3 mins, 95ºC for 30s, 55ºC for 30s, 72ºC for 45s and a final extension at 72ºC for 5 mins for 20-30 cycles. Optimal annealing temperature for each length was determined and the effect of number of cycles needed for PCR cycling was compared. The chip PCRs were subjected to the same thermal cycling profile as the conventional protocol.

4.1.6 Surface Passivation

Prior to chip based PCR experiments, the inner surface of the reaction chamber was passivated to avoid any non specific adsorption of the reagents, enzymes and DNA which would cause poor or no amplification. The 2-Mask chip was covered with SiO_2 through PECVD for passivation. The SiO_2 pre-coating was a reproducible and inexpensive

standard MEMS process and could be accomplished in a batch fashion. In addition, dynamic coating using BSA was also evaluated.

PCR reaction based on symmetric PCR protocol as described previously was performed using 2-Mask PCR chip on Attocycler™ genetic analyser (Attogenix). PCR were also carried out concurrently on Applied Biosystem 2720 thermal cycler to compare the device's efficiency against the conventional thermal cycler. The target sequence for PCR was approximately 500 bp fragment (using reverse primers R2 and forward primers F1) of 16S rRNA gene of *E.coli* genome. Different concentrations of BSA were added to the PCR master mix to determine the optimal concentrations. The detailed operating procedures for this experiment are attached in Appendix J.

4.1.7 µPCR Chip Amplification

With the addition of the titrated amount of BSA based on the surface passivation experiments, symmetric PCR amplification using the µPCR chip was carried out. A comparison between PCR efficiency between conventional PCR and the µPCR chip was also done.

4.2 DNA Microarray

4.2.1 Fabrication of DNA Microarray

Similar to µPCR chip, the substrate for device fabrication was an 8 inch silicon wafer. The device was fabricated using microfabrication techniques, which consisted of photolithography and dry etching. The channel depth of DNA microarray was etched to 50 µm. The bare silicon wafer was covered by a layer of SiO_2 through PECVD.

The DNA microarray wafer was diced to size (16 mm × 11 mm) and was spotted with probes (see next section on micro spotting) on the serpentine channels. The DNA microarray was capped by glass which was diced to the size.

In order to enclose the DNA microarray with glass, a thin poly (dimethylsiloxane) (PDMS) intermediate layer was used as an adhesive (Figure 4.5). PDMS prepolymer (SYLGARD 184 Silicone Elastomer Kit, Dow Corning) was mixed with its curing agent in the volume ratio of 10:1. A layer of PDMS prepolymer mixture was applied onto the glass surface and thinning of the PDMS was done through the use of nitrogen gas N_2. The glass coated with PDMS was partially cured at 80ºC for about 20-30s before it was bonded to the DNA microarray. The glass-DNA microarray bonding was completed after curing at 80ºC for about 15 mins.

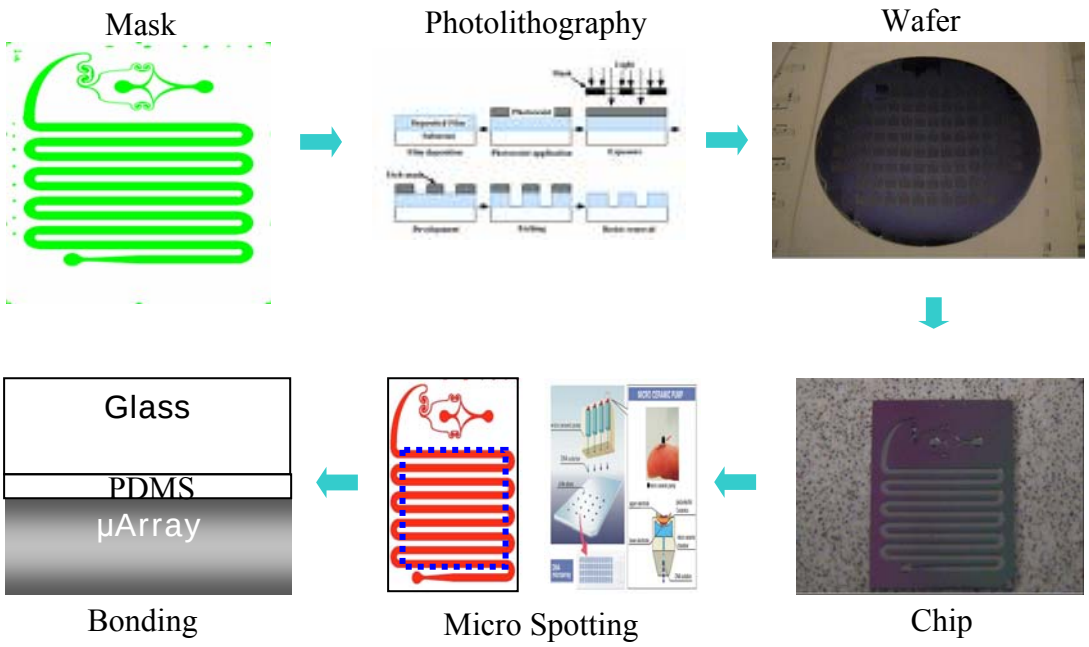


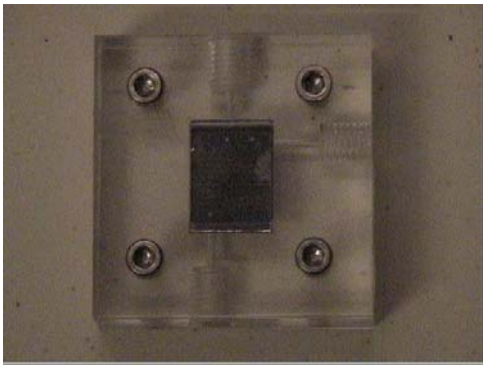
Figure 4.5: DNA microarray process

4.2.2 System Setup for DNA Microarray

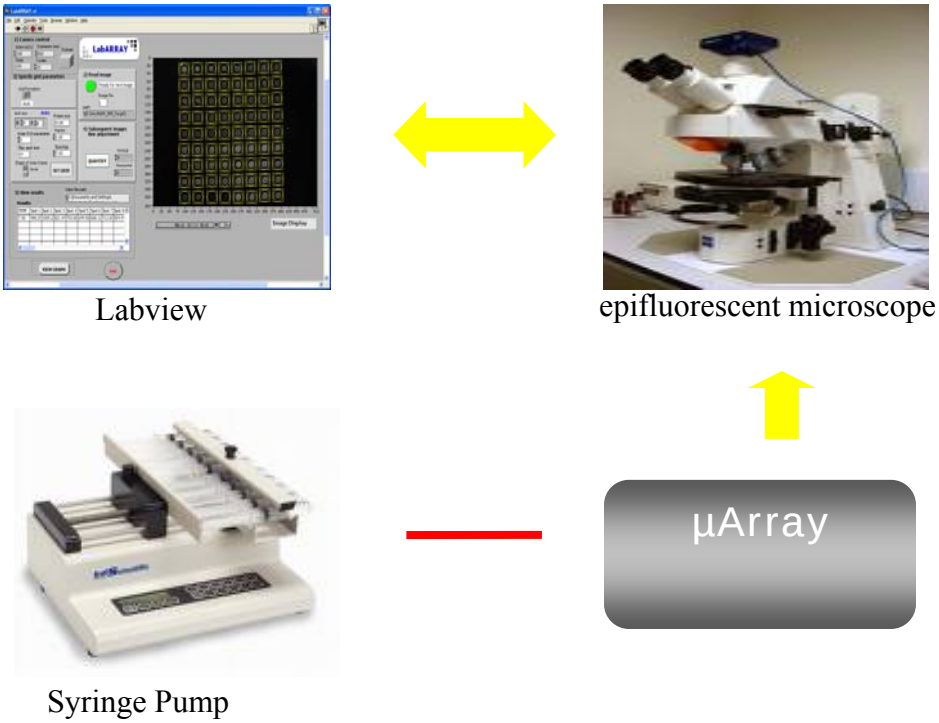
The DNA microarray was housed in an acrylic housing (Figure 4.6a) as described previously. The oligonucleotides/PCR amplicons and hybridization buffer mixture that were introduced into the device were contained in a 1ml plastic syringe (BD, Franklin Lakes, NJ). A syringe pump (KDS100, KD Scientific, Boston, MA) provided a constant force to discharge contents of the syringe at a steady flow rate.

The imaging system used to monitor the hybridization signals comprised of an epifluorescent microscope (BX51, Olympus, Singapore), a 100W mercury lamp and fluororescence filter set 41007 (Chroma technology, Rockingjam, VT). A SPOT-RT Slider cooled-charged coupled device (CCD) camera (Diagnostic Instrument, Sterling Heights, MI) was used to capture 12-bit monochrome images. Image acquisition was controlled with the MetaMorph 5.0 software (Molecular Devices, Sunnyvalve, CA). In all experiments, the exposure time was 1500 ms and images were captured at 60s intervals.

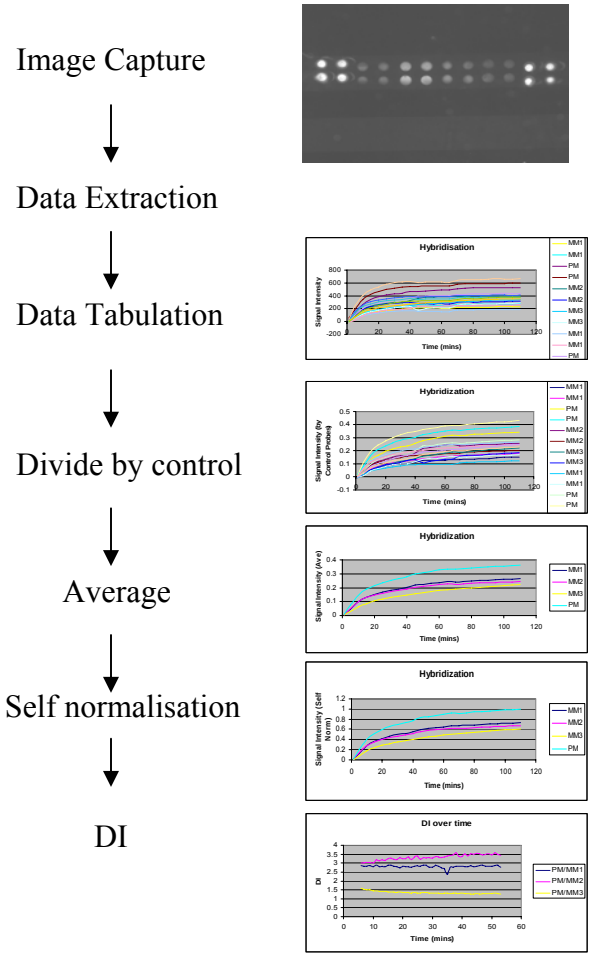
A Labview –based program (v7.1, National Instrument, Austin, Tx) developed in house previously was modified to batch analyzed the captured images [195]. For each captured image, the mean intensity was determined for all probes. The final results were then exported to Excel (Microsoft, Redmond, WA) and the variations of the fluorescence intensity over the hybridization period were plotted. Figure 4.6d shows the data analysis sequence for DNA microarray hybridization experiments.The setup is shown in Figure 4.6b. Detail operating procedures are attached in Appendix K.



a) DNA microarray in acrylic housing



(b) System setup



(c)

Figure 4.6: DNA microarray setup. (a) DNA microarray in acrylic housing; (b) System setup;(c) Data analysis flow for DNA microarray hybridization experiments

4.2.3 Mixer Testing

In order to verify the passive mixer's efficiency, FITC and water were pumped through the two inlets of DNA microarray respectively and the mixing efficiency was monitored. The imaging system used to monitor the mixing comprised of an epifluorescent microscope (IX71, Olympus, Singapore), a 100W mercury lamp and fluororescence filter cube#3 for FITC. A Q-Imaging cooled-charged coupled device (CCD) camera was used

to capture 12-bit monochrome images. Image acquisition was controlled with software Image Pro.

4.2.4 Surface Treatment and DNA Microarray Printing

Prior to DNA microarray experiments, the oxidized silicon wafer was silanized and modified with crosslinkers as per protocol attached in Appendix L. The 5' SH-terminated oligonucleotides purchased from Proligo were used as probes and were immobilized on silicon based microarray by covalent attachment [193] through micro spotting. Oligonucleotides were diluted to a concentration of 5 µM in autoclaved ddH_2O and spotted with a diameter of approximately 200 µm at intervals of 250 µm. All experiments using DNA microarray were spotted with oilgonucleotides using the BioChip Arrayer (BCA) (Perkin-Elmer). BCA is a non-contact micro dispensing system designed specifically for pipetting sub-microliter volumes to dense arrays controlled by an external computer. The operating procedures of BCA are attached in Appendix M.

4.2.5 Hybridization using Synthetic DNA Targets

The oligonucleotide probes and synthetic DNA targets used for hybridization were purchased from Proligo (Singapore). The 18-mer oligonucleotide probes consisted of a perfect match probe PM (5'-GCCCTCACGATCTCTTCC-3') and three other mismatch probe namely MM1 (5'-GCCCTCACTATCTCTTCC-3'), MM2 (5'-GCCCTCACAATCTCTTCC-3') and MM3 (5'-GCCCTCACCATCTCTTCC-3'). Probes had identical sequences except for a single nucleotide variation (underlined) and were modified with thiol at 5' end for immobilization onto DNA microarray. Target oligonucleotides were perfectly complementary to the PM probes and were labeled with a

Cy3 fluorophore at the 5' end as a reporter for the hybridization activity. These oligonucleotide probes were spotted on DNA microarray with control probes (5'-TTTTTTTTTTTTTTTGGGG-3') labeled with Cy3 at 3' end and modified with thiol at 5' end as illustrated in Figure 4.7. The use of control probes was to allow a common base for result comparison between different chips

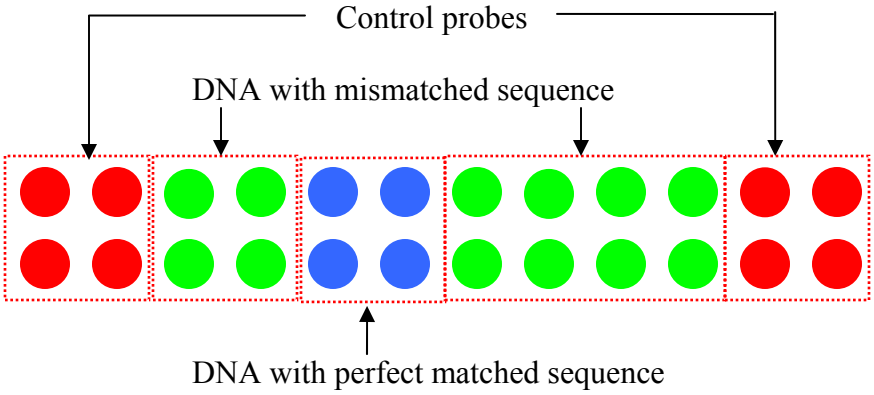


Figure 4.7: Probes format

4.2.5.1 Hybridization Conditions

To optimize discrimination between PM and MM, different concentrations of sodium chloride (NaCl) and formamide (FA) in the hybridization buffer were evaluated for their ability to differentiate PM and MM hybridization kinetics. The set of experiments conducted was repeated for three different NaCl concentration (100 mM, 300 mM, 900 mM) while FA concentrations remained at 0%. Another set of experiments was repeated for three different FA concentrations (30%, 50%, 60%) while NaCl concentrations remained at 300 mM. The hybridization buffer was introduced into the microarray at a fixed flow rate, target concentrations and Tris HCl pH 0.8 concentrations (0.02 M). The PM and MM hybridization kinetics were monitored temporally for each parameter studied. To obtain a quantitative estimate for the difference between the PM and MM

hybridization, a maximum discrimination index (DI) was derived based on the following formula:

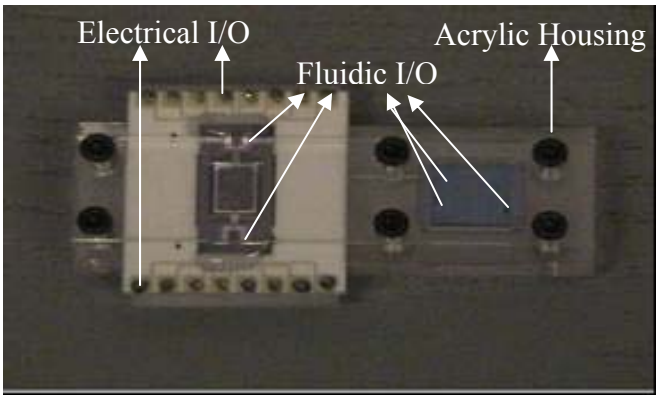
$$DI = [\frac{PM}{MM}]_{max} \quad (4.2)$$

4.3 Experimental Procedures for Micro Total Analysis System

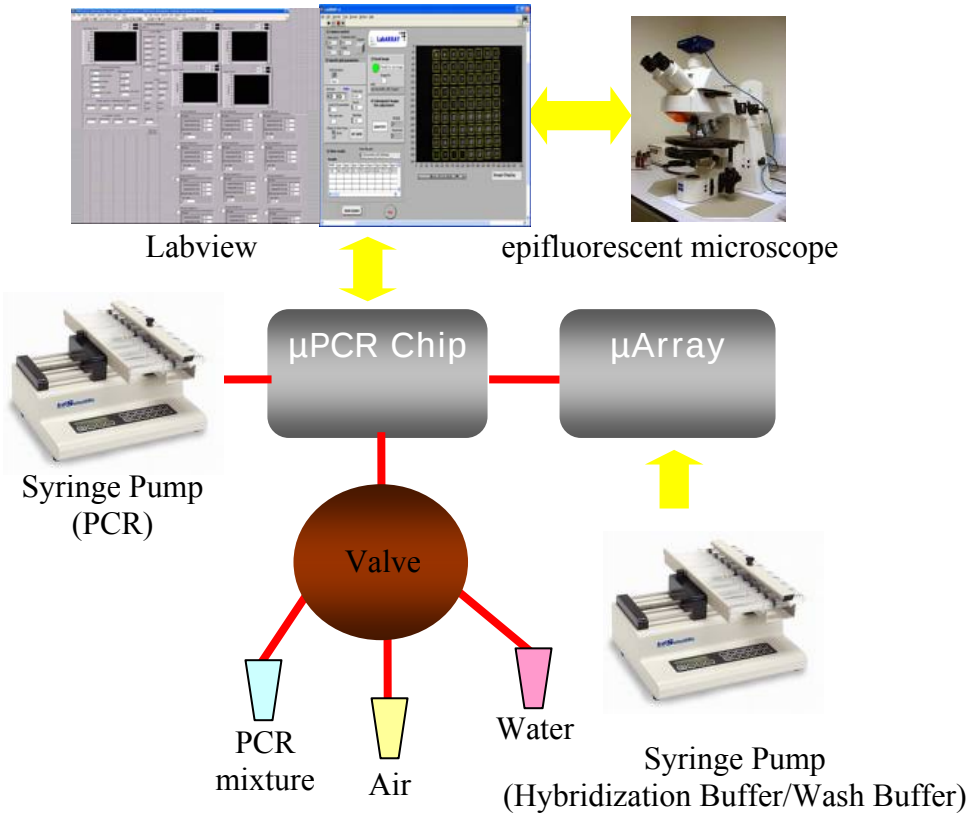
4.3.1 System Set Up for Micro Total Analysis System

The µPCR chip and DNA microarray were housed in an acrylic housing with the system set up (Figure 4.8a). The movement and positioning of fluid in the system was controlled by a syringe pump. The use of a three way valve was to generate the water /air /sample /air /water configurations as described previously. Stoppers were used to cap the inlets and outlets of the acrylic housing to enclose the system before running PCR.

The µPCR chip was electrically connected to data acquisition and temperature control hardware and thermal cycling was controlled by Labview. The stoppers were removed after the completion of PCR, to allow the transfer of sample and hybridization buffer to the DNA microarray at 1 µl/min for about 30-40 mins. The DNA microarray was imaged under fluorescent microscope used in DNA microarray system setup. The system set up is shown in Figure 4.8b. Detail operating procedures are found under appendix N.



(a)



(b) System set up

Figure 4.8: Micro total analysis system set up; (a) Micro total analysis system; (b)System setup

4.3.2 Asymmetric PCR protocol

Asymmetric PCR protocol was used for PCR amplification in the micro total analysis system. For each PCR experiment, 100 µl of master mix was prepared and aliquot to 10 tubes of 10 µl each. All PCR reagents were purchased from Qiagen (Taq PCR Core Kit 201223). The mixture contained 10µl of 10 × PCR buffer solution (Tris HCl, KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$; pH 8.7 (20 ºC)), 4µl of 25 mM $MgCl_2$, 4µl of 10 mM dNTP mix (200 µM of each dNTP), 50 µl of 10 µM 5' end Cy 3 labelled forward primer and 0.5µl of 10 µM reverse primer, 1µl of 5 units/reaction Taq DNA polymerase, 30.5µl of autoclaved double-deionized water (ddH_2O). The template DNA was based on 16S rRNA of *E.coli* genome. Amount of BSA added was based on the experiments conducted for surface passivation. Asymmetric PCR was carried out on conventional thermocycler and µPCR chip with thermal cycling profile as follows: Initial denaturation at 95ºC for 3 mins, 35 cycles of 95ºC for 30s, 55ºC for 30s, 72ºC for 45s and a final extension at 72ºC for 5 mins. This thermal cycling profile was based on previous study [183] which was successful. The amplified products from both conventional thermocycler and µPCR chip were applied on DNA micorarray (next section) to determine successful conditions for asymmetric PCR.

4.3.3 Hybridization with Microbial Targets (*E.Coli*)

E coli. was used as the microbial targets in this experiments. The oligonucleotide probes used for hybridization with microbial targets were purchased from Proligo (Singapore). The oligonucleotide probes consisted of a perfect match probe PM Esc447 (5'-ATT AAC TTT ACT CCC TTC CTC CCC-3') and three other mismatch probes including 3MM Esc TCA (5'-ATT AAC TTT ATC ACC TTC CTC CCC-3'), 2MM Esc 2C (5'-ATT AAC

CTC ACT CCC TTC CTC CCC-3') and 1MM Esc MM12 (5'-ATT AAC TTT ACA CCC TTC CTC CCC-3'). Probes had identical sequences except for variations as underlined and were modified with thiol at 5' end for immobilization onto DNA microarray. According to the RDP and a BLAST Search, the probe Esc447 targets specifically the genus *Escherichi*a and *Shigella.* The oligonucleotide probes with the positive (5'- GCT GCC TCC CGT AGG AGT-3') and negative (5'-ACA TCC TAC GGG AGG C-3') control probes for bacteria (modified with thiol at 5' end) were spotted on DNA microarray. The other set of control probes (5'-TTTTTTTTTTTTTTGGGG-3') labeled with Cy3 at 3' end and modified with thiol at 5' were also spotted on the DNA microarray as illustrated in two different formats in Figure 4.9. The Labview based imaging system as described in DNA microarray setup was used to monitor the hybridization signals of microbial target with the oligonucleotide probes.

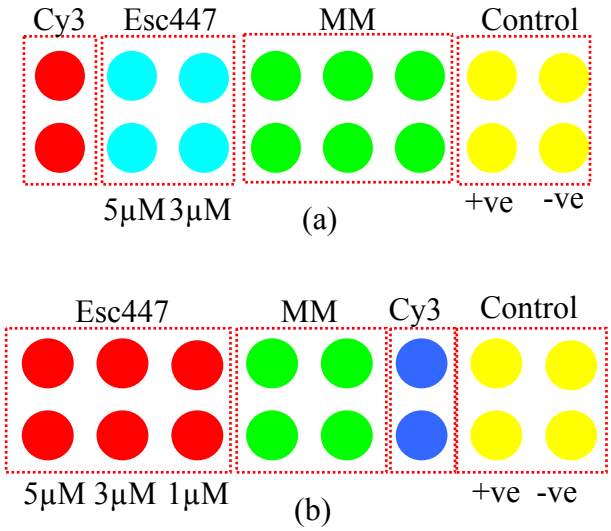


Figure 4.9: Layout of probes for hybridization with microbial target. (a) Probe Layout; 1 (b) Probe layout 2

CHAPTER 5 RESULTS

5.1 µPCR Chip

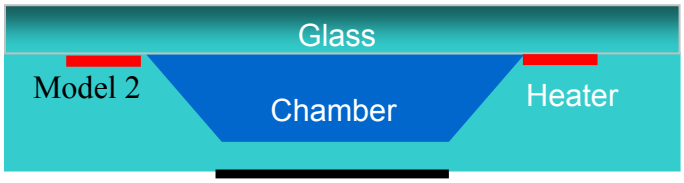
5.1.1 General Design

Silicon was chosen as a base material because of its well known characteristic and its well established microfabrication processes [196]. Silicon as a structural material had both high thermal conductivity which facilitated fast thermal cycling as well as mechanical strength [90]. It allowed for precise design and micromachining of components with well defined thermal conductance. Each reaction chamber had a silicon membrane as its floor and a glass sheet as its roof. The advantage of using glass was that it was transparent so that sample filling and flowing could be seen clearly. A thermal isolation design was implemented by etching through thermally conductive silicon membrane, to eliminate cross talk between the reaction and the substrate (which is also a heat sink) and to reduce parasite heat capacitance. In addition, a side symmetrical heating scheme was implemented to achieve good temperature uniformity. Heating structures were only placed strategically at the sides of the chamber linked by the small joints made by silicon beams to reduce the temperature deviation across the chamber. These results will be further elaborated in the next sections.

5.1.2 Numerical Analysis

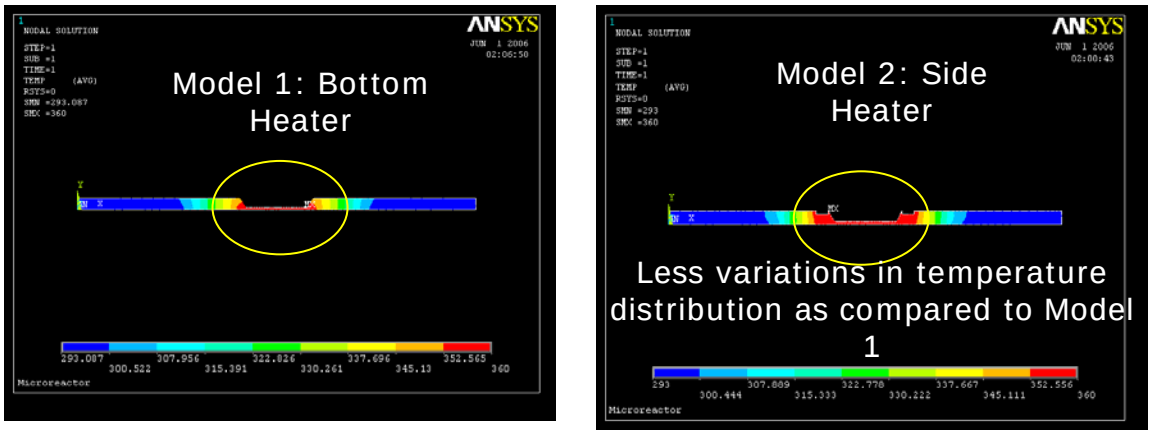
5.1.2.1 Side Heaters vs Bottom Heater

The 2D simulation results (Figure 5.1a and b) demonstrated that the side heating scheme gave a much better uniform heating than the bottom heater.



Model 1

(a)



(b)

Figure 5.1: Thermal models for heating scheme for µPCR chip. (a) Side view of model for heater position comparison; (b) Thermal model comparison between model 1 bottom heater and model 2 side heaters

5.1.2.2 Heater Position

The 3D thermal model was used to investigate five different heaters' positions (Figure 5.2a) to determine its optimal placement. The ANSYS simulation results showed that heater position 5 gave the least sample temperature variation of 1 ºC in comparison to the rest of the heater positions. Heater position 3 gave a relatively small temperature variation of 1.5 ºC while other heater position 1,2,4 gave a variation of 6.5 ºC, 4 ºC and 3 ºC respectively. These results implied that the placement of heater at a central region between the chamber and the silicon beam would allow better heat distribution. In additional, simulation results of three different heater configurations (Figure 5.2b,c and d)

showed that the "L" shaped heater (configuration 3) gave the least variation in sample temperature of 0.5 ºC in comparison to the other two heater configurations. Heater configuration 1 and 2 gave a variation of 1 ºC, 1.4 ºC respectively. The final design was based on heater configuration 3 which gave the best temperature uniformity.

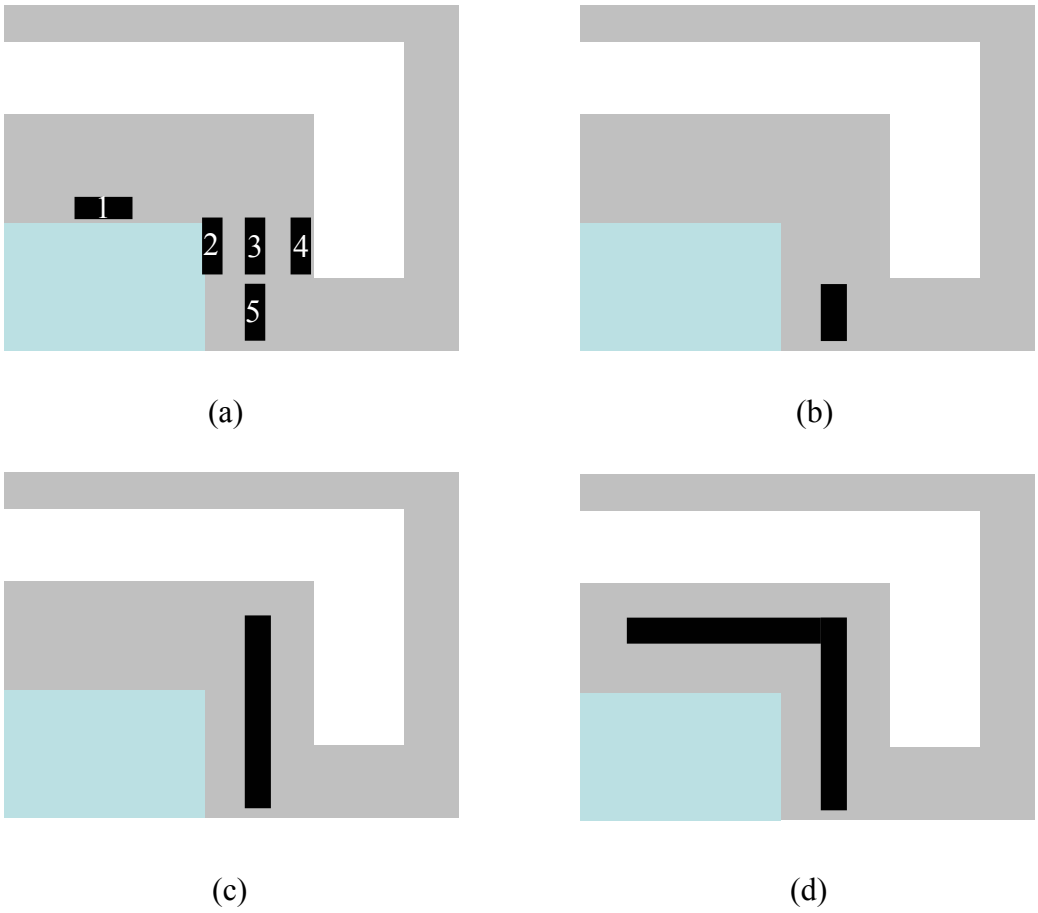


Figure 5.2: Different heater configuration. (a) 5 different heater positions; (b) Heater configuration 1; (c) Heater configuration 2; (d) Heater configuration3

5.1.2.3 Air Gap

The air gap's size (La) (Figure 5.3a) was investigated to determine the impact of thermal isolation to the reaction channel. As air is a poor conductor of heat, a larger air gap will provide a better thermal isolation to the reaction chamber. In this simulation, it could be

seen that there was little cross talk between the substrate and reaction chamber at all air gap length (La_1). This was shown by the large difference in colour contours as illustrated in Figure 5.3b-e. However with a shorter air gap (La_1), a higher heat flux/power was required to heat up the substrate to the desirable temperature.

Another consideration was the critical force of the silicon beam given by Euler's formula:

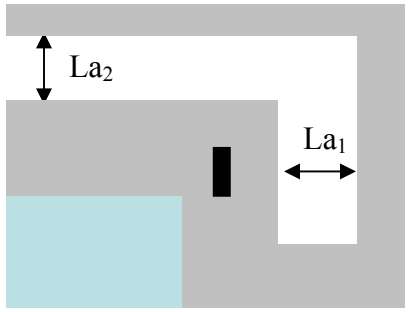
$$F_{cr} = \frac{EI\pi^2}{L^2} \tag{5.1}$$

where E = Young's modulus of the column material

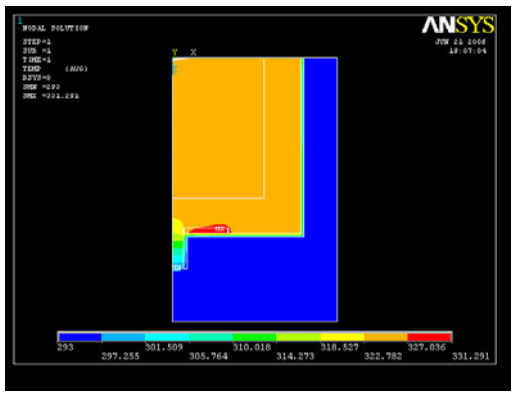
I = area moment of inertia of the cross-section

L = the length of the column

As air gap (La_1) increased, the silicon beam L would increase. Based on equation 5.1, this would imply a large drop in the critical force of the structure. This would mean that the chip would become mechanically weaker and more vulnerable to breakages during processing and handling. Simulation results further suggested that the difference between adopting a 2 mm and 2.5 mm air gap was insignificant. With consideration to simulation results, mechanical stability and previous similar studies done [115], an air gap of length of 2 mm with width of 0.6 mm silicon beam was adopted for the μPCR design. Numerical simulation on the other air gap length (La_2) (see also Figure 5.3a) were also done. The result demonstrated that the side air gap length (La_2) was not a critical parameter as changes in La_2 did not affect the temperature uniformity significantly. La_2 was given a random value of 0.3 mm.



(a)



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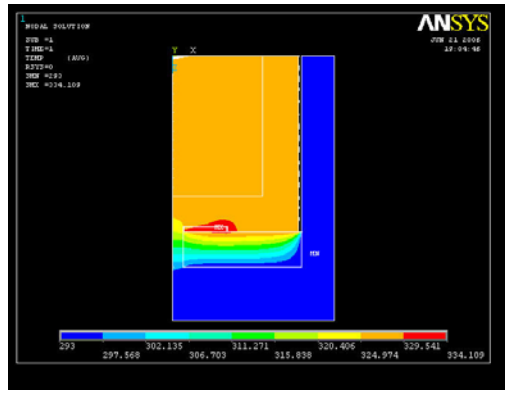
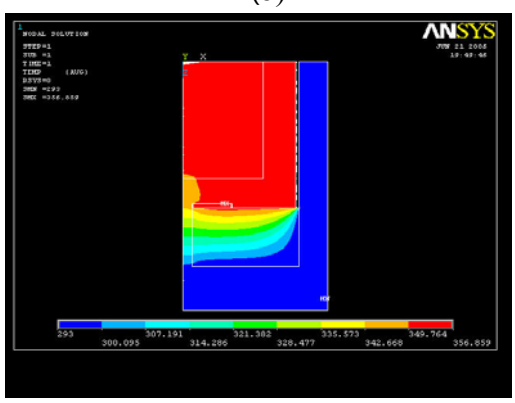
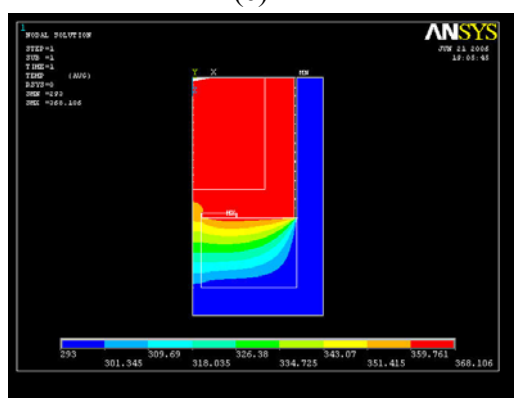


(c)



(d)



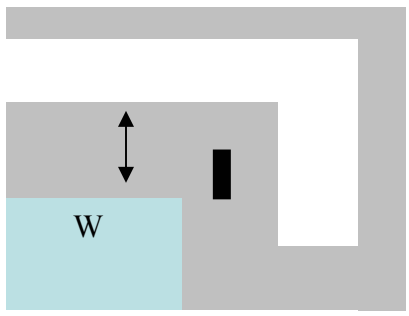
(e)

Figure 5.3: Thermal model with different air gaps La. (a) Length La of air gap; (b) La_1 = 0.1mm; (c) La_1 = 1 mm; (d) La_1 = 2 mm; (e) La_1 = 2.5mm

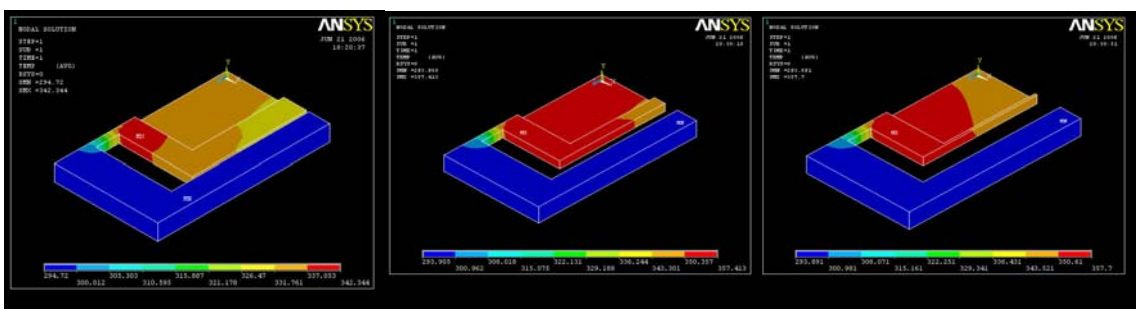
5.1.2.4 Thermal Mass

Numerical simulations of the change in thermal mass would demonstrate the impact on sample temperature distribution during heating. The change in thermal mass was simulated by changing length (W) as indicated in Figure 5.4a. It was expected that heat

was able to spread more uniformly at a faster rate with a smaller thermal mass. Simulation results showed that the least steady state temperature deviation of 0.5 ºC was achieved when the length W was reduced to 0.5 mm. In comparison, the sample temperature deviations were 1.5 ºC and 1 ºC when length W was 1.5 mm and 1 mm respectively. Length 0.5 mm was chosen as the dimension W of reaction chamber. The length W was not further reduced to less than 0.5mm as this would probably cause a reduction in mechanical strength unsuitable for chip usage.



(a) Width W of device



(b) W = 1.5 mm (c) W = 1 mm (d) W = 0.5 mm

Figure 5.4: Thermal model for change in thermal mass, (a) Width W of device; (b) W = 1.5 mm; (c) W = 1 mm; (d) W = 0.5 mm

5.1.2.5 Chamber Geometry

Three different chamber configurations of different length (x,y, depth) with total volume of 10 µl were investigated (Figure 5.5). The steady and transient state simulations on

chamber geometry 1 (3.35 mm, 2.5 mm, 0.3 mm) and geometry 2 (2.5 mm, 3.35 mm, 0.3 mm) showed similar thermal performances. The steady state temperature deviation of geometry 1 and 2 was 1.25 ºC and both geometries required 5 s to heat up (room temperature 25ºC to denature temperature of 95 ºC) and 6 s to cool down (95 ºC to annealing temperature 55 ºC). While chamber geometry 3 (5 mm, 5 mm, 0.1 mm) showed the least temperature deviation across the sample at 1 ºC, its heating/cooling time was twice slower than geometry 1 and 2 due to greater thermal path. The final geometry chosen was chamber geometry 1 (3.35 mm, 2.5 mm, 0.3 mm) due to the fast heating and cooling time. Although results of geometry 1 showed that its sample's temperature deviation was slightly more than geometry 3, it could be corrected through the use of other parameters.

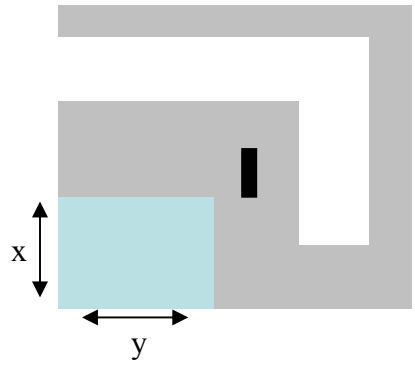
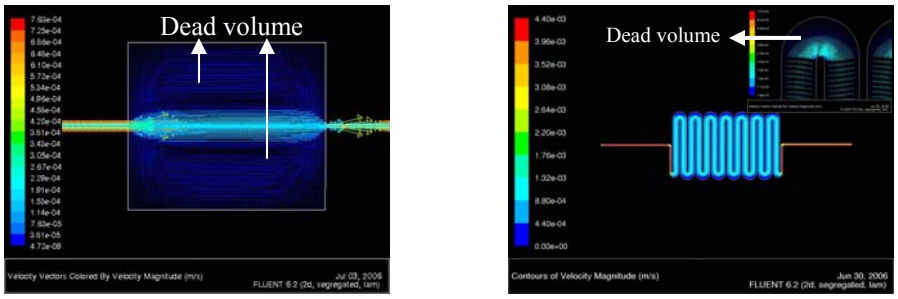


Figure 5.5: Chamber geometry x,y

5.1.2.5.1 Chamber Geometry and Dead Volume

Numerical analysis using Fluent 6.1 was used to study the chamber configuration and to minimize dead volume. This was especially important for future integration work as maximum sample recovery would allow more sample volume to be available for reactions downstream. Serpentine channel geometry was adopted in the µPCR design as

recovery of sample in a serpentine channel was better than a single rectangular chamber (Figure 5.6a and b).

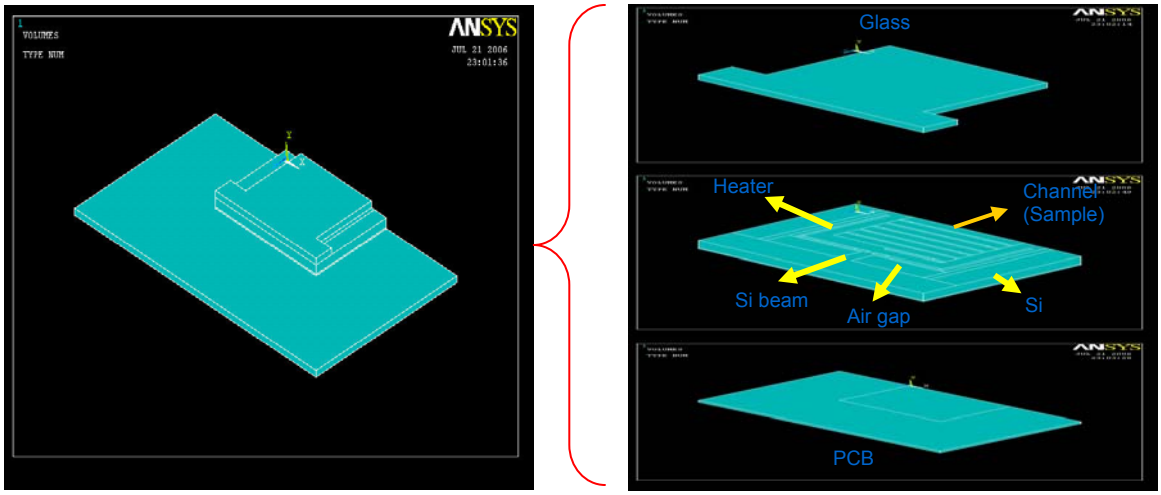


(a) (b)

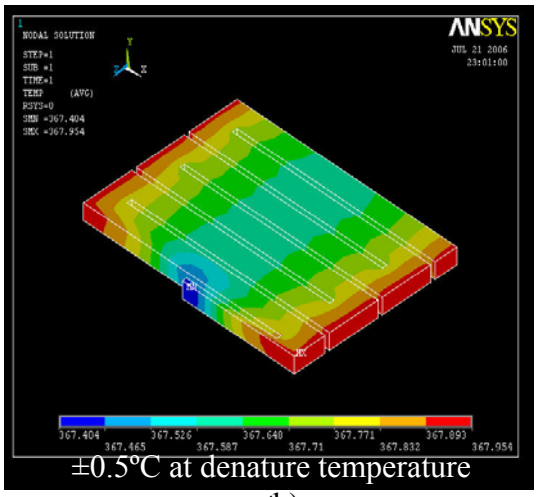
Figure 5.6: Comparison of 2 different channel geometries. (a) Rectangular chamber; (b) Serpentine channels

5.1.2.6 Final Design

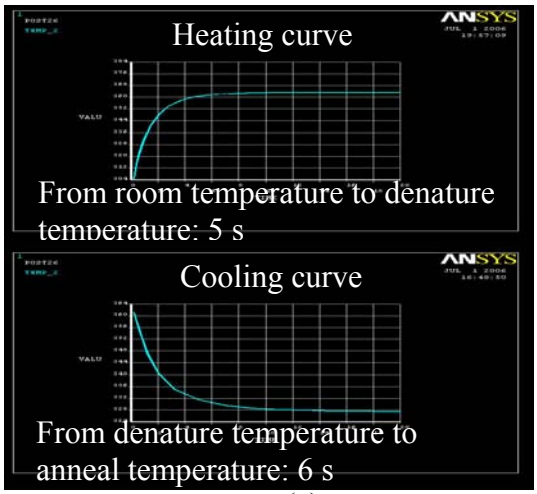
The final design was based on results from numerical analysis covered in section 5.1.2.1 to 5.1.2.5. The dimensions were slightly adjusted to accommodate the chip's features. ANSYS simulations of the final design (Figure 5.7a) showed that the design had a good temperature uniformity of ± 0.5ºC (Figure 5.7b) with a rapid heating and cooling rate of 15 ºC/s and 8 ºC/s (Figure 5.7c) respectively. This was much faster when compared to conventional thermal cycler with heating and cooling rates of 1 ºC/s. The final dimensions of the µPCR chip are provided in the Appendix A.



(a)



(b)



(c)

Figure 5.7: Thermal model of final design of µPCR chip. (a) Half thermal model used due to symmetry; (b) Steady state of sample; (c) Transient state at sample

5.1.3 Aluminium Heater

Aluminium thin film resistors were designed based on the numerical analysis results. Aluminium with resistivity of 2.65×10^{-8} ohm (m) was patterned on the silicon chip as heaters. The thickness of alumininum heater was 0.8 µm with a total length of 44 mm. In order for the hardware to support the heater, careful considerations were taken to ensure

that the maximum voltage required would be less than what could be supplied. The following calculations were done to verify this.

In order for the sample to reach the desired denature temperature, numerical simulation suggested that a heat flux of 4 x 10^6 W/m^2 was required to be applied onto a total heater area of 0.0006 x 0.00015 m^2

Using Power = Heat flux × Area (5.2)

Theoretical power required per heater = 4 x 10^6 W/m^2 × 0.0006 × 0.00015 m^2

= 0.36 W

$$\text{Using Resistance} = \frac{Resistivity \times Length}{Thickness \times Width} \quad (5.3)$$

$$\text{Resistance of aluminium heater} = \frac{2.65 \times 10^{-8} \times 44 \times 10^{-3}}{0.8 \times 10^{-6} \times 10 \times 10^{-6}}$$

= 145.75 Ω

$$\text{Using Power} = \frac{Voltage^2}{Resistance} \quad (5.4)$$

Rearranging equation 5.4, Voltage = $\sqrt{Power \times Resistance}$

Voltage required per heater = $\sqrt{0.36 \times 145.75}$

= 7.2 V.

The maximum voltage supplied by the NI PXI 4110 triple output power card used for the control of μPCR chip was ± 20 V. The calculations showed that the voltage required was less than the supply source. Another factor that was considered was electromigration. In order to prevent ions/atoms in metals from moving and thus resulting in defects such as

open circuit due to voids during heating, the current density value J must not exceed the limit where electromigration occurs (1 x 10^6 A/cm^2).

Based on µPCR chip design parameters

Using current density value $J = \left(\frac{Power}{Thickness \times resistivity \times Area}\right)^{0.5}$ (5.5)

$$J = \sqrt{\frac{0.36}{0.8\times10^{-6}\times2.65\times10^{-8}\times44\times10^{-3}\times10\times10^{-6}}}$$

$$J = 0.6 \text{ x } 10^6 \text{ A/cm}^2 < 1 \text{ x } 10^6 \text{ A/cm}^2$$

The calculated density value was less than the critical value of electromigration.

The µPCR chip's heaters were designed within hardware's limitation to provide heat to the chip without exceeding electromigration value.

5.1.4 Alumininum Sensors

Alumininum were patterned to a thickness of 0.8µm at four corners of the chip's channels (Figure 5.8). The total length of each sensor was 35 mm. This allowed the sensors to provide real time temperature feedback to the system. In order to ensure the voltage requirement of the sensor would not exceed the hardware source, the following calculations were done.

Using equation 5.3: Resistance = $\frac{Resistivity \times Length}{Thickness \times Width}$

Resistance of aluminium sensor Ro = $\frac{2.65\times10^{-8}\times35\times10^{-3}}{0.8\times10^{-6}\times10\times10^{-6}}$

$= 116\ \Omega$

Maximum temperature measured by sensor for PCR = 100ºC

Using equation 4.1: R = Ro[1+ α(T-To)]

where R = sensor resistance (Ω) at temperature T (ºC)

Ro = reference resistance (Ω) at reference temperature To(ºC) = 116 Ω

α = temperature coefficient of resistance (TCR) of the sensor = 0.0043

T = 100 ºC

To = 20 ºC

Therefore R = 116[1+0.0043(80)]

= 399.04 Ω

Based on power card used for temperature feedback in the hardware, current supply was 1 mA and maximum voltage that could be measured by hardware was 2.5 V.

Using Voltage = Current x Resistance (5.6)

Voltage output by aluminium sensor = $1 \times 10^{-3} \times 339.04$

= 0.399 V (<< 2.5 V hardware source)

The voltage output by the sensor was 0.399 V which was very much less than the hardware limitation. Thus hardware was able to measure voltage for the sensor to function as feedback to the system for temperature control.

The reliability and performance of the fabricated aluminium sensors were also evaluated. The temperature-coefficient-of resistance (TCR) of each of the four sensors (Figure 5.8a) was measured. The measurements showed very high temperature-coefficient-of resistance (TCR) and the sensors had a consistent linear response over the whole temperature range (Table 5.1 and Figure 5.8b). Using equation y = 0.924x + 212.14 obtained from graph (Figure 5.8b).

The TCR value of aluminium sensor, $\alpha = \dfrac{Slope\_of\_graph\_Resistance\_vs\_temp}{Ro}$

$$= \frac{0.925}{212.14} = 0.00436$$

The value calculated was almost identical to the theoretical value of 0.0043 which implied that the sensor was stable and able to measure temperature accurately.

To further evaluate the sensitivity of the alumininum sensors, change in resistance per degree change in temperature was calculated.

Using equation 4.1: $R = Ro[1+\alpha(T-To)]$

where R = sensor resistance (Ω) at temperature T (ºC)

Ro = reference resistance (Ω) at reference temperature To (ºC) = 116

α = temperature coefficient of resistance (TCR) of the sensor = 0.0043

At T = 20 ºC = To

R = Ro = 116 Ω

At T = 30 ºC

$R = 116[1+ 0.0043(10)]$

= 120.988 Ω

Difference between changes in resistance due to change in 10 ºC

=120.988 -116 Ω

= 4.988 Ω

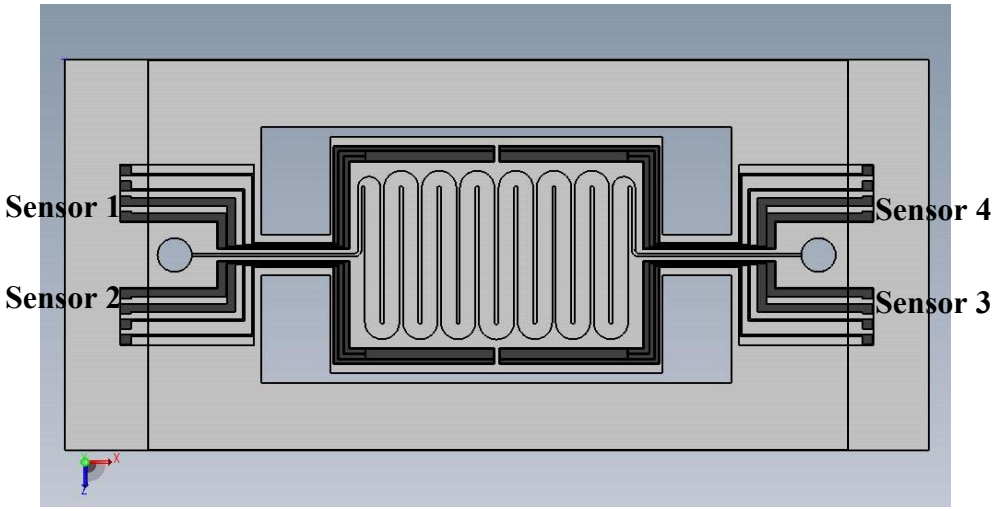
Theoretical change in resistance per change in degree = $\frac{4.988}{10}$

= 0.4988 Ω/ºC

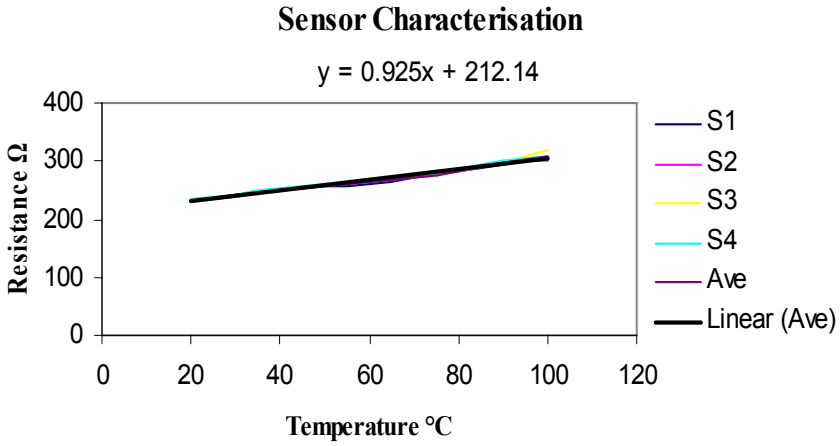
This small change in resistance in relation to 1ºC would enable the sensor to give accurate feedback to the system.

Table 5.1: Resistance of aluminium sensor over temperature

Temperature ºC	Sensor 1(S1)	Sensor 2 (S2)	Sensor 3(S3)	Sensor 4(S4)	Ave
20	232	233	232	234	232.75
30	240	242	240	242	241
40	248	248	249	253	249.5
50	256	256	258	262	258
60	262	264	267	269	265.5
70	272	273	272	275	273
80	281	282	284	285	283
90	294	296	296	302	297
100	306	302	318	310	309



(a)

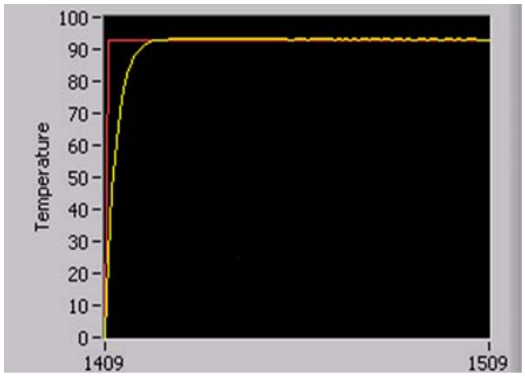


(b)

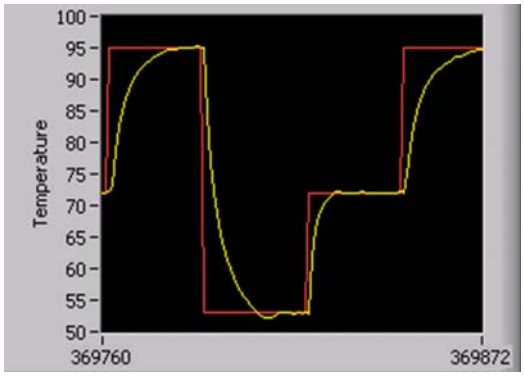
Figure 5.8: Characterization of aluminium sensor. (a) Aluminium sensors position; (b) Plot of resistance over temperature based on Table 5.1

5.1.5 Temperature Control

The side symmetrical heating scheme with the high thermal conductivity of silicon provided fast heating (10-15 º C/s) and cooling (8 º C/s) (Figure 5.9a and b) as well as good temperature homogeneity of the µPCR chip during thermal cycling. The integrated sensors showed that the temperature distribution around the reaction chamber was less than ± 0.3°C (Figure 5.9c).The thermal isolation feature incorporated into the PCR chip had also helped to confine heating within the reaction chamber. However the heating and cooling rates were slightly slower than numerical simulated results and the voltage required was more than the theoretical value of 7 V(the required voltage is still within voltage supply source). This may be attributed to the extra mass (acrylic housing) that the µPCR chip came in contact with (this was not taken into account during numerical simulation) and the imperfect conditions of the aluminium heaters (sometimes present of small voids) due to fabrication.



(a)



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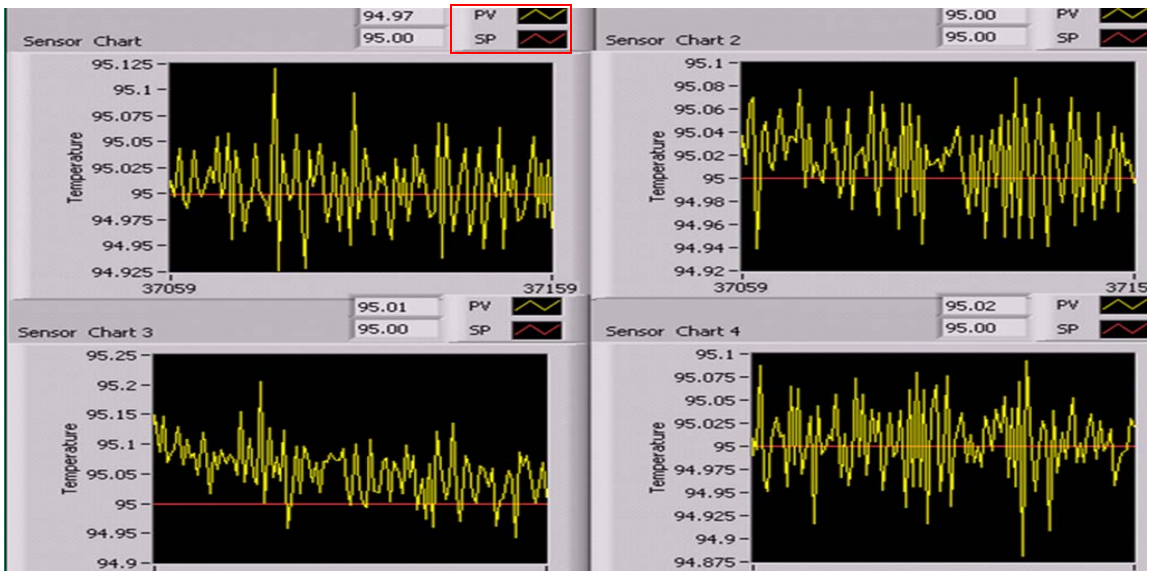


(c)

Figure 5.9: Temperature performance of µPCR chip. (a) Heating curve: 6s to heat from 25 ºC to 94 ºC; (b) Cooling curve portion: 8s to cool from 94 ºC to 55 ºC; (c) Feedback from 4 on chip aluminium sensors located at 4 locations around the reaction channels. PV = Present value; SP = Set point

5.1.6 Cleansing

The chip cleansing step which included the use of ethanol and sodium hypochlorite was performed to remove PCR inhibitors and carryover from previous PCR runs to allow chips reuse. Figure 5.10 shows that without the cleansing step, subsequent PCR cannot be carried out successful.

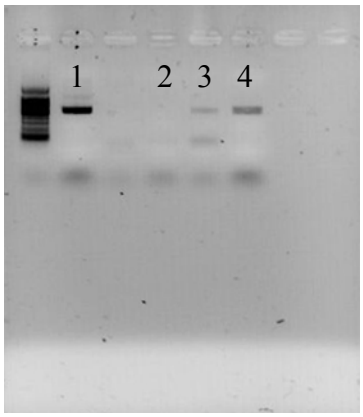


Figure 5.10: Gel Electrophoresis (1.5% Agarose Gel) to compare difference in washing protocol and effect on PCR on chip when it is used for 3rd time. Lane 1: Conventional PCR. Lane 2: Chip PCR with no washing. Lane 3: Chip PCR with 70% ethanol washing step. Lane 4: Chip PCR with 70% ethanol and 0.3% NaOCl washing step

5.1.7 Symmetric PCR protocol

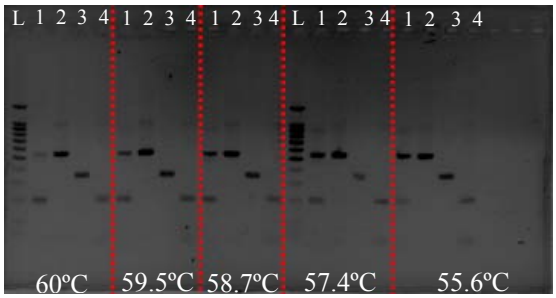
5.1.7.1 Thermal Cycling Profile

Thermal cycling profile was arbitrary determined based on typical PCR cycling program: 95ºC for 3 mins, 95 ºC for 30s, 55 ºC for 30s, 72 ºC for 45s and a final extension at 72 ºC for 5 mins for 20-30 cycles.

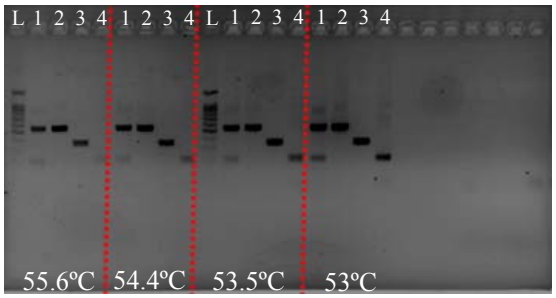
5.1.7.2 Annealing Temperature

Optimization of annealing temperature was required as it would contribute to the purity and yield of the reaction products. The optimal temperatures of the three sets of primers were determined by using the temperature gradient PCR thermal cycler. Different annealing temperature was used to compare the product intensity on gel (Figure 5.11). Multiplex PCR were also run to determine feasibility of using three sets of primers together for different pathogen detection for future works. However as the regions of 300 bp and 500 bp overlapped, the multiplex PCR was only successful for 500 bp and 200 bp regions. Re-design of unique primers that amplifies different regions is necessary for

multiplex PCR to function properly, However as this work is out of the scope of this thesis, this area of work will not be covered in this thesis



(a)



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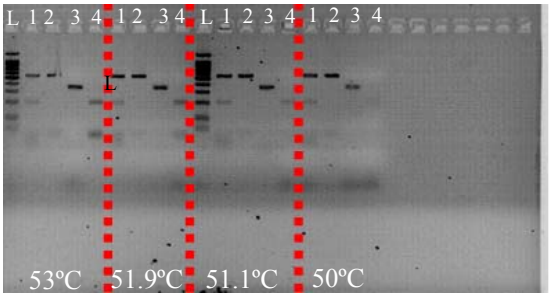

(c)

Figure 5.11: 1.5% agarose gel of PCR using different annealing temperature.(a) Gel for PCR products using annealing temperature from 55.6ºC to 60ºC; (b) Gel for PCR products using annealing temperature from 55.6ºC to 60ºC;(c) Gel for PCR products using annealing temperature from 55.6ºC to 60ºC. Lane L: 100bp ladder. Lane 1: Multiplex PCR of 500 bp, 300 bp and 200 bp products. Lane 2: 500 bp products. Lane 3: 300 bp products. Lane 4: 200 bp products.

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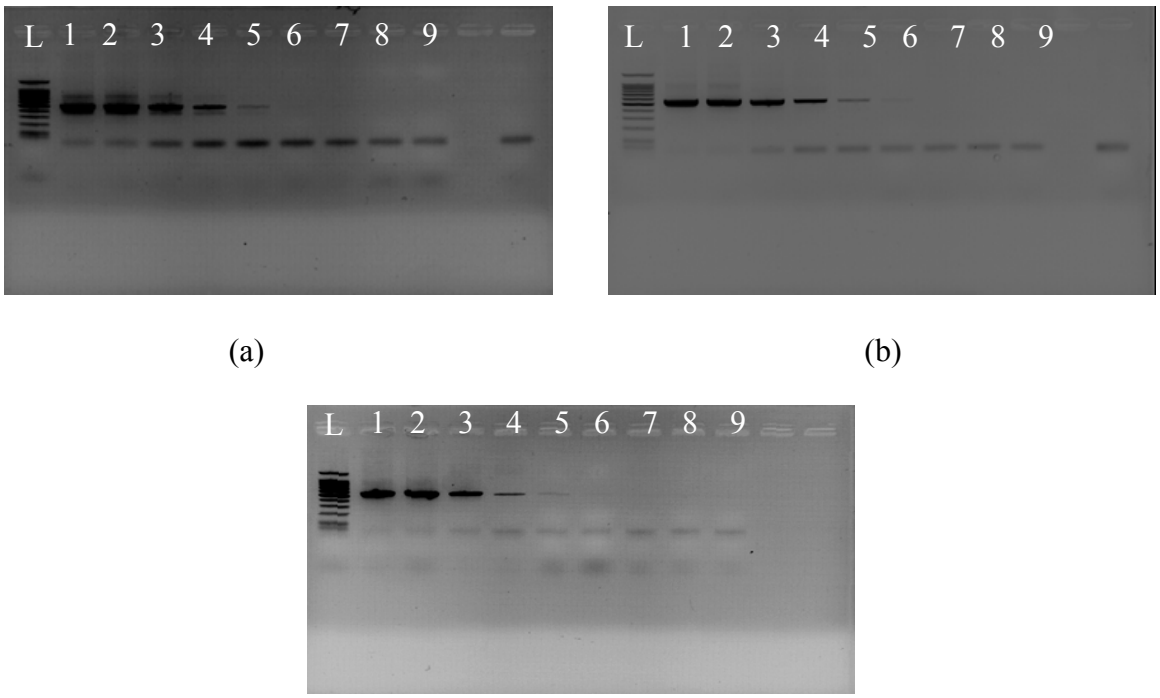
5.1.7.3 PCR on different *E coli* dilutions

DNA templates from *E coli* with concentration ranging from 1 to 10^8cfu/ml were used as template for PCR. Conventional PCR showed that the detection limit was 10^4 cfu/ml for 20 to 30 cycles (Figure 5.12). In order to minimise PCR experiment time, 20 cycles were used for the rest of the symmetric PCR experiments unless otherwise stated.



(a) (b)

(c)

Figure 5.12: 1.5% agarose gel of PCR using different template concentration and number of cycles. (a) 30 cycles; (b) 25 cycles; (c) 20 cycles. Lane L: 100 bp ladder. Lane 1: 10^8 cfu/ml. Lane 2: 10^7 cfu/ml. Lane 3: 10^6 cfu/ml. Lane 4: 10^5 cfu/ml. Lane 5: 10^4 cfu/ml. Lane 6: 10^3 cfu/ml. Lane 7: 10^2 cfu/ml. Lane 8: 10 cfu/ml. Lane 9: 1 cfu/ml

5.1.7.4 Surface Passivation

Adsorption phenomena played a leading role in the inhibition of PCR in non flow through silicon based chips [118] because of their large surface to volume ratios [145]. This would contribute to the PCR inhibition through non-specific sequestering of Taq polymerase [197]. One method for reducing Taq polymerase adsorption on the chip walls was the use of BSA as a standard chemical deterrent of polymerase inhibition and enhancer of PCR in various reports [198,199]. Previous reports [198,199] had shown that the addition of titrated amount of BSA could effectively counteract the polymerase adsorption. Using the 2-Mask PCR chip on Attocycler^{TM} genetic analyser based on PCR

protocol as described previously, titration experiments to determine the optimal BSA concentration for the chip's operation were conducted.

The experiment results were obtained from a series of conventional and chip based PCR experiments carried out with different BSA titration (Figure 5.13). The amplification efficiencies at all BSA concentrations for conventional PCR were very similar, whereas, the efficiencies for chip PCR were only comparable with the conventional thermal cycler at a final BSA concentration of 1µg/µl. The use of higher concentrations of BSA resulted in lower yield [204]. Similarly, lower concentration of BSA also resulted in lower amplification efficiency as the BSA concentration was not sufficient to counter the polymerase adsorption.

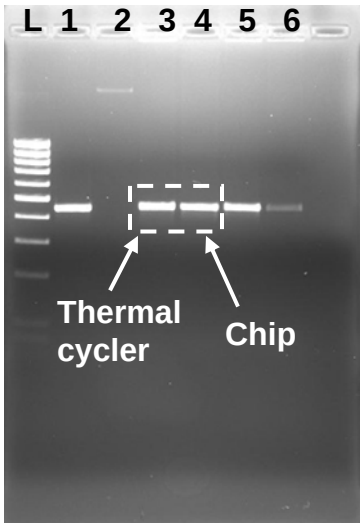


Figure 5.13: Gel Electrophoresis (1.5% Agarose Gel) to compare between conventional PCR and chip PCR. Lane L: 100 bp ladder. Lane 1: Conventional PCR with BSA 0.1 µg/µl. Lane 2: Chip PCR with BSA 0.1 µg/µl. Lane 3: Conventional PCR with BSA 1µg/µl. Lane 4: Chip PCR with BSA 1µg/µL. Lane 5: Conventional PCR with BSA 10µg/µl. Lane 6: Chip PCR with BSA 10 ug/ul.

5.1.8 µPCR Chip Amplification

With the addition of 1µg/µl of BSA into the PCR protocol, successful amplification of PCR using the µPCR chip was demonstrated. A comparison between PCR efficiency by conventional PCR and the µPCR chip (same PCR protocol) showed a lighter intensity band for the µPCR chip in the 1.5% agarose gel (Figure 5.14). This implied that the µPCR chip had a lower efficiency than conventional thermal cycler which corresponded to a study done [200]. These reports showed that even together with titrated BSA, additional amount of Taq polymerase (at least 2x) had to be added to the PCR master mix to allow efficiency to be comparable with PCR products from the conventional thermal cycler.

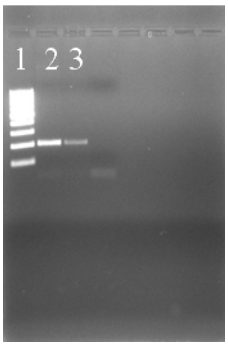


Figure 5.14: Gel Electrophoresis (1.5% Agarose Gel) to compare PCR between conventional thermal cycler and µPCR chip. Lane 1: 100 bp DNA ladder; Lane 2: PCR product from conventional thermal cycler; Lane 3: PCR product from µPCR chip.

5.1.8.1 µPCR Operating Procedures

The cleansing step was added in the operating procedure to ensure repeated use of the µPCR chip was achieved. With the cleansing step after each PCR reaction on our chip, successful amplification at different denature temperatures and different lengths (500 bp and 200 bp) were demonstrated consecutively (Figure 5.15). This showed that there was no cross contamination between reactions and cleansing protocol was effective. In

addition, the use of water/air/sample/air/water interface during PCR helped to reduce evaporation without using oil. This would eliminate the need of an additional device for oil removal and was especially crucial during the integration of the µPCR and DNA microarray. Any additional device used would not only complicate the operating procedure but also caused loss of sample. The water zone was spatially separated from reagent and sample by an air zone, providing a barrier to cross contamination and dilution.

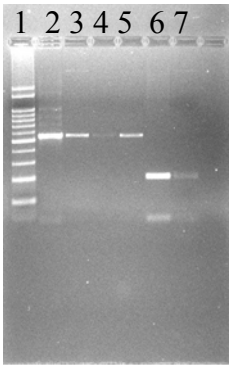


Figure 5.15: Gel Electrophoresis (1.5% Agarose Gel) to compare PCR between conventional thermal cycler and µPCR chip for consecutive amplification. Lane 1: 100 bp DNA ladder; Lane 2: Thermal Cycler: 500 bp; Lane 3: µPCR chip: 500 bp 94ºC; Lane 4: µPCR chip: 500 bp 96ºC; Lane 5: µPCR chip: 500 bp 95ºC; Lane 6: Thermal Cycler: 200 bp; Lane 7: µPCR chip:200bp 94ºC

5.2 DNA Microarray

5.2.1 DNA Microarray Design

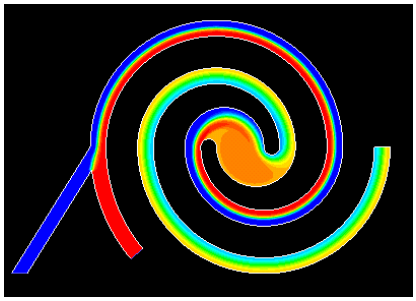
Several features were added into the DNA microarray design to speed up the hybridization reaction. One of the features was the use of low channel height of 50 µm. Study [205] had shown that with continuous flow of DNA target to probes through a small channel depth of 100 µm in contrast to bigger channel depth could help to reduce the time to reach hybridization equilibrium. This was attributed to faster convection near

the surface in smaller channels, due to parabolic velocity profile, which tend to reduce the magnitude of the target depletion zones and increases the overall rate of reaction.

The other feature was the DNA microarray's serpentine channels which allowed the use of continuous flow concept where DNA targets could be constantly sent to the probes. The passive mixers were also incorporated into the design to allow efficient mixing of hybridization buffer and PCR amplicons for hybridization to work.

5.2.2 Numerical Stimulation of Mixer Design

Four different mixer designs were evaluated using Fluent 6.1. Based on simulation as shown in Figure 5.16a-d, design 4 showed the best mixing efficiency. However there was still incomplete mixing for design 4 as shown by the presence of different layers of colours at the end. To further improve on mixer's efficiency, two sets of such mixers were included in the DNA microarray to enable complete mixing. The final design is shown in Figure 5.16e.



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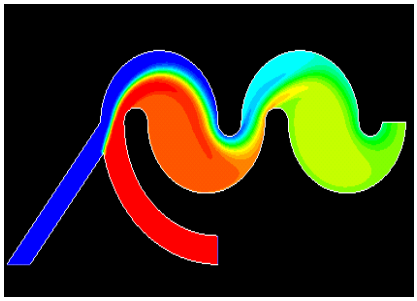

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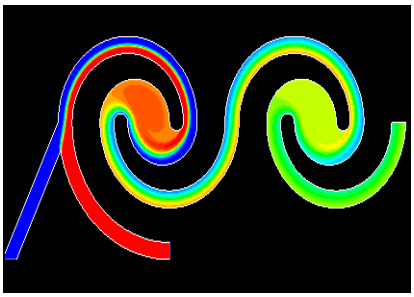

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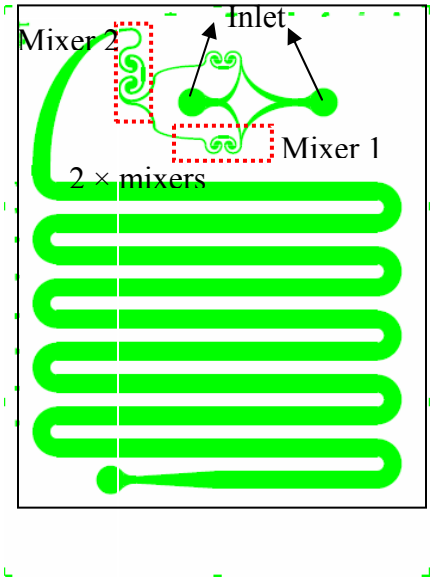
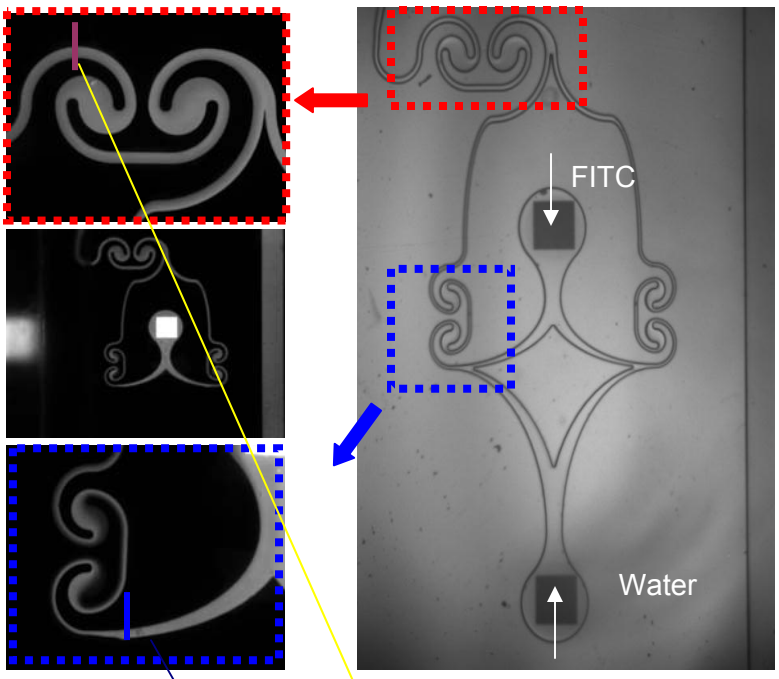


(e)

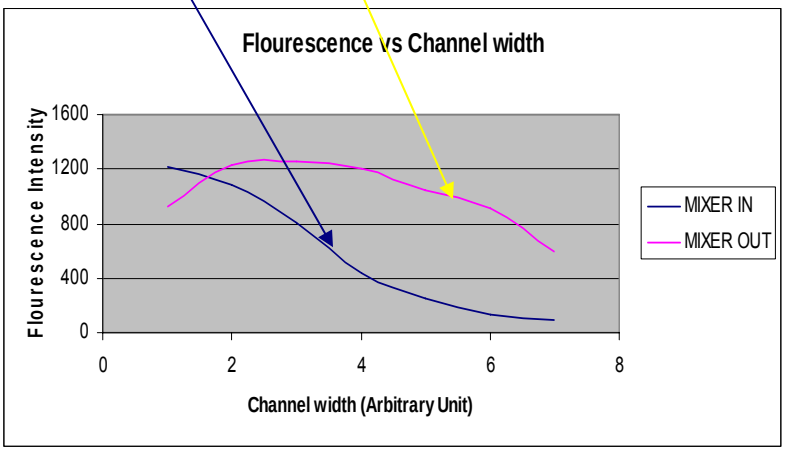
Figure 5.16: DNA microarray mixer design. (a) Mixer design A; (b) Mixer design B; (c) Mixer design C; (d) Mixer design D; (e) Final design

5.2.3 Mixer Testing

To further evaluate and compare the efficiency of the DNA microarray mixers, FITC and water were input into the two inlets of microarray to determine the mixing capability. The fluorescence intensity across the width of the channel started off with approximately high intensity (≈ 1200) at one end(due to FITC) of the channel and low intensity (0) at the other end (due to water) but it leveled off after passing through the two mixers (Figure 5-17a and b). This result demonstrated that the mixer was able to perform its function.



(a)



(b)

Figure 5.17: DNA microarray mixer testing. (a) DNA microarray mixer with water and FITC; (b) Intensity of fluorescence across the channel width at 2 positions

5.2.4 Hybridization with Synthetic DNA Target

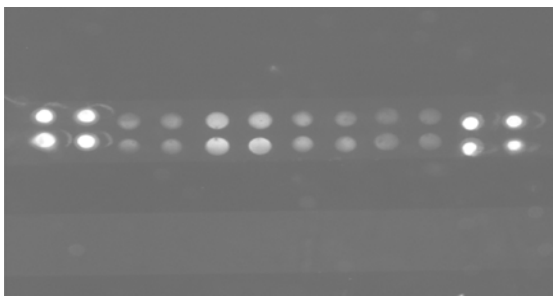
Ultimately the use of hybridization between DNA and DNA probes for bacterial identification relies on good discrimination between perfect match (PM) duplexes and those containing mismatch nucleotides (MM). As complete discrimination is often difficult to achieve, non specific hybridization must be quantified in order to establish its contribution to signal intensity. One approach has been to compare hybridization of the complementary probe with probes having one or two MM nucleotides. Optimization of hybridization conditions was done to establish good discrimination between PM and MM.

Even though the ultimate goal of our DNA microarray was to apply it on microbial targets, the use of synthetic DNA target before the use of PCR amplicons would enable the understanding of the general trend and possible optimal conditions required to differentiate between single nucleotide variations. This study was effective in providing the relevant information as shorter strands would allow more efficient hybridization results [206]. It was expected that with longer strand of PCR products, the optimal hybridization conditions may differ slightly.

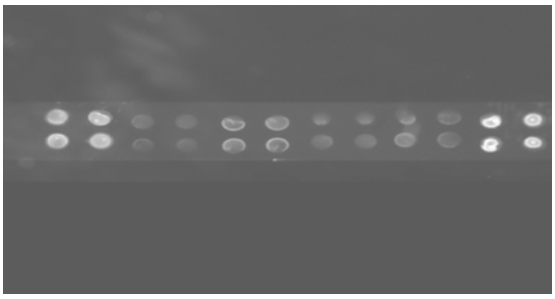
The study on concentration of synthetic DNA target and flow rate was determined as initial experiment conditions and was fixed to study the concentration of NaCl and formamide (FA). Different concentrations of NaCl and FA in the hybridization buffer were separately evaluated for their ability to maximise the difference between the PM and MM hybridization kinetics. Concentration of DNA target and flow rates were fixed as these factors were not critical and they would be totally different when microbial DNA targets were used.

5.2.4.1 Determination of Synthetic DNA Target Concentration

Arbitrary values of 0.02µM and 0.1µM targets were compared in this experiment. The use of synthetic target of concentration of 0.1 µM did not cause over saturation of the background and there was no problem during continuous data acquisition during the hybridization process (Figure 5.18a and b). It was estimated that the hybridization required 1 hour to completion. Comparing the graphs from Figure 5.18a and b, the use of 0.02 µM targets would require a longer time to reach hybridization equilibrium when compared to the usage of 0.1µM target. 0.1 µM synthetic DNA target was chosen as the fix parameter in the hybridization experiment.



(a)



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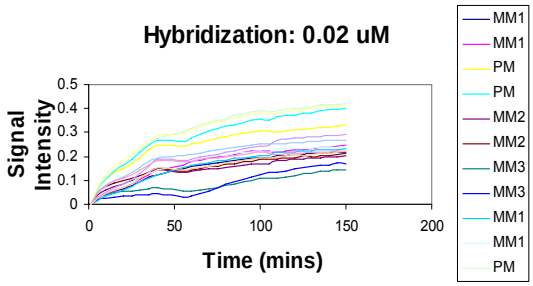

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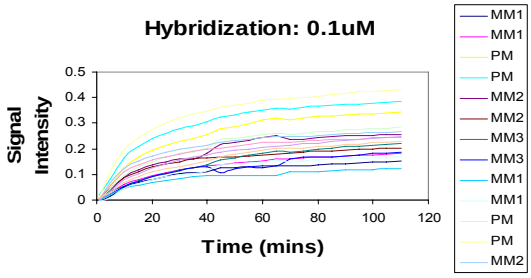


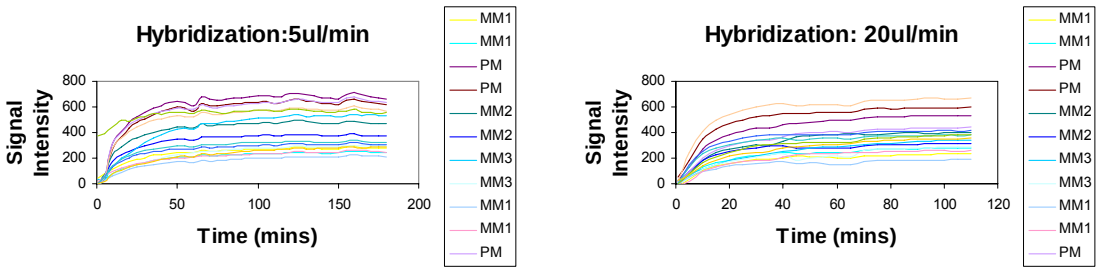
(d)

Figure 5.18: Hybridization using different synthetic target. (a) Hybridization using 0.02 µM Target; (b) Hybridization using 0.1 µM Target

5.2.4.2 Determination of Flow Rate

A higher flow rate required a shorter time for hybridization to complete [194], however this reduction in hybridization time was offset by a greater increased in hybridization buffer required. An arbitrary value of 20 µl/mins was chosen based on studies done [194]. In order to ensure sufficient time was given for interaction of target and probes, a slower flow rate 5 µl/mins was used for comparison.

Experimental results (Figure 5.19a and b) showed similar maximum intensity attained for both speeds. This implied that there was no significant difference in providing sufficient time for proper interaction between the matching targets and probes for hybridization at the flow rate of 5 µl/mins and 20 µl/mins. The flow rate of 20 µl/mins was chosen for hybridization experiments for faster reactions. Thus the initial experiments settings for hybridization were run for 1 hour to near hybridization completion using 0.1µM DNA target at 20µl/min for different concentrations of NaCl and FA.



(a) (b)

Figure 5.19: Hybridization at different flow rate (a) Hybridization with flow rate of 5 µl/min;(b) Hybridization with flow rate of 20 µl/mi

5.2.4.3 Determination of NaCl Concentration

Monovalent cations in this case sodium ions interact electrostatically with nucleic acids (mainly at phosphate groups) so that the electrostatic repulsion between the two strands

of the duplex decreases with increasing salt concentrations. i.e. higher salt concentrations increase the stability of the hybrid but reduce the specificity. Experimental results (Figure 5.20a-c) showed that the optimal concentration of 100 mM NaCl gave the best DI of 1.6 (Figure 5.20d). It was also noted that with lower concentration of NaCl, the hybridization buffer becomes more stringent resulting in better discriminating factor but lower signal was generated.

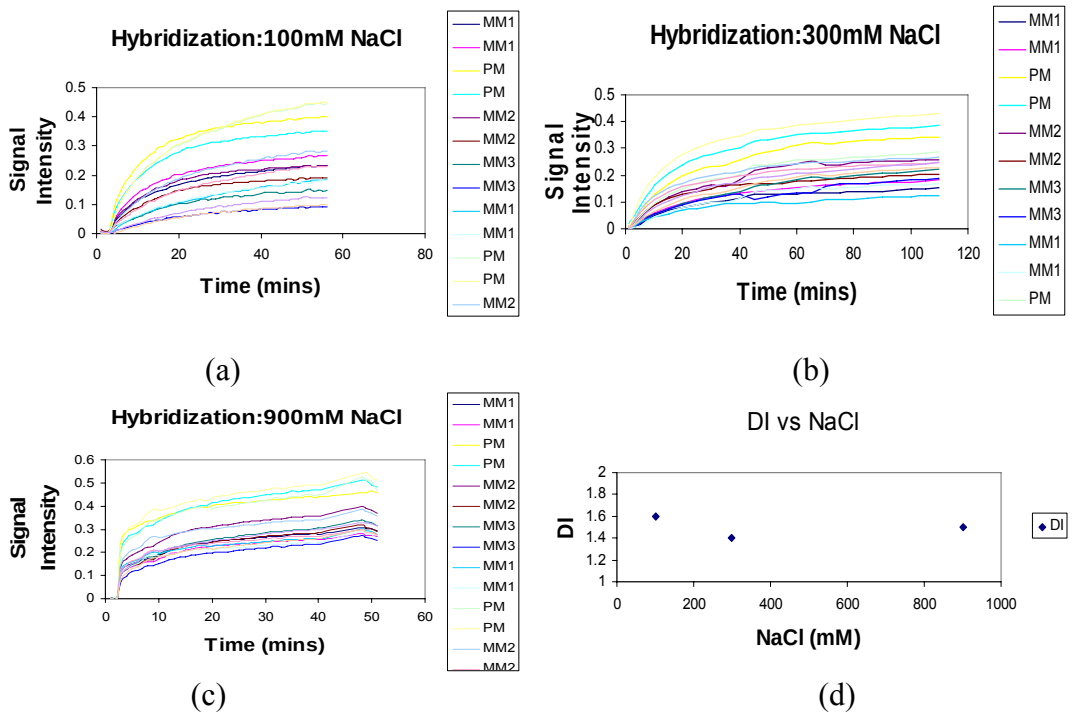
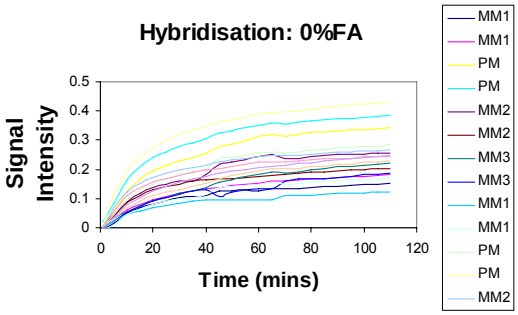


Figure 5.20: Hybridization curves of different NaCl concentration. Signal intensity normalized using Cy3 control probes (a) NaCl 100 mM;(b) NaCl 300 mM; (c)NaCl 900 mM; (d) DI at different NaCl concentration

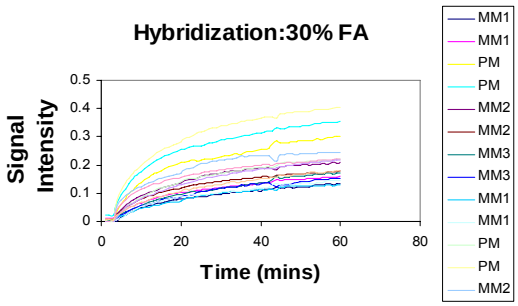
5.2.4.4 Determination of FA Concentration

FA is able to reduce the melting temperature of DNA-DNA and DNA-RNA duplexes in a linear fashion by 0.72 ºC for each percent FA. Thus hybridization can be performed at room temperature with FA present in the hybridization buffer. The renaturation rate

decreases in the presence of FA. A suitable concentration of 30% based on the experiments (Figure 5-21e) was chosen to allow hybridization with high specificity. From data obtained from hybridization curves (Figure 5.21a–c), it was also observed that as the FA concentration increases, the hybridization buffer became more stringent. However at the same time, the signal intensity dropped as FA concentration reached a critical value. For this set of experiments, this occurred at 60% FA as the signal to background ratio (S/B) was only slightly more than 1. The optimal conditions used for synthetic DNA target was 100 Mm NaCl and 30% FA. More importantly, these studies had provided the general trend of the two parameters which could be applied when hybridization was carried out with microbial targets



(a)



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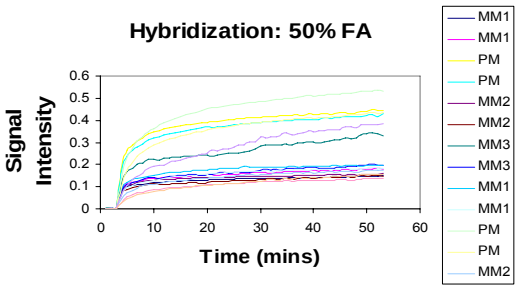

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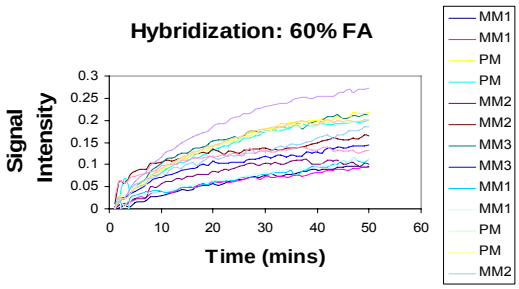

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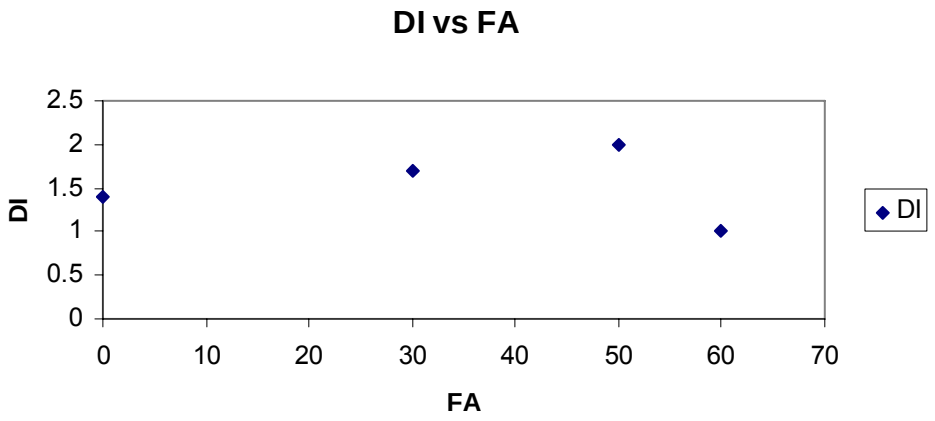


(e)

Figure 5.21: Hybridization with different FA concentrations. (a) 0% FA; (b) 30% FA; (c) 50% FA; (d) 60% FA; (e) DI with respect to FA concentration

5.3 Micro Total Analysis System

The concept of the micro total analysis system based on nucleic acid based detection of pathogen consisted of a μPCR chip integrated with a continuous-flow based micro-array as illustrated in Figure 5.22. Sample preparation was done off chip because targeted pathogens were often too diluted in environments and thus large sample volumes were needed, depending on the infectious dose and minimal detection requirements. The sample volume necessary to filter or concentrate sample volumes could range from 100 ml for bacteria and up to 1000 L for viruses. A secondary purification and concentration step was also necessary to selectively capture and separate target pathogens from the rest of particles in sample.

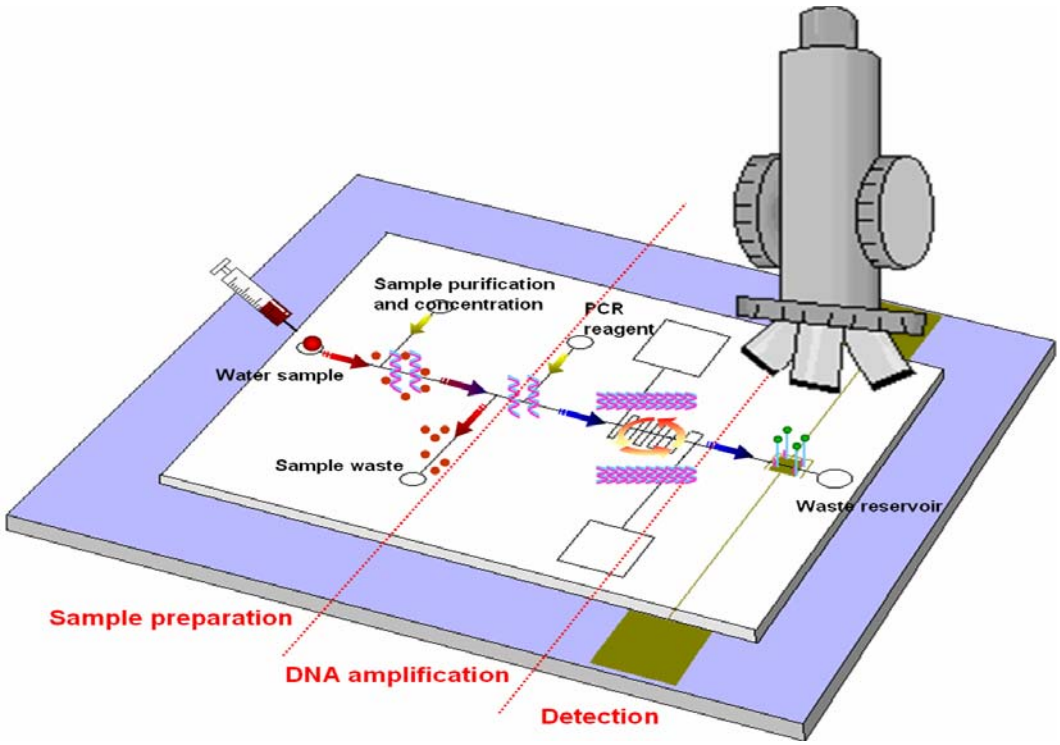


Figure 5.22: Concept of nucleic acid based microsystem

5.3.1 Asymmetric PCR

5.3.1.1 Asymmetric PCR using Thermal Cycler

Asymmetric PCR is a PCR process in which the predominant product is a single-stranded DNA, as a result of unequal primer concentrations. As asymmetric PCR proceeds, the lower concentration primer is quantitatively incorporated into double-stranded DNA. The higher concentration primer continues to primer synthesis, but only of its strand. Single stranded DNA can be produced for this purpose using asymmetric PCR, in which the two primers are used in 100:1 ratio, so that after 20-25 cycles of amplification, one primer is exhausted so that single stranded DNA is produced in the next 5-10 cycles [207]. Thermal cycler was used to run the asymmetric PCR. Volume of PCR mixture used was 10 µl and 35 cycles were used during thermal cycling. The changes in PCR protocol are reflected in Table 5.2.

In our experiments, a high concentration of the labeled primers was required to generate enough single strands for detection. The ratio of forward labelled primers: Reverse primers were 100:1. The increase in DNA polymerase Taq together with the use of dynamic coating BSA was used to counter adsorption of Taq by the large surface of silicon chip as explained previously. Additional DNTP was also added in consideration of the large number of cycles needed for asymmetric PCR.

Studies had also shown that microarray hybridization efficiencies and specificity decreased with long PCR amplicons (200 bp) [206]. Thus to improve on hybridization, a high concentration of NaCl (900mM) was used with the total hybridization buffer volume of 10 µl to reduce DNA target dilution. End point detection instead of continuous data acquisition was carried out as high background generated by the high concentrations of labeled primers prevents continuous data taking as shown in Figure 5.23. The asymmetric PCR protocol was analyzed through hybridization on DNA microarray (next section).

Table 5.2: Asymmetric PCR protocol

Primers Conc	Other Reagents	Hybridization buffer volume	Signal	Hybridization time
4uM;0.4 uM	As per protocol in section 4.3.2	100ul	No	1 hr
4uM;0.4 uM	As per protocol in section 4.3.2	100ul	Yes	Overnight
4uM;0.4 uM	Taq 2x	100ul	No	1 hr
5uM;0.5 uM	Taq 2x; DNTP 2x	10ul	Yes	30 mins

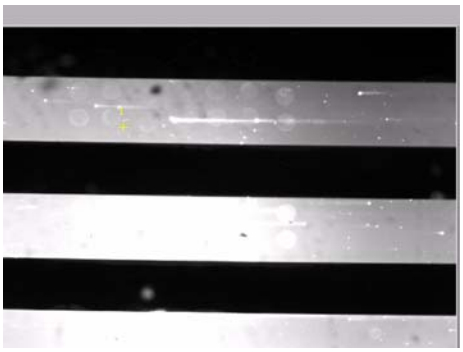


Figure 5.23: High background during hybridization

5.3.1.2 Asymmetric PCR using µPCR Chip

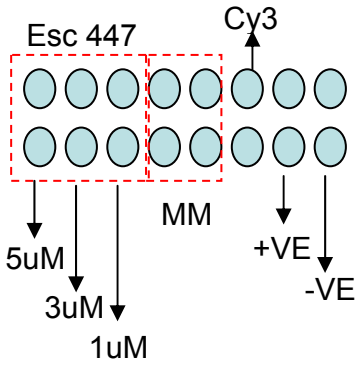
Based on conventional asymmetric PCR protocol, the µPCR chip was used to run asymmetric PCR. However due to the adsorption of polymerase by the PCR chip, the number of cycles used was increased from 35 to 45 (as in contrast with 35 cycles in thermal cycler) to improve the chip's efficiency. On the other hand, due to the fast heat transfer of silicon, the denature time was reduced to 10s, annealing time to 30s and extension time to 30s. The PCR product was analyzed by hybridization using DNA microarray. The results will be shown in the next section.

5.3.2 Hybridization with Microbial Target

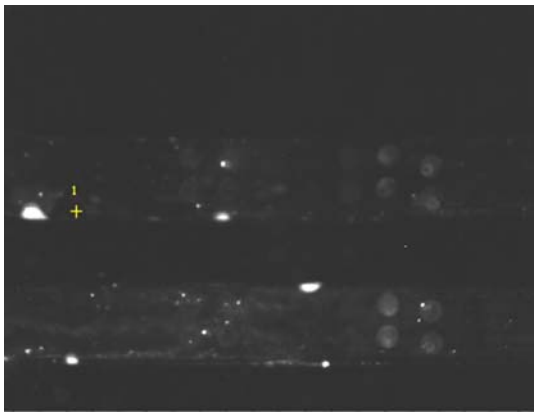
5.3.2.1 Hybridization with Microbial Target from Thermal Cycler

In order to facilitate direct transfer of PCR amplicons from PCR chip to microarray, asymmetric PCR was adopted. Before asymmetric PCR was conducted on the µPCR chip, conventional asymmetric PCR was done to study the protocol. The success of the asymmetric PCR was based upon the hybridization signals from the DNA microarray when the PCR amplicons were applied to the DNA microarray.

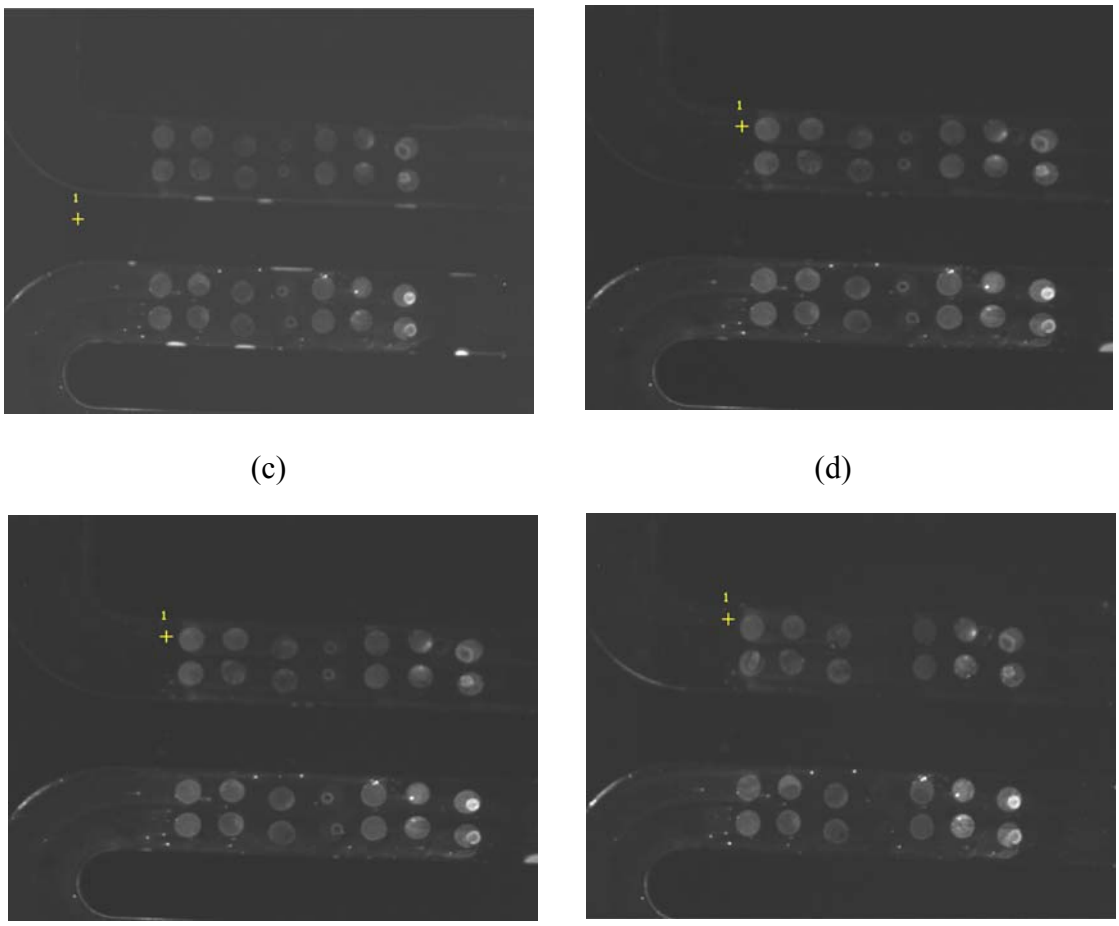
Hybridization results from DNA microarray showed positive signals of hybridization with 500 mM and 900 mM NaCl. The signals to background noises (S/B) were 16 and 28 respectively. The need for higher concentration of NaCl was most probably due to the longer PCR amplicons trying to hybridize to their matching probe. Effects such as steric/repulsive effect became more prominent in longer strands as compared to shorter strand. However at high NaCl concentration, the hybridization conditions became less stringent and additional washing steps became necessary to generate a better DI value. Thus additional washing steps were carried out after hybridization for about 10 mins after hybridization using 900 mM NaCl and 30% FA. A stringent washing solution comprising of 50 mM NaCl and 30% FA was able to raise the DI from 1 to 2. Figure 5.24f shows that with addition of washing steps, the DI becomes higher.



(a)



(b)



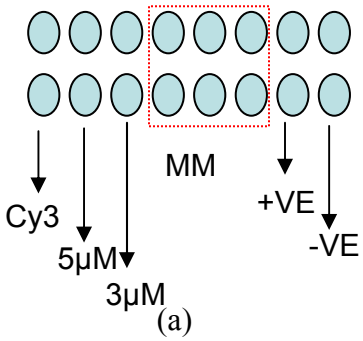
(c) (d)

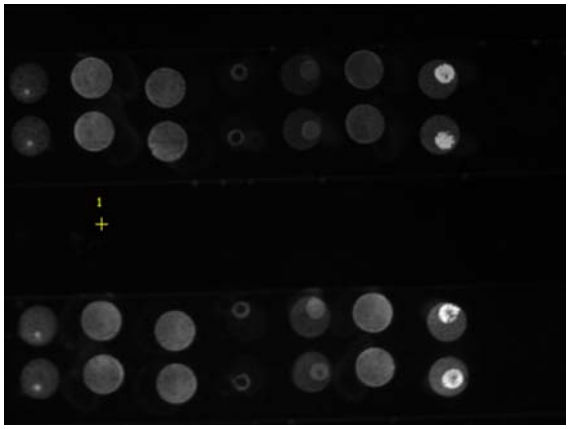
(e) (f)

Figure 5.24: DNA microarray microbial target hybridization. (a) Probes format in each row; (b) NaCl: 300 mM; FA:30%: DI<1 Signal/Background (S/B) <1; (c) NaCl:500Mm; FA:30%;DI = 1.6 S/B=16; (d) NaCl: 900Mm: FA: 30%; DI = 1.1 S/B=26; (e) Washing:NaCl: 100 mM DI = 1.3;S/B=22; (f)Washing: NaCl: 50 mM DI = 2.3;S/B=18

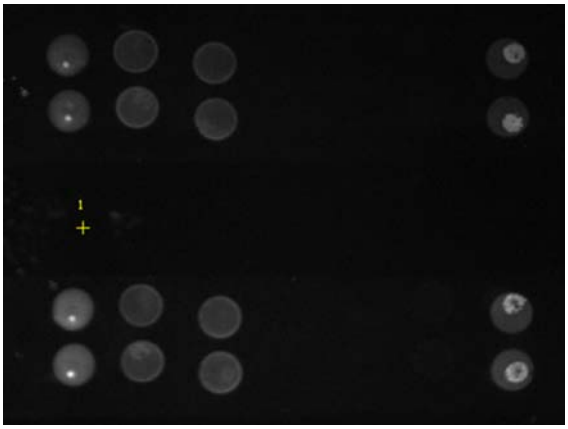
5.3.2.2 Hybridization with Microbial Target from µPCR Chip

Asymmetric PCR were conducted on the µPCR chip which contained 10 µl of sample. The hybridization buffer volume was 10 µl and was made up of 900mM NaCl, 30% FA and 0.02 M Tris HCl pH 8. Successful hybridization (Figure 5.25) with DI factor of more than two was achieved after washing with 50 mM NaCl and 30% FA for 10 mins.





(b)



(c)

Figure 5.25: Hybridization with targets from μPCR chip. (a) Probe format in each row; (b) After hybridization; DI=1.6; S/B=2.07; (c) After washing DI=14; S/B = 25

5.3.3 Integration of μPCR Chip with DNA Microarray

Successful amplification by the μPCR chip and hybridization and detection of microbial target *E coli on* DNA microarray were demonstrated separately in the previous sections. However the ultimate objective was to combine both chips into an integrated device to form a micro total analysis system which could perform amplification to detect water borne pathogen.

To enable integration, an acrylic housing as described previously was used to house both chips together. The chips were linked together through embedded channels in the acrylic housing where PCR amplicons can be transfer to the DNA microarray through the use of syringe pump. The hybridization buffer was also pumped into the microarray device for passive mixing with PCR amplicons before flowing towards the DNA microarray for hybridization. Both chips adopted the use of serpentine channels to facilitate continuous flow which provided the means of mass transportation, through convection and flow induced increment of diffusion coefficient, expecting to reduce the hybridization time and overall analysis time. This would also enable maximum recovery of samples from the PCR chip to DNA microarray. Asymmetric PCR was use to enable direct transfer of product from PCR chip to DNA microarray for hyrbridization. In additional, the use of water/air/sample/air/water fluidic zone also facilitates the above.

E coli was used as the target pathogen sample and amplification of DNA using the PCR chip was completed within 100 mins. Thereafter the amplified PCR products of 10 µl were transferred to DNA microarray at 1 µl/min and were passively mixed with the hybridization buffer (900 mM NaCl 30% FA) which was pumped into the DNA microarray through another inlet at 1 µl/min. The hybridization was allowed to run for 30 to 40 mins and end point detection was done using fluorescent microscope.

Successful detection with DI value of more than two was achieved with *E coli's* sample (Figure 5.26) using hybridization buffer and washing buffer based on previous results. The total analysis time was completed within the 3 hours.

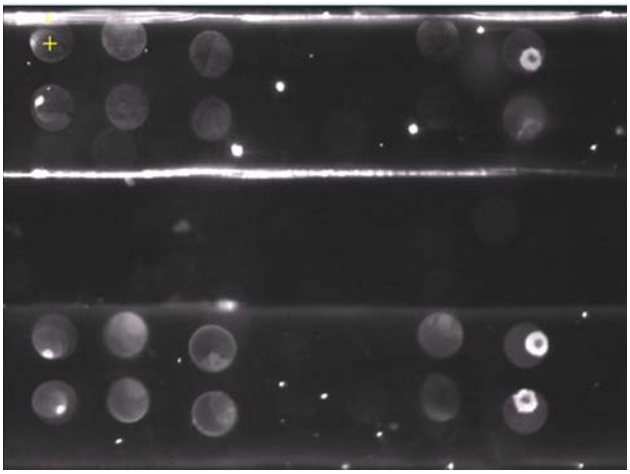


Figure 5.26: Hybridization results from micro total analysis system; D.I = 2; S/B = 18

CHAPTER 6 DISCUSSIONS

6.1 µPCR Chip

6.1.1 PCR Speed

In many cases such as waterborne pathogen detection, amplification is essential for nucleic acid analysis. Although there are various nucleic acid amplification techniques available, PCR has been the most popular due to its simplicity; however traditional PCR instruments are characterised by huge thermal mass and thus slow heating and cooling rates (1-2ºC/S) [217]. But the slow PCR speed can be improved by miniaturization using MEMS technology [12,15]. The miniaturized PCR chip described in this study was fabricated with MEMS technology using silicon substrate integrated with side heaters. These features had enabled fast heat transfer (15 ºC/s) and cooling (8 ºC/s) but in comparison with some of the reported works [36, 132, 209] the heat transfer was slightly slower by about 5 ºC/s. This slower thermal speed was most probably attributed to the use of the acrylic block which increased the overall thermal mass of the PCR system. The use of the acrylic block was necessary as it was a convenient way to inject and extract sample to and from the PCR chip. This method of injection and extraction provided a more consistent recovery method than other means [78] which included the use of silicon tubes/ pipette tips bonded to the access port of the chip.

Another advantage of PCR chips over conventional systems was their efficient heat transfer, which ensured that the heat source temperature was distributed almost instantly across the whole PCR chamber [210,211]. This fact was exploited to reduce the denature and extension time to 10s and 30s respectively for the PCR chip. However annealing time

remained unchanged as study [200] had demonstrated that PCR specificity improved when annealing times were decreased, but the overall performance of the reaction seemed to be negatively affected by fast cycling, producing inferior products than control reactions. A feasible explanation for this effect might reside in the fact that, in conventional reactions, extended annealing takes place during the relatively slow cooling and heating transients immediately before and after the annealing hold period. Conversely, in chip PCR with very fast transients, this principle did not hold and reaction efficiency became hampered due to low annealing rates [211]. Therefore, to obtain yields comparable to those of standard reactions, primers must be carefully selected to ensure that effective annealing took place during the short holding times, and the annealing temperature must be carefully titrated to obtain optimal results. Thus not all PCR reactions may be effectively adapted for fast PCR [200]. In view of these reasons, a straight comparison between different PCR devices in terms of total time and consequently yield was difficult.

6.1.2 Temperature Control

The thermal performance of the PCR chip is not only dependent on the chip design but also its temperature control. Although PCR can be carried out with relatively low accuracy (±1.5 ◦C [78]), the perceived understanding is that a more precise temperature control will improve both reaction yields and specificity [211]. Moreover, given the low thermal mass of the μPCR chips and the fast transients sought, reported control methods that consisted of simple PID/PI [214] algorithms implemented would tend to cause overshoots that could be, particularly at denaturing temperatures, severely detrimental to

the PCR. In order to achieve precise temperature in this study, the application of gain scheduling along with digital feedback PI control was used. As PCR requires repetitive thermal cycling at three temperature set points, gain scheduling was a good approach to provide satisfactory control for a different operating point of the system [216]. With this system, an average precision of as low as ± 0.2 ºC precision with minimal overshoot (approximately 0.2ºC) was achieved. The adopted control strategy was able to achieve at least comparable results to those previously reported [95, 211].

6.1.3 Chip Design: Chamber Geometry

The issue of reagent insertion/extraction is of critical importance in PCR chip since PCR is often a preliminary step in many analytical procedures and the amplified product must be recovered for further analysis. Chamber geometry plays an important factor in this aspect. A serpentine like PCR chamber was used in this study and was able to consistently recover 70 to 90% of the sample from the PCR chip. In comparison, other chamber geometries, such as rectangular and rhomboidal shaped chamber could only recover a maximum of 60% of the sample [200]. This extraction limit was probably caused by the formation of a low-resistance air conduit between the input and output ports. In essence, once a certain amount of the inserted liquid had been extracted, the formation of an airway between input and output ports becomes practically unavoidable and, once in place, the air conduit would impede the further extraction of reagents, which remain stuck at the chip walls [214].

6.1.4 Evaporation

Sample evaporation is often problematic because PCR volumes are usually very small. This will occur especially at denaturation temperatures which approach 100°C. At this temperature, the sample evaporation is so rapid that the sample would dry up quickly under standard atmospheric pressure. To circumvent such evaporation, the application of water/air/sample/air/water interface was used in the PCR chip in this study. The use of such method saw a consistent recovery of 70% to 90% of the sample for gel analysis and was also applied as part of the integration solution. This approach was different from the use of mineral oil cover layer which was frequently used as a vapour barrier to prevent evaporation [90, 93, 99, 156]. The mineral oil is a suitable liquid cover because it has a boiling point far above 100°C and a density slightly below 1.0 g/cm^3. However, its applicability is questionable for highly integrated PCR systems. Another approach is the use of a solid cover or valve to resist the internal pressure generated during PCR [60, 94,104, 105]. However the use and fabrication of valve is complex and may be difficult to control and maintain [215].

6.1.5 PCR Amplification

In order to enable successful PCR amplification by the PCR chip, the fundamental issue of adsorption phenomena in silicon chip must be overcome [145]. Previous findings [198, 199] had shown that the addition of titrated amounts of BSA could effectively counteract this adsorption [118]. The use of a final concentration of 1µg/µl of BSA in this study was able to lead to successful amplification. This was comparable to various studies reported [200,213]. Nonetheless, even when using the titrated BSA concentration, the experiments

on PCR chip amplification showed lower product yield than those from the conventional thermal cycler. This effect had been previously reported [198] and was probably due to the residual polymerase adsorption (by the chip surface) that persisted under BSA blocking that may be enough to hinder PCR efficiency. However in general, there was consecutive successful amplification of *E. Coli* in the µPCR chip and were in agreement with previously reported amplifications in PCR chips [200]. An additional washing protocol was also implemented between PCR experiments to help to prevent cross contamination and inhabitation which allow the reuse of chip.

6.1.6 Active PCR system (µPCR Chip) vs Passive PCR system (2-Mask PCR Chip)

With appropriate BSA concentration, the PCR product produced by 2-Mask PCR chip with external heat source (passive PCR chips[4]) was as efficient as the conventional PCR. In this aspect, the µPCR chip with integrated heater and sensors (active PCR chips) did not outperform the passive PCR chip in terms of PCR-product efficiency. Although a straight comparison was not done between the two systems, the results were inferred from individual experiments to compare product efficiency between each system and conventional PCR separately.

The above results corresponded to a study done on comparison between passive and active system [200]. A feasible explanation proposed for this effect could be sought in the poorer transition rates and the slightly heavier temperature overshoots of passive PCR chips. Slower transitions and longer stabilization times due to increased overshoots

[4] Passive PCR chip: PCR chip which uses external heat source
Active PCR chip: PCR chip with integrated heater and sensor

generate prolonged extension and annealing periods both during the temperature stabilization phase and the initial stages of transition. Since Taq polymerase is partially active (some two orders of magnitude less) at annealing temperatures and annealing may begin to take place (depending on primer length and composition) up to 5 ºC off the estimated annealing temperature [201], both factors can significantly contribute to an increase of PCR efficiency (though at the loss of some specificity).

In light of the above results, the use of which thermal cycling systems is application dependent. Active PCR chips still presents far better speed (15–20% shorter analysis times in active PCR chips than passive PCR chips[200]) and power consumption rates than passive PCR chip and these are highly desirable in the applications of rapid pathogen detection [202] and the possibility of using our system as a portable PCR [203] in future. In addition, the integration of heating circuitry provides a more scalable technology for the development of multi-chip modules or the integration of additional mechanisms (e.g. sensors or control circuitry).

6.2 DNA Microarray

6.2.1 DNA Microarray Design

Since the diffusional mobility of DNA fragments is very low [173], static hybridization requires a long period (usually overnight) to run to completion. In order to speed up hybridization reaction, several features as described previously were added into the DNA microarray design. The use of such features was able to bring the hybridization of synthetic targets to run to completion within 60 mins and asymmetric PCR was able to

generate signals for detection within 30 mins. These features were easy to implement in comparison to some of the methods reported [174-176] such as sample oscillations and chaotic advection.

6.2.2 Hybridization

The use of formamide as the denaturant in the hybridization buffer allowed hybridization and disassociation to be temperature-independent thus avoiding the need for precise thermal control that would otherwise required for melting curve analysis. It also minimizes the bias that arises from the temperature dependency of common fluorophores such as the Cy3 dye used in probes/target labeling [212]. But one disadvantage associated with the method is that real-time monitoring of the hybridization/disassociation process will cause photobleaching of the fluorophore due to continuous imaging and repeated exposure of the Cy3-labeled targets to ultra-violet light. This reduces the signal intensity of the PM duplex over both the hybridization and disassociation process. But the impact on the MM duplex is less as most of the targets are dissociated within very short time from the start during dissociation. Overall, the discrimination power will be diminished thus to minimize the effect of photobleaching on the PM and MM kinetics in this study, end point detection was used on hybridization/disassociation process for microbial targets experiments.

6.3 Micro Total Analysis System

The main objective of this project was to develop a micro total analysis system that could potentially be applied for waterborne pathogen detection. The μPCR chip was combined

with a DNA microarray as PCR was necessary to amplify the target DNA to provide adequate PCR amplicons to hybridize in DNA microarray to facilitate detection. This is especially important as water pathogens are usually in low concentration which makes it very difficult to detect.

With this focus, both the μPCR chip and DNA microarray were designed with features to facilitate integration of both chips**.** One of the features included the use of serpentine channels on both chips which enabled smooth transition of sample between both chips. Asymmetric PCR was used as protocol with the water/air/sample/air/water interface to enable the direct transfer of fluid from one chip to another. Another important feature that was considered was thermal isolation**.** Most DNA-based assays are highly temperature sensitive and require precise temperature control. When integrating these analytical components, thermal crosstalk will deteriorate chip device's performance and the thermal insulation is often required. In this study, the μPCR chip was isolated through etching of air gaps in the device itself and in the acrylic housing that combines both chips. Finally both chips were combined using an acrylic block with embedded channels to form the micro total analysis system. This approach of integration was simple and can be easily reproduced.

While there are many works directed at PCR chip and DNA microarray separately, only a few PCR and microarray combined systems has been described and none has shown its application in water pathogen monitoring. One of the reported works [183] was to perform PCR and DNA hybridization sequentially in a micro chamber thus no sample

transfer was needed. However, conducting hybridization following PCR in the same chamber took a long time ranging from several hours to overnight as mass transportation was the rate-determination step for static hybridization. In contrast, the use of continuous-flow hybridization after PCR in the micro total analysis system facilitated mass transportation, through convection and flow induced increment of diffusion coefficient, reducing the hybridization time to less than 1 hour, resulting in the overall analysis time to about 3 hours. This method was also much faster than the detection method based on culture based method which usually will take more than a day.

With this concept, the application of this micro total analysis system for fast detection of water pathogen becomes possible. In light of health (drinking water is typically distributed and consumed before testing) and environmental policies, the need for a faster method than the current culture methods becomes more apparent. The micro total analysis system introduced in this study has the potential to bridge this gap.

CHAPTER 7 CONCLUSION AND RECOMMENDATIONS

7.1 Conclusion

Methods 9131 and 9132 are regulated by EPA to access the microbial safety of water. However, they are time consuming (more than 1 day) as they rely on cultivation of indicator bacteria in laboratory conditions followed by analysis. This project works on a faster method for detection of water pathogen using the concept of micro total analysis system.

In this project, a micro total analysis system based on nucleic acid detection of water pathogen detection has been developed. The system consisted of silicon/glass hybrid based bio devices namely the μPCR chip and DNA microarray. In this system, the PCR device was miniaturized and combined with a DNA microarray. Both of the μPCR chip and DNA microarray were fabricated based on MEMS technology and were integrated at packaged-level using acrylic housing. The μPCR chip consisted of a serpentine channel suspended on a silicon membrane and silicon beams which provided air gaps that isolate PCR reactor from rest of device during integration. Metal heaters and temperature sensors were integrated on chip to allow quick and accurate temperature control (15ºC/s for heating and 8ºC/s for cooling) with high thermal uniformity (± 0.1 to 0.3ºC). The DNA microarray consisted of a passive micro mixer for mixing of DNA amplicons and hybridization buffer and a channel where pathogen genes' capture probes were spotted. Sample was pressured driven and transferred between two chips using embedded channel in the acrylic housing. The serpentine channels of both chips allowed maximum sample recovery from the μPCR chip to DNA microarray.

During PCR, the sample was encapsulated by air zones on both sides and kept in place by water on each end of the air zone. This water/air/sample/air/water zone arrangement helped to reduce evaporation. Moreover as asymmetric PCR produced single-strand DNA, the amplicons were delivered directly to the DNA microarray for hybridization reducing operation steps and time. *E. coli.* was used as the target pathogen sample as it is used in the EPA approved methods. The DNA microarray was spotted with probe Esc447 (specifically targets the genus of *Escherichi*a and *Shigella)* and several other referenced probes with at least 1 base-pair difference. Final results showed that the fluorescence signal of the Esc447 probes was stronger than the referenced probes with a ratio more than two. This represents that *E.coli* can be differentiated from other species even with 1 base-pair difference. In addition to one species monitoring, our system showed potential in direct monitoring of a range of pathogens at the same time through PCR and different probes immobilized on DNA microarray.

In conclusion, the pathogen sample has been successfully detected in our micro analysis system through DNA amplification by the μPCR chip follow by direct transfer of the amplicons to the microarray for detection. The analysis time for our system was completed within 3 hours compared with the cultivation methods.

7.2 Recommendations

7.2.1 μPCR chip

7.2.1.1 Dead Volume in Acrylic Housing

The use of acrylic housing as the interface to link the "world to chip" offers a cheap and simple solution. However it has its disadvantages. The main disadvantage is the dead volume generated in the embedded channels in the acrylic housing especially at 90 º

bends due to the limitation of the fabrication process. Although this issue was not critical as the device was still able to fulfill its objectives, this problem was raised for awareness. Even though it could not be solved currently as the vendor did not have the necessary technical capabilities, it provided information to consider other material/fabrication method or even integration solution alternatives.

7.2.1.2 Operation Procedures

Another issue was the operation effectiveness of the device. As illustrated in the operating procedure protocol as attached in Appendix, the set up of the device to run the PCR required a number of steps. One of the most problematic portions of operation was to control fluid movement and the sequence of fluid flow. A possible alternative method is to make use of passive valve incorporate into the device/housing which is self operating or automated valves to minimize human intervention and thus improve consistency during operation. This may also help to bring the solution a step closer to a fully automated solution. However the use of integrated valves may imply the need to consider the change of the total design of housing as suitable fabrication process and material have to be considered.

7.2.2 DNA Microarray

7.2.2.1 DNA Microarray Bonding

The DNA probes were spotted on the microarray's channels by micro spotting before the device was enclosed by the glass wafer on top. This bonding was done manually by usually PDMS as an "adhesive to bond glass and silicon chip. The bonding was able to

fulfill the purpose of encapsulation and was feasible for protyping. However a standardized and consistent bonding procedure may be required for mass production to ensure repeatability and good quality control. One possible method is to use UV cure bonding between wafer and glass after probes are micro spot on the DNA microarray. The region to where the probes are spotted can be covered by using a mask to prevent UV exposure. But in order to do this, micro spotting has to be done at wafer level. Currently micro-spotting can only be done at chip level. Micro spotting at wafer level may require modification on fixture of micro spotting machines and software.

7.2.2.2 Hybridization Efficiency

The probes that were used for hybridization were approximately 18 bp oligonucleotide. Possible improvement of hybridization efficiency could include the use of spacers to the probes to increase the height and thus decrease the distance between the targets and probes or using bead based device [194] which could improve hybridization.

7.2.2.3 Detection Limit

Detection limit was not recorded in this thesis due to the difficulty of getting an accurate value. This difficulty lies in the accurate quantification of single strand (DNA) and this was demonstrated through the use of spectrophotometer. The data obtained showed wide variations and non repeatability in single strand quantification. Moreover this is further complicated by different parameter settings (e.g. exposure time) during fluorescence imaging which could lead to false interpretation of results, Thus to truly reflect the detection limit of the device, a more suitable approach has to taken. One suggestion for

accurate quantification might be the use of synthetic DNA strand which can be used as a quantity reference during detection limit testing. In addition to this, one should also determine the correct data (intensity) and formulation to extract and use after fluorescence imaging to obtain the true detection limit of the device.

7.2.3 Micro Total Analysis System

The peripheral equipment required to run the PCR chips were large. These equipment included a personal computer and Labview control hardware. There are further avenues to miniaturize these equipment to form a portable system. One such example is the use of microprocessor instead of labview. Smaller pumps or passive pumps may be used instead of syringe pump. However these works involved system development and would required many considerations before proceeding.

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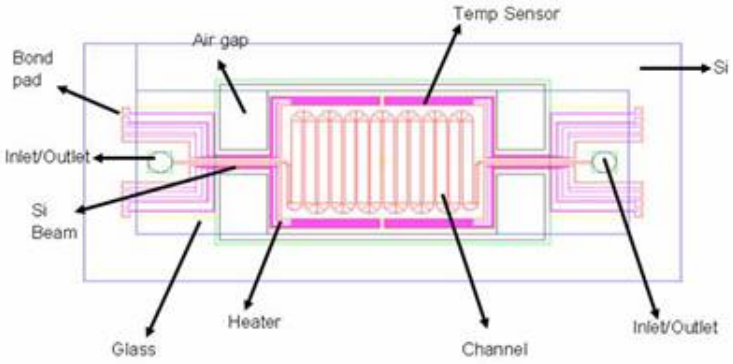
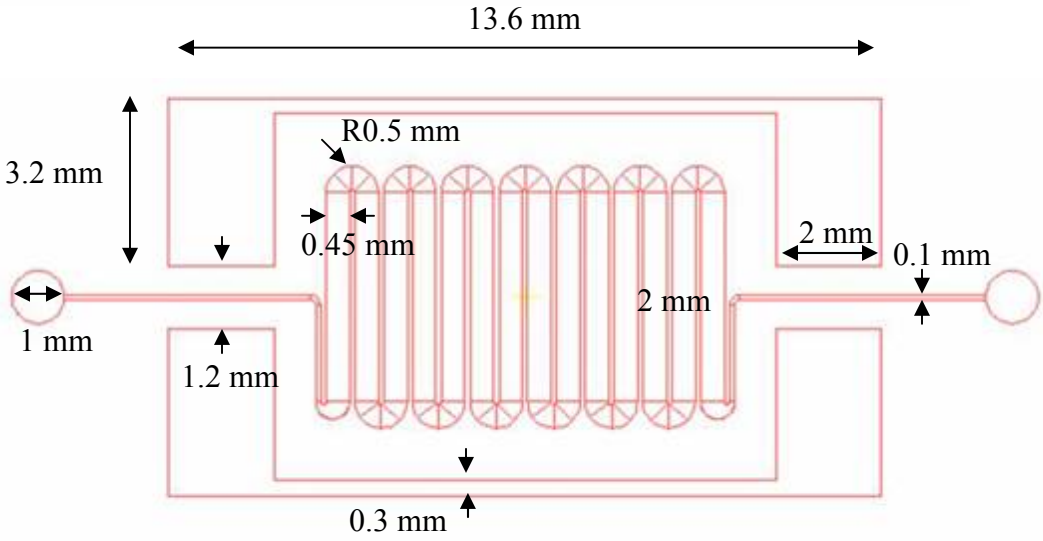
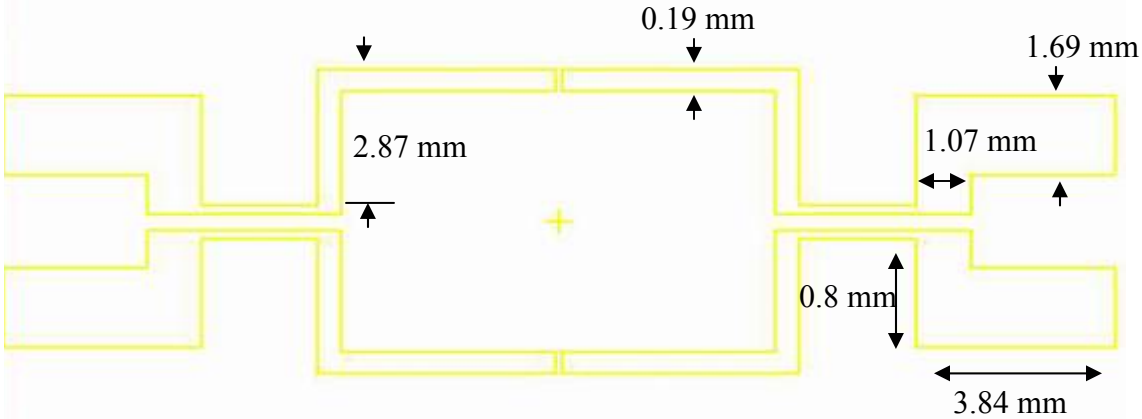
APPENDICES

APPENDIX A µPCR Chip Mask Design

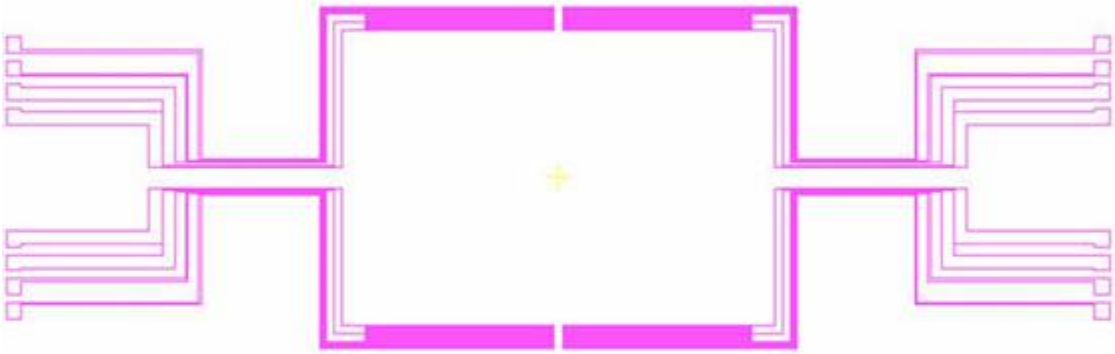




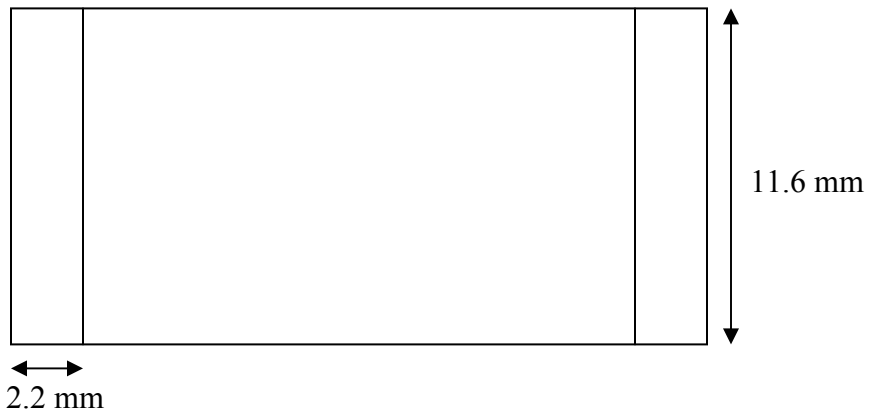
Mask Layer: Air gap + Channels



Mask Layer: Etch slot for metal lines

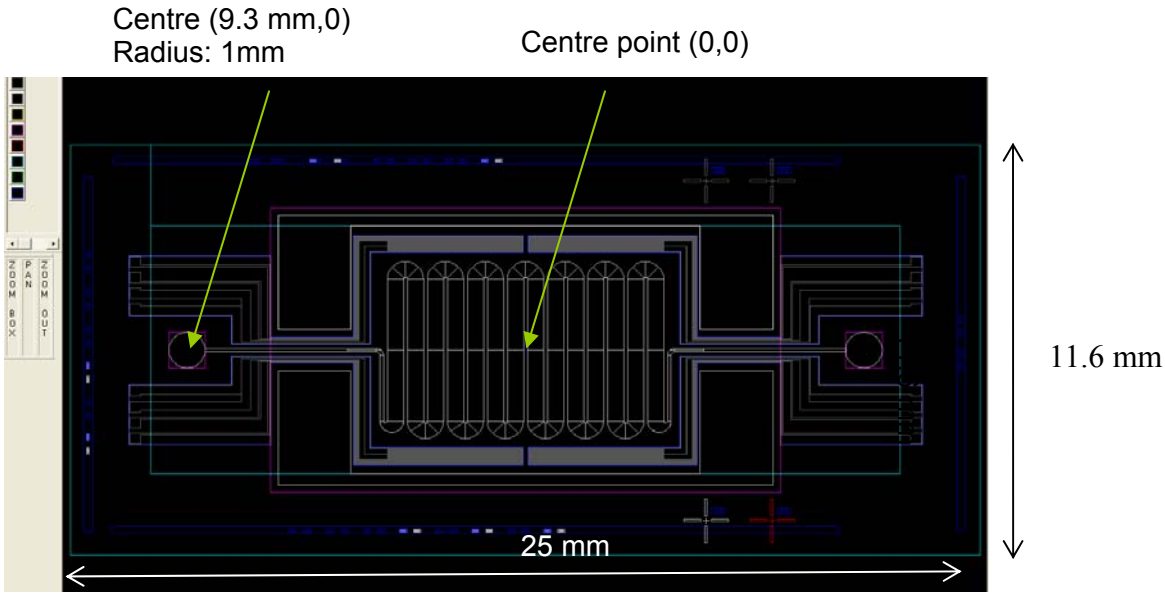


Mask Layer: Metal lines for heater and sensor



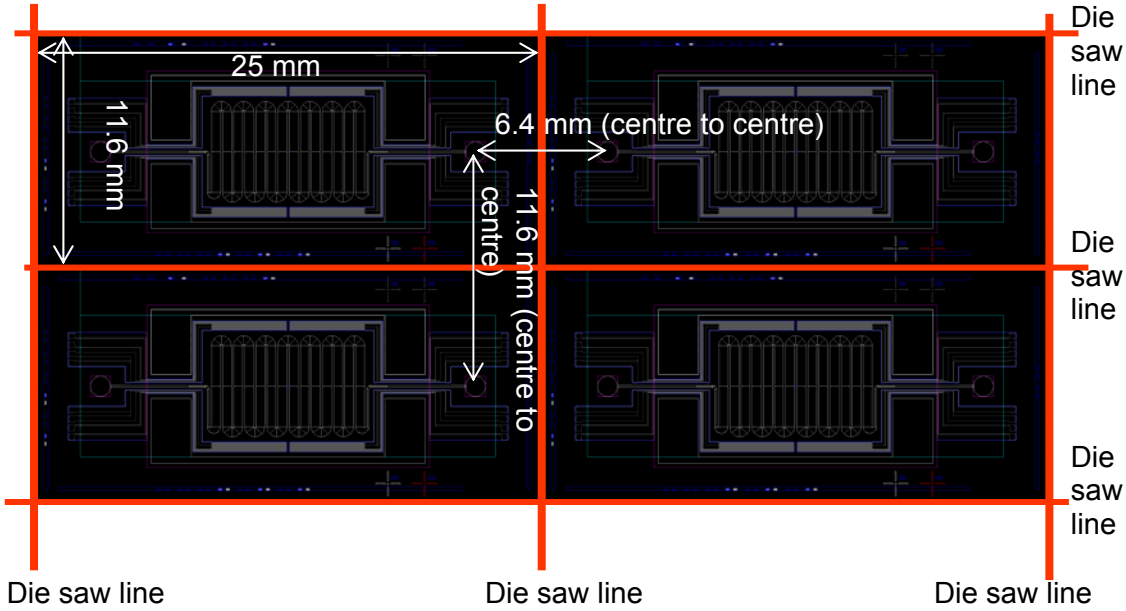
Mask Layer: Etch for glass layer for pre-cut

µPCR Chip Dicing Information

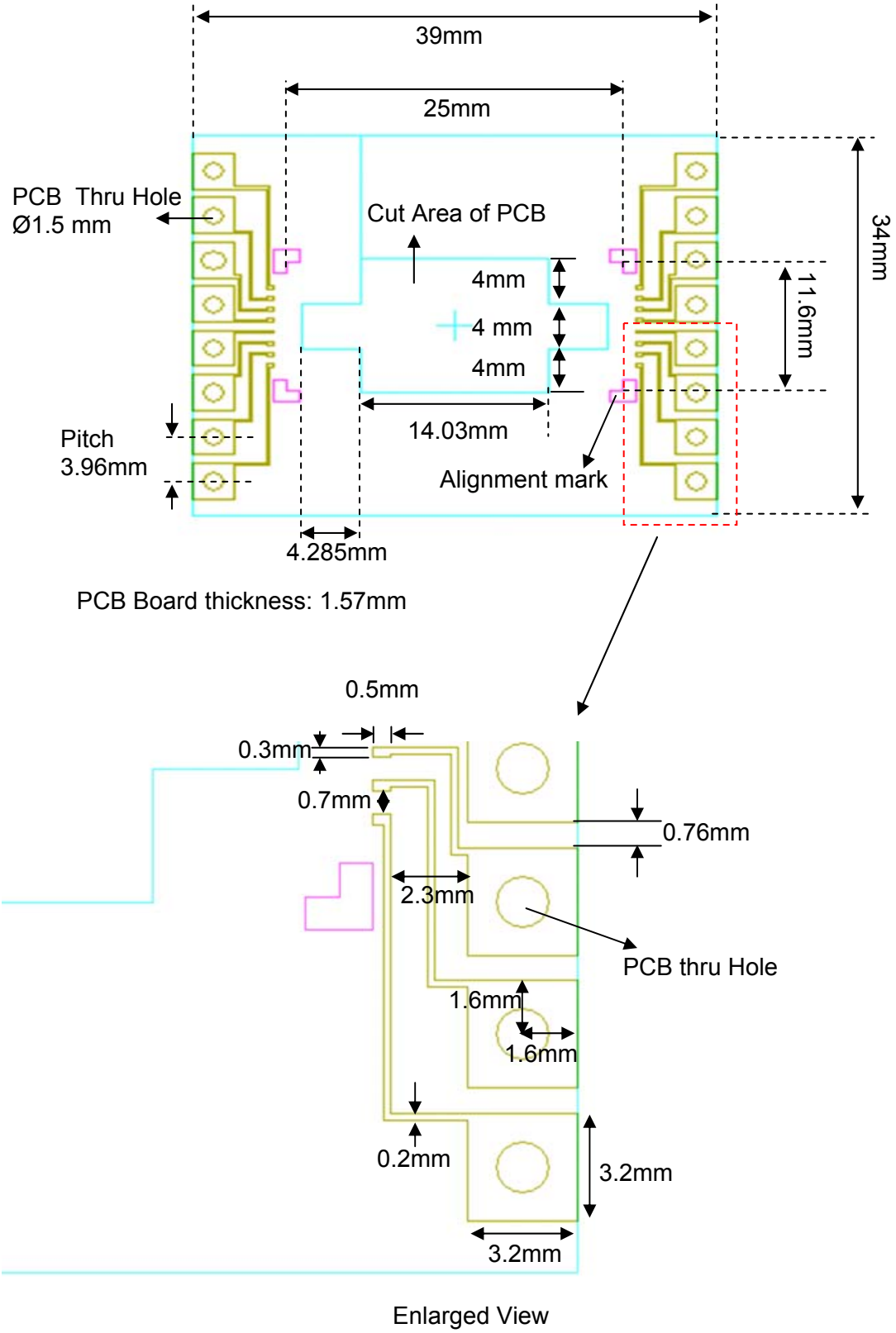


Dimension of chip and chip position for dicing

Glass Pre cut Blade Depth	0.47 mm
Glass removal from Silicon/Glass Chip at bond pad	0.9 mm



APPENDIX B Printed Circuit Board (PCB) Dimensions



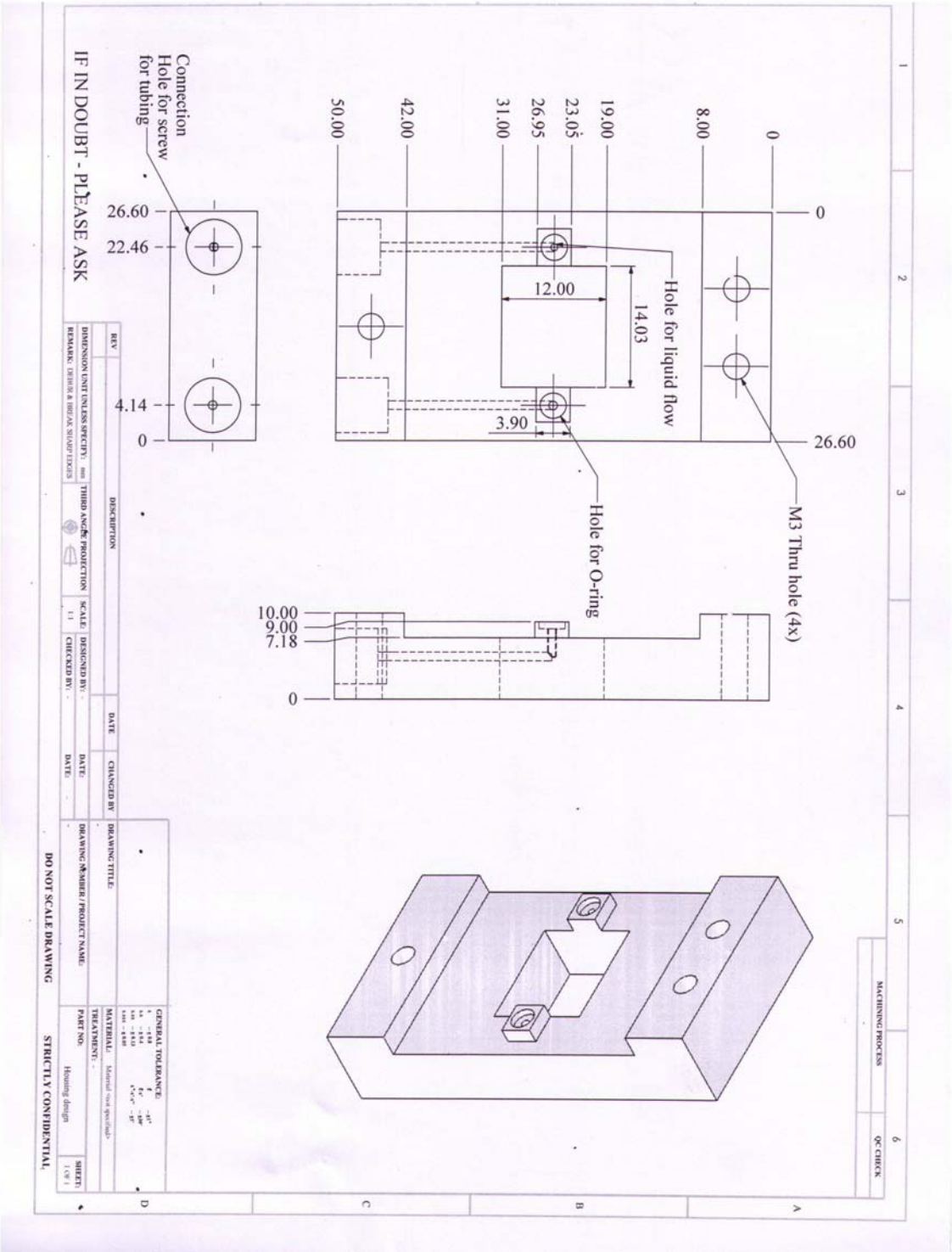
Material properties of PCB

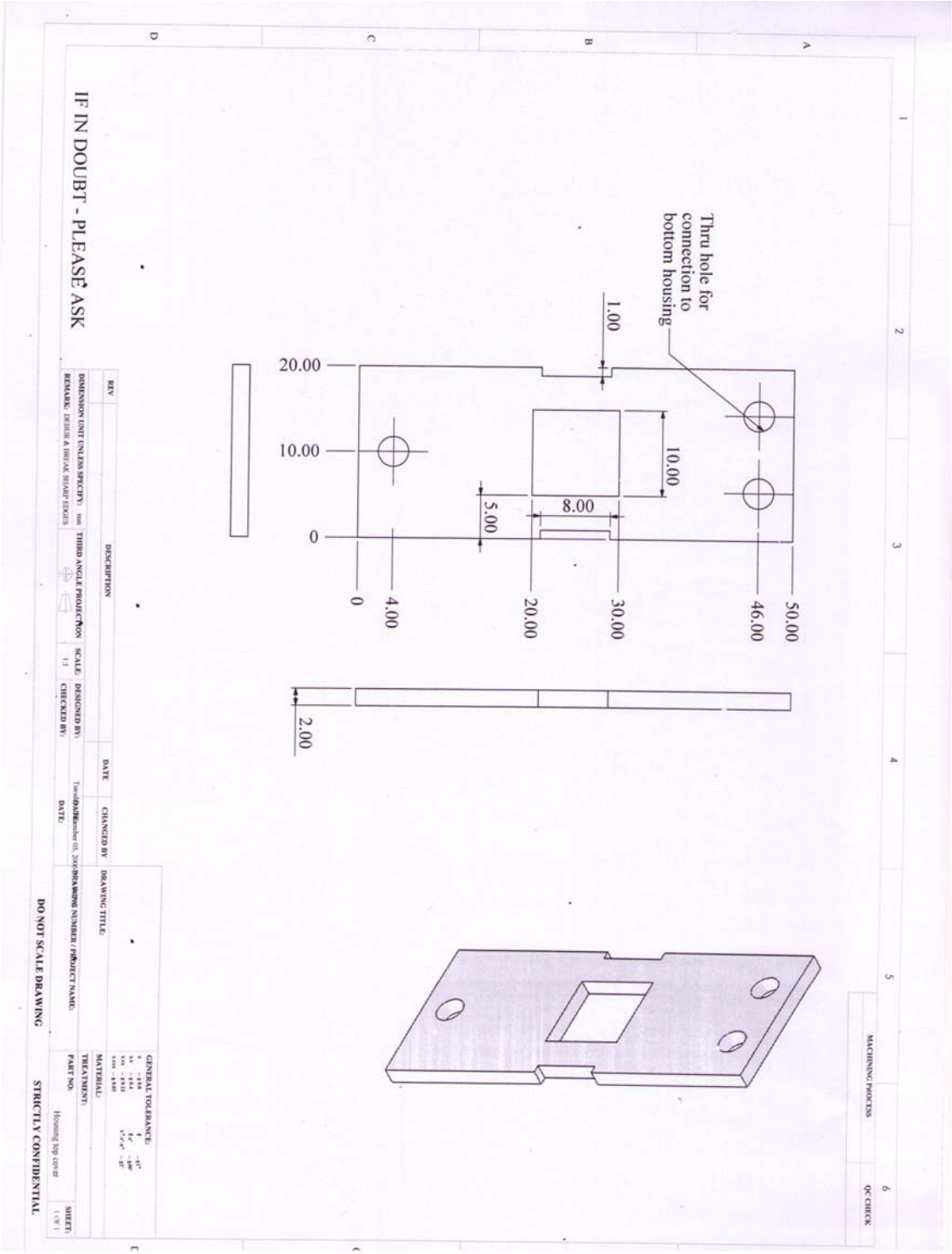
Material Characteristics of RUBALIT® and ALUNIT®

6

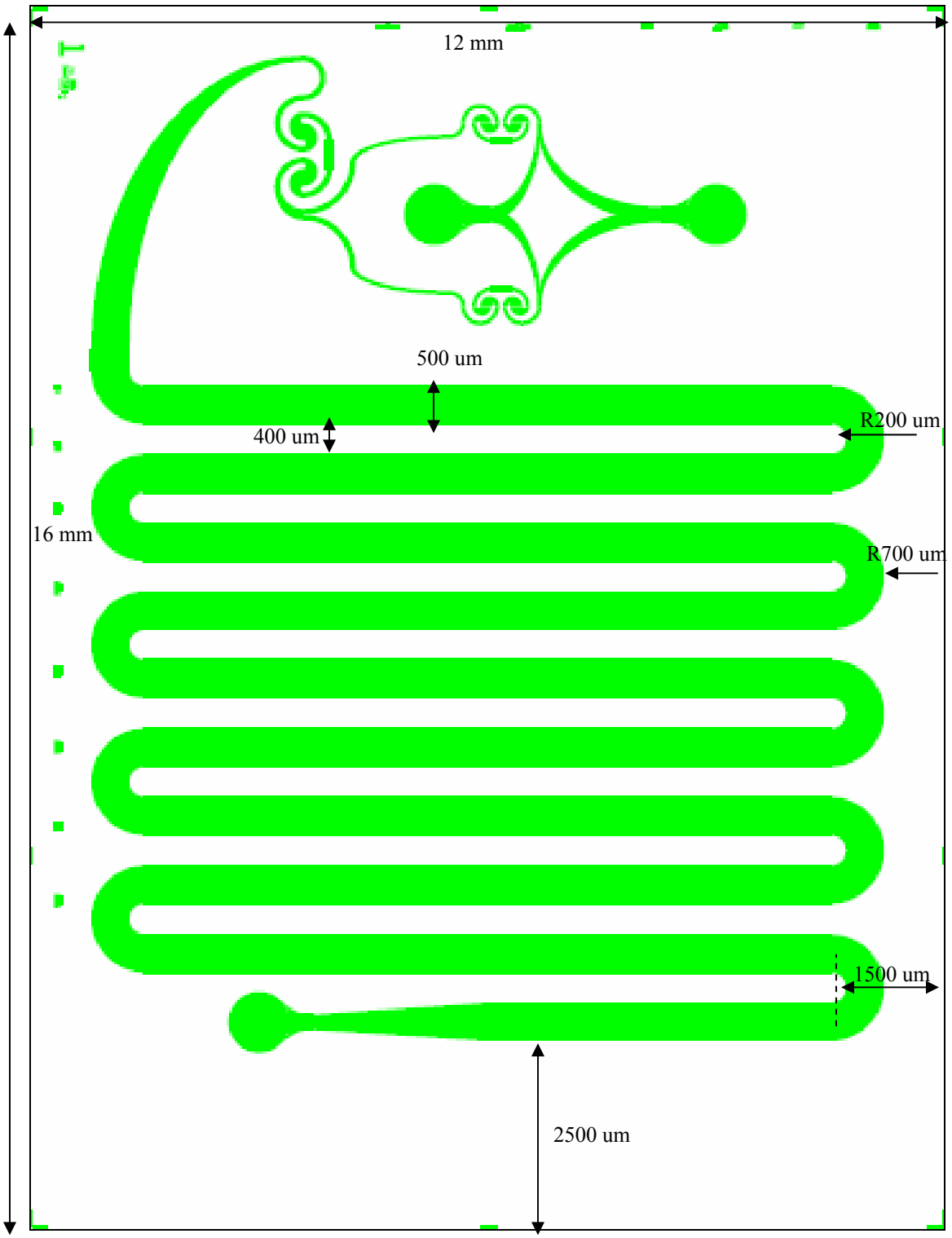
Property	Units	RUBALIT® 708S 96% Al_2O_3	RUBALIT® 710 99.6% Al_2O_3	ALUNIT® AlN	Test per
Color	–	white	white	translucent medium gray	–
Medium grain size d_{50}	µm	3-5	2	4-5	–
Surface roughness R_a	µm	0.6 max	0.1	0.6 max	Profilometer (0.8 mm cutoff)
Bulk density	kg/m³	3780	3900	3330	ASTM C 20
Water absorption capacity	%	0	0	0	ASTM C 373
Bending strength					
– 4-point method (40 x 4 x 3 mm³)	MPa	400		360	ASTM F 417
– dual-ring method (0.63 mm substrate thickness)	MPa	500	400		DIN 52292
Modulus of elasticity	GPa	340	350	320	ASTM F 417
Thermal conductivity 20–100 °C	W/m °K	24	28	180	ASTM C 408
Specific heat	J/kg°K	800	800	738	
Coefficient of linear expansion	10^{-6}/°K				ASTM C 373
20–300 °C		6.8	6.8	4.7	
20–600 °C		7.3	7.5	5.2	
20–1000 °C		8.0	8.5	5.6	
Dielectric constant					ASTM C 150
– 1 MHz		9.8 ± 10%	10.1 ± 10%	9.0 ± 10%	
– 1 GHz		10.0 ± 10%	10.1 ± 10%		
Dielectric loss factor (1 MHz)	10^{-3}	0.3	0.2	0.4	ASTM D 150
Breakdown voltage	KV/mm				ASTM D 149
– 1 mm substrate thickness		15		16	
– 0.63 mm substrate thickness		20	>10		
– 0.25 mm substrate thickness		28			
Volume resistivity	Ohm x cm				ASTM D 257
– 20 °C		10^{13}	10^{13}	10^{13}	
– 200 °C		10^{12}	10^{13}		
– 400 °C		10^{11}	10^{12}		
– 600 °C		10^{8}	10^{9}		

APPENDIX C µPCR Chip Acrylic Housing Design

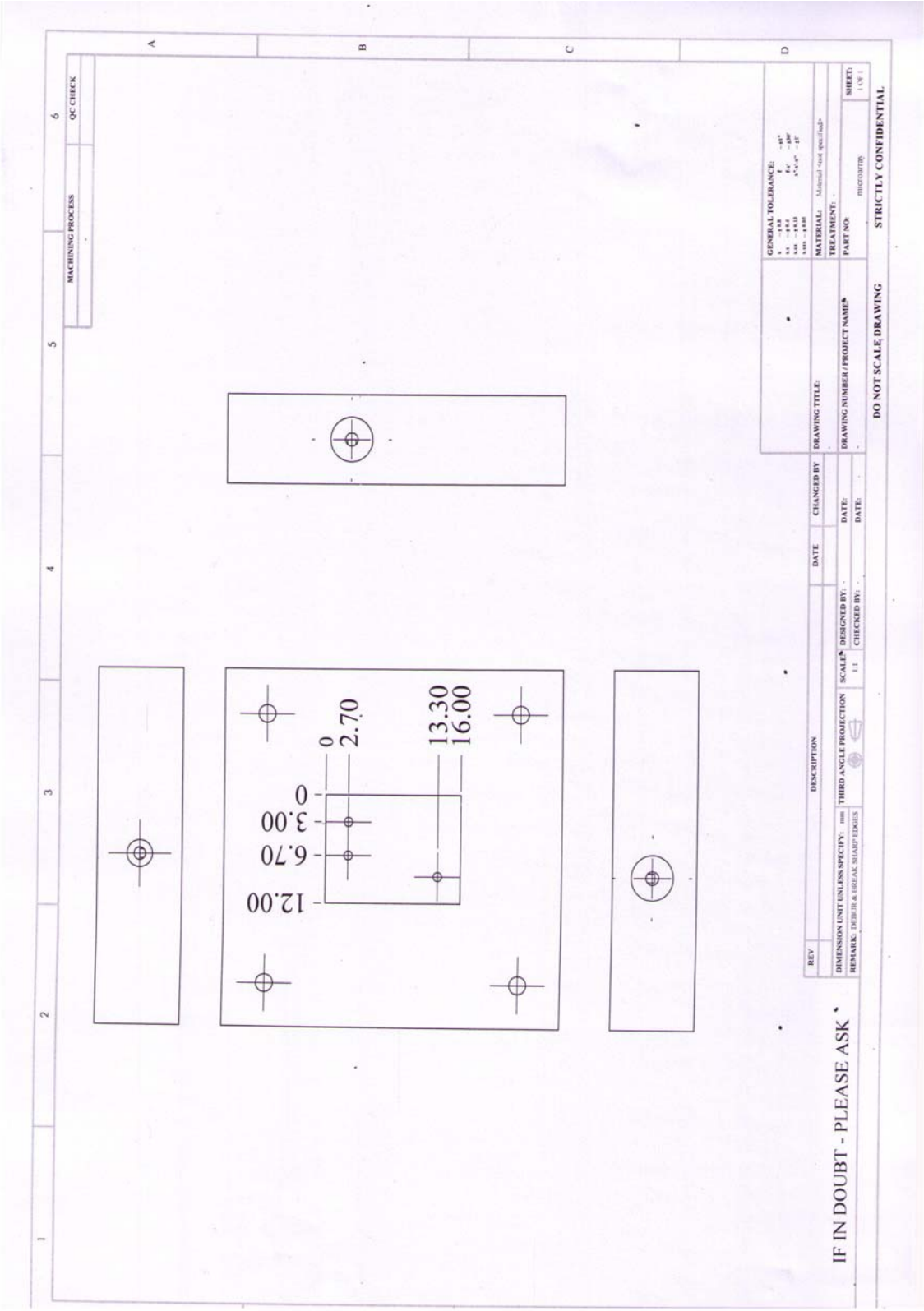


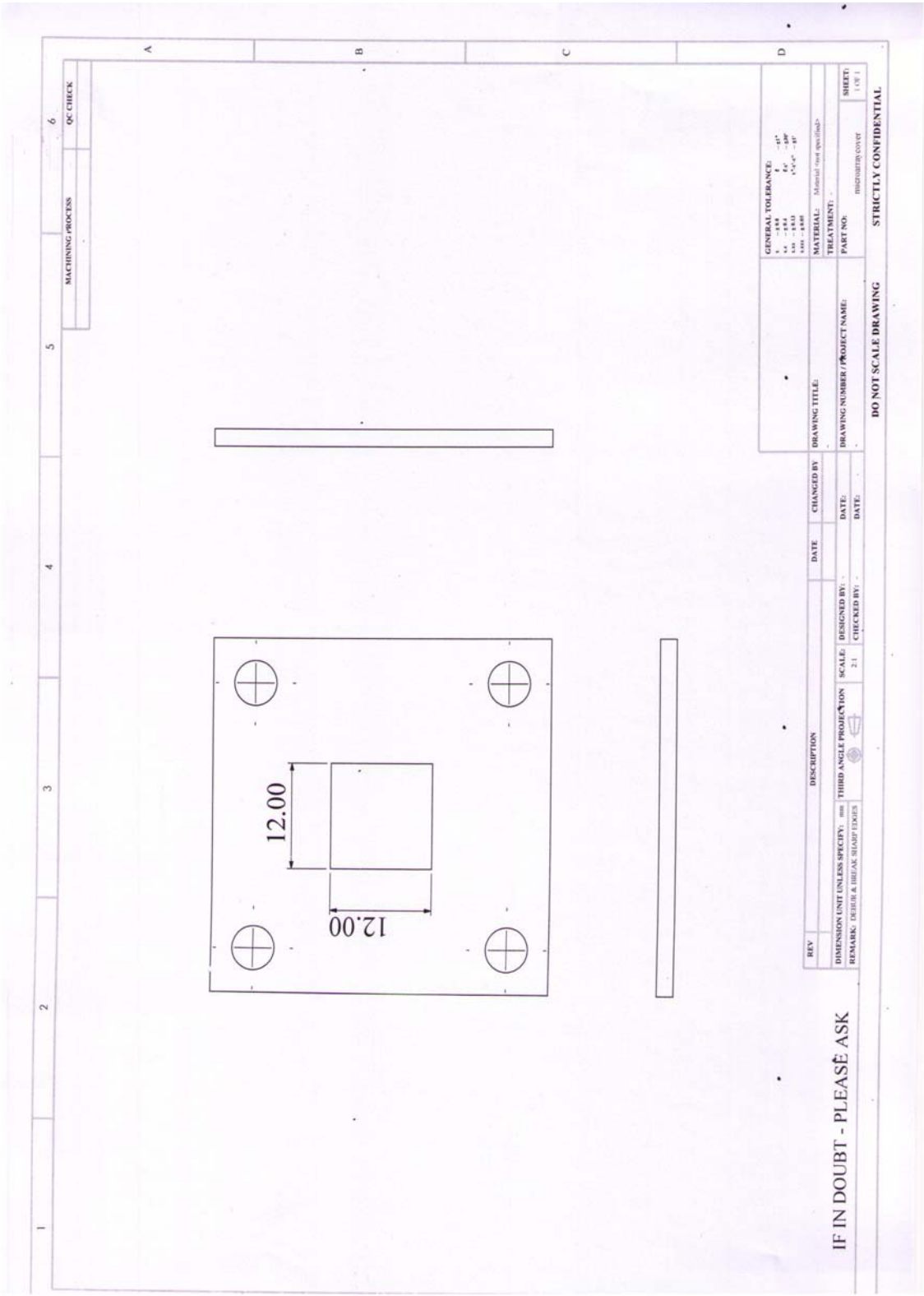

Thru hole for connection to bottom housing
1.00
10.00
8.00
5.00
20.00
10.00
0
50.00
46.00
30.00
20.00
4.00
0
2.00
IF IN DOUBT - PLEASE ASK
REV
DESCRIPTION
DATE
CHANGED BY
DIMENSION UNIT UNLESS SPECIFY: mm
THIRD ANGLE PROJECTION
SCALE: 1:1
DESIGNED BY:
CHECKED BY:
DATE:
DRAWING TITLE:
MATERIAL:
TREATMENT:
PART NO:
Housing top cover
SHEET: 1 OF 1
DO NOT SCALE DRAWING
STRICTLY CONFIDENTIAL
MACHINING PROCESS
QC CHECK

APPENDIX D DNA Microarray Dimensions

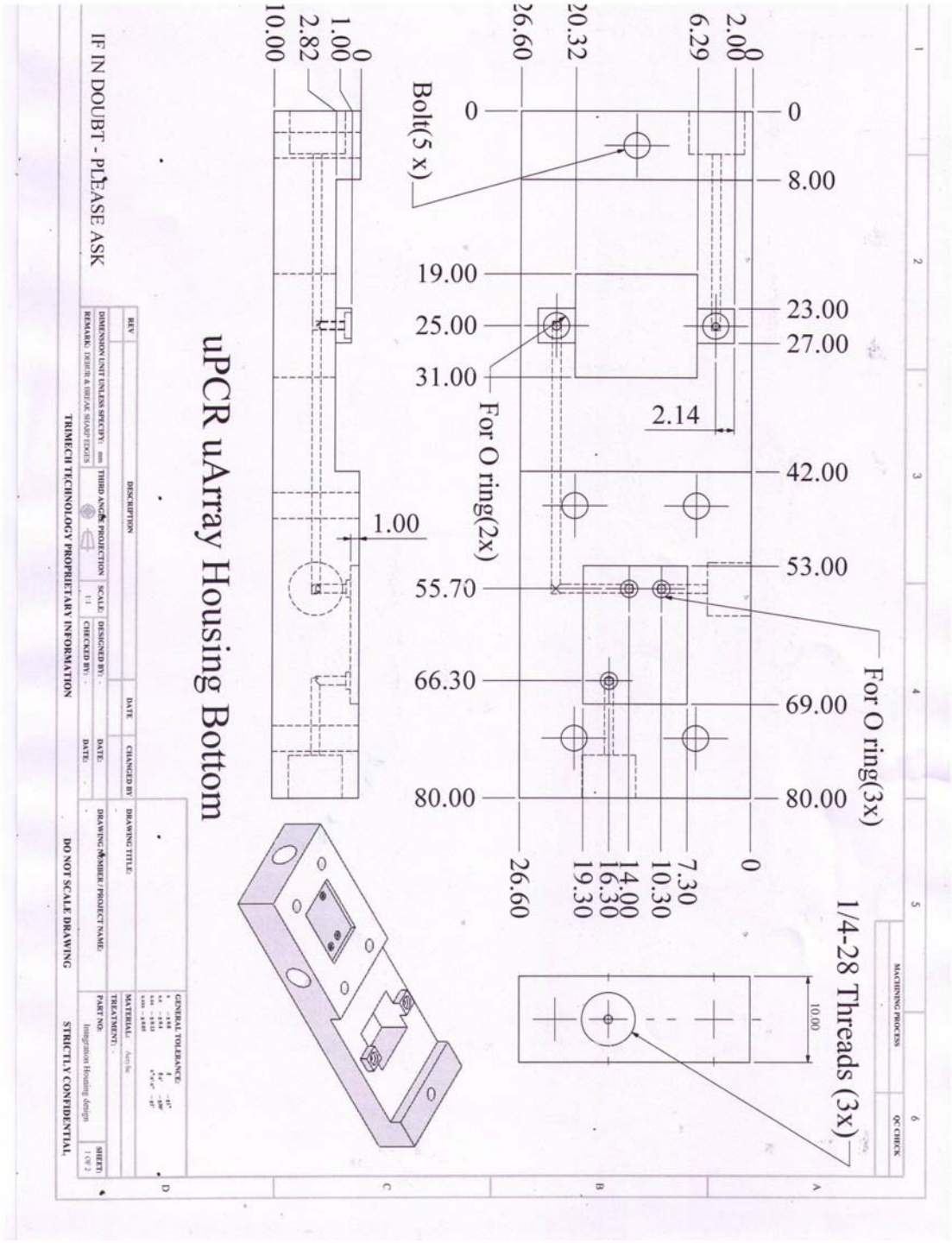


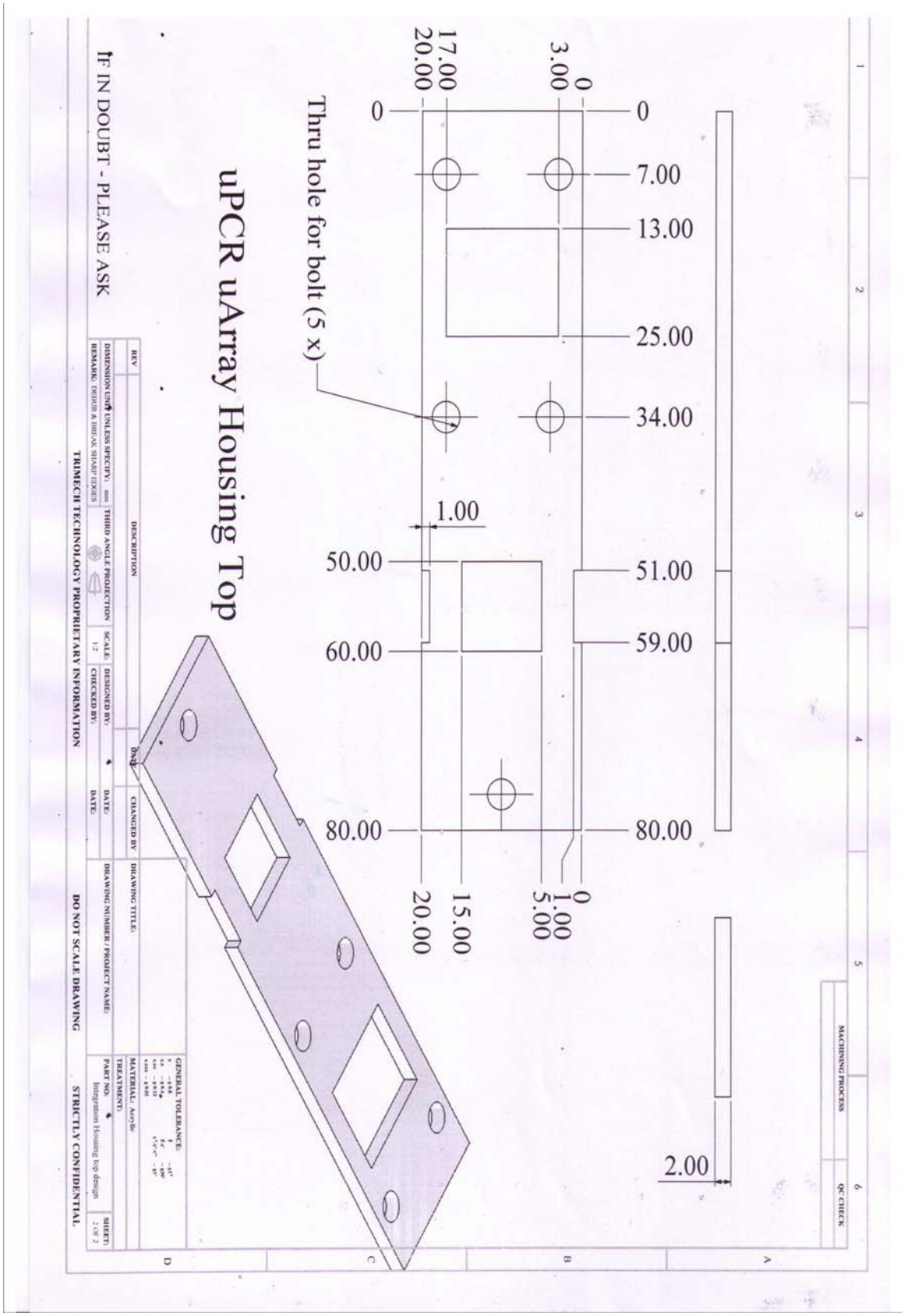
APPENDIX E DNA Microarray Acrylic Housing




12.00
12.00
MACHINING PROCESS
QC CHECK
REV
DESCRIPTION
DATE
CHANGED BY
DRAWING TITLE:
GENERAL TOLERANCE:
MATERIAL: Material <not specified>
TREATMENT: -
DRAWING NUMBER / PROJECT NAME:
PART NO:
mechanting cover
SHEET: 1 OF 1
DIMENSION UNIT UNLESS SPECIFY: mm
THIRD ANGLE PROJECTION
SCALE: 2:1
DESIGNED BY:
CHECKED BY:
DATE:
REMARK: DEBUR & BREAK SHARP EDGES
DO NOT SCALE DRAWING
STRICTLY CONFIDENTIAL
IF IN DOUBT - PLEASE ASK

APPENDIX F Micro Total Analysis System Acrylic Housing




uPCR uArray Housing Top
Thru hole for bolt (5 x)
0
3.00
17.00
20.00
0
7.00
13.00
25.00
34.00
1.00
50.00
60.00
51.00
59.00
80.00
80.00
0
1.00
5.00
15.00
20.00
2.00
IF IN DOUBT - PLEASE ASK
TRIMECH TECHNOLOGY PROPRIETARY INFORMATION
DO NOT SCALE DRAWING
STRICTLY CONFIDENTIAL
DESCRIPTION
CHANGED BY
DRAWING TITLE:
DRAWING NUMBER / PROJECT NAME:
THIRD ANGLE PROJECTION
SCALE:
DESIGNED BY:
CHECKED BY:
DATE:
MATERIAL: Acrylic
TREATMENT:
PART NO.
Integration Housing top design
SHEET
2 OF 2
MACHINING PROCESS
QC CHECK

APPENDIX G Wire bonding for µPCR Chip

FNK Wire Bonding Operating Procedures

1. "On" button to start machine
2. Clamp device in place with 2 metal bars
3. "Load Program"-Press Ok
4. "Move bond head to init"-Press Ok
5. File → Load→OK→ Use Program for 16 mm bond cap
6. Page → BHC Parameter:Flame off z:9100; gap:120 to check
7. Esc
8. Mode Single bond; Mode 11
9. Determine position of bond 1 and bond 2
10. Adjust Focus Height
11. Measure bond height
12. Check ball size and ball current settings
13. If wire breaks, use "clamp off" to bring in new wires
14. To shut down

FNK Wire Bonding Parameters

FNK Wire Bonder Parameters		
Wire: 1.2 mil		
Current	4.5	
Ball size	5.8	
	1st bonding setting	2nd bond setting
UT	55	80
UP	65	80
BF	30	40
CF	2	0
VF	0	
	Parameter at 1st bond	Parameter at 2nd bond
Bond delay	20	20
TD Flag	1	1
Force	30	30
Ramp	500	500
TH	15	15
OD	500	500
Steps	0	0
Spd	0	0
Accel	0	0
	Tail Parameter	
Tear Flag	1	

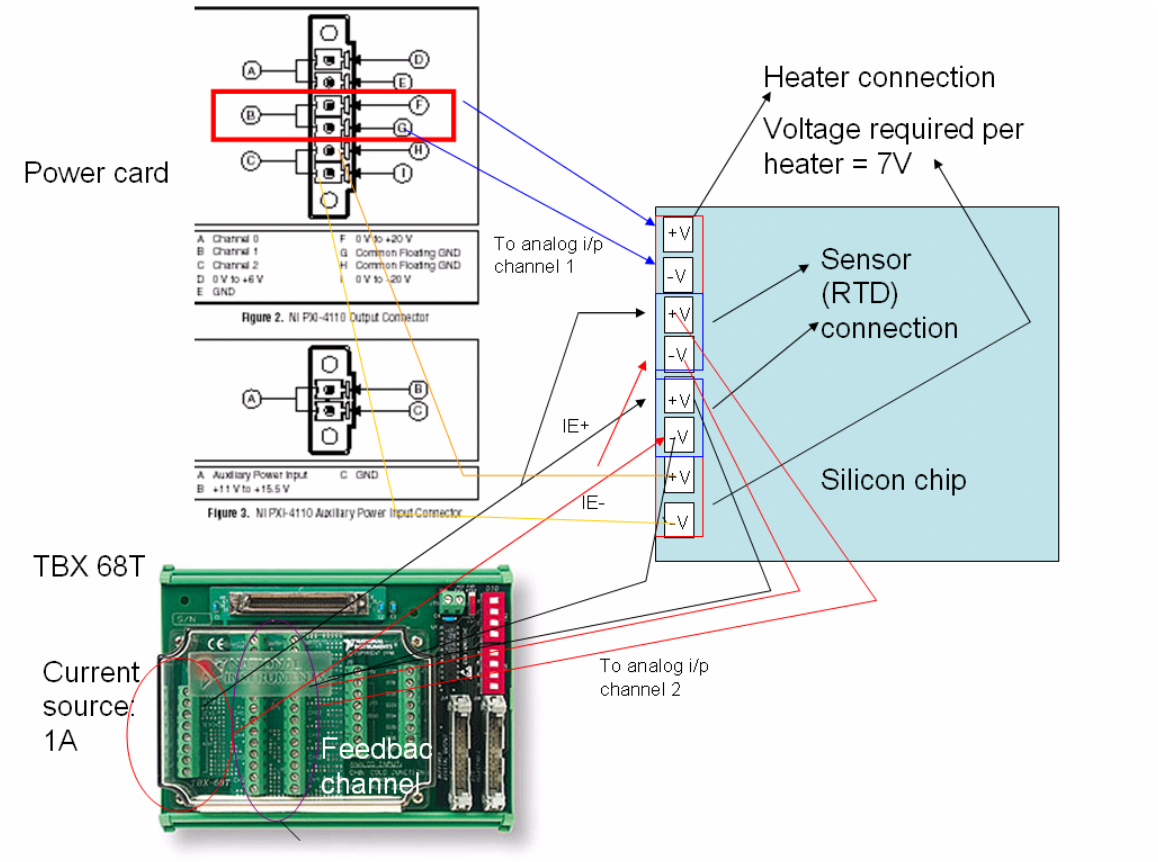
Relief Force	35	
Tear US Power	25	
XY Movng counter	0	
Tail Height	150	
Tear Height	150	
	Loop Parameter	
Loop form	1	
Loop mode	11	
Z Loop predesign	200	
Ab loop ht	0	
LH Factor 1	60	
RH	300	
RF	0	
XY	40	
Z loop delay	0	
-	70	
-	70	
-	70	

Wire bonding DOE

No	Parameters									
	Loop Type	RH	Z Loop	Loop Ht factor	RF	XY Factor	Loop ht at corner	ht at ball	ht at stitch	Remarks
1	Rectangle	300	50	60	15	40				
2	Rectangle	300	70	60	15	40				
3	Rectangle	300	70	80	15	40				
4	Rectangle	300	90	60	15	40				
5	Rectangle	350	90	60	15	40				Break
6	Rectangle	300	50	40	15	40				
7	Rectangle	300	30	60	15	40				Break
1	Rectangle	300	70	60	15	40	0.18	0.1	0.03	
2	Rectangle	600	70	60	15	40	0.17	0.07	0	
3	Triangle	300	70	60	15	40	0.02	0.02	0.6	
4	Triangle	600	70	60	15	40	0.006	0.006	0.87	
5	Triangle	1200	70	60	15	40	0.066	0.066	0.06	
6	Rectangle	1200	70	60	15	40	0.07	0.066	0.012	
7A	Rectangle	2000	70	50	15	40	--	--	--	No bond
7B	Rectangle	2000	70	30	15	40	0.09	0.0545	0.02	
8	Rectangle	2000	200	30	30	40	0.08	0.05	0.04	Slack
9	Rectangle	300	200	60	15	40	0.17	0.06	0.048	
10	Rectangle	300	300	60	30	40	0.16	0.05	0.04	
11	Rectangle	300	300	60	15	40	0.15	0.07	0	
1	Rectangle	300	300	60	15	40	0.16	0.07	0.06	
2	Rectangle	300	300	60	15	80	0.17	0.09	0.04	
3	Rectangle	300	300	60	15	80	0.15	0.06	0.04	
4	Rectangle	300	70	60	15	100	0.14	0.08	0.06	
5	Rectangle	300	300	60	15	20	0.1	0.07	0.064	
6	Rectangle	300	300	60	15	100	0.19	0.08	0.05	Z loop max: 300
7	Rectangle	10000	300	60	15	100	--	--	--	Too long: Max

										10000
8	Rectangle	5000	300	60	15	100	0.22	0.07	0	
9	Rectangle	5000	300	60	15	100	0.21	0.06	0.04	
10	Rectangle	5000	300	60	15	100	0.15	0.05	0.04	
11A	Rectangle	5000	300	60	15	20	--	--	--	Break
11B	Rectangle	5000	300	60	15	40	0.14	0.09	0.03	
12	Rectangle	5000	100	60	15	40	0.16	same plane	0.03	
13	Rectangle	5000	300	60	15	40	0.06	0.19	0.03	

APPENDIX H Electrical Connections for µPCR Chip

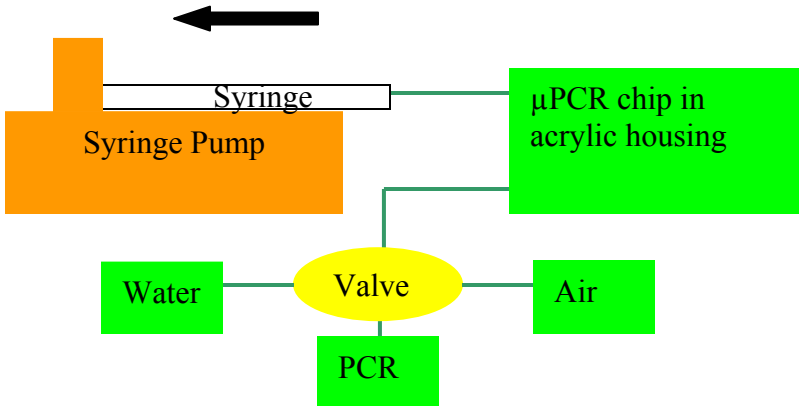


Wire are soldered directly on gold bond pads of µPCR chip are connected to the corresponding hardware respectively

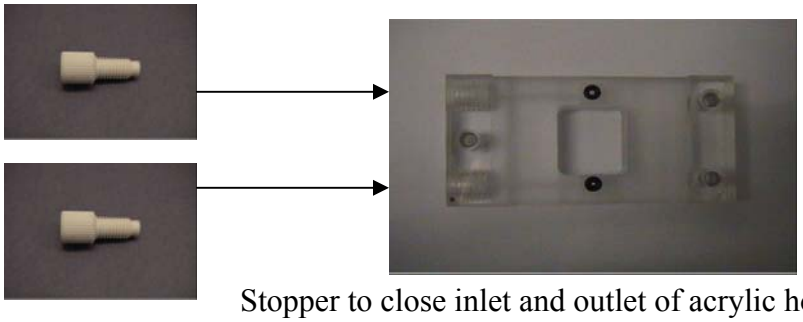
Operating Procedure for µPCR chip

System Set Up

1. Set up connections between syringe pump, syringe , telfon tubings and acrylic housing (with µPCR chip).



2. Syringe pump is used in the withdrawal mode
3. Prime all flow line and chip with ultrapure water
4. Choose port of valve to turn to determine type of fluid flow into PCR chip
5. Sequence should be as follows:
   A. port for air (Volume: 1µl)
   B. port for PCR (Volume: 10 µl)* About 13µl sample is prepared for input to chip
   C. port for water (Continuous volume)
6. Position the sample zone into the reaction channels using syringe pump.(Positioning is done by setting pre set volume in syringe pump)
7. Enclosed inlet and out of acrylic housing with stopper
8. Run PCR

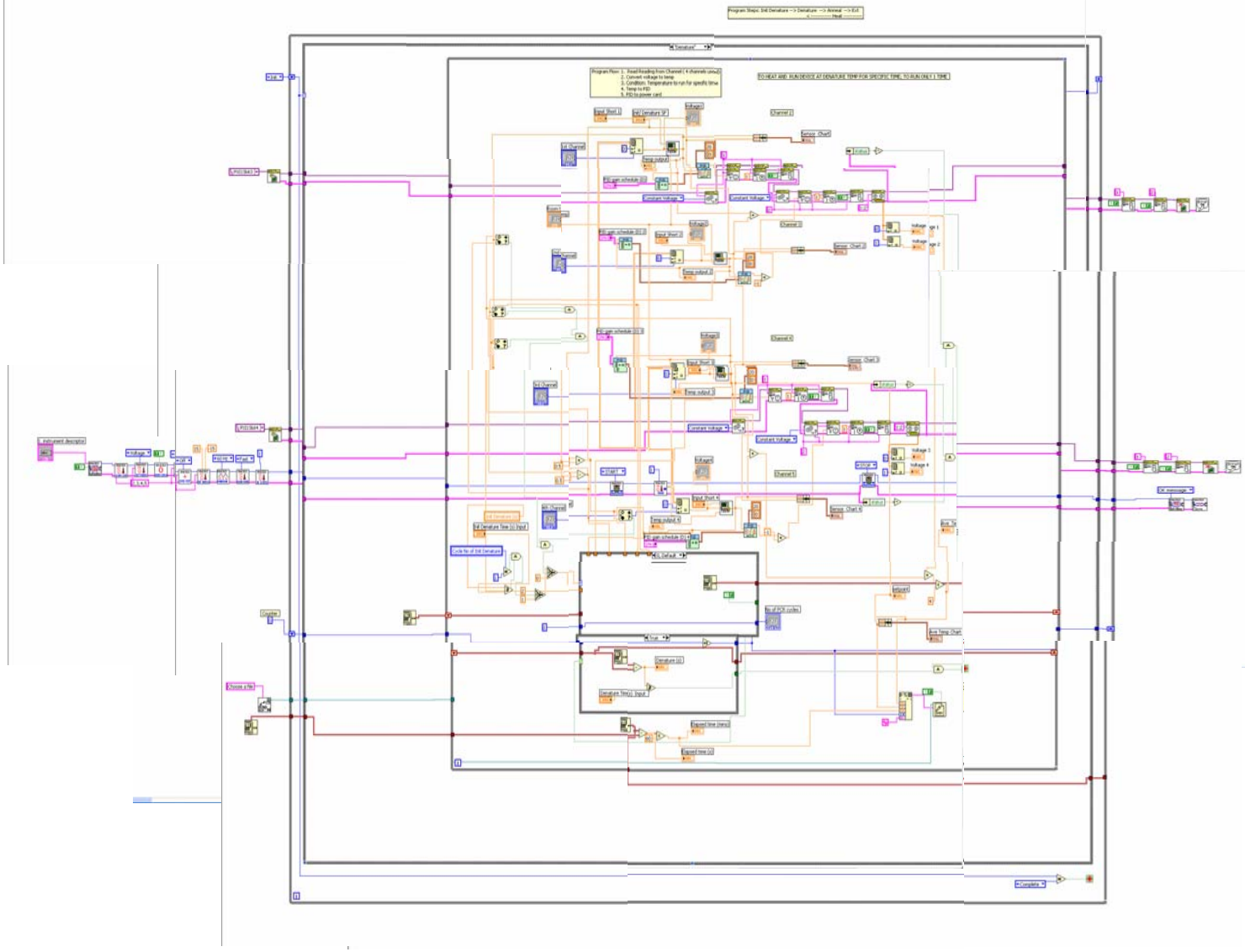


Stopper to close inlet and outlet of acrylic housing

Washing step

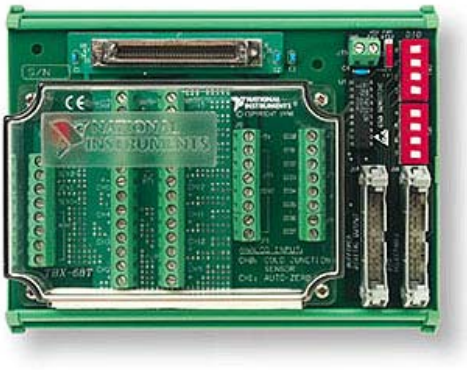
1. After PCR, wash chip in acrylic housing
2. Flush device with ultrapure water
3. Incubate device with 70% Etoh for 2 mins
4. Flush device with 3 volumes of ultrapure water
5. Incubate device with 0.3% NaOCl
6. Flush device with 3 volumes of ultrapure water

A typical Labview Code for PCR

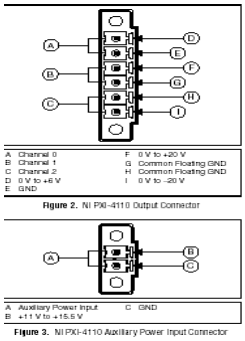


Labview Hardware

No	Equipment Name	Qty	Purpose
1	Labview 8.0/8.2	1	Graphical programming software
2	PID Toolset	1	PID control
3	TBX- 68T Terminal block with one cold sensor and 88	1	To read sensor reading
4	NI 4351 for PXI	1	As interface between TBX-68T and Labview
5	NI PXI 4110 Triple Output DC power supply	2	Power supply to heater
6	NI PXI-PCI8331, MXI-4 Kit with copper cable	1	Interface between
7	APS 4100 auxiliary power source for NI DC power	2	External Power source
8	Power Cord, 240,10A	2	
9	PXI-1031, 4 slot 3U chasis with universal AC Power	1	To house all cards



TBX 68T



NI PXI 4110 Triple Output DC power supply

NI 4351 for PXI

PXI-1031, 4 slot chasis with universal AC Power

APPENDIX I Protocol for Bacteria DNA Extraction

Protocol from QIAamp® DNA Mini Kit and QIAamp DNA Blood Mini Kit Handbook

Isolation of genomic DNA from bacterial cultures
a) Plate cultures
1. Remove bacteria from culture plate with an inoculation loop and suspend in 180 µl of Buffer ATL (supplied in the QIAamp DNA Mini Kit) by vigorous stirring.
2. Follow the Tissue Protocol (page 33) from step 2.
b) Suspension cultures
1. Pipet 1 ml of bacterial culture into a 1.5 ml microcentrifuge tube, and centrifuge for 5 min at 5000 x *g* (7500 rpm).
2. Calculate the volume of the pellet or concentrate and add Buffer ATL (supplied in the QIAamp DNA Mini Kit) to a total volume of 180 µl.
3. Follow the Tissue Protocol (page 33) from step 2.
2. Add 20 µl Proteinase K, mix by vortexing, and incubate at 56°C until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample, or place in a shaking water bath or on a rocking platform.
Note: Proteinase K must be used. QIAGEN Protease has reduced activity in the presence of Buffer ATL.
Lysis time varies depending on the type of tissue processed. Lysis is usually complete in 1–3 h. Lysis overnight is possible and does not influence the preparation. In order to ensure efficient lysis, a shaking water bath or a rocking platform should be used. If not available, vortexing 2–3 times per hour during incubation is recommended.
3. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid. Continue with step 3a, or if RNA-free genomic DNA is required, continue with step 3b.
Transcriptionally active tissues, such as liver and kidney, contain high levels of RNA which will copurify with genomic DNA. RNA may inhibit some downstream enzymatic reactions, but will not inhibit PCR.
3a. Add 200 µl Buffer AL to the sample, mix by pulse-vortexing for 15 s, and incubate at 70°C for 10 min. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.
It is essential that the sample and Buffer AL are mixed thoroughly to yield a homogeneous solution.
A white precipitate may form on addition of Buffer AL, which in most cases will dissolve during incubation at 70°C. The precipitate does not interfere with the QIAamp procedure, or with any subsequent application.
OR
3b. First add 4 µl RNase A (100 mg/ml), mix by pulse-vortexing for 15 s, and incubate for 2 min at room temperature. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid before adding 200 µl Buffer AL to the sample. Mix again by pulse-vortexing for 15 s, and incubate at 70°C for 10 min. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.
It is essential that the sample and Buffer AL are mixed thoroughly to yield a homogeneous solution.

A white precipitate may form on addition of Buffer AL. In most cases it will dissolve during incubation at 70°C. The precipitate does not interfere with the QIAamp procedure or with any subsequent application.
4. Add 200 µl ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.
It is essential that the sample, Buffer AL, and the ethanol are mixed thoroughly to yield a homogeneous solution.
A white precipitate may form on addition of ethanol. It is essential to apply all of the precipitate to the QIAamp Spin Column. This precipitate does not interfere with the QIAamp procedure or with any subsequent application.
Do not use alcohols other than ethanol since this may result in reduced yields.
5. Carefully apply the mixture from step 4 (including the precipitate) to the QIAamp Spin Column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Spin Column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.
Close each spin column to avoid aerosol formation during centrifugation.
It is essential to apply all of the precipitate to the QIAamp Spin Column.
Centrifugation is performed at 6000 x *g* (8000 rpm) in order to reduce noise.
Centrifugation at full speed will not affect the yield or purity of the DNA. If the solution has not completely passed through the membrane, centrifuge again at a higher speed until all the solution has passed through.
6. Carefully open the QIAamp Spin Column and add 500 µl Buffer AW1 without wetting the rim. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Spin Column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.
7. Carefully open the QIAamp Spin Column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 min. Continue directly with step 8, or to eliminate any chance of possible Buffer AW2 carryover, perform step 7a, and then continue with step 8.
Note: Residual Buffer AW2 in the eluate may cause problems in downstream applications. Some centrifuge rotors may vibrate upon deceleration, resulting in the flow-through, which contains Buffer AW2, coming into contact with the QIAamp Spin Column. Removing the QIAamp Spin Column and collection tube from the rotor may also cause flow-through to come into contact with the QIAamp Spin Column. In these cases, the optional step 7a should be performed.
7a. (Optional): Place the QIAamp Spin Column in a new 2 ml collection tube (not provided)
and discard the collection tube containing the filtrate. Centrifuge at 20,000 x *g* (14,000 rpm) for 1 min.
8. Place the QIAamp Spin Column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Spin Column and add 200 µl Buffer AE or distilled water. Incubate at room temperature for 1 min, and then centrifuge at 6000 x *g* (8000 rpm) for 1 min.
9. Repeat step 8.
A 5 min incubation of the QIAamp Spin Column loaded with Buffer AE or water,

before centrifugation, generally increases DNA yield.
A third elution step with a further 200 µl Buffer AE will increase yields by up to 15%.
Volumes of more than 200 µl should not be eluted into a 1.5 ml microcentrifuge tube because the spin column will come into contact with the eluate, leading to possible aerosol formation during centrifugation.
Elution with volumes of less than 200 µl increases the final DNA concentration in the eluate significantly, but slightly reduces the overall DNA yield (see Table 3, page 16). Eluting with 4 x 100 µl instead of 2 x 200 µl does not increase elution efficiency.
For long-term storage of DNA, eluting in Buffer AE and placing at –20°C is recommended, since DNA stored in water is subject to acid hydrolysis.
Yields of DNA will depend both on the amount and the type of tissue processed.
25 mg of tissue will yield approximately 10–30 µg of DNA in 400 µl of water (25–75 ng/µl), with an *A*260/*A*280 ratio of 1.7–1.9.

APPENDIX J 2-Mask PCR Chip Operation

Attocycler™ operation

1. Open laptop link to Attocycler™
2. Click on "Attos exe" to open control software
3. Switch on machine using switch located behind Attocycler™
   A "clicking: should be heard: this indicates the detection of controller laptop by the machine
4. Load chip onto loading plate
5. Close chamber
6. Choose "reaction" from software
7. Input thermal cyclng parameters
8. Start reaction
9. After completion of reaction, extract PCR mixture from chip using acrylic housing for µPCR chip and run product on 1.5% agarose gel for analysis using gel electrophoresis.
10. Flush acrylic housing with DI water after every extraction to prevent cross contamination.

2-Mask PCR Chip preparation

1. Prepare PCR master mix as per required for experiment.
2. Load 10 µl of PCR mixture to PCR chip using pipette through the inlet/outlet located at back of chip. PCR mixture will flow into reaction channel through capillary force.
3. Once channels are completely filled, covered the inlet/outlet of the chip using PCR tape cut to size.
4. Ensure PCR mixture pump into chip is without bubble.
5. Load chip to loading plate of Attocycler™

APPENDIX K Operating Procedures for DNA Microarray

DNA Microarray

External Mixing
-PCR amplicon/oligonucleotide and hybridization buffer were mixed before input into microarray. Initial experiments were done this way to reduce unknown factors (passive mixer) if experiment fail.

Procedures
1.Set up device in housing and connect housing to syringe pump through telfon tubings.(Note: 2 syringe pumps are used to input hybridization buffer and PCR amplicons/oligonucleotides separately into microarray if passive mixer is used;for external mixing, only 1 syringe pump is used and the other inlet is enclosed with a stopper)
2. The mixture is pumped into the device at the appropriate speed and data acquisition is carried out continuously using the epiflourescent microscrope.
3. Flush acrylic housing with filter TT solution and DI water after each experiment.

APPENDIX L DNA Microarray Surface modification Protocol

Protocol for functionalization of silicon oxide and immobilization of DNA

Cleaning of SiO wafers
1.Rinse with 100% ethanol. Dry under nitrogen stream.

Reagent preparation

5% silanes (v/v)((3-Aminopropyl)triethoxysilane) in 95% ethanol

Silanization

1. Dry wafer in nitrogen stream before adding silanes. Add 50 ul of silanes to each wafer. Incubate for 3-5 hrs at room.temperature.
2. Rinse in 100% ethanol (Remember to rinse the back of the chips as well, otherwise they will stick to the hot plate later), dried in N2 and baked on hotplate at 120 deg C. Cool the wafers before adding cross linker solution. (Remove cross linker from fridge to room temperature because it is moisture sensitive. Prepare cross linker solution)

Reagent preparation
2mM cross linkers in DMSO + ethanol
Recipe for SMPB* = 0.712mg in 100 ul (= 200 ul DMSO + 800ul ethanol)
Cover cross linker with Al foil id not used immediately after preparation as cross linker is light sensitive. The solution should be prepared fresh each time.
*4-(4-Maleimidophenyl)butyric acid N-hydroxysuccinimide ester

Adding Cross linkers

1. Add 50 ul of cross linker solution to each wafer and incubate for 2 hrs (Prepare ethanol for rinsing)
2. 2. Rinse wafer with ethanol

Bioarrayer is used to spot DNA immobilization

APPENDIX M Operating Procedures for Biochip Arrayer

Maintenances of machine to be carried out 3 times a week

1) Switch on the power for the machine
2) Go to "Utilities" and select "Prime Tips" (This function is to be carried out twice)
3) After completion of the second Prime Tips function, go to "Utilities" and select "Start of Day". Place the 96 well plate into the designated plate area and fill up the first four wells of the plate with 70% methanol. The "Start of Say" function is to calibrate the pressures in the tips (look out for the standard deviation of the tips: should not be more than 3 to 4)
4) After doing "Start of Day". Carry out "Primer Tips" once.
5) Go to "Utilities" and click on " Dispense Verification"; this is to see what kind of spot, volume and morphology of the spot from each tip. After the tips have picked up the buffer from the trough and move itself in front of the camera, press Shift 1 to dispense followed by shift 2 to equilibrate the pressure in the tips. The optimized volume is around 0.287 to 0.333 nl/drop. After you are satisfied with the drop volume. Press ".". This would terminate the testing for the tip and move on to the next tip. Repeat the operation for the rest of the tips.
6) ** If there is no test to run, go to "Utilities and execute 'End of Day" function. Remember to fill up 96 well plate with 70% methanol.

** If there is a test to run, go to "Tool" and "Define Position File"

- This would allow the user to set the dimension of the array that is going to be printed. The x- and y- values show the centre to centre distance of the two spots in the array.
- After putting in the array information, save the file under a recognizable name and exit.

Next, go to "Tool" and " DEFINE Rack type"

- Select HL for the first rack(and SHAH for the second rack if there are a lot of chips to print) and change the file name to "test" and click on "Evaluate". This would bring you to DOS mode and from there , you can specify where you want to start spotting your array.
- The first command you would come up which says "Adjust camera Height"
- Just press"." And "y"
- The second command would then ask you to specify your base position (first spot of your array) for your array, After maneuvering the tips using the x- and y-aixs. Press ".". And "y"
- Save the file

Next go t to "File" and "load test"

- Select on the first 3 files.
- Change only the number of drops per spot and number of replicates and save the file.

- Execute the file by pressing >, and click on align racks before pressing run test (* Remember to place your 384 well plate in the sample trough)
- This would allow you to align your chips before printing just in case the position is not correct initially.
- After finishing the re-alignment of chips, click “done: and “run test”
- Just wait and see your chip printed.
- At the end of the test, click on the option “view error”
- If command says there are no errors to view, means the printing has gone on smoothly.

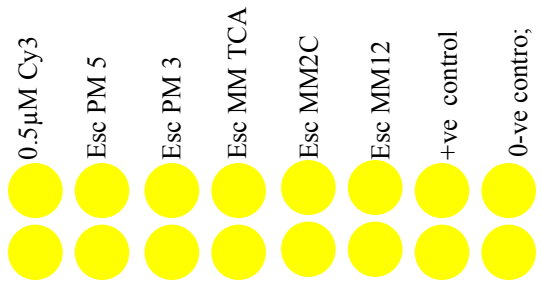
In the case where are errors in printing, save the error file (name.err) and close the error message.

- Go to “Tools” and choose option “Convert error to map file:
- Open folder and choose and open the error file that was saved
- The error file is now converted into a .map file
- Close the window and save the map file
- Go to “file”: and ”load test”
- ;Load the same test that was used for printing and click on the box use MAP based test.
- Save the table and click > Do not click on align racks before start
- The MAP based test would print the missed spots
- After printing has ended Just click on the view dispense error and see if any more errors are present.
- If not, select the “End of Day: option from “Tools”. Fill up the 70% methanol and wait till the machine finishes the function and return to the original interface before shutting down the machine.

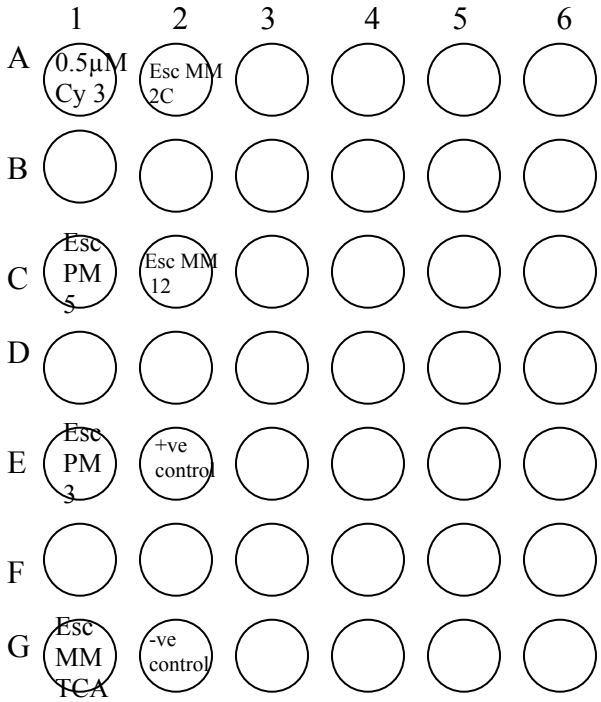
DNA Micro Spotting Layout

An example of spotting is shown below

With spotting of 2 spots per probe for probe format, the layout on the plate looks like this



Probe format



384 plate format

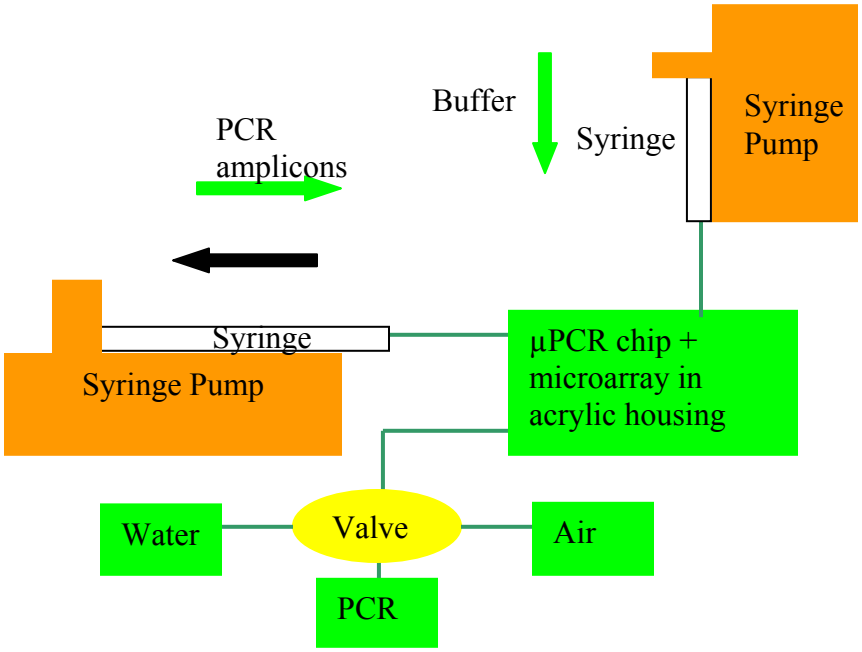
DNA Probe Concentrations for Hybridization

No	Probes	O.D.	Conc(ng/ul)	MW	Conc (µM)	Probe Conc (µM)	Total Vol (µl)	Probe	Water
1	PositivecontrolRev	0.24	792	6312.2	125.4713	5	100	4.0	96.0
2	NegativecontrolRev	0.224	739.2	6327.2	116.8289	5	100	4.3	95.7
3	Enc131Rev	0.223	735.9	6336.2	116.1422	5	100	4.3	95.7
4	Bac303Rev	0.23	759	6352.2	119.4862	5	100	4.2	95.8
5	Ecs447_PM23	0.32	1056	7306.2	144.5348	5	100	3.5	96.5
6	Esc447MMTCA	0.323	1065.9	6410.16	166.2829	5	100	3.0	97.0
7	Esc447MMCTC	0.265	874.5	6425.16	136.1056	5	100	3.7	96.3
8	Esc447MM2C	0.325	1072.5	6434.3	166.6848	5	100	3.0	97.0
9	Esc447MMCG	0.303	999.9	6450.16	155.0194	5	100	3.2	96.8
11	Ecs447_MM12	0.32	1056	7315.2	144.357	5	100	3.5	96.5
12	EFA126_PM21	0.27	891	6511.2	136.8411	5	100	3.7	96.3
13	EFA126_MM12	0.125	412.5	6496.2	63.49866	5	100	7.9	92.1
14	Cy3 Control				82.5	1	100	1.2	98.8
15	Cy3 Control				82.5	0.5	100	0.6	99.4
16	Ecs447_PM23	0.32	1056	7306.2	144.5348	3	100	2.1	97.9
17	Ecs447_PM24	0.32	1056	7306.2	144.5348	1	100	0.7	99.3

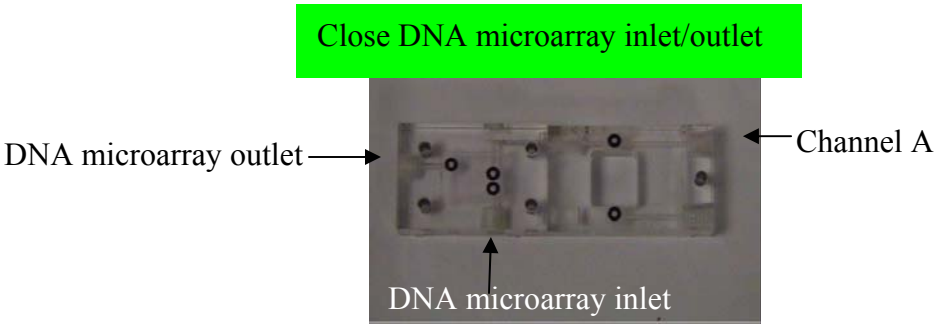
Hybridization buffer (Microbial target)				Washing buffer
	900 mM NaCl	500 mM NaCl	300 mM NaCl	50 mM NaCl
Water	0	1.6	2.4	67
Tris HCl (0.02M)	0.4	0.4	0.4	2
NaCl	3.6	2	1.2	1
FA(30%)	6	6	6	30
Template	10	10	10	-
Volume in µl	10	10	10	10

APPENDIX N Operating Procedures for Micro Total Analysis System

1. Set up connections between syringe pump, syringe, telfon tubings and acrylic housing (with µPCR chip and DNA microarray).



2. Syringe pump is used in the withdrawal mode (black arrow) during setup for PCR
3. Enclosed DNA microarray inlet/outlet using stopper during this phase/use dummy DNA microarray(without inlets) to seal of channels leading to DNA microarray



4. Choose port of valve to turn to determine type of fluid flow into PCR chip
5. Sequence should be as follows:
   A. port for PCR (Volume: 10 µl)* About 13µl sample is prepared for input to chip
   B. port for water (Continuous volume)
6. Position the sample zone into the reaction channels using syringe pump.(Positioning is done by setting pre set volume in syringe pump)
7. Remove stoppers of DNA microarray inlet/outlet or slightly unscrew region of microarray region if dummy microarray is used
8. Fill Channel A with water

9. Enclosed all inlet and outlet of acrylic housing with stoppers and tighen any lossen screws of housing
10. Run PCR
11. Upon completion of PCR, Remove all stoppers and change actual DNA microarray with dummy DNA microarray if used. Attached 2nd syringe pump to inlet of DNA microarray. Start syringe pump at 1µl/min to inject hybridization buffer to DNA microarray chip.
12. In this phase, all syringe pumps are used in "pushing" mode (Green arrow)
13. The PCR sample in µPCR chip is pushed out of the PCR chip at a faster speed. Once PCR sample moves out of channel, syringe pump speed will be change to 1µl/min.
14. When the reaction is complete, DNA microarray is removed and image under microscope