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Design of a microfabricated device for Ligase Detection Reaction (LDR)

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DESIGN OF A MICROFABRICATED DEVICE FOR LIGASE DETECTION
REACTION (LDR)

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science in Mechanical Engineering

in

The Department of Mechanical Engineering

by

Dwhyte Omar Barrett
B.S., Louisiana State University, 2001
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LIST OF SYMBOLS

ρ	Fluid density
ε	Thermal emissivity
α	Radiation Absorption
Δt	Time difference
ν	Kinematic viscosity
β	1/Temperature
ΔT	$T_{\text{Hot}} - T_{\text{Cold}}$
σ	Boltzman's constant
ΔP	Pressure difference, psi
ΔT	Temperature difference, °C
μ	Dynamic viscosity, kg/m s
A	Cross-sectional area, m ²
A_{surf}	Surface area, m ²
a	Seebeck Coefficient (V/ K)
C	Thermal capacitance
c, c_p	Specific heat capacity
c	Convergence rate
D	Width of block, m
d_e	Equivalent diameter, m
d_h	Hydraulic diameter, m
d_f	Diffusion coefficients, m ² /s
Dt	Temperature difference, °C

f	Friction factor
G	Area / Length of T.E. Element (cm)
g	Acceleration due to gravity, m/s^2
h	Channel depth, μm
h_{comb}	Comcined heat transfer coefficient, $\text{W/m}^2\text{-K}$
h_{con}	Convective heat transfer coefficient, $\text{W/m}^2\text{-K}$
h_r	Radiation heat transfer coefficient, $\text{W/m}^2\text{-K}$
I	Current, A
k	Thermal conductivity, W/m-K
K_p	Proportional gain
K_i	Integral gain
K_D	Derivative gain
l	Length of fluid path, m
L	Length of channel, m
L_c	Characteristic length, m
m	Mass, kg
M_{wt}	Molecular weight
N	Number of thermoelectric couples
Nu	Nusselt Number
P	Fluid pressure, N/m^2
Pr	Prandtl number
P_T	Thermal power, W
q	Heat flux

Q	Fan Flow Rate, m ³ /s
Q_{in}	Heat flux in, W
Ra	Rayleigh number (Ra),
R_{cond}	Resistance for conductive heat transfer, Ohm
R_{conv}	Resistance for convective heat transfer, Ohm
Re	Reynolds number
R	Thermal resistance, Ohm
S_e	Effort source for input into or output from a bond graph
S_f	Flow source for input into a bond graph, m ³ /s
T_{∞}	Ambient temperature, °C
T_c	Cold side temperature, °C
T_c	Maximum temperature of coarse grid, °C
T_e	Exact temperature, °C
T_f	Maximum temperature of fine grid, °C
T_g	Glass temperature, °C
T_h	Hot side temperature, °C
T_i	Initial temperature, °C
T_m	Maximum temperature of medium grid, °C
T_{max}	Maximum temperature, °C
T_o	Initial temperature, °C
T_s	Final temperature, °C
t	Time, s
t_l, t_3	Residence Time, s

t_2, t_4	Ramp Time, s
u	Fluid velocity, m/s
u_m	Mean fluid velocity, m/s
v	Mean fluid velocity, m/s
x_2-x_1	Length of channel, m
x	Slit size, m
$x_{fd,h}$	Hydrodynamic development length, m
$W(t)$	Power from controller (W)
w	Channel width, μm
Z	Figure of Merit (K^{-1})

ABSTRACT

The Ligase Detection Reaction (LDR) is a mutation detection technique used to identify point mutations in deoxyribonucleic acid (DNA). Developed by Francis Barany and associates at Cornell University it is used to find specific low abundant point mutations that may lead to colorectal cancer in the early stages of disease development.

The research objective was to design and manufacture a microscale Ligase Detection Reaction (LDR) device in polycarbonate. The LDR module will be incorporated with other microdevices such as: Continuous Flow Polymerase Chain Reaction (CFRCR) and Capillary Electrophoresis (CE) in modular lab-on-a-chip technology. In making the microdevice, the duration of original reaction had to be scaled down from the current 2 ½ hours for 20 cycles for the macroscale reaction. It was found that an excess of primers in relation to PCR product was needed for efficient ligation. By changing the concentrations, volumes and time for the process the current time is down to 40 minutes for 20 cycles with indications that further time reductions are possible on the microscale.

There are two mixing stages involved in the reaction. Micromixers were simulated in Fluent (v5.4, Lebanon, NH) and several test geometries selected for fabrication. Passive diffusion mixing was used based on obtaining high aspect ratios, 7 to 20. The mixers were made by SU-8 lithography, LIGA, laser ablation, and micromilling to characterize each fabrication method. It was found that LIGA was best for making the micromixers, but was the longest process. The micromixers are fabricated and tested using chemi-luminescence technique.

For a successful reaction, temperatures of 0°C, 95°C and 65°C were needed. A stationary chamber was used for thermal cycling in which the sample sits while the

temperature is cycled. Finite element analysis showed uniform temperatures in the rectangular 1.5 μ l chambers and that air slits can effectively separate the thermal cycle zone from the 0°C cooling zone and also isolate the mixing region. A test device was laid out and micromilled with the temperature zones maintained and fluid flow controlled. A commercial thin film heater and a thermoelectric module were used with PID controls to obtain the required process temperatures. Heating from 65°C to 95°C took 10 seconds, while cooling from 95°C to 65°C also took 10 seconds. The residence times at the required temperatures can be adapted to changes in the LDR.

CHAPTER 1: INTRODUCTION

The Human Genome Project began formally at the start of the 1990's as an international, collaborative research program between the U.S. Department of Energy, the National Institutes of Health and several universities (Ommen et al, 1999). The stated goals of the project included identification of the approximately 36,000 genes and three billion base pairs that comprised the human genome. The project also funded innovative research in biotechnology and fostered the development of new medical applications. This promise holds considerable implications for medical advances and improved health care. Together, these goals have the potential to produce a vast body of knowledge and comprehensive understanding of DNA variation among individuals. Revolutionary methods for the diagnosis, treatment and prevention of disorders and diseases may result. It was found that several diseases are attributed to defects in genes. Some cancers and sickle cell anemia have been linked to genetic defects.

Deoxyribonucleic acid (DNA) is a double-stranded helix of nucleotides, which carries the genetic information of a whole organism. The genetic information of all living organisms, including plants, animals, bacterium and viruses have sequences of nucleotide building blocks that are uniquely present in its respective species. Complex organisms such as human beings possess DNA sequences that are uniquely and specifically present only in particular individuals (Saiki, et al. 1988). These unique variations make it possible to trace genetic material back to its origin, identifying with precision the species of organism and often a particular member of that species. In order to study the encoded information, DNA analysis is needed. There are several steps in the analysis of DNA. First a sample has to be retrieved from the individual. The cells have to be opened to release the DNA. This process is known as cell lysis. The released

DNA is amplified to increase the quantity. This is done using the polymerase chain reaction (PCR) (Mullis, 1983). The resultant amplified sample is then investigated by mutation methods, depending on the original aim. The results of this test or the PCR are then quantified during an identification step. As is the case for all clinical tests at this time, these involve the use of large samples (10-50 μ l), expensive equipment that is relatively slow and requires expert operators.

There has been significant research to miniaturize the steps involved in DNA analysis and to incorporate them in a micro total analysis system. These systems require less reagent sample volume, have improved efficiency, and are very cost effective. The advent and development of microfabrication techniques such as LIGA (an acronym from the German words for lithography, electroplating, and molding), laser ablation and micromilling along with improved materials have made these micro total analysis both attractive and feasible.

Currently, Louisiana State University research groups are working to develop a micro total analysis system (Mitchell et al, 2002, Wang et al, 2003, Wabuye et al, 2003, Feng et al, 2004, Henry et al, 2000, Thomas et al, 2002, Soper et al, 2001). The different components will be interconnected with each other or can be stand alone devices. A continuous flow PCR device has been manufactured, while capillary electrophoresis identification and hybridizations chips are currently in use. Most of the chips are made using PMMA (poly methyl methacrylate) or PC (polycarbonate) which are disposable, easy to work with, and readily available. These components were microfabricated and have significantly reduced the time for analysis. There is ongoing research in interconnections, cell capture, and cell lysis micro devices. All of the

devices either use pressure-driven or electrokinetically-driven fluid flow depending on the application.

The focus of the work presented in this thesis is the development of a disease mutation detection step for the micro total analysis system. This reaction is known as the ligase detection reaction (LDR) that can be used to detect colorectal cancer mutations in the early stages of the disease (Barany et al, 1991). The main objective was to design and microfabricate a molded polycarbonate LDR device capable of the required temperature sequence in the lowest possible time. Presently, the reaction is done using tubes that are limited by their size (5 μl), rate of heating and cooling (2-3 $^{\circ}\text{C s}^{-1}$), and cost. Several issues had to be dealt with in order to accomplish this task. The time for the current macroscale reaction had to be reduced for the device to be compatible with other devices in clinical applications in order for timely decisions to be made by a doctor and patient. The device must be able to produce the desired temperature cycles and maintain these temperatures for the necessary time durations in order to obtain sufficient product. The reaction has several reagents that require micromixers. By making DNA mutation analysis quicker and less expensive, the use of the analysis could become more widespread in medicine, leading to an improvement in medical diagnostics.

This thesis is arranged as follows: Chapter 2-The background of mutations, detection, PCR, LDR and a review of the literature on LDR including the reaction time reduction experiments; Chapter 3-Design, simulation, microfabrication, and testing of test micro mixers. The most feasible device mixer configuration was selected based on the results; Chapter 4- Detailed heat transfer simulations using ANSYS (v5.7, ANSYS, Inc., Houston, PA) were performed. Models of the fluid and thermal characteristics of

the LDR chip were developed and used to determine the thermal power input requirement and construct the necessary control circuit to achieve the temperatures required. Chapter 5-This chapter includes the discussion, conclusions, and recommendations for future development of this project.

CHAPTER 2: BACKGROUND

This chapter will provide the background information on mutations, genes, colorectal cancer, the polymerase chain reaction (PCR), and the LDR. It was necessary to find out how mutations occur, any links they have to diseases, and how they are identified. A full knowledge of the LDR process was required including the reaction conditions, reagents, and detection methods in order to investigate if the reaction time and volume could be reduced.

2.1 MUTATION DETECTION

A *mutation* is any heritable change in the DNA sequence (Friedberg et al, 1995). Only a permanent change in the sequence of DNA constitutes a mutation, as some changes such as DNA damage are repaired so that the original sequence is maintained. Deoxyribonucleic acid or DNA carries the genetic information of the cell (Stryer, 1995). This information is processed within the cell during replication and transcription. During replication an enzyme, DNA polymerase, copies the information and during transcription an enzyme, RNA polymerase, transcribes the DNA into messenger ribonucleic acid, mRNA. Transcription only occurs on portions of DNA that are genes. The locations of transcription are governed by specific sequences termed promoters. Exons are portions of genes where transcription of DNA occurs while the other areas are termed introns. The information of DNA is stored within a sequence that is made up of four nucleotides, each containing a different aromatic base: adenine (A), thymine (T), guanine (G), and cytosine (C). Any error, or mutation, that occurs in the strings of nucleic acids may cause the process to fail. Therefore, the study and detection of mutations is very important to understanding the links between genetics and disease.

Mutations are classified based on the products formed after transcription and translation (Friedberg et al, 1995). During translation codons, groups of three bases, code for amino acids. If the amino acid that is encoded does not change, even though the codon sequence is changed, the mutation that occurred is termed a silent mutation. Mutations that are not silent are classified into frameshift mutations and point mutations (Friedberg et al, 1995). Frameshift mutations occur when a portion of the DNA sequence is deleted or another sequence is inserted. These types of mutations lead to a complete change of codon sequences throughout the length of the exon. Point mutations are characterized by the substitution of a single base for another at a specific location.

Mutations that occur within the genome can be spontaneous or can be induced. Spontaneous mutations result from the natural process of the cells, while induced mutations are caused by the interaction of DNA with an outside agent or mutagen (Friedberg et al, 1995). Errant DNA replication is the most important and frequent cause of spontaneous mutations. If a DNA polymerase inserts an incorrect base that is not subsequently corrected the mutation is conserved throughout further rounds of replication and transcription, affecting the sequence produced. Spontaneous mutations can also be caused by natural structural alterations of the bases and by damage to the bases caused by metabolites of other cellular processes (Friedberg et al, 1995).

Mutagens are natural or man-made chemicals that alter DNA bases (Friedberg et al, 1995). Intercalating dyes such as acridine orange, proflavin, and ethidium bromide are mutagens due to their ability to insert themselves between the bases of a DNA strand causing the DNA polymerase to insert an extra base across from these intercalating agents (Stryer, 1995). Ionizing radiation is energetic enough to cause the formation of reactive ions when reacting with biological molecules. This type of

radiation also causes the formation of free radicals, formed from water molecules, and peroxide, formed during cellular respiration, that can then act as mutagens. UV radiation is less energetic than ionizing radiation but the bases of DNA preferentially absorb these wavelengths (Friedberg et al, 1995).

While there are repair pathways for most mutations, not all are repaired and are left to replicate (Friedberg et al, 1995). Mutations that are not repaired can possibly lead to disease if they are located within the coding region of a gene. Many diseases such as cancer, asthma, myocardial infarction, diabetes, atherosclerosis, cystic fibrosis, Alzheimer's, and sickle cell anemia, are associated with genetic alterations in genomic DNA (www.ncbi.nlm.nih.gov/disease, 2004). Identifying particular genetic mutations is very important in order to utilize presymptomatic diagnosis and to individualize the treatment. Many forms could be prevented with early detection of genetic alterations that cause the disease. Detection of genetic predispositions to, for example, heart disease and cancer, would revolutionize clinical diagnosis and permit significant progress in preventative medicine. Once the molecular basis of any disease is understood, analysis of the relevant genetic material requires robust tools to uncover its presence.

An important problem in detecting point mutations is the occurrence of low abundant mutations or mutations that occur in low concentrations in the early development of a disease. Techniques that are sensitive enough to detect low concentrations of mutations in very high concentrations of normal (wild type) DNA are a requirement for the early detection of disease. Point mutations that are associated with certain diseases may fall consistently in one position or several different positions along a large span of a gene. For this reason two basic types of methods for mutation

detection have been developed, scanning and diagnostic techniques. Diagnostic methods are employed when the location of the mutation of interest is already characterized, while scanning or screening methods are used for an unknown location.

While scanning methods potentially allow for the detection of all mutations in a specific region of DNA, the presence of the mutation is the only information obtained and further analysis is required. Diagnostic methods detect an already characterized mutation and give the specific sequence composition of the mutation. Although direct DNA sequencing, determining the primary structure of the DNA fragment under investigation, is the best standard for diagnostics, the immense time and labor required to directly sequence DNA has led to the development of alternative methodologies. In addition, direct sequencing cannot determine the presence of low abundant mutations and is generally ineffective at analyzing samples containing both normal and mutant DNA. The detection of low abundant mutations has become a major goal of mutation detection for early detection of disease or the recurrence of disease.

2.1.1 SCANNING METHODS

One of the first scanning methods developed was restriction fragment length polymorphism (RFLP) (Schumm et al, 1988). RFLP's consists of fragments of DNA produced after restriction enzyme sites found in the target are cut by restriction endonucleases that recognize short sequences. Point mutations, which must occur in the cutting site of the restriction endonuclease, change the pattern of fragments produced by these polymorphisms allowing separation and analysis via electrophoresis to uncover the presence of the mutation. The technique is limited in that the mutation must occur at an existing restriction site or an additional restriction site must be created by the mutation.

Many other scanning methods use the fact that changes in the properties of DNA occur when a mismatch is present in the structure due to the presence of the mutation. These properties include modifications in electrophoretic mobilities and/or changes in stability. One such example is heteroduplex analysis (HDA) which is a detection strategy that detects mutant DNA molecules by thermal denaturation and reannealing to form homo- and heteroduplexes of wild type and mutant alleles that have different electrophoretic mobilities due to bulges or thermodynamic instabilities induced by mismatches (Nagamine et al, 1989).

2.1.2 DIAGNOSTIC METHODS

The majority of diagnostic techniques use amplification via the polymerase chain reaction (PCR). PCR can be done either non-specifically, in which primers flank the coding region containing the mutation or specifically, in which allele specific primers that hybridize to the mutation site are used in the PCR step. If allele specific PCR is not used, the primary PCR reaction must be followed by another reaction that can discriminate between the wild type and mutant DNA. A major concern associated with mutation diagnostic methods is the ability to detect low abundant mutations. Diagnostic techniques must be sensitive enough to detect the mutant DNA without generating false positives or false negatives.

Allele specific PCR is accomplished with specially designed primers of defined lengths, which hybridize to the target at the mutation potentially producing mismatches. The premise is that mismatches in the primers impede or reduce the efficiency in the amplification step (Smooker et al, 1993). Other diagnostic methods use a DNA ligase enzyme. A DNA ligase is an enzyme that will catalyze the formation of a phosphodiester bond preferentially in oligonucleotides hybridized to a target DNA

strand. DNA ligase has very high selectivity to “seal” DNA in which there is perfect complementarity (match). A single base mismatch at the junction inhibits ligation. The ligase detection reaction is one such diagnostic method (Barany, 1991). In low level mutation detection of genetic diseases such as cancer, PCR/LDR detection reactions have been used to detect mutations with a sensitivity of 1 mutant in 1000 normal alleles (Wang et al, 2003).

2.1.3 GENE MARKERS - kras GENE

Researchers know of DNA mutations that often affect certain genes, such as the APC gene, kras oncogene, and p53 tumor suppressor gene in colorectal cancer cells. Studies are testing new ways to recognize these DNA mutations in cells found in stool samples, to see if this screening approach is useful in finding large polyps and colorectal cancers at an earlier stage. Early detection of cancers requires the ability to detect mutations in DNA.

Colorectal cancer has been used as a model system for mutation detection since genetic variations associated with the cancer involve progressive alterations in APC, hMSH2, hMLH1, kras, DCC and P53 genes. Point mutations in the kras gene are found in 35-50% of colorectal adenomas and cancers and they also occur early in the development of colorectal neoplasm. Most of these mutations are localized on codon 12, and to a lesser extent at codons 13 and 61 (Khanna et al, 1999). kras mutations are preserved throughout the cancer development and can serve as excellent biomarkers for diagnostic testing. However, in the early stages of tumor progression, cells that contain somatic mutations represent a small fraction compared to the normal cells, which makes their detection difficult. Therefore, highly sensitive assays have to be developed in order to improve the efficiency in the diagnosis.

Direct sequencing has been applied for detection of kras mutations in primary tumors, although it is limited due to its low sensitivity (Ruiz-Martinez, 1998). In addition, allele-specific oligonucleotide hybridization or restriction digestion can also be applied. Highly sensitive techniques, such as phage cloning or allele-specific PCR, have been used to detect kras mutations in stool or lymph nodes of cancer patients at early stages (Singh et al, 1996). Since some of the techniques developed are not able to detect the full spectrum of kras mutations, new screening detection strategies are being developed to overcome this limitation.

2.2 COLORECTAL CANCER

Cancer is the second leading cause of death in the United States, comprising 23% of all deaths. Cancer occurs when cells in a part of the body begin to grow out of control. Normal cells divide and grow in an orderly way. Cancer cells do not. Colon and rectal cancers begin in the digestive system, also called the GI (gastrointestinal) system. This is where food is changed to create energy and rid the body of waste matter. Colorectal (colon and rectal) cancer is the second leading cancer killer in the United States. In 2004, the American Cancer Society estimated that 146,940 people in the U.S. will be diagnosed with colorectal cancer and 56,730 people will die of the disease (www.cancer.org, 2004).

Most of these cancers begin as a polyp--a growth of tissue into the center of the colon or rectum. Polyps are also known as adenomas. Removing the polyp early may prevent it from becoming cancerous.

2.2.1 CAUSES OF COLORECTAL CANCER

No definitive cause has been identified for colorectal cancer and everybody is susceptible to the disease. However, there are several risk factors that will increase the

Figure 2.2: Diagram of anatomy showing location of colon and rectum

chances of a person catching the disease. People with a personal history of colorectal cancer, personal history of colorectal adenomatous polyps, or a history of chronic inflammatory bowel disease are at high risk of developing the disease. The disease may recur if it was removed or may develop from the polyps or the inflamed bowel (www.cancer.org, 2004).

Colorectal cancer is hereditary. A family history of colorectal cancer increases the risk for the disease. People who have two or more close relatives with colorectal cancer make up about 20% of all people with colorectal cancer. About 5%-10% of patients with colorectal cancer have an inherited genetic abnormality that causes the cancer. Familial adenomatous polyposis is a disease where people typically develop hundreds of polyps in their colon and rectum. This usually occurs between the ages of 5 and 40. By age 40 almost all people with this disorder will have developed cancer if preventive surgery is not done. Jews of Eastern European descent (Ashkenazi Jews) are

thought to have a higher rate of colorectal cancer due to a specific genetic mutation (www.cancer.org, 2004).

Aging, physical inactivity, obesity, a diet mostly from animal sources, diabetes, smoking, and alcohol intake all increase the chances of developing colorectal cancer. More than 90% of people found to have colorectal cancer are older than 50. People with diabetes not only have 30% to 40% increased chance of developing colorectal cancer, they also tend to have a higher death rate after diagnosis. It is imperative that everyone at some point in time be screened for the disease.

2.2.2 DETECTION OF COLORECTAL CANCER

Screening tests are used to spot a disease early, before there are symptoms or a history of that disease. Screening for colorectal cancer means that it can be found at an early curable, stage, and it can also be prevented by finding and removing polyps that might eventually become cancerous (www.cancer.org). There are several tests used to screen for colorectal cancer:

Fecal occult blood test: Used to find occult (hidden) blood in feces. Blood vessels at the surface of colorectal polyps or adenomas or cancers are often fragile and easily damaged by the passage of feces.

Flexible sigmoidoscopy: A sigmoidoscope is a slender, flexible, hollow, lighted tube about the thickness of a finger. It is inserted through the rectum into the lower part of the colon.

Colonoscopy: A colonoscope is a longer version of a sigmoidoscope. It is inserted through the rectum and allows a doctor to see the lining of the entire colon. The colonoscope is also connected to a video camera and display monitor so that the doctor can closely examine the inside of the colon. If your doctor sees a large polyp or tumor

or anything else abnormal, a biopsy will be done. In this procedure, a small piece of tissue is taken out through the colonoscope. Examination of the tissue can help determine if it is a cancer, a noncancerous (benign) growth, or a result of inflammation.

Barium enema with air contrast: Barium sulfate, a chalky substance, is used to partially fill and open up the colon. When the colon is about half-full of barium, the patient will be turned on a x-ray table so that the barium spreads throughout the colon. Air will be pumped into the colon to make it expand. This produces the highest contrast pictures of the lining of the colon.

The best treatment method for the disease will be selected by a physician, with input from the patient, based on the results of these screenings,

2.2.3 TREATMENT OF COLORECTAL CANCER

According to the American Cancer Society the death rate from colorectal cancer has been decreasing for the past 15 years. One reason is that there are fewer cases. Also, they are being found earlier, and treatments have improved. The three main types of treatment for colon cancer and rectal cancer are surgery, radiation therapy, and chemotherapy. Depending on the stage of the cancer, two or more of these types of treatment may be combined at the same time or used after one another (www.cancer.org).

Stage 0: Cancer has not grown beyond the inner lining of the colon. Surgery to take out the cancer is all that is needed. This may be accomplished in many cases by polypectomy or local excision through the colonoscope. Colon resection may be necessary if the tumor cannot be removed by local excision.

Stage I: Cancer has grown through several layers of the colon, but has not spread outside the colon wall itself. Surgical resection to remove the cancer is the standard treatment.

Stage II: Cancer has grown through the wall of the colon and may extend into nearby tissue, but has not yet spread to the lymph nodes. Surgical resection is usually the only treatment needed, but if it was growing into other tissues, radiation therapy or chemotherapy may be recommended.

Stage III: Cancer has spread to nearby lymph nodes, but not yet spread to other parts of the body. Surgical resection is the first treatment, followed by chemotherapy.

Stage IV: Cancer has spread to distant organs and tissues such as the liver, lungs, peritoneum, or ovaries. Surgery, segmental resection or diverting colostomy, in this stage is usually to relieve or prevent blockage of the colon and to prevent other local complications. Surgery in stage IV is usually not done with the expectation of curing the colon cancer.

Recurrent colon cancer: Cancer has returned after treatment. Recurrence may be local (near the area of the initial tumor) or it may affect distant organs. If metastases cannot be removed, chemotherapy is the primary treatment (www.cancer.org, 2004).

2.3 PCR

The polymerase chain reaction, PCR, was invented in 1983 by Dr. Kary Mullis. He was awarded the Nobel Prize in Chemistry ten years later. It is a powerful technique, which results in the rapid production of multiple copies of a target DNA sequence. PCR requires a template molecule (the desired DNA) and two primer molecules to begin the copying process. The primers are short chains of four different chemical components that make up any strand of genetic material. These four components, commonly known

as nucleotides or bases (Mullis et al, 1994), are the building blocks used to construct genetic molecules. Since its discovery by Kary Mullis in the mid 1980s (Saiki et al, 1998), PCR has become one of the most important techniques available for genetic analyses (Mullis et al, 1994]. PCR takes analysis of minute amounts of genetic material to a new level of precision and reliability while being far simpler and less expensive than previous techniques for duplicating DNA (Saiki et al, 1998).

2.3.1 PCR STEPS

The first step in PCR is to separate the two DNA chains of the double helix. As the two strands separate, DNA polymerase makes a copy using each strand as a template. The adenine (A), on one strand always pairs with the thymine (T), on the other, whereas cytosine(C), always pairs with guanine (G) [<http://www.accessexcellence.org>, 2003]. The products of one PCR cycle can act as templates for the next PCR cycle, so the number of new identical molecules produced doubles with each repetition of the cycle.

The three parts of the PCR are carried out at different temperatures. At 95 °C the two DNA chains are split. This is done for about 30 seconds. The primers cannot bind to the DNA strands at such a high temperature, so the vial is cooled to 55 °C. At this temperature the primers anneal to the ends of the DNA strands for about 20 seconds. Two different primers are used to bracket the target sequence to be amplified. One primer is complementary to a strand at the beginning of the target sequence while the second primer is complementary to the strand at the end of the sequence. The final step of the reaction is to make a complete copy of the templates at around 75 °C. The Taq polymerase begins adding nucleotides to the primer and eventually makes a complementary copy of the template. If the template contains an A nucleotide, the

enzyme adds on a T nucleotide to the primer. If the template contains a G, it adds a C to the new chain, and so on to the end of the DNA strand. This completes one PCR cycle. The three steps in the polymerase chain reaction - the separation of the strands, annealing the primer to the template, and the synthesis of new strands can be completed in less than 12 seconds (Innis, 1988) or may be repeated or cycled for the target sequence to be amplified exponentially.

2.3.2 LITERATURE REVIEW OF PCR DEVICES

Typical laboratory equipment and techniques for performing PCR are very expensive, time consuming and are not robust. They usually consist of large thermal cyclers with heating and cooling rates of 2-3 °C/s, and 10-50µl tube vials that have to be moved from the amplification to the detection stage. Operation requires expertise, the use of large quantities of reagents and can take on the order of hours to complete. The performance of microfluidic devices capable of doing these functions are significantly better in terms of speed, costs, flexibility and performing multiple functions on the same device. New manufacturing techniques such as LIGA (an acronym from the German words for lithography, electroplating, and molding) for building microfluidic components have led to an increase in the development of microscale biological and chemical analysis systems. Table 2.3.2 shows some publications that have dealt with miniaturized PCR. The table identifies the different groups, sample volume used, reaction time, the primary materials used for device construction, and the number of cycles incorporated on the chip.

In 1989, Wittwer et al. reported a thermocycler that was similar to conventional models and used air for heating and cooling (Wittwer, et al. 1989 and 1990). Capillary tubes, having sample volumes of 10µl, were used. This was different from the

Table 2.3.2: Cited Works of Prior MicroPCR Devices

Groups	Sample Volume	Reaction time	Material	Cycles
Landers, et al. 1998	5-15 μ l	14min	Glass	30
Kopp, et al. 1998	5-20 μ l	50min	Glass-copper	30
Friedman, et al 1998	5 μ l	20 min	Glass	35
Ferrance, et al. 2000	10 μ l	7 min 44sec	Silicon	30
Lagally, et al. 2000	10 μ l	15 min	Glass	20
Khandurina, et al. 2000	7 μ l	20 min	Cr-Glass	10
Giordano, et al. 2000	5 μ l	4 min 50 sec	Polyimide	15
Lin, et al. 2000	50 μ l	30 min	Silicon/Glass	20
Nagai, et al. 2001	1.3 pl -32 μ l	120min	Silicon	40
Schneegass, et al. 2001	50 μ l	35 min	Glass	7
Chou, et al. 2002	19 μ l	40 min	Cofired Ceramics	30
Mitchell, et al. 2002	5 - 20 μ l	5-15 min	Polycarbonate	20
Yoon, et al. 2002	3.6 μ l	62 min	Silicon	30
Shin, et al. 2003	2 μ l	30 min	PDMS	30

conventional method of sample blocks. Air was pumped into a cycling chamber at the appropriate temperature. This work showed that cycle times could be optimized and transition times between temperatures could be reduced by lowering the thermal capacitance of the system.

PCR may occur either with the sample stationary, as in the case of the conventional method, where the temperature of the batch changes, or continuously, by

moving the sample between temperature zones. Lin et al demonstrated a stationary microPCR in a silicon diced microchamber on a glass substrate with a volume of 50 μ l (Lin et al., 2000). The required temperature profile was achieved using a thermoelectric unit with a power supply controlled by Labview software. The heating and cooling rates were 4°C/s and 2.2°C/s respectively with a total reaction time of 30 minutes for 30 cycles.

Recently, Mitchell et al produced a disposable continuous flow PCR chip in molded polycarbonate (Mitchell et al, 2002). The layout of their device is shown in Figure 2.3.2.

Made with the LIGA process, the chip consisted of three zones heated to the required temperatures by Kapton thin film heaters. The channels were 50 μ m by 150 μ m and 1.8 m long with flow rates ranging from 2 mm/s to 15 mm/s. This produced a total PCR reaction time of 15 minutes for 20 cycles. Hot embossing polycarbonate is a repeatable process that is crucial for mass production of PCR chips.

Figure 2.3.2: PCR Spiral device conjugation designed by Yannick Bejat (Bejat, 2001) and fabricated by Michael Mitchell (Mitchell, 2002). Two devices are in the layout.

2.4 LDR

The ligase detection reaction (LDR) was designed as a point mutation detection strategy in order to identify low abundant mutations (Khanna et al, 1999). It involves processing a mixture of buffers, template DNA (PCR product), and the primers. Oligonucleotide primers, a common and a discriminating primer, are annealed adjacently to one strand of the target DNA (Figure 2.4a & 2.4b). The adjoining discriminating and common primers are then covalently joined by a thermostable DNA ligase to form an LDR product if the target nucleotide at the mutation site is complementary to the 3'-end of the discriminating primer. All possible mutations at a specific site can be analyzed by including all possible discriminating primers corresponding to the possible nucleotide substitutions.

Generation of an LDR product indicates the presence of a mutation while the size of the product indicates which specific substitution is present. The temperatures that are typically used in the LDR reaction include 92-96°C for denaturation of the double-stranded DNA molecule, and 64-66°C for ligation of primers to the single-stranded DNA template. This temperature sequence is then cycled, typically 10-50 times to increase the probability of finding mutations.

2.4.1 PCR/LDR

The polymerase chain reaction (PCR) has become the most widely used technique for DNA analysis and is thus the centerpiece of most mutation detection strategies. Most methods used are not sensitive enough to detect genomic DNA therefore sections containing mutations to be analyzed must be amplified by PCR prior to analysis.

Figure 2.4(a): With a point mutation the ligase attaches the czip-11 and dye labeled com-2 together alongside the DNA at the mutation point;
 (b) When there is no mutation the com-2 does not attach.

The PCR amplifies both mutant and wild-type DNA in a sample. The advantages of a PCR/LDR assay are that it can be configured to do highly multiplexed assays by using the ligase enzyme to linearly amplify the LDR product and can potentially detect 1 mutant DNA in 1000 copies of normal DNA (Wang et al, 2003). Doing LDR on PCR products improves the detection specificity.

2.4.2 STEPS AND REQUIREMENTS FOR LDR

Figure 2.4.2 shows the full schematic representation of the LDR reaction. After mixing the PCR products, primers, buffers and salts the resulting cocktail is preheated to 95°C and held at this temperature for 2 minutes. After the initial preheat the ligase enzyme, which is stored at 0°C, is added and mixed. The mixture then goes through 20-30 thermal cycles of 95°C and 65°C for 30 seconds and 4 minutes respectively (Barany, 1991). This gives a nominal total reaction time of over 2 ½ hours. The reaction is stopped by cooling to 0 °C, at which time the products are analysed.

Figure 2.4.2: Schematic representation of the ligase detection reaction (LDR)

2.4.2.1 PRIMERS AND BUFFERS

The reaction uses the amplified PCR products and four primers: dye labeled com-2, czip-11, DTT and NAD. These are in a buffer solution and mixed together and heated to 95°C for 90 seconds. Primers are designed and selected based on the specific mutation that is being sought. Usually a fluorescence-labeled discriminating primer and a phosphorylated common primer, both in excess of the target DNA to which the primers are annealed, is used. This excess of discriminating primers and common primer is required to ensure complete annealing of the target DNA and efficient ligation (Wang et al., 2003). This surplus of primers causes annealing competition when the assay is used for analysis of multiple mutations or multiplexing.

2.4.2.2 LIGASE ENZYME

Ligase is a thermo-stable enzyme. This enzyme will catalyze the formation of a phosphodiester bond preferentially in oligonucleotides hybridized to a target DNA strand (Wang, et al., 2003). DNA ligase has very high selectivity to “seal” DNA in which there is perfect complementarity (match). A single base mismatch at the junction inhibits ligation. If a mutation is present the ligase enzyme is able to anneal the dye labeled com-2 to the DNA strand. The enzyme is not able to anneal the com-2 if no mutation is present; making detection of a mutation possible.

2.4.2.3 TEMPERATURE PROFILE

The first part of the reaction is to separate the DNA chains by denaturing the genetic material through heating to 95°C. The primers cannot bind to the DNA at this temperature, so the vial is cooled to 65°C, where the czip-11 and com-2 primers anneal alongside the DNA strand.

2.4.3 CURRENT PROTOCOL FOR LDR

Conventional LDR is typically performed by thermocycling the LDR mixture in a thin-walled plastic vial embedded in a temperature controlled aluminum heat sink (also known as a block thermal cycler). Due to the high thermal capacitances of the materials, the ramp rate of the temperature is typically on the order of 2°C per second for heating and cooling. For the temperature transition between the 95 °C and 65 °C zones the time would be approximately 15 sec. The cycle speed is limited by the thermal capacitance of the heat sink and the heat transfer rate between the heat sink and the LDR sample. To complete this analysis in a conventional LDR block thermal cycler takes approximately 2½ hrs. The process also involves mixing different reagents which requires experience. As in the case of conventional PCR there is significant reagent consumption and the reaction takes on the order of hours to complete.

2.4.3.2 EQUIPMENT AND PROCEDURES

Allele-specific ligation primers for identifying point mutations in codon 12 were designed based on the known sequence of the kras gene. Com-2, common primer (5'-pTGGCGTAGGCAAGAGTGCCCGCTGCGCCGC-Cy55-3', near-IR dye-labeled oligonucleotide molecules were purchased from LI-COR Biotechnology (Lincoln, NE).

Figure 2.4.2.3: The desired temperature profile for a single LDR cycle

The czip-11 discriminating primer (5'-Cy5-GCGGCGCAGCAAACTTGTGGTAGT TGGAGCTGA-3' was obtained from Integrated DNA Technologies (IDT, Coralville, IA)). 20 mM Tris-HCl (pH 8.3), 25 mM KCl, 10 mM MgCl₂, 0.5 mM NAD⁺, 0.01 % Triton X-100, 10 pM of LDR primers and a mixture of genomic DNA, DTT, NAD, buffer, and PCR product (G12V synthesized in Cornell University Lab), were first mixed together for a total volume of 20 µl in 50 µl tubes using an Eppendorf centrifuge (Westbury, NY), then heated to 95°C and held for 2 minutes in a Techne thermal cycler (Burlington, NJ). The ligase (0.25 U thermostable DNA ligase (Ampligase™, Epicenter Technologies, Madison, WI)) stored in the refrigerator until use was then added to the mixture using the Eppendorf centrifuge (Westbury, NY). The vial was placed in the Techne thermal cycler (Burlington, NJ) that was used for the desired temperature cycling by running a preset control program. After the thermal cycle the vial was cooled to 0°C to stop the reaction and storage. The products were then placed in a LiCor DNA analyzer (Lincoln, NE) as specified by the manufacturer for analysis to determine the composition of the analyte.

2.5 LITERATURE REVIEW FOR LDR

A review of the literature shows several uses of LDR. Barany has teamed with other researchers to utilize the PCR/LDR assay. Belgrader et al used a multiplex PCR-ligase detection reaction assay for human identity testing (Belgrader et al, 1996). Day et al demonstrated a methodology for genetic diagnosis of 21-hydroxylase deficiency, the most common cause of congenital adrenal hyperplasia, an inherited inability to synthesize cortisol that occurs in 1 in 10,000-15,000 births. The method uses gene-specific PCR amplification in conjunction with thermostable DNA ligase to discriminate single nucleotide variations in a multiplexed ligation detection assay (Day

et al, 1995). The assay was designed to use either fluorescent or radioactive detection of ligation products by electrophoresis on denaturing acrylamide gels. Favis et al developed a harmonized protocol involving multiplex polymerase chain reaction/ligase detection reaction (PCR/LDR) with Universal DNA microarray analysis and endonuclease V/ligase mutation scanning for identifying TP53 mutations in 138 stage I-IV colorectal adenocarcinomas and liver metastases without first enriching for tumor cells by microdissection. In the study, sequences were verified using dideoxy sequencing. Data analysis comparing colon cancer entries in the TP53 database (<http://p53.curie.fr>) with the results reported in this study showed that distribution of mutations and the mutational events were comparable (Favis et al, 2004).

Other researchers have also used the LDR. Busti et al showed the combined use of selective probes, ligation reaction and a universal microarray approach yielded an analytical procedure with a good power of discrimination among bacteria. (Busti et al, 2002). McNamara et al developed a multiplex PCR-ligase detection reaction (LDR) assay that allows the simultaneous diagnosis of infection by all four parasite species causing malaria in humans. This assay exhibits sensitivity and specificity equal to those of other PCR-based assays, identifying all four human malaria parasite species at levels of parasitemias equal to 1 parasitized erythrocyte/microl of blood. The multiplex PCR-LDR assay goes beyond other PCR-based assays by reducing technical procedures and by detecting intra individual differences in species-specific levels of parasitemia (McNamara et al, 2004). Gaffney et al found a method to identify and differentiate two homologues of the SSX gene (SSX1 and SSX2) in synovial sarcomas (SS). It was accomplished by reverse-transcription polymerase chain reaction (RT-PCR) followed by fluorescent thermostable ligase detection reaction (f-LDR), microparticle bead

capture and flow cytometric detection. It showed the f-LDR method with flow-based detection is a robust approach to post-PCR detection of specific nucleotide sequences in synovial sarcomas (SS) and may be more broadly applicable in molecular oncology. (Gaffney et al, 2003). Delrio-Lafreniere et al developed a low-density DNA array for the detection and typing of human papillomavirus (HPV) DNA. The gene chemistry strategy involves using a combination of the polymerase chain reaction (PCR) with the consensus oligonucleotide primers MY09/MY11 followed by a ligase detection reaction (LDR). Fluorochrome-labeled HPV-specific primers are joined to a common primer modified with a unique anchoring sequence called a zip code on its 3' end. The result is a series of 60-70 base pair and single-stranded ligation products that are then hybridized to their respective zip code complements affixed to glass slide based arrays. These consensus primers were shown to detect over 40 different HPV types. The purpose of this study was to evaluate the analytic performance of this low-density microarray based assay for HPV (Delrio-Lafreniere et al. 2004).

The LDR has also been used for food applications. Bordini et al applied the ligation detection reaction (LDR) combined with a universal array approach to the detection and quantitation of the polymerase chain reaction (PCR) amplified cry1A gene from Bt-176 transgenic maize. The study demonstrated excellent specificity and high sensitivity as little as to 0.5 fmol (nearly 60 pg) of PCR amplified transgenic material was unequivocally detected with excellent linearity within the 0.1-2.0% range with respect to wild-type maize. (Bordini et al, 2004).

The model for LDR in this thesis is identifying rare point mutations in codons 12, 13, and 61 of the kras gene that occur early in the development of colorectal cancer and are preserved throughout the course of tumor progression. Khanna et al, also in

collaboration with Barany, have shown that these mutations can serve as biomarkers for shed or circulating tumor cells and may be useful for diagnosis of early, curable tumors and for staging of advanced cancers. They developed a multiplex polymerase chain reaction/ligase detection reaction (PCR/LDR) method that identifies all 19 possible single-base mutations in kras codons 12, 13, and 61, with a sensitivity of 1 in 500 wild-type sequences (Khanna et al, 1999).

Wabuye et al coupled LDR to single pair fluorescence resonance energy transfer (spFRET) to rapidly indentify single base point mutations in codon 12 of a kras gene without the use of PCR for amplification. The results showed that it was possible to discriminate single based differences in the kras gene in less than 5 minutes at a frequency of 1 mutant DNA per 10 normals using a single LDR thermal cycle on a microchip with genomic DNA (Wabuye et al, 2003).

2.6 LDR MICRODEVICE DESIGN ISSUES

Even though much of the research demonstrated the use of microarrays for end analysis, the ligase detection reaction was done using the conventional protocol and equipment as discussed earlier. Only Wabuye used a microchip, but this was only for a single cycle and the reagents were prepared off chip. The principal objective of this work was to design and microfabricate a LDR device capable of executing the required temperature sequence in the shortest possible time. To achieve a microdevice for LDR several design issues were addressed:

- A suitable material had to be chosen for the device, with a means for mass-producing such a device;
- Reduce the reaction time and reagent volumes;
- Mixing the primers, PCR product and ligase on the device;

- Obtaining and maintaining the required temperatures of 95°C, 65°C, 0°C (storage of ligase) on the device.
- Include an end analysis step on the device such as electrophoresis or a zip code array.
- The device should be compatible and be able to interconnect with other devices, such as a microPCR, of a micro total analysis system.

It was shown that the miniaturization of PCR has improved the functionality and feasibility of that reaction. By addressing these design issues similar improvements are expected.

2.7 LDR TIME AND VOLUME REDUCTION

Experiments were conducted to quantify whether the reaction time and reagent volumes could be reduced from the nominal values in the standard protocol. A test matrix was developed to systematically evaluate which factors affected the reaction in order to reduce the duration of each cycle. Table 2.7 shows the volume and composition of the reactants for some representative test runs. Column 1 shows the original reaction and the sample volumes and concentrations of the different reagents.

Table 2.7: Example of test matrix used to quantify LDR time reduction.

RUN 1	RUN 2	RUN 3
0.1 μ M G12 V 1 μ l 1 μ M czip-11 2 μ l 1 μ M com-2 2 μ l 200 mM DTT 1 μ l 10 mM NAD 1 μ l 2 x buffer 12 μ l Ligase 1 μ l	0.1 μ M G12 V 1 μ l 1 μ M czip-11 2 μ l 1 μ M com-2 2 μ l 200 mM DTT 1 μ l 10 mM NAD 1 μ l 2 x buffer 12 μ l Ligase 1 μ l	0.1 μ M G12 V 1 μ l 1 μ M czip-11 2 μ l 1 μ M com-2 2 μ l 200 mM DTT 1 μ l 10 mM NAD 1 μ l 2 x buffer 12 μ l Ligase 1 μ l
94 C 30 s 65 C 4min X 20 cycles	94 C 30 s 65 C 2min X 20 cycles	94 C 30 s 65 C 1min X 20 cycles

In the current macroscale reaction the reagents are mixed in 50 μ l tubes and a Techne thermal cycler (Burlington, NJ) is used to obtain the desired temperature profile. Com-2, czip-11, DTT, NAD, buffer and PCR product (G12) were first mixed together in the tubes, using an Eppendorf centrifuge (Westbury, NY), then heated to 95°C and held for 2 minutes. The ligase was added and mixed before the thermal cycle. The products were analyzed using a LiCor DNA analyzer (Lincoln, NE) to determine the composition of the analyte. Two main tests were conducted. In the first set the volume of the primers was changed with the other reagents volume remaining the same over four runs. In the second set the time for annealing at 65°C was changed from 4 minutes through to 1 minute over four runs with the reagent volumes constant.

2.7.1 LDR PRODUCT DETECTION METHODS

There are three main methods used for analyzing LDR products, DNA sequencer, microchip electrophoresis, and zip code array. The end method is selected based on the quantity of products and the ratio of wild type to mutant DNA in the original sample.

2.7.1.1 DNA SEQUENCER- SLAB GEL ELECTROPHORESIS

LDR products were first analyzed by slab gel electrophoresis. Slab gel electrophoresis is the conventional electrophoretic format used to separate biomolecules on the basis of their sizes. Several samples are analyzed on a flat, horizontally or vertically oriented gel containing wells at one end for sample introduction. The gel, agarose or polyacrylamide, is submersed in buffer and sample solutions are placed in the wells at the top of the walls. A voltage is then is supplied using an external source. The DNA fragments, being negatively charged, move toward the positive electrode. The

sieving matrices are chosen because they are chemically inert, readily formed to provide mechanical stability, and limit solute dispersion due to convection and diffusion.

The gel is prepared by pouring a liquid containing either melted agarose or unpolymerized acrylamide between two glass plates a few millimeters apart. As the agarose solidifies or the acrylamide polymerizes, a gel matrix is formed. Polyacrylamide produces smaller pore sizes that are able to separate small DNA fragments (1 nucleotide to 2 kb)(Thomas et al, 2004). Agarose contains pores larger than those in polyacrylamide gels and is used to separate large DNA fragments (500 bp to 20 kb). Typical slab gels range from five to 25 cm in width and length and 1 to 2 mm in thickness. Electric field strengths between 100 and 250 V/cm are used with analysis times running from a couple of hours to overnight. Multiple lanes can be run on a single slab gel simultaneously giving the technique high parallel processing capacity.

There are commercially available devices for doing slab gel analysis. A LiCor 4000 automated DNA sequencer (Lincoln, NE, USA) has been used to perform sequencing of the PCR samples at Louisiana State University by this method. This system was coupled to an IBM computer with an OS/2 operating system, which performed image analysis.

Even though the technique is simple and capable of resolving a broad molecular weight range of DNA fragments, it is time consuming, labor intensive, and non-quantitative when on-line detection is not employed.

2.7.1.2 MICROCHIP ELECTROPHORESIS

Microdevice platforms offer improvements in cost, resolution, speed and automation in genetic analyses over slab gel electrophoresis. The first microdevices developed for electrophoretic purposes were constructed on glass microscope slides and

produced comparable resolution to conventional CE but at a fraction of the time (Gao et al, 2000). Electrophoresis on a planar chip builds on the inherent advantages associated with CE, including high surface-to-volume ratios produced by using very small channels for the separation, which permit the application of higher electric fields for faster separations (Thomas et al, 2004). The simplest device format for performing electrophoresis is the twin T design, which contains a cross-like structure (Zhang et al, 2001). The side branches of the T are used for electrokinetic injection of sample and are offset to define a fixed plug length, while the longer channel is used for separation. The underlying mechanism for DNA separation is essentially the same as in capillary electrophoresis, thus the separation matrices used in capillary electrophoresis may also be employed in the microdevice formats. The switching of voltages from well to well allows the loading and separation of samples. Microdevice injections are volume-based. This is particularly important for samples that may be subject to injection biases, such as low abundant mutations, or which contain high mobility contaminants such as salts and primers, such as PCR products.

In an effort to better analyze products of the LDR strategy, microdevice electrophoresis was employed by Thomas et al. for their study to analyze low abundant point mutations in certain gene fragments (kras) with high diagnostic value for colorectal cancer (Thomas et al, 2004). The microdevice used in those studies were of the modified twin T design shown in Figure 2.7.2.2 in which the separation channel was lengthened and contained two turns. The microchannel was hot embossed in poly (methyl methacrylate) (PMMA) using a mold insert produced by the LIGA process. The separation channel was 10 cm in length and the side channels were 0.5 cm.

Figure 2.7.1.2: Twin T microdevice. A is the waste well, B is the sample well, C is the buffer reservoir, and D is the separation well (Thomas et al, 2004).

A Laser Induced Florescence (LIF) detector designed in the research lab of Dr. Steven Soper accomplished the detection. The study found that when the reaction contained a 100-fold molar excess of wild-type DNA compared to a mutant allele, the ligation product could be effectively resolved from unligated primers in 120 seconds. (Thomas et al, 2004).

2.7.1.3 ZIP CODE ARRAY

A DNA microarray is a hybridization-based assay in which the affinity of surface immobilized DNA probes for binding their complements in solution is utilized to detect and quantify targets. Probes are spotted on a surface and are designed to match the primers for a successful LDR product. The allele-specific probe contains on the 5' end, a zip code complement that directs the ligation product to a particular site on

the array. The common probe contains a fluorescent dye on its 3' end. If the mutation is present, LDR ligates the two probes together and generates a fluorescence signal at that particular element of the array. In the absence of the mutation, the discriminating probes hybridize to their complements, but do not generate a fluorescence signature. The challenge in this assay format is that unligated discriminating probes compete with ligated product for a fixed number of sites at the particular element of the array (Wang et al, 2003). It can be a high throughput technology with ordered arrays of oligonucleotides or DNA molecules with known sequences attached to the solid support. In addition, the hybridization rate is enhanced on flat surfaces since the labeled targets do not need to diffuse into and out of the pores in membrane materials.

Figure 2.7.1.3 shows the process of the PCR/LDR and zip code hybridization used by Wang et al in their study of low-density arrays assembled into microfluidic channels. The channels were hot embossed in PMMA to allow for the detection of low-abundant mutations in gene fragments (kras) that carry point mutations with high diagnostic value for colorectal cancers. (Wang et al, 2003) After hybridization a near IR scanner was used to look at the fluorescence signal. Using this analysis method a 1:1000 ratio of mutant to wild type sequences could be discriminated (Wang et al., 2003).

2.7.2 CURRENT PROTOCOL FOR TEST: MACROSCALE AND MICROSCALE EXPERIMENTS

The macroscale or standard LDR was conducting using the protocol of section 2.4.3.2. In obtaining microscale results the spiral channel platform developed by Mitchell et al for the PCR reaction was used. The polycarbonate microchip was made by hot embossing polycarbonate from the PCR mold insert. It was then sealed with

Figure 2.7.1.3: Schematic showing the LDR process and the hybridization to zip code oligonucleotide probes arrayed on a solid surface (Wang et al, 2003)

polycarbonate cover slip with Watlow Kapton heaters (Watlow, St. Louis, Missouri, USA) placed, along with the required thermocouples, at the heating zones according to Mitchell's design. The only difference was that instead of three zones of 95°C, 72°C, 55°C, required for PCR, two zones of 95°C, 65°C were created for the LDR. A premixed cocktail of the LDR primers, buffers and PCR product was made off the chip and the ligase was also added and mixed off chip after preheating at 95°C. The resulting mixture was then pressure-driven at a flow rate of 3 mm/s in the spiral channel to achieve the LDR. Again different mixtures were put through the chip according to

the test matrix (Table 2.7) in order to see which factors affected the reaction. Both macroscale and microscale reactions were analysed by slab gel electrophoresis.

2.7.3 RESULTS

Representative results of the LDR time reduction study on the macroscale are shown in Figure 2.7.3 (a) & (b). In each case the different test runs are compared to the original composition of the successful macroscale reaction. Typical LDR products (50-70 bp) are longer than normal DNA (20-50 bp). Markers were used to show where the products should be on the DNA ladder.

Figure 2.7.3 (a): Results of changing primer volumes: Column 1 - normal reaction. Column 2 - volume of com2- czip-11 doubled. Column 3 - volume of PCR product were doubled. Column 4. - volume com2, czip-11and PCR product were doubled; (b): Results of changing annealing time (at 65°C): 1 minute, 2 minutes, 3 minutes and 4 minutes, respectively, from left to right.(c) Results of changing the total reaction sample volumes: Column 1 - 5µl, Column 2 - 10µl, Column 3 - 20µl

(d)

Figure 2.7.3 (d): Scanner image of microscale LDR done on Mitchell's chip with 30 second residence time at 65°C

In Figure 2.7.3(a) doubling the volume of com-2 and czip-11 increased the intensity of the product in Column 2. There was no significant increase in product if the PCR product was doubled (Column 3). The intensity also increased in column 4, when the entire mixture was doubled. Column 1 shows the standard macroscale reaction results. All reactions in this test were done at a nominal 30 seconds at 95°C followed by 4 minutes at 65°C repeated for 20 cycles. In looking at the results it is evident that an excess of primers produces better product intensity. This is in accordance with the findings of Wang et al that an excess of discriminating primers and common primer is required to ensure complete annealing of the target DNA and efficient ligation (Wang et al., 2003)

In Figure 2.7.3(b), the times for the reaction were changed and the baseline mixture was used. In all four columns the mixture was held at 95°C for 30 seconds, but the annealing process at 65°C was done for 1 minute, 2 minutes, 3 minutes and 4 minutes, respectively, for each cycle. The reaction yielded detectable product after only one minute of annealing. In Figure 2.7.3 (c) the total reaction sample volume was changed. All reactions in this test were done at a nominal 30 seconds at 95°C followed by 4 minutes at 65°C repeated for 20 cycles. The reagents were mixed as a percentage

of the original reaction, keeping the ratios the same. The original reaction used 20 μ l, but product can be obtained with 10 μ l and 5 μ l as indicated by the image. These results show that it should be possible to reduce the time and volume for the reaction even further on the microscale, by adjusting experimental factors.

Figure 2.7.3(d) shows the LDR product for the microscale reaction done on Mitchell's chip. Residence times in the two heated zones were approximately 30 seconds for each. Even at the 30 seconds residence time at 65°C the primers were able to anneal.

2.8 CONCLUSION

In this chapter, genetic mutations were shown to be linked with different diseases. These mutations are of different types and have come about by different means either internally or externally in the genes of the human body. One such gene is the *kras* gene that has a high diagnostic value for colorectal cancer. The statistics for colorectal cancer, along with the risk factors, detection and treatment were presented.

It was also shown that miniaturization of the PCR reaction has increased the functionality and feasibility of that reaction. Different methods are being used to identify gene mutations, but for the case of low abundance of the mutant to the wild type, it was evident that the PCR/LDR strategy could find the mutants with high specificity, especially for the *kras* gene.

The conventional LDR was not without its limitations and it will be the focus of the rest of this paper to miniaturize the LDR reaction platform. The LDR reaction time has been investigated and it was found that it is possible to do LDR on the microscale with positive results, excess primers are needed for efficient ligation, and that the residence times in the temperature zones can be reduced.

CHAPTER 3: DESIGN AND FABRICATION OF THE MICROMIXERS

Depending on how the system is configured, there are several mixing stages involved in the LDR. Micromixers had to be fabricated for use on the microchip. Their performance had to be simulated and designed to produce efficient mixing in the shortest possible time and with the minimum possible footprint. There are several microfabrication methods that can be used to make the micromixers. This chapter will characterize four different methods in terms of the quality of microchips and speed of the process. The micromixers will then have to be tested and implemented on the prototype device.

3.1 LITERATURE REVIEW ON MICROMIXING

It is difficult to mix solutions in microchannels as flows in these channels are laminar and diffusion across the channels is slow. The literature on micromixing includes two classifications of mixers, active mixers and passive mixers. Active mixers employ external forces or active control of the flow field by moving parts or varying gradients, while in passive mixers no energy is input except for the mechanism used to cause the fluid to flow at a constant rate. Although active mixers may effectively provide rapid mixing, the actuators used in these mixers need extra energy and may be difficult to fabricate. Additionally, the electrical field and heat generated by active control may damage biological samples (Chung et al, 2004). Different methods and substrates have been used to fabricate each, but it is generally agreed that passive mixers are easier to fabricate and simpler in design than active mixers.

Stroock et al (2002) presented a passive method for mixing streams of steady pressure-driven flows in microchannels at low Reynolds number by chaotic advection. This method used bas-relief structures on the floor of the channel that were fabricated in

polydimethylsiloxane (PDMS) using soft lithography. Using this method, the length of the channel required for mixing grows only logarithmically with the Peclet number, and hydrodynamic dispersion along the channel is reduced relative to that in a simple, smooth channel. Liu et al (2000) developed a three-dimensional serpentine microchannel design using three-dimensional polydimethylsiloxane (PDMS) microfabrication and a plastic micromolding technique to fabricate the L-shaped micromixers for enhancing the mixing of biological sample preparation. Wong et al (2004) fabricated micro T-mixers on a silicon substrate covered with a Pyrex glass plate to enable observation and characterization of mixing performances. The goal was to test the feasibility of using T-mixers for rapid mixing. It was shown that for a micro T-mixer with a mixing channel having a hydraulic diameter of 67 μm , an applied pressure of 5.5 bar was sufficient to cause complete mixing within less than a millisecond after the two liquids made contact. Chung et al (2004) proposed microfluidic self-circulation in a mixing chamber to improve mixing performance. The device was constructed with two PMMA (polymethyl methacrylate) layers. The upper PMMA layer was blank and the structures of the components were built on the lower PMMA layer using a high-speed CNC engraving and milling machine. The mixing chamber was 4 mm in diameter and 500 μm deep, and the two channels, 500 μm x 500 μm in cross-section, for a total volume of 20 μL . The self-circulation of microfluid in the mixing chamber was achieved by the forward and backward pumping of the working fluids.

Bertsch et al (2001) studied two geometries, a series of stationary rigid elements that formed intersecting channels to split, rearrange and combine component streams and a series of short helical elements arranged in pairs, each pair comprised of a right-handed and left-handed element arranged alternately in a pipe. Micromixers were

designed by CAD and manufactured by microstereolithography, a microfabrication technique that allowed the manufacturing of complex three-dimensional objects in polymers. Volpert et al (1999) developed an active micromixer for improving the mixing of two fluids in a microchannel. The flow through the main channel of the micromixer was unsteadily perturbed by three sets of secondary flow channels, enhancing the mixing. Lee et al (2001) designed a micromixer, which employed unsteady pressure perturbations superimposed on a mean stream to enhance the mixing. The channels of the mixer were etched into a silicon wafer using deep reactive ion etching (DRIE) and anodically bonded to Pyrex cover plates. Glasgow et al (2003) demonstrated the merits of flow rate time dependency through periodic forcing. Mixing in a simple "T" channel intersection was studied by means of computational fluid dynamics (CFD) as well as in physically mixing two aqueous reagents. The channels segments were 200 μ m wide by 120 μ m deep.

3.2 MIXING CRITERIA AND MIXING FORMAT SELECTION

3.2.1 DESIGN ISSUES

Passive diffusional micromixers were considered for onboard combination of the reagents and analyte in the LDR (Maha, 2004). In making the micromixers several issues had to be addressed:

- The micromixers had to be implemented on the microchip and isolated from the temperature cycling zones;
- High aspect ratio, between 5 to 20, structures were needed for efficient mixing;
- Mixing should be accomplished in the shortest possible time and there should be a small pressure drop (Maha, 2004);

- Microfabricated mixers should maintain strict dimensional integrity;
- Mixing processes should not affect the biological and chemical function of the reagents of the LDR

Isolating the mixers ensures that no thermal effects occur in the mixing stage that could affect the efficiency of mixing. High aspect ratios increase the contact area of the fluids and enhance mixing while the dimensional integrity allows for valid comparisons to be made to simulations.

3.2.2 SIMULATIONS

Preliminary simulations were done using Fluent (v5.4, Lebanon, NH) on different micromixer geometries (Maha, 2004). The four basic configurations are shown in Figure 3.2.2.1: Y-mixers, T-mixers, jets in cross flow, and the separation wall mixer, all leading to a diffusion length. The simulations were used to evaluate the designs, test the degree of mixing, analyze the pressure drops, and to define the size and geometry of the prototype micromixers for fabrication (Maha, 2004).

Figure 3.2.2.1: The four main configurations of the mixers with the diffusion channel at the bottom

3.3 MICROFABRICATION OF MIXERS

Different microfabrication methods were used to make the candidate micromixers to assess their capabilities. The four methods used were:

- SU-8 lithography (direct);
- Laser Ablation (direct);
- Micromilling (indirect);
- LIGA (indirect).

There were several issues with each method. The first approach in making the micromixers for experimental evaluation was to make them in SU-8 photoresist (MicroChem Corp., Newton, MA) using both ultraviolet (UV) lithography and X-ray lithography. SU-8 is a negative photoresist, that when exposed to UV or X-ray light, crosslinks and hardens. The section that is not exposed can be washed away with developer. As the name suggest, the micromilling process uses a cutting tool to directly mill a mold insert for hot embossing using brass disks. Laser ablation was used to direct write the patterns in polycarbonate using a high intensity pulsed excimer laser. Finally, LIGA was used to fabricate a mold insert for hot embossing. In order to obtain the aspect ratios needed, the heights of the channels had to be greater than 140 μm as the diffusion section of the mixers was 20 μm in width. Two heights were used in this study, 150 μm and 300 μm for aspect ratios of 7 and 15, respectively.

3.3.1 DESIGN AND MASK LAYOUT

The first step was to fabricate an optical lithography mask that could be used in UV lithography or that would enable transfer of the pattern to an X-ray mask [Desta, 2000]. For UV light based optical lithography, 300 Å of chromium forms the radiation-absorbing layer. Dark field regions on the mask are covered with chrome and block the

UV light. Clear field regions allow the UV light to pass through and modify the photoresist. The light either polymerizes a negative resist into chains or causes molecular scissions in a positive resist. The masks where dark field regions define the pattern are called dark field masks, while those where the clear areas define the pattern are called clear field masks. Dark field and clear field masks for the same pattern have chrome on complementary areas and are in opposite tone to each other.

An optical lithography mask pattern of micromixers was laid out using AutoCAD 2000 (AutoDesk, San Rafael, CA) as in Figure 3.3.1. The AutoCAD drawing was converted to the .dxf file format and sent out for bids. The minimum feature size on the mask was 5 μ m width and 800 μ m length. Two optical masks, a dark field and a clear field, were purchased from Advance Reproductions (North Andover, MA). The majority of the mixers had dimensions ranging from 10 to 100 μ m. The industry standard chrome on soda lime glass, 0.09 inches thick and with each side, 5 inches by 5 inches length, was used for the masks.

3.3.2 LITHOGRAPHY OF SU-8 MICROMIXERS

SU-8 is a negative photoresist that cross links when exposed to radiation. It is normally used as an intermediate step for microfabrication to make other structures. It comes in a liquid form and only hardens after processing. The surface that it is processed on must be flat, normally silicon wafers, graphite masks, or Kapton masks. The hardened structure has good physical and thermal properties and can be used to make permanent structures on a suitable substrate. The steps involved in doing this are easier than the full LIGA process.

For this application SU-8 was processed on a substrate must be transparent and that enabled the microchannels to be accessed by easily drilling through the substrate.

Figure 3.3.1: Mixers pattern from Autocad 2000

This limited the material selection to polymers and omitted the materials that SU-8 is more normally processed on such as silicon, graphite, and glass. Both polycarbonate and PMMA were used in our application.

3.3.2.1 UV LITHOGRAPHY OF SU-8

Polycarbonate and PMMA sheets, 26"x 26", were bought from Goodfellow (Goodfellow, UK). These were cut into 4 inch disks, cleaned in an IPA solution, and air-dried. The cutting of the disks was done on a CNC mill for a smooth finish. All of the other processes were done in the cleanroom (www.camd.lsu.edu). The disks were then placed in the oven, 80°C for PMMA and 110°C for polycarbonate, for 3 hours and then cooled to room temperature. This helped to lower the internal stresses. After annealing a disk was placed on a spin coating machine, and fixed into place by a vacuum chuck. Approximately 15 ml of SU-8 50 (MicroChem Corp., Newton, MA) negative photoresist was placed on the disk and spun at 1200 rpm for 20 seconds to give a uniform coat of the resist. A thickness of 150-170 μm of resist was obtained on the

disk. The spin curve speed for SU-8 50 is given in the Appendices. All visible bubbles were removed from the SU-8 layer using a small pin. The layer was then allowed, while being covered, to relax for 5 minutes to lessen any internal stresses and to prevent cracking after processing.

The SU-8 was pre-baked in a convection oven, starting at a temperature of 65°C, then ramped up to 95°C at a rate of 4°C/min, maintained at this temperature for about 2 hours, then slowly cooled down to 30°C at 2°C/min in the oven. Final cooling from 30°C to room temperature (~23°C), took place outside the oven. The slow cooling was necessary to reduce the internal stresses generated in the SU-8 layer during solidification.

After pre-baking the surface of the SU-8 was not fully flat, with 5-10 µm variation over the surface. In order to get good exposure, a thin film of glycerol was placed between the layer and the optical mask. This served to even the surface and allowed the SU-8 layer to get a uniform dosage. In order to test the effect of the glycerol and also to find the optimum exposure dosage a test matrix was developed. For a 150 µm thick layer of SU-8 the required dosage should be 700 mJ/cm² of UV light (Appendices), but in the matrix five different intensities were used, at different location on the same substrate to find the optimum exposure dosage for development. Dosages were selected at 20% and 40% above and below the table specified value. The substrate was divided and subjected to the dosages as shown in Figure 3.3.2.1. The coated disks were exposed to ultraviolet light through the optical mask for 50.3 seconds using the Oriel UV exposure station at CAMD. The UV station has an intensity of 12.8 mW/cm². An exposure time of 50.3 seconds was used to expose the disk to 720 mJ/cm² of UV light. The time and intensity were related by Equation 3.1.

Figure 3.3.2.1: Test substrate exposure dosage

$$Time(s) = \frac{Dosage(mJ/cm^2)}{Intensity(mW/cm^2)} \quad 3.1$$

After exposure the disks were post-baked in a convection oven, starting at a temperature of 65°C, ramped up to 95°C at a rate of 4°C/min, maintained there for 15 minutes, after which they were cooled down to 70°C at 1°C/min. Cooling to room temperature was done in ambient air in the cleanroom.

The post-baked, patterned disk was developed in the SU-8 developer (MicroChem Corp., Newton, MA) for a total of 10-20 minutes, moving back and forth from a cleaning bath to a rinse in IPA in 3 steps. The appearance of a white residue indicated incomplete development of SU-8. Whenever there was any white residue the disk was reimmersed in the solution for more development. After complete development it was rinsed and dried with compressed air.

The micro-PIV CCD camera capture system (IDT Tech, Florida, USA) required the use of 150 μm glass cover slips in order to focus inside the channels. In order to bond the slips to the SU-8 layer of the microchannels a layer of SU-8 had to be spin coated on the slips. The 3" square glass slips were cleaned in IPA solution and air-dried. Approximately 5 ml of SU-8 25 (MicroChem Corp., Newton, MA) negative photoresist was placed on the glass and spun at 2000 rpm for 20 seconds to give a uniform coat of the resist. A thickness of 5-10 μm of resist was obtained on the slips. All bubbles were removed from SU-8 layer using a small pin. The SU-8 coat was pre-baked in a convection oven, starting at a temperature of 65°C, then ramped up to 95°C at a rate of 4°C/min and maintained at this temperature for about 20 minutes, then slowly cooled down to 30°C at 2°C/min in the oven. Final cooling from 30°C to room temperature ($\sim 23^\circ\text{C}$), took place outside the oven. The coated slips were flood exposed to ultraviolet light for 1 minute with 720 mJ/cm^2 of UV light. After exposure the slips were post-baked in a convection oven, starting at a temperature of 65°C, ramped up to 95°C at a rate of 4°C/min, maintained there for 10 minutes, after which they were cooled down to 70°C at 1°C/min. Cooling to room temperature was done in ambient air.

UV glue (SK9-40CPS, Summer Optics, USA) was used to bond the glass slips. Approximately 5 ml of glue was placed on the glass and spun at 2000 rpm for 20 seconds to give a uniform coat. A thickness of 5-10 μm of glue was obtained on the slips. The coated slips were flood exposed to ultraviolet light for 5 minutes with 320 mJ/cm^2 of UV light for pre-curing. After exposure the slips were attached to the SU-8 covered disk and cured for one hour under UV light.

3.3.2.2 X-RAY LITHOGRAPHY OF SU-8

Similar to the process of UV lithography, X-ray lithography was also used to develop SU-8 micromixers. X-ray lithography uses synchrotron X-ray radiation as a lithographic light source. The highly parallel X-rays from the synchrotron impinge on a mask patterned with X-ray high radiation absorbers. The absorbers on the mask are thick enough to prevent the penetration of X-rays. The synchrotron ring at the Center for Advanced Microstructures and Devices (CAMD, www.camd.lsu.edu) at Louisiana State University was used for this application. This is an electron storage ring with electron storage energies of 1.3 GeV and 1.5 GeV. Due to the use of shorter wavelengths X-ray lithography is far superior to optical lithography for producing aspect ratios on the order of 20:1 or higher.

The disks were prepared and processed in the same fashion as with UV lithography. The only difference was that after the pre-baking stage the substrates were covered with foil, for transfer from the cleanroom to the X-ray station for exposure. An X-ray mask was fabricated by the Center for Advanced Microstructures and Devices (CAMD) staff. The process for making the mask will be discussed later. It was made using a Kapton membrane with a gold absorber layer on the surface. It had a similar tone to the UV clear field optical mask with the structures being gold.

The required dosage for the exposure of SU-8 was calculated using DOSE SIM [<http://www.camd.lsu.edu>, 2000]). The exposure dose was carefully calculated based on the exact beamline being used, thickness of resist, thickness of the Kapton membrane and the size of the exposure area. For this application the XLRM-1 beamline (<http://www.camd.lsu.edu>, 2000) was used for the 160 μm SU-8 layer, with a 13 μm

Kapton membrane and an exposure scan length of 9 cm. After exposure the disks were developed as discussed before with the UV lithography.

3.3.3 MICROMILLING

3.3.3.1 MACHINE AND TOOLS

The micromilling machine (Kern MMP – Microtechnic, Murnau-Westried, Germany) is shown in Figure 3.3.3.1. It consists of a moveable stage, tool holder, computer control, and a microscope. The spindle is able to achieve the 40000 rpm necessary for clean cuts with the small tools. The coordinates of the structures are input on the computer using GIBBS CAM/CAD software (GibbsCAM 2004, Moorpark, CA) to convert the CAD drawings to CAM files for machining. The substrate is placed on the stage and fixed in place. The stage moves to generate patterns. Compressed air is blown at the cutting tool to remove debris, while a microscope monitors the cutting process in real time.

Figure 3.3.3.1: Micromilling machine at the Center for Bio-Modular Microsystems at Louisiana State University (www.lsu.edu/cbmm)

Various tool bits are available. These are usually made of solid carbide and are selected based on the detail of the structures and the material of the substrates (www.kern-microtechnic.com). The bits have a typical tool life of 20 hours and need to be changed frequently in order to continue to give a good finish. The smallest bit size available had a 25 μ m radius, so that any inside 90-degree junctions would have a minimum fillet of at least 25 μ m, with typical aspect ratios being 10:1 (www.lsu.edu/cbmm).

3.3.3.2 SUBSTRATES

Any machineable surface is a potential substrate for micromilling, from metals to polymers. Brass (353 brass alloy) was selected for this application based on its good machineability. It is not as hard (Rockwell B = 62, www.matweb.com) as stainless steel (316 stainless steel, Rockwell B = 95, www.matweb.com), hence it preserves tool life. Brass does not require the use of lubrication, allowing for easier setup and cleanup. Furthermore, it can withstand the temperatures in the hot embossing process. Brass was cut into ¼ inch thick 5 inch round disks with six counter sunk holes drilled around the edges (appendix). The holes are used for matching in the PHI (City of Industry, CA) hot embossing machine.

It was possible to directly mill the micromixers into polycarbonate but it was more economical to make a mold insert with brass, as numerous test chips were required for evaluating the micromixers. Furthermore the channels would have to be cut into the surface of the polycarbonate. This would be a problem especially for the smaller 10 – 20 μ m structures since the smallest tool size is 25 μ m radius. These dimensions are easily obtained in the mold insert, as it is the negative and made with the

tool coming from the outside. It took three hours to micromill the mixers at a height of 180 μ m.

After milling, burrs on the edges of the brass were removed by lapping and polishing. Crystal bond acrylic (Crystalbond 509, Structure Probe Inc., West Chester, PA) was applied to the surface to protect the microstructures during the lapping process. The brass was heated to 150°C and the acrylic, which melts at 140°C, was applied evenly and leveled. After cooling the bond hardened for use during lapping and polishing. Once polishing was completed the acrylic was removed with acetone, leaving a mold insert ready for hot embossing.

3.3.4 LASER ABLATION

Excimer laser ablation involves the use of a pulsed energetic UV laser to break the chemical bonds of a material (www.resonetics.com). Each pulse removes a certain quantity so depth control is achieved by the number of pulses, while the size of the area is controlled by a stainless steel mask. Typical materials used are polymers and ceramics (www.resonetics.com). A custom Resonetics (Nashua, NH) excimer laser machine was used at the Center for Bio-Modular Microsystems at Louisiana State University (www.lsu.edu/cbmm) to direct write the micromixers into polycarbonate without contact shown in Figure 3.3.4(a).

The main components of the system are the excimer laser generator, two computer-controlled moveable stages, a stainless mask holder, and the demagnifying lens. The polycarbonate was placed on the substrate stage and fixed in place. A stainless steel mask containing different geometries such as circles and squares was placed in the moveable mask holder stage and shown in Figure 3.3.4(b). Only a single geometry can be used at a time and selection depended on the size and the type of structures needed.

Figure 3.3.4(a): Resonetics laser machine at the Center for Bio-Modular Microsystems at Louisiana State University (www.lsu.edu/cbmm)

The laser beam was scanned through the mask to transfer that pattern to the substrate. The demagnifying lens focused the beam, after it passed through the mask; hence a $100\mu\text{m}$ circle on the substrate was achieved at 20X demagnification through a 2mm circle on the stainless steel mask.

Figure 3.3.4(b): Stainless steel mask with different geometries

After focusing the desired beam at the required pulse rate, the stage with the substrate is moved according to the input geometry on the computer to obtain the prescribed pattern. There were two methods by which the patterns were achieved. One was direct writing using the focused beam to trace the pattern of the channel with the stage moving and the other was using a stainless mask with the actual patterns included on the mask. In the latter case the laser was scanned through the mask to obtain that pattern on the substrate.

Only some of the mixers could be fabricated by laser ablation. The design of the micromixers called for changing widths along the diffusion length connected to an angled line. This could not be achieved with the single beam. These micromixers, such as the Y-mixers and T-mixers, could only be made by making a stainless steel mask of the patterns shown in Figure 3.3.4(c).

Figure 3.3.4(c): Stainless steel mask with different mixer patterns

Figure 3.3.4(d): Inside section of the stainless steel masks have to be supported

The laser would then scan through this mask to ablate the polycarbonate by the pattern on the mask. The jets in cross flow could not be made by just one scan as the inside section of the stainless mask has to be supported as shown in Figure 3.3.4(d). The supports would block the laser. After the initial scan the support section left unablated in the polycarbonate was removed with a single beam. This was difficult, as the channel lines had to be aligned properly.

3.3.5 LIGA MOLD INSERT

Mold inserts for micromixers were fabricated using X-ray LIGA microfabrication techniques. X-ray LIGA was used because of the ability to fabricate high, smooth, vertical sidewalls at high aspect ratio. The X-ray mask was used to reproduce the desired features in poly methyl methacrylate (PMMA), which was attached to a stainless steel base. Following X-ray exposure, the exposed PMMA was dissolved in a chemical developer (GG developer) and nickel was electroplated into the pattern to produce the desired mold insert structure. The structure was then lapped and polished to the desired final height. These mold inserts were used to hot emboss high aspect ratio microstructures of polycarbonate.

3.3.5.1 BACKGROUND OF LIGA FABRICATION

LIGA (an acronym from the German words for lithography, electroplating, and molding) is a micromachining technology used to produce micro-electromechanical systems (MEMS) mainly in metals, ceramics or plastic (Kovacs, 1998). This process uses synchrotron X-ray radiation as a lithographic light source. X-rays from the synchrotron pass through a patterned mask with high radiation absorbers that are thick enough to prevent the penetration of X-rays. In the open areas of the mask, the radiation passes through and exposes the PMMA photoresist. The PMMA is attached to a substrate that is used later as an electroplating base. Bond scissions occur in the region of the PMMA that is exposed to the X-ray, which are selectively dissolved in a chemical developer. Once the PMMA is developed, the resulting pattern is filled with metal by electrodeposition from its conductive base (Madou 1997).

LIGA processing contains two applications of electroplating. The X-ray mask is made by electroplating gold on a substrate to form the absorber during the synchrotron exposure and a final electroplating of nickel into the developed PMMA pattern to form the mold insert structures. The gold thickness is typically about ten to twenty micrometers and must be uniformly thick to provide an adequate contrast ratio. The absorber stress must also be minimized otherwise it can cause pattern displacement errors.

The use of PMMA as an X-ray resist has been documented (Pan et. al, 2001; Madou, 1997). The exposure and development of PMMA resists can be done as long as the X-ray source provides 4 to 25 KJ/cm³ of energy for a maximum top to bottom dose ratio of 5 (Madou, 1997). The exposure dose was carefully calculated using DOSE SIM (<http://www.camd.lsu.edu>, 2000). Overexposure can cause swelling and cracking which

may result in deformation or delamination of the desired structures. Underexposure may cause PMMA to be undeveloped, resulting in the inability to electroplate metal on the surface of the substrate (Mitchell, 2002). The full LIGA process with conditions is given in Appendix C.

3.3.5.2 X-RAY MASK FABRICATION TECHNIQUES

There are several different materials than can be used for making X-ray masks. Silicon (Si), Silicon Carbide (SiC), Silicon Nitride (Si_xN_y), Beryllium (Be), Titanium (Ti), Graphite (C), and Kapton have been used (Madou, 1997; Desta et al., 2000). For this application graphite was used as a substrate for the mask.

Graphite wafers were purchased from Poco [Poco, Decatur, TX, DFP-3] in 4 inch disks that were 200 μm thick. They were polished and cleaned thoroughly using acetone, IPA, and DI water to remove all unwanted particles. Graphite acts as a filter for X-rays of shorter wavelength, thus transmitting a specific bandwidth of X-ray radiation to the PMMA (Desta, 2000). Approximately 5 ml of SU-8 50 (MicroChem Corp., Newton, MA) negative photoresist were spun to give a uniform coat of the resist. A thickness of 50-60 μm of resist was obtained on the disk. The graphite was then processed as shown in the Appendix.

Radiation absorption is proportional to the molecular weight cubed. Gold, with a high molecular weight and density ($M_{\text{wt}}=196$, $\rho=19300 \text{ kg/m}^3$), has an absorption coefficient, α , ranging from 0.8 – 11 $\mu\text{m/m}$ (Madou, 1997), and was electrodeposited for this application. A commercially available, ready-to-use neutral gold sulfite solution (Technic Gold E25, Technic Inc, Irving TX), containing 1 troy oz gold per gallon was used for gold plating with conditions given in the Appendix. It was important that the graphite disk remained flat and no warping was allowed during plating in order for the

gold to plate evenly. It was recommended not to stop electroplating mid-process so it should be secured prior to the process.

After electroplating, the graphite wafer was bonded to a standard NIST (National Institute of Standards and Technology) aluminum adapter ring to avoid sagging of the graphite wafer under its unsupported weight plus the weight of the resist. This was done using super glue (Loctite 4210, Loctite Corporation, Newington, CT). Sagging generally causes underexposure because of the change in gap between the mask and the wafer. This leads to resist being underdeveloped. Figure 3.3.5.5 shows the finished graphite X-ray mask for the micromixers.

Figure 3.3 5.5: Fabricated graphite X-ray mask for PMMA resist exposure

3.3.5.6 PREPARATION OF STAINLESS STEEL MOLD INSERT SUBSTRATE

Stainless steel was cut and machined into 5-inch diameter base plates $\frac{1}{4}$ inch thick. Six counter sunk screw holes were placed around the edges for attachment to the hot embossing machine. It is imperative that the plating base be flat with top and bottom faces parallel. All stainless steel bases were measured on the profilometer to characterize flatness. The profilometer stylus was moved across the surface along the diameter line, then the disk turned 180 degrees to repeat the profile in the orthogonal direction. This was done on both sides of the disk. If the profile varied by 2- 7 μ m the disk was lapped using diamond slurries on an ENGIS lapping machine (Model 15LM11V Engis Wheeling, IL) until the required profile was obtained. This ensured uniform mold inserts. The stainless steel was then sandblasted to create a roughened surface with an rms value of approximately 2 μ m. It is then activated in C-12 activator (Puma Chemical, Warne, NC) and Wood's strike as given in the Appendix. The chromium atoms in stainless steel have a tendency to form an oxide layer across the surface of the substrate (Stefanov, 2000). This oxide layer creates a weak bond between the substrate and the electroformed nickel. Debonding is a significant problem when structures are plated directly onto this oxide layer.

3.3.5.7 PMMA BONDING

PMMA resist, 3000 μ m thick sheet stock (CQ grade Vista Optics, UK) was cut into 4 inch disks using a CNC mill and bonded to the stainless steel base with an MMA glue as detailed in the Appendix. The solution was then poured in the center surface of the cleaned stainless steel. The 3000 μ m thick PMMA resist was pressed onto the substrate so that no bubbles are made in the glue. Once this was done, the resist should

not be moved. The sample was placed in a pneumatic press under a pressure of 42 psi at room temperature overnight. After bonding, the PMMA was fly cut to the final desired height of the microstructures, plus 50 μ m to allow for surface grinding and polishing. The thickness of the PMMA resist needed to be known to calculate X-ray exposure dosage.

3.3.5.8 X-RAY EXPOSURE OF PMMA

X-ray exposures were performed on the XRLM-1 beamline at CAMD. A 125- μ m Beryllium filter was installed in the beamline to act as an initial filter for low energy X-rays. The exposure dose was carefully calculated using DOSE SIM (<http://www.camd.lsu.edu>, 2000). Overexposure can cause swelling and cracking resulting in deformation of desired structures, while underexposure results in the inability to electroplate a metal at the substrate surface (Madou, 1997). For full exposure of PMMA, the bottom surface of the resist must receive at least 4000 mJ/cm³ of X-ray energy (www.camd.lsu.edu). In this application, there were two mold insert samples with 350 μ m and 200 μ m thick PMMA resist.

3.3.5.9 PMMA RESIST DEVELOPMENT

X-ray radiation causes scissioning of the polymer chains (Madou, 1997). The broken chains have lower molecular weight and can be dissolved in a molecular weight-sensitive organic solvent, hence the reason for initial high molecular PMMA. For every 200 μ m of PMMA thickness, the wafer was run through a development cycle of 20 min in the GG developer (Appendix) and a rinse cycle of 40 min in the GG rinse at room temperature for three repetitions (Mitchell, 2002). The GG developer was filtered continuously. After development the microstructures were dipped in DI water and dried with compressed air before electrodeposition.

3.3.5.10 NICKEL ELECTRODEPOSITION

A nickel sulfamate electroplating solution (Alfa Aesar, Ward Hill, MA), mixed with other reagents as in the Appendix, was used to electrodeposit nickel into the developed PMMA pattern. Six liters of nickel sulfamate electroplating solution was prepared for the bath. Nickel sulfamate has the advantage of low stress in electroplated microstructures, as compared to a nickel sulfate or nickel chloride electroplating solutions.

The manufacturer of the nickel solution specified a pH in the range of 3.6-4.3. After mixing, the pH was below this value so sodium hydroxide was used to bring it up to the required range. Boric acid was used as a buffering agent to minimize the pH rise of the solution while electroplating. Lauryl sulfate served as a wetting agent reducing the surface tension of the solution, which helped the solution to reach deep recesses in microstructures. In the case of the 350 μ m resist plating took 24 hours for the 5 cm² micromixers pattern. It was in the solution until the nickel formed a mushroom shape on the surface meaning the pattern is filled was overfilled. The 200 μ m resist was overplated.

3.3.5.11 NICKEL OVERPLATING

In direct plating, the substrate was different from the plated material. Even though Wood's strike was done, small structures <10 μ m do not get good adhesion and rarely survive to the embossing process. In order to ensure good adhesion of these structures overplating is used.

During overplating only the C-12 activator was used before PMMA bonding. No Wood's strike was done as the microstructures will be separated from the plating base. The same procedure and conditions for plating nickel were applied. When the PMMA

pattern is filled with nickel a thin layer of gold (500 Å) is sputtered on the surface of the electrodeposited nickel and the PMMA surface. This acts as a new plating base for the nickel to plate evenly so the finish of the mold insert will be as smooth as the PMMA surface. The current density was adjusted for the new area. Plating continued for one week until the nickel above the PMMA was about 3 mm thick. By tapping the backside of the stainless steel the nickel bonds were broken and separated. The remaining PMMA resist was removed by acetone. The bulk electrodeposited nickel was machined to fit a stainless steel holder then lapped and polished.

3.3.5.12 LAPPING AND POLISHING

Diamond slurries were used with an ENGIS lapping machine (Model 15LM11V Engis Wheeling, IL) to lap the microstructures to the final height and then polished for a smooth finish. The LIGA mold inserts and the micromilled insert were lapped in a similar manner. The smoother surface enables easier demolding, and the top of the structures formed the base of the microchannels and should be smooth.

The PMMA was flood exposed at CAMD and developed in the GG developer to remove the remaining PMMA. The mold insert was then complete and ready for hot embossing.

3.3.5.13 HOT EMBOSSING

Embossing of the micromixers was done on a PHI (City of Industry, CA) press embossing machine equipped with a vacuum chamber (Figure 3.3.5.13). Polycarbonate sheets, ¼ inch thick (McMaster Carr, USA) were cut into 5 inch diameter disks. This was the size of the vacuum embossing chamber. The disks were cleaned with isopropanol and distilled water then air-dried. The polycarbonate plates were pre-baked in an oven at 80°C overnight to dry out excess moisture and residual monomer. Each

plate was then placed in the vacuum fixture and held in place with an aluminum ring. The mold insert was attached to the opposite side of the vacuum fixture with screws. This side of the fixtures had an independent heating element and control for better control of temperatures near the molded polycarbonate. The complete vacuum fixture was then placed between the two plates of the PHI press with the side of the vacuum fixture containing the molding tool in contact with the top plate. The vacuum was drawn and the press closed.

The vacuum fixture was heated to 180°C (above the glass transition temperature of the polycarbonate), while the upper plate and lower plates were heated to 190°C and 170°C, respectively. A pressure of 900 psi was applied for 4 minutes, then the press was released. These were the optimized conditions for hot embossing developed by the staff at the Center for Bio-Modular Microsystems at Louisiana State University (www.lsu.edu/cbmm). Air assisted in demolding. After molding, the polycarbonate disks were placed between two glass plates and kept in an oven at 130°C for one half hour, then allowed to slowly cool at 2 °C /min to room temperature to reduce the residual internal stress in the polycarbonate chips and prevent warping.

3.4 MICROFABRICATION RESULTS

The different microfabrication methods produced varied quality micromixers. After each process the samples were visually inspected for any noticeable defects and analyzed under the light microscope. Scanning electron microscope (SEM) images of the mold inserts were taken at the Material Characterization facility in the Department of Mechanical Engineering at LSU. The depth profiles of the fabricated mixers and mold inserts were done at the Center for Bio-Modular Microsystems and are also presented in the following sections.

Figure 3.3.5.13: PHI press equipped with a machined vacuum chamber used for hot embossing of the micromixers

3.4.1 SU-8 UV & XRAY LITHOGRAPHY

The first substrate that was used with SU-8 was polycarbonate. The SU-8 developer (MicroChem Corp., Newton, MA) attacked the surface of the polycarbonate by breaking the molecular bonds causing swelling and eventually peeling of the outer polycarbonate layers. These results are seen in Figure 3.4.1(a). SU-8 developer also attacks PMMA, but takes a much longer time to do so. To protect the PMMA, a thin 10 μ m base layer of SU-8 was spun unto the surface. This was also done to make channel walls all uniformly SU-8. This process produced better structures as seen in Figure 3.4.1(b). Figure 3.4.1(c) shows the profilometer trace across the SU-8 channels. It was observed that the channels were 150 μ m deep with smooth side walls and smooth bases.

When X-ray structures were compared to UV structures not many differences were found in quality of the finish. One problem that was found with X-ray was that approximately 50 J/cm³ of X-rays pass through the gold absorber layer. For other resists

Figure 3.4.1 (a): SU-8 mixers on polycarbonate

Figure 3.4.1 (b): SU-8 mixers on PMMA

Figure 3.4.1 (c): Profilometry of SU-8 Mixers

such as PMMA this is no problem, but for small SU-8 structures this hardened the top 1-3 μm of the surface (www.camd.lsu.edu). This sealed the top of the channels preventing the developer from cleaning the unexposed resist properly.

3.4.2 MICROMILLING

Micromilling was one of the easier methods for fabricating the mold insert. However, from the SEM's of Figure 3.4.2(a) and Figure 3.4.2(b) two problems are evident. For this application straight intersections are imperative (Maha, 2004). This could not be achieved because of the tool size limit. Secondly the sidewalls of the brass structures were rough and would produce rough channel walls. The profilometer trace (Figure 3.4.2(c)) shows a height of 180 μm for the mold insert. This was before lapping and polishing to remove the brass burrs and to get the 150 μm height needed.

Figure 3.4.2 (a): SEM of mixers mold insert by micromilling

Figure 3.4.2(b): SEM of mixers mold insert by micromilling

Figure 3.4.2 (c): Profilometry of mixers mold insert by micromilling

3.4.3 LIGA

The full LIGA process was by far the most time consuming and complicated of the manufacturing methods. There were several stages involved that have to be error free or else the whole process would have to be done again. It, however, gave the desired dimensional integrity, smooth sidewalls, and smooth channel bases. Figure 3.4.3(b) is an SEM image of the nickel structures on the stainless steel substrate. Figure 3.4.3(c) shows the molded polycarbonate. Structures on the top surface, are the 2µm sandblasted pits on the stainless steel base. The profilometer traces (Figure 3.4.3(c) & (d)) shows that both the mold insert and the molded polycarbonate were 300µm in height hence the hot embossing was deemed a success.

3.4.4 DRILLING AND THERMAL BONDING

To gain access to the wells of the molded micromixers, holes had to be drilled before thermal bonding of the cover slips. In initial runs, it was difficult to drill the 700µm

Figure 3.4.3 (a): SEM of LIGA mixers mold insert

Figure 3.4.3 (b): Polycarbonate molded using LIGA mold insert

Figure 3.4.3 (c): Profilometry of LIGA mixers mold insert

Figure 3.4.3 (d): Profilometry of mixers molded in polycarbonate using LIGA mold insert

holes of the mixers through the 5mm thick polycarbonate. It was hard to get good alignment, the tool would break even with lubrication, and burrs would be left that blocked the channels. This problem was solved by drilling larger 2 mm counter sink holes from the backside of the reservoirs to within 200-300 μ m of the reservoir base. A smaller 500 μ m drill bit was then used to drill from the reservoir to the countersink. This alleviated the alignment problem, the small bit only had to drill through 200-300 μ m of polycarbonate, and there were fewer burrs.

After drilling, the molded mixers were cleaned by ultrasonics in DI water for 1 hour and air-dried. Polycarbonate sheets (McMaster Carr, USA) of thickness 0.125 mm were cut according to the area to be sealed. The hot embossing machine was used for thermally bonding the cover slips to the disks. Immediately after drying the two surfaces to be bonded were placed together in the vacuum fixture. The vacuum was drawn and the press closed. The vacuum fixture was heated to 155°C (above the glass transition temperature of the polycarbonate), and the upper plate and lower plates were also heated to 155°C. The pressure on the press was 150 psi. Bonding was allowed to proceed for 10 minutes, at which time the press was released. Figure 3.4.4 show cross-sections of channels with the cover slip bonded on top.

Figure 3.4.4: Cross-sections of bonded regions

3.5 EXPERIMENTAL TESTING AND PERFORMANCE OF MIXERS

After the micromixers were prepared, they were taken to the Micro-PIV Laboratory for testing. Chemi-luminescence was used to test the performance and efficiency of the mixers with the results being compared to the simulations (Maha et al, 2004). Chemi-luminescence is a technique where two or more reagents are mixed to produce photons. For this application Elisa Supersignal Femto maximum sensitivity substrate (Pierce, Rockford, IL) was used as the reagent. The reagent contained two parts that were mixed together to produce photons. The intensity of the photons was captured and measured by the CCD camera to quantify mixing efficiency (Maha et al, 2004).

3.5.1 EXPERIMENTAL SETUP

Polycarbonate female adapters with 0.5 cm inside diameter were designed and built for the connection of the mixers. These adapters were bonded to the microchip with epoxy. Fluidic assemblies from Upchurch Scientific (Oak Harbor, WA) were used to connect the micromixers to a syringe pump for pressure driven flow. The syringe pump (Harvard 22, Harvard Apparatus, Holliston, MA) was used to pump the chemi-luminescent reagents through the micromixers. The microchip was placed over the upward facing CCD camera which was focused into the channels (Figure 3.5.1).

Figure 3.5.1: Experimental setup for micromixer testing

3.6 CONCLUSIONS

The microfabrication of the mixers dealt with the comparison of different manufacturing methods. SU-8 lithography and laser ablation were more suited for individual microchips and simpler geometries. From the results LIGA is the best for mass-producing quality repeatable microstructures for the mixers. Micromilling was also viable for mass-producing microstructures, but the tool radius limits its capacity to give orthogonal intersections, which was a necessity in the micromixer designs. The time for the manufacturing process was fastest for laser ablation (2-3 hrs.), followed by micromilling (3-5 hrs.). SU-8 lithography (24 hours) was a longer process than the aforementioned but it was even shorter than the full LIGA process (one week).

From simulation results, the jets in cross flow were the most efficient micromixers (Maha et al, 2004). The microfabricated mixers would have to produce similar efficiency not only during the test by chemi-luminescence but also when the actual LDR reagents were used. No heating effects were considered in the design as it was assumed the mixing region would be isolated and insulated from the temperature zones on the chip.

CHAPTER 4: DEVICE LAYOUT WITH FLUID AND THERMAL ANALYSIS

Temperatures of 0°C, 65°C and 95°C had to be obtained in order to have a successful LDR. The analyte would have to pass through 95°C zone for a hot start, then the ligase enzyme, stored at 0°C, would be added and mixed. The 95°C to 65°C temperature cycle would be repeated 20 times, at which time the reaction was stopped by cooling to 0°C. All of the temperature regions had to be isolated and insulated from each other and the other sections of the chip, such as the mixing stages and the identification section, in order to ensure that no thermal effects occurred in those regions. The original macroscale residence time at each temperature were assumed for the microchip, hot start for 2 minutes, 95°C for 30 seconds and 65°C for 2 minutes, but the thermal design had to be able adapt to lower residence times during the testing stages.

The device was to be made of polycarbonate (Mitchell et al, 2002) whose properties are given in Table 4. (<http://www.matweb.com>).

Table 4: Properties of Polycarbonate

Density (ρ)	1.2 g/cc (1200 kg/m ³)
Heat capacity (c)	1.2 J/g-K (1200 J/kg-K)
Thermal Conductivity (k)	0.2 W/m-K
Glass temperature (T _g)	143-150°C

4.1 EXISTING THERMAL METHODS (LITERATURE)

Even though there were no miniaturized LDR devices, researchers had used a variety of methods to obtain thermal cycles of 95°C, 72°C and 55°C for microPCR devices. The various methods could be characterized under two main headings, a

continuous flow approach with the sample moving through temperature zones and the chamber approach with the sample at rest while the temperature is cycled.

Kopp and Manz developed the first flow through type of PCR (1998). A glass chip with a 40 μm by 90 μm by 2.2 m channel was built using a lithographic and etching technique. The PCR mixture was pumped along the channel through three different temperature zones heated by thermostatic copper blocks (Kopp et al 1998). Sun et al fabricated a microchannel on quartz glass using photolithography and wet etching techniques for continuous flow PCR. Two indium-tin-oxide films were deposited as the heating sources (Sun et al 2002). Chou et al developed a miniaturized cyclic PCR in low temperature co-fired ceramics (Chou et al, 2002). Schneegass et al created a silicon and glass flow through PCR device. The silicon and glass chip were heated by thin film transducers (Schneega, et al. 2001). Recently, Mitchell et al produced continuous flow PCR chip in molded polycarbonate (Mitchell et al, 2002). Made by the LIGA process the chip consisted of three zones heated to the required temperature by Kapton thin film heaters. The channels were 50 μm by 150 μm by 1.2 m with flow rates ranging from 2 mm/s to 15 mm/s.

Landers and his group also used micro-capillaries as the reactor, but performed infrared-mediated temperature control, in which a tungsten lamp generated IR-radiation to excite IR-active vibration modes in the aqueous solvent (Landers, et al. 1998, Giordano, 2001). Friedman and Meldrum used thin-walled micro-capillaries to contain the PCR mix and then coated the micro capillaries with a thin-film of indium-tin oxide (ITO) that not only acted as the heating element, but also as a temperature sensor (Friedman and Meldrum, 1998). In 1989 Wittwer et al. developed a thermocycler that is similar to conventional models that use air to heat and cool the analyte (Wittwer, et al.

1989 and 1990). Capillary tubes, having a sample volume of 10 μ l, were used, which is different from the conventional method of sample blocks. Air was pumped into the cycling chamber at the appropriate temperature. Shin et al developed a polydimethylsiloxane (PDMS) microPCR heated by cartridge heaters and cooled by a fan. Heating and cooling rates of 2°C/s and 1.2°C/s were reported (Shin et al, 2003). Lin et al demonstrated a stationary microPCR in a diced silicon microchamber on a glass substrate with a volume of 50 μ l (Lin et al., 2000). The required temperature profile was achieved using a thermoelectric unit with a power supply controlled by Labview software. The heating and cooling rates were 4°C/s and 2.2°C/s. Khandurina et al also used Peltier modules on glass substrates for microPCR. Heating and cooling rates of 2°C/s and 3-4°C/s were reported for the sample while cooling to 4°C could be achieved for storage of the sample following cycling (Khandurina et al, 2000). Rapid cycling times of 16 s/cycle were demonstrated by Rodriguez et al. using a silicon microfabricated PCR chamber with aluminum heaters and temperature sensors that were integrated onto the glass cover (Rodriguez et al, 2003). Yoon et al. fabricated Si-based micromachined PCR chips with an integrated platinum thin-film microheater with heat sinks and a temperature sensor allowing for heating and cooling rates of 36 and 22°C/s, respectively (Yoon et al, 2002).

4.2 DEVICE CONFIGURATION

In looking at the literature it was seen that heating, 2-36°C/s is much faster than cooling, 1-22°C/s. Materials such as silicon with high conductivity can easily be air cooled, but this was not possible with polycarbonate unless the thermal capacitance was reduced. It was also observed that flow through type devices have larger footprints, an optimum velocity is required and might need an initial time for start up.

4.2.1 DIFFERENT GEOMETRIES CONSIDERED FOR THERMAL CYCLING

Three alternative designs were considered for the LDR device. These were:

- Continuous flow LDR;
- Shuttle LDR;
- Stationary LDR.

The continuous flow LDR would be similar to the CFPCR demonstrated by Mitchell et al (2002). The sample would flow through preheated zones at an optimum velocity, for thermal cycling, to achieve maximum heat transfer in order for the residence times and temperature transition times to be well defined. The required temperature profile for the PCR is well defined in a time ratio of 1:1:4 for 95°C, 55°C and 72°C respectively. In this application work was still ongoing to optimize the LDR reaction residence times. The manufactured device needed to be able to adapt easily to changes. This would be difficult to do with a continuous flow method as the flow velocity would have to be changed mid process and would affect the transition bands. The continuous flow setup also requires the device to be filled completely, using large sample volumes, 5µl-13µl.

Similar to the continuous flow device the shuttle LDR would move the sample through preheated zones for thermal cycling. The major difference would be the sample would move back and forth between the zones. A schematic of the shuttle LDR is shown in Figure 4.2.1.

The sample plug would flow from left starting in the 95°C zone, shuttle to the 65°C where the flow would be stopped for the required residence time. The length of the zones would be designed so that the plug would be fully contained in each zone.

Figure 4.2.1: Shuttle LDR temperature zones

Air slits around the temperature zones would isolate them from each other and from the rest of the device. The shuttling would occur 20 times for the reaction. This design would use less reagents than the continuous flow approach and would be able to adapt to changes in the residence time of the reaction. The significant problem was the diffusion of the plug, making it spread after 20 cycles and might not be contained in the zones in order to get the required temperature profile.

The stationary LDR was much simpler in design. The analyte would flow into a chamber and be held while the temperature was cycled. This would lead to a smaller footprint device, use less reagents, and would be able to adapt easily to changes in temperature residence times. The major issue with this approach would be to minimize the thermal capacitance of the thermal cycle zone in order for rapid transitions between the two temperatures.

The above designs all dealt with achieving the thermal cycle for the reaction. The rest of the device would have to be implemented around and isolated from this section.

4.2.2 PROTOTYPE LAYOUTS

In the first prototype of the full device, the design shown in Figure 4.2.2(a) was laid out. The sample would be put in the reagent wells then proceed to the first mixing stage. The analyte would then pass through the 95°C zone for preheating. The ligase, which was stored in the 0°C zone, would then be mixed as the sample proceeded to the thermal cycling zone. After thermal cycling the analyte would pass through the 0°C zone to stop the reaction and out to the identification section.

This design was laid out with the intention of using a small plug of analyte for flow through the device. This would be the first preliminary device for testing LDR at the microscale. All three detection methods, slab gel electrophoresis, microchip electrophoresis and zipcode array, would be used for testing. The zipcode array developed by Wang et al (2003) needs a minimum product volume of 168nl and the microchip electrophoresis used by Thomas et al (2004) requires a minimum volume of 50-200nl. Getting these volumes from the layout was feasible using a plug, but the product volume for slab gel electrophoresis, which requires a volume of 1µl, would be

Figure 4.2.2(a): First prototype layout of LDR device

difficult to obtain as this would require a large plug, so a chamber or reservoir had to be used. Another problem with the first layout was that the zones were not properly isolated from each other and the rest of the microchip.

Chambers were implemented on a modified design and the second prototype layout is shown in Figure 4.2.2 (b). Similar to the first layout the reagents would be loaded and mixed in the first mixing stage. The sample would be transferred to the first chamber for preheating. The ligase, which was stored in the 0°C zone would be added and the sample driven to the thermal cycling chamber. After thermal cycling the analyte would be pumped through the 0°C chamber to stop the reaction and out to the identification section. Apart from having the chamber to provide the needed product volume for slab gel electrophoresis the thermal isolation should be improved.

Figure 4.2.2(b): Layout of second prototype LDR device

4.3 FLUID ANALYSIS

The micromixers used pressure driven flow by syringe pumps for operation. Other types of flows such as electrokinetics, using an electric field to move the sample, could be used. However, in keeping with the micromixer design, pressure driven flow was used for the LDR device.

4.3.1 CHAMBER DESIGN AND HYDRODYNAMIC CONSIDERATIONS

In designing the chambers and microchannels for pressure-driven flow there should be minimum pressure drop and the fluid should remain stationary in the chambers during the thermal processes with minimal diffusion. Since the slab gel electrophoresis required 1 μl of analyte for product detection, a chamber volume of 1.5 μl was used as a preliminary size that should compensate for any fluid loss by diffusion or dead volumes as the fluid moved through the system. The size of the chamber also depended on reducing the thermal area for heating and cooling as a smaller area would reduce the thermal capacitance of the system, enhancing thermal cycling. The connecting microchannels needed to be as small as possible in width to increase the resistance between the chambers ensuring that the majority of the fluid remains in the chambers. There was however a limit on reducing the width as in too small a dimension blockage might occur from the reagents. Figure 4.3.1 shows the chamber design selected.

Figure 4.3.1: Chamber design

It was 1 mm x 5 mm x 0.3 mm connected to 0.1 mm x 0.3 mm microchannels. The deflection posts serve to spread the flow uniformly into the chamber and also increase the resistance at the ends of the chambers.

For an internal flow there are entrance and fully-developed flow regions. When a fluid enters a channel with uniform velocity and makes contact with the side walls the fluid closer to the channel walls has zero velocity due to the no-slip boundary condition caused by viscous forces (Incropera, 1985). The boundary layer develops with increasing distance into a channel and development concludes with the boundary layers merging at the centerline and the flow becoming fully developed. For this application with a fluid flowing at an average velocity of 2 mm/s, the Reynolds (Re) Number, equivalent diameter and hydraulic diameter for a rectangular duct were calculated using the following equations:

$$d_e = \frac{1.3(wh)^{0.625}}{(w+h)^{0.25}} \quad 4.1$$

$$d_h = \frac{4wh}{2*(w+h)} \quad 4.2$$

$$\text{Re} = \frac{ud_h}{\nu} \quad 4.3$$

where Re was the Reynolds Number, w was the width, h was the height, d_e was the equivalent diameter, d_h was the hydraulic diameter, u was the fluid velocity and ν was the fluid viscosity. The hydraulic diameter was used to determine the Reynolds number, while the equivalent diameter was used in pressure drop calculations. The calculated values of hydraulic diameter, and Reynolds's (Re) Number for the microchannels were

175 μ m and 0.37, respectively. As $Re < 2300$, the flow was laminar. For laminar flow, the hydrodynamic development length in the entrance region, $x_{fd,h}$, was:

$$\left(\frac{x_{fd,h}}{d_h} \right)_{lam} \approx 0.05 Re \quad 4.4$$

The velocity development length was calculated to be 2.54 μ m which was negligible when compared to the length of the microchannels, so the flow was assumed to be fully developed throughout.

The pressure drop throughout the system was calculated from the following equations:

$$f = \frac{64}{Re} \quad 4.5$$

$$\Delta P = f \frac{\rho u_m^2}{2d_e} (x_2 - x_1) \quad 4.6$$

where f was the friction factor, ρ was the fluid density, Re was the Reynolds number, u_m was the fluid mean velocity, d_e was the equivalent diameter, $x_2 - x_1$ was the length of the channel and ΔP was the pressure drop. For this application the pressure drop was only 300 Pa across the device.

The fluid in the chamber experiences random diffusion, and would spread to a length L , represented by the following equation:

$$L = \sqrt{d_f t} \quad 4.7$$

where d_f was the diffusion coefficients and t was the time for diffusion. The reagents in the LDR had diffusion coefficients of 10^{-9} - 10^{-11} m²/s, hence if the sample was stationary in the chamber the diffusion length would be 0.15 mm to 4 mm due to the duration of the thermal cycle.

4.4 THERMAL ANALYSIS

4.4.1 FINITE ELEMENT ANALYSIS

Finite Element Analysis (FEA) is used to simulate loading conditions on a design and determine the response to those conditions. The model is composed of discrete building blocks called elements. Each element has exact differential equations which describe how it responds to a certain load. The sum of the responses of all of the elements in a model gives the total response of a system. A finite element analysis package, (ANSYS vs. 5.7, Canonsburg, PA), was used to simulate the temperature distribution of the LDR layout. A steady-state thermal analysis was performed for the device that calculated the effects of steady thermal loads on a system or component.

The FEA package solves the general heat equation

$$\frac{\partial}{\partial x} \left(k \frac{\partial T}{\partial x} \right) + \frac{\partial}{\partial y} \left(k \frac{\partial T}{\partial y} \right) + \frac{\partial}{\partial z} \left(k \frac{\partial T}{\partial z} \right) + \dot{q} = \rho c \frac{\partial T}{\partial t} \quad 4.8$$

with variable thermal conduction in the rectangular coordinate system (Kakac, 1993).

Since there was no heat generation in the system and steady state conditions: $\dot{q} = 0$ and

$\frac{\partial T}{\partial t} = 0$ respectively, hence for steady state the equation simplified to:

$$\frac{\partial^2 T}{\partial x^2} + \frac{\partial^2 T}{\partial y^2} + \frac{\partial^2 T}{\partial z^2} = 0 \quad 4.9$$

for three dimensional heat flow.

Radiation, conduction and convection effects were taken into account for the simulations. The focus of the simulations were to look at the temperature distribution of the prototype layout, size the air slits, determine the power inputs needed to maintain the temperatures, and to investigate the temperature uniformity in the thermal regions.

4.4.1.1 SETUP

A thermal simulation was specified, using the h-method. The selected ANSYS element for the steady-state heat conduction model was PLANE70. It can be used as an axisymmetric element with three-dimensional thermal conduction capability. The element has eight nodes with a single degree of freedom, temperature, at each node. The required units and properties of polycarbonate and the fluid were written into the package. The properties were temperature dependent; this had to be modeled accordingly. There are two main methods for running simulations using the ANSYS package, Graphic User Interface (GUI) and the command line. A command line text file was written for the simulations. This allowed for easier changes to be made to the models (Appendix).

The model was developed according to Figure 4.4.1.1. Both a zipcode array and a microelectrophoresis chips have been developed. Fluid interconnects would be used to connect the LDR device to these other microchips if it was needed. This simplified the layout as only the section to the left of the dashed vertical line needed to be simulated.

Figure 4.4.1.1: FEA model for simulation

The convective heat transfer coefficient was assumed to be $25\text{W/m}^2\text{k}$ and the ambient air temperature was 25°C . The emissivity of polycarbonate was 0.9. The thermal cycle region was $4.5\text{ mm} \times 7\text{ mm} \times 1\text{ mm}$ with two chambers, while the cooling zone was $4\text{ mm} \times 7\text{ mm} \times 1\text{ mm}$ with a single chamber. The rest of the microchip such as the mixing regions were drawn much larger than they would be. After the simulations were complete the size would be reduced based on the results. From the remaining geometry the horizontal line running across the figure acts as a line of symmetry. This further reduced the size of the model as only a half, above the line, was simulated. After the model was drawn it was meshed to obtain the finite elements.

Thermal loads and boundary conditions were applied. The entire chip had convective boundary condition except for the area below the thermal zone and the cooling zone. The thermal zone had a constant heat flux into to the model, while the cooling zone had a constant heat flux from the model. The line of symmetry had an adiabatic boundary condition. After loads and boundary conditions were applied the model was solved.

4.4.1.2 STATIONARY FLUID IN MICROCHAMBER

Water was used as the working fluid as a large percentage of the LDR sample is composed of water. In the FEA the fluid was assumed as stationary. For a layer of fluid of thickness, L , confined between two parallel, isothermal plates and heated from below, the heat transfer through the layer is governed by the difference in temperature between the two plates, $(T_H - T_C)$, Figure 4.4.1.2. The criterion for instability and the onset of cellular convection is a critical value of the Rayleigh number (Ra) (Incropera 1985), which is a dimension less parameter:

$$Ra = g\beta\Delta TL^3 \rho^2 C_p / k\mu = 1708 \quad 4.10$$

where:

g = gravitational acceleration (m/s^2)

β = $1/T$ ($1/K$)

ρ = density (kg/m^3)

ΔT = $T_{Hot} - T_{Cold}$ ($^{\circ}C$)

μ = viscosity ($kg/m \cdot s$)

k = thermal conductivity ($W/m \cdot K$)

c_p = specific heat ($J/kg \cdot K$)

If the Rayleigh number was below this value convection effects could be ignored inside the chamber and there would be pure conduction across the fluid.

After the properties of water were used in the equation along with the chamber size, it was found that in order to get a Rayleigh number greater than 1708 the temperature difference would have to be much greater than $200^{\circ}C$. This temperature difference was much larger than the largest planned temperature difference ($95^{\circ}C - 25^{\circ}C = 70^{\circ}C$) for this application thus the fluid was simulated as a solid block with properties of water.

Figure 4.4.1.2: Rayleigh number schematic for chamber

4.4.1.3 CONVERGENCE CHECK AND GRID PATCH TEST

The FEA solution analysis should be investigated to check if the model was meshed properly and to ensure that the simulated results obtained are independent of element grid size. A patch grid and a convergence grid test were used. In the patch grid test a uniform boundary condition, in this case a uniform temperature, was applied to the model. If there was connectivity in the grid then all elements in the model should show the same temperature. It was evident that the meshed grid had good connectivity from the result shown in Figure 4.4.1.3. The convergence test uses three different mesh sizes, fine, medium and coarse, with each having approximately 4 times as many elements as the preceding mesh size, Figure 4.4.1.3.

Figure 4.4.1.3: Convergence and Patch grid test results

Loads and boundary conditions were applied to the model and the maximum value of temperature is obtained for each mesh. The results for the LDR layout are shown in Table 4.4.1.3.

Table 4.4.1.3: Convergence test summary

	Coarse Grid	Medium Grid	Fine Grid
$T_{\max}$ (°K)	368.23	368.17	367.95
# of elements	3467	13806	55091
Element edge length (m)	$50e^{-6}$	$25e^{-6}$	$12.5e^{-6}$

Using the following equations the convergence was determined:

$$|T_c - T_m| > |T_m - T_f| \quad 4.11$$

$$T_e = \frac{T_m^2 - T_f T_c}{2T_m - (T_f + T_c)} \quad 4.12$$

$$c = \ln \left(\frac{T_m - T_e}{T_f - T_e} \right) / \ln(2) \quad 4.13$$

where:

c = convergence rate

T_c = maximum temperature of coarse grid(°K)

T_m = maximum temperature of medium grid(°K)

T_f = maximum temperature of fine grid(°K)

T_e = exact temperature(°K)

From the calculations the simulation was converging. This proved that the results obtained were independent of the mesh size, with the medium mesh size selected for the rest of the simulations.

4.4.1.4 RESULTS – AIR SLIT SIZING AND TEMPERATURE PROFILE

The purpose of the simulation was to size the air gaps needed for temperature isolation, which would give the footprint of the device, and to check the temperature uniformity in the chambers. Figure 4.4.1.4(a) shows the temperature profile along the symmetry line of the device for 2 mm air slits. Zone 1 is the mixing region, Zone 2 is the thermal region and Zone 3 is the cooling region. Simulations were done using the highest temperature of the thermal cycle, 95°C. It was evident that the air gaps gave excellent thermal isolation. Figure 4.4.1.4(b) gives the actual contour plot of the LDR layout simulation for the 2 mm slits. From the plot it was observed that the temperature contour propagated slightly outside the thermal cycling zone but there was still no significant cross talk between the regions.

Side cuts were introduced into the model at the end of the slits to see if further improvements could be made. Figure 4.4.1.4(c) shows the temperature profile along the line of symmetry for the device for 2 mm air slits with side cuts. Similar to the previous result temperature isolation was obtained. In Figure 4.4.1.4(d) it was observed that the temperature contour again propagated outside the thermal cycling. The major improvement with the side cuts was that the temperature distribution become more uniform inside the regions and the heat flux required to heat and cool the zones was reduced. The width of the air slits was reduced to 1 mm as this would further reduce the footprint of the device. Figure 4.4.1.4(e) shows the temperature profile for the 1 mm slits with side cut. Even with 1 mm slits, the target temperatures were obtained and isolated. A closer look at the temperature revealed that uniform temperatures were

achieved, within the temperature tolerance, both across the thermal zone, Figure 4.4.1.4(g) & (h), and along the chambers, Figure 4.4.1.4(i).

Figure 4.4.1.4(a): Temperature profile across LDR layout with 2 mm slits.

Figure 4.4.1.4(b): Contour plot showing temperature distribution on LDR model for 2mm slits.

Figure 4.4.1.4(c): Temperature profile across LDR layout with 2 mm slits with side cut.

Figure 4.4.1.4(d): Contour plot showing temperature distribution on LDR model for 2mm slits with side cuts.

Figure 4.4.1.4(e): Temperature profile across LDR layout with 1 mm slits with side cut.

Figure 4.4.1.4(f): Contour plot showing temperature distribution on LDR model for 1mm slits with side cuts.

Figure 4.4.1.4(g): Temperature profile across the thermal zone.

Figure 4.4.1.4(h): Temperature contours across the thermal zone.

Figure 4.4.1.4(i): Temperature profile along the centerline of the chamber

4.4.2 COOLING METHODS

The FEA simulations showed that temperature isolation could be achieved with uniform temperatures in the zones for the steady state. Heating and cooling elements needed to be selected in order to obtain the required temperatures and thermal cycling. Cooling elements were needed to cool from 95°C to 65°C for the thermal cycle and to cool to 0°C, while heating elements were needed for heating from 65°C to 95°C for the thermal cycle and the initial startup of the device.

4.4.2.1 FORCED CONVECTION

The simplest cooling method for use in the thermal cycle would be passive cooling of the zone under ambient conditions. This would require a low thermal capacitance and would depend on the ambient conditions. If the ambient conditions

changed then the cooling rate would also change and repeatability would be an issue. In order to have active control, different cooling elements were investigated. Table 4.4.2 gives a summary of different cooling schemes used for active microcooling.

Using forced convection by a fan was similar to passive cooling with the only difference being an increase in the heat transfer coefficient. The advantage of this method would be the ability to control the fan speed and the rate of cooling. If the thermal cycle region is modeled a block 4.5 mm x 7 mm x 1 mm, Figure 4.4.2. Equations 4.14 - 4.18 can be used to determine a forced convection coefficient.

Table 4.4.2: Different cooling elements used for active microcooling.

Cooling Mechanism	Thermoelectric	Microjet	Cryo coolers	Fans/ fins
Reliability	High	Low	High	High
Fabrication	High	Low	High	Low
Performance	Low	High	Moderate	Moderate
Cost	High	Low	High	Low
Integration w/ electronics	Yes	Yes	Yes	Yes
Vibration level	none	high	low	High
Life-time	long	moderate	long	High
Enviromentally-Benign	yes	no	Depends on fluid	yes

Figure 4.4.2: Forced air blowing over a block

$$u_{\infty} = \frac{Q}{A} \quad 4.14$$

$$Re = \frac{u_{\infty} D}{\nu} \quad 4.15$$

$$Nu_D = 0.3 + \frac{0.62 Re_D^{1/2} Pr^{1/3} D}{[1 + (0.4/Pr)^{2/3}]^{1/4}} \left[1 + \left(\frac{Re_D}{282000} \right)^{5/8} \right]^{4/5} \quad 4.16$$

$$h = \frac{Nu_D k}{D} \quad 4.17$$

$$\frac{T - T_{\infty}}{T_i - T_{\infty}} = e^{-\frac{hA_s t}{\rho V c}} \quad 4.18$$

where:

Re = Reynolds number

Pr = Prandtl number

Nu = Nusselt number

u_{∞} = speed of air (m/s)

Q = Flow rate of fan (m^3/s)

A = Fan area (m^2)

D = width of block

Air was blown from a fan with the velocity calculated using Equation 4.14. The Reynolds number was calculated and used with the properties of air to estimate the Nusselt number (Incropera 1985). The heat transfer coefficient was calculated and used

with the lump capacitance method. For this application if a fan rated at 10 CFM were used the time to cool from 95°C to 65°C would be ~5 seconds.

4.4.2.2 THERMOELECTRIC MODULE

Thermoelectric modules were used to obtain the 0°C zone, as cooling below ambient was required. The modules were also used for heating during the preliminary tests. Thermoelectric modules use the Peltier effect to move heat through doped bismuth telluride couples when a DC current is applied (Goldsmid, 1986). They are small solid-state devices that function as heat pumps and are able to give long term temperature stability for both cooling and heating applications. The typical module is a sandwich formed by two ceramic plates with an array of parallel small bismuth telluride couples in between, Figure 4.4.2.2.

When a DC current is applied, heat is moved from one side of the device to the other where it must be removed with a heat sink. The heat sink should have a low thermal resistance in order to quickly dissipate the pumped heat and may need some form of active cooling (www.marlow.com, 2004; www.melcor.com, 2004). The governing equation for the cold side temperature of the thermoelectric is given by Equation 4.19.

Figure 4.4.2.2: Thermoelectric modules (www.melcor.com, 2004)

$$Q_c = 2N[aIT_c - \left(\frac{I^2 p}{2G}\right) - kDtG] \quad 4.19$$

$$Dt_{\max} = T_h - \left[\frac{(1 + 2ZT_h)^{1/2} - 1}{Z} \right] \quad 4.20$$

$$Q = 0$$

where:

- T_h = Hot side temp
- T_c = cold side temp
- Z = Figure of Merit ($a^2 / (p k)$) (Kelvin⁻¹)
- Dt = $T_h - T_c$
- G = Area / Length of T.E. Element (cm)
- N = Number of Thermocouples
- I = Current (amps)
- p = Resistivity (ohm cm)
- a = Seebeck Coefficient (volts / Kelvin)
- k = Thermal Conductivity (watt / (cm Kelvin))

It is imperative that modules are selected based on the overall operating range of temperatures as during operation heat will be pumped until a maximum temperature difference occurs across the module (Equation 4.20). At this stage no heat will be pumped. Careful attention should also be placed on mounting the module to reduce contact resistance, enhance heat transfer, and to prevent localized hot spots on the module surface.

4.5 THERMAL SYSTEM DYNAMICS

The system dynamics of the LDR device was studied to determine the required thermal input into the thermal regions, to determine appropriate control parameters and to check the overall performance of the system.

4.5.1 PSEUDO BOND GRAPH

A pseudo-bond graph was developed in order to study the dynamics of the system (Karnopp, 2000). The thermal zone was modeled as a 4.5 mm x 7 mm x 1 mm block, Figure 4.5.1. Before this method could be used it was required to check if the block could be treated as a single lump. Both convection and radiation heat transfer were taken into consideration. The Biot number, Equation 4.21, was found for the system and was less than 0.1, so the block could be treated as a single lump (Incropera 1985).

$$bi = \frac{hl_c}{k} \quad 4.21$$

In pseudo-bond graphs temperature is an effort while heat flux is a flow (Karnopp, 2000). The model shown in Figure 4.5.1(b) was developed for the system.

Figure 4.5.1(a): Thermal zone model used for pseudo bond graph

Figure 4.5.1(b): Pseudo Bond Graph of system

A heat flux, S_f , was applied to the underside of the block, passing through the thermal capacitance, C , of the block and across the combined convection and radiation thermal resistances, R , to an ambient temperature, S_e .

The thermal capacitance was evaluated using Equation 4.22.

$$C = mc_p \quad 4.22$$

where c_p was the heat capacity of polycarbonate and m was the mass of the block.

The heat flux raises the temperature of the block and at equilibrium heat in must be equal to heat out by convection and radiation, as defined by Equation 4.23:

$$Q_{in} = h_{comb} A_{surf} (T_1 - T_\infty) \quad 4.23$$

Where Q_{in} was the heat flux, h_{comb} was the combined heat transfer coefficient of radiation and convection, A_{surf} was the surface area, T_1 temperature of the block and T_∞ was ambient temperature. The linearized radiation heat transfer coefficient is given by Equation 4.24 (Incropera, 2000):

$$h_r = \varepsilon \sigma (T_s + T_{sur}) (T_s^2 + T_{sur}^2) \quad 4.24$$

where ε is emissivity of polycarbonate, σ was Stefan-Boltzmann constant (5.67×10^{-8} W/m² K⁴), T_s was block temperature, T_{sur} was ambient temperature. It was added to the convection heat transfer, h_{conv} , coefficient given the combined coefficient.

The total resistance to heat flow out of the block was hence given by equation 4.25:

$$R = \frac{1}{h_{comb} A_{surf}} \quad 4.25$$

By developing and solving the state equation for the bond graph model the transfer function, Equation 4.26, was obtained.

$$TF = \frac{Sf}{C} - \frac{T}{RC} = \frac{Sf/C}{s + (1/RC)} = \frac{1.853}{s + 0.02726} \quad 4.26$$

The transfer function of a system relates the output to the input of that system (Ogata, 1990). This enables predictions to be made about the response of the system to a given input. Solving the denominator of the transfer function gave the system pole. A system is stable if its poles have negative real parts (Franklin, et al., 1994). The pole of the system was -0.027265 which was negative and real so the time response will decay.

4.5.2 OPEN LOOP RESPONSE

The open loop response gave the response of the system to a step input. Figure 4.5.2 shows the temperature response of the system to step inputs in the heat flux for both analytical and experimental trials. The analytical solution was obtained by applying a step input to the transfer function in Matlab (ver. 5.4, The Mathworks, Natick, MA). Experimental results were obtained by heating a polycarbonate block, with model dimensions, with a Watlow Kapton heater (Model K005020C5, Watlow, Dallas, TX). The power input was obtained by solving Equation 4.23.

Figure 4.5.2: Open Loop Response of System Model

The results show that the experimental solution was within 1-2°C of the analytical solution, validating the model and assumptions. A power of 0.06W was needed to obtain 95°C and 0.034W was needed for 65°C. The time constant for the system was 37 seconds, with steady-state temperature being reached after 160 seconds. For this application the time constant for the open loop system was too large for thermal cycling hence a closed loop system needed to be implemented.

4.5.3 PID TUNING

Temperature was the output of the system. In an ideal case an input to the system would produce the exact temperature required, with the required performance in terms of the ramp rate and the time the system takes to reach steady state for the open loop. The real process did not operate in this way as seen in the open loop response. The output responded too slowly to changes in input. In this situation, it was necessary that the output of the system be measured and regulated by a controller. This configuration, called a closed loop feedback control system, is illustrated in Figure 4.5.3. Closed loop control handles disturbances better than open loop and the time constant can be adjusted (Ogata, 1990).

4.5.3.1 PID CONTROLLER PARAMETERS

A controller normally involves the use of a combination of three gains to modify the output of a system, K_P , K_I and K_D , that are referred to as the proportional, integral and the derivative gains (Ogata, 1990). The power (W) from a PID controller is

Figure 4.5.3: Typical system under closed loop control.

Table 4.5.3.1: The effects of each of the controller gain on a closed-loop system

CLOSED LOOP RESPONSE	RISE TIME	OVERSHOOT	SETTLING TIME	STEADY-STATE ERROR
K_P	Decrease	Increase	Small change	Decrease
K_I	Decrease	Increase	Increase	Eliminate
K_D	Small change	Decrease	Decrease	Small change

given by Equation 4.27:

$$W(t) = K_p \times \left((T_s - T_o) + K_D \frac{d}{dt}(T_s - T_o) + K_I \times \int (T_s - T_o) dt \right) \quad 4.27$$

where T_s and T_o are the final and initial temperature of the system, respectively. The effects of increasing each of the controller gains on a closed-loop system are summarized in Table 4.5.3.1.

Figure 4.5.3.1 shows the Simulink (ver. 5.4, The Mathworks, Natick, MA) model used to determine the required gains for controlling the temperature output. Selecting optimal values of K_P , K_I and K_D parameters of the closed loop control system was an iterative process called PID tuning. The effects on the system will be discussed later.

Figure 4.5.3.1: Simulink model of Control system

4.6 TEST DEVICE ASSEMBLY

A test device was made for preliminary evaluation. It was made using the entire device platform but only included the thermal cycle and the cooling chambers as shown in Figure 4.6. Water was used as the working fluid.

4.6.1 FABRICATION

For rapid fabrication, micromilling was used to fabricate the test device at the Center for Bio-Modular Microsystems at Louisiana State University (CBMM). Similar procedures were followed as discussed in previous sections for micromilling. The chambers were 1 mm x 5 mm x 0.3 mm connected by 100 μ m channels, with the entrance and exit reservoirs being 1mm in diameter. Figure 4.6.1(a) shows the micromilled test LDR device in 1 mm thick polycarbonate.

Figure 4.6: AutoCAD Layout of test LDR

Figure 4.6.1(a): Test LDR micromilled in polycarbonate

4.6.2 EXPERIMENTAL SETUP

After fabrication a 125 μ m polycarbonate cover slip was thermally bonded to the device to seal the channels. The device was then assembled with a heater, a thermoelectric module and thermocouples. A commercial thin film resistance heater (HK5565R5.3L12D, Minco, Minneapolis, MN), with a resistance of 5.3 ohms, was attached to the thermal cycle zone with a high thermal conductivity glue (Omegatherm 201, Omega, Stamford, CT). The thermoelectric cooler (OT1.5-17-F1A, Melcor, Trenton, NJ), with a maximum heat pumping capacity of 1.5W was attached under the cooling region, with the appropriate heat sink, as shown in Figure 4.6.1(b). Four K type thermocouples (HYPO-33-1-T-G-60-SMP-M, Omega, Stamford, CT) were used for monitoring the zone temperatures, two in each zone. The thermocouple ends were placed in small 300 μ m holes drilled as close as possible to the microchambers, at two different locations in the zone. The heater was controlled by a Watlow PID controller (Series 96, Watlow, Winona, MN), while the thermoelectric used a Wavelength (HTC-3000, Wavelength Electronics, Bozeman, MT) PI controller with an evaluation board. Figure 4.6.2 shows the entire setup for the experiments.

Figure 4.6.1(b): Test LDR assembly

Figure 4.6.2: Experimental setup

4.6.3 THERMAL CYCLING AND COOLING PROFILE

During the experiments both the thermal cycle and the cooling zone were operated at the same time. It was observed that using 2 mm air slits instead of 1 mm air slits allowed for easier implementation of the electronics. The air slits provided the required thermal isolation between the temperature zones and the two thermocouple readings were within 1°C of each other for the respective thermal zones. Figure 4.6.3(a) shows the cooling profile achieved in the cooling zone.

The system was turned on with the proportional and integral gains set from the Simulink model. It was seen that the system could be cooled from room temperature to 0°C in approximately 15 seconds. There was a 1°C overshoot, with the system reaching steady state after 250 seconds. The PI controller provided the long term temperature stability needed for LDR product storage.

Figure 4.6.3(a): Cooling region Temperature profile

Figure 4.6.3(b) and 4.6.3(c) shows the temperature profile for the thermal cycling zone. The heater was controlled by the PID controller. It should also be noted that this test was done with passive cooling by ambient air. In the Figure 4.6.3(b), the proportional gain was 112, integral gain was 1.2, and the derivative gain was 0.11. It was evident that these gains gave the required thermal cycle but with oscillation at the setpoint temperatures. Further PID tuning using the Simulink model a proportional gain of 112, an integral gain of 0.11 and a derivative gain of 0.02 were used to obtain the temperature profile in Figure 4.6.3(c) for both heating and cooling. Even though there was a slight overshoot there were no oscillations with the temperature well within the tolerance. Further improvements can be made by further PID tuning. The selected controller allows for setting a different set of gains for heating and cooling which should enhance the performance of each method, especially when active cooling is used.

Figure 4.6.3(b): Thermal cycling with PID controller

Figure 4.6.3(c): Thermal cycling with PID gains adjusted

A unique feature of the controller allows the residence times for each temperature setpoint to be set. During heating and cooling the temperature would be increased or decreased but the countdown time at each setpoint would not start until the temperature reaches the preset value of the tolerance around that setpoint. For this test the residence time at 95°C was set at 25 seconds, while the residence time at 65°C was set at 50 seconds, which was obtained in Figure 4.6.3(d) for t_1 and t_3 . The time in moving from setpoint to setpoint was approximately the same, t_2 and t_4 , at 10 seconds. The residence times can be adjusted to adapt to changes in the speed of the reaction, and to allow for getting the initial preheat temperature.

4.7 CONCLUSIONS

A final prototype for the LDR device was laid out using a chamber method for cycling the sample temperature. Pressure driven flow was used to move the analyte through the chip. The chambers and connecting microchannels were designed to

Figure 4.6.3(d): Thermal cycle

minimize the pressure drop, with the chamber sizes selected in order to ensure that all three LDR product detection methods could be used. It was shown the fluid dynamics could be controlled with the majority of the fluid remaining inside the chambers during the thermal processes.

FEA simulation results showed that a thermal profile of room temperature, 95°C and 0°C could be obtained on the prototype layout using air slits to separate the temperature zones. The mixing zones would be isolated, hence no thermal effects occurs in these regions. There was also no cross-talk between the heated and cooled thermal zones. The simulations showed good temperature uniformity inside the chambers. These results were validated on a preliminary test device fabricated by micromilling polycarbonate. It was seen that the thermal profiles could be achieved using a heater and a thermoelectric module with PI and PID control to enhance the performance. The system will be able to adapt to changes in the LDR reaction time.

CHAPTER 5: CONCLUSION AND RECOMMENDATIONS

The focus of this work was to develop a microdevice for doing the Ligase Detection Reaction (LDR). The LDR was developed by Francis Barany (Barany et al, 1991) and consists of several mixing stages for the combination of six reagents. In order to accomplish mixing, micromixers had to be developed and microfabricated. Temperatures of 65°C, 95°C and 0°C had to be maintained on the microchip in a particular order to get a thermal cycle for a successful reaction. The device had to be compatible with other devices such as microPCR for implementation unto the micro total analysis system.

5.1 CONTRIBUTION TO MICROLDR DEVELOPMENT

This was the first microdevice developed solely for LDR. It was shown that it is possible to microfabricate a LDR device from polycarbonate. Polycarbonate is a moldable polymer that is readily available and easy to be hot embossed for mass-producing microchips. It has a glass transition temperature of 140°C that is more than the highest temperature (95°C) used in the LDR. The time of 4 minutes at 65°C for the original macroscale reaction was investigated by changing the concentrations and volume of the reagents. It was found that an excess of primers in relation to the PCR product increased the LDR product. It was possible to get LDR product after even 30 seconds at 65°C and also with smaller samples. This showed that the time and sample volumes for the reaction can be reduced. Wang et al have used 2 minutes at 65°C and have discriminated mutant DNA from normal DNA in ratios of 1000:1. There are several methods that can be used for LDR identification with the zip code array being the most sensitive.

Micromixers were designed and simulated by Fluent (v5.4, Lebanon, NH) (Maha, 2004). Different sizes of four geometries were selected for microfabrication, Y-mixers, T-mixers, jets in cross flow and separation wall mixer with the smallest feature size being 5 μ m. X-ray & UV lithography on SU-8, micromilling, laser ablation and the LIGA process were used to make the micromixers. Heights of 150 μ m and 300 μ m were achieved for the micromixers. SU-8 lithography and laser ablation were more suited for individual microchips and simpler geometries. LIGA was the best for mass-producing quality repeatable microstructures for the mixers with straight and smooth sidewalls by giving a mold insert for hot embossing. Micro milling was also viable for mass-producing microstructures, but the tool radius always leaves a fillet even if straight intersections are needed. In terms of time for the manufacturing process, laser ablation was the fastest followed by micromilling. SU-8 lithography was a longer process than the aforementioned but it was even shorter than the full LIGA process. From the simulations, the jets in cross flow were the most efficient micromixers (Maha et al, 2004). There was no way to efficiently test the mixing of the LDR reagents, only after the full device was assembled and the reaction carried out could a performance assessment be made.

Thermal aspects of the microchip were investigated. Two methods were considered to obtain the thermal cycle, continuous flow and a stationary sample. Finite element simulations (vers. 5.7, ANSYS, Inc., Canonsburg, PA) were done for both cases, with both being feasible. However the continuous flow approach resulted in a larger footprint for the device and required more reagent volume. By reducing the device thermal capacitance, commercial heaters with passive cooling were used to get the thermal cycling with the appropriate controls. A preliminary test device was

fabricated by micromilling. It was shown that the transition from 65°C to 95°C could be done in 10 seconds and from 95°C to 65°C also in 10 seconds. The thermal chamber could hold 1.5µl of analyte. A thermoelectric cooler is used to obtain the 0°C temperature zone for ligase storage and for stopping the reaction. This zone was isolated from the thermal cycle zone. All the thermal zones were isolated from the rest of the microchip and thermal effects do not affect the mixing stage or the identification stage. The final device was laid out and has a total footprint of 3 cm x 3 cm. Based on the similarity of the experimental and analytical transient temperatures response, the assumptions made during the system dynamics simulation were validated.

5.2 RECOMMENDATIONS FOR FUTURE WORK

The chemistry involved in the LDR still needs further investigation in order to find low abundant mutations from small sample sizes in quicker processing times. Finding the exact chemical parameters may also enable the shuttle LDR design to be used. The heating element should be deposited directly on the polycarbonate. Nichrome heaters have been deposited on ceramic substrates and have reached temperatures of 350°C (Pasupuleti, 2004). Depositing heaters will increase the accuracy of the approximation of one-dimensional heat flow and will make the device more robust.

Full testing of the device needs to be done with micromixers implemented. Microfluidic interconnects should be developed for the attachment of the PCR to the LDR device and other devices with full analysis of a sample being investigated. The major obstacle in having fully functional microdevices is to standardize the off-chip components. Different microchips have different functions, but if there were standard equipment, then experimental setup would be simpler and more cost effective.

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APPENDIX A: DETAILED DRAWING OF MICROMIXERS

Figure A1: Detailed drawing of micromixers

APPENDIX B: SU-8 PROCESS SPEED AND EXPOSURE TABLE

SU-8 50 Spin Speed Curve

Figure A2: SU-8 Process Speed Curve (www.camd.lsu.edu)

Table A1: SU-8 Exposure dosages (www.camd.lsu.edu)

Exposure Dose vs. SU-8 Thickness /CAMD UV-Station							
Thickness (μm)	Dose (mj/cm^2)	Thickness (μm)	Dose (mj/cm^2)	Thickness (μm)	Dose (mj/cm^2)	Thickness (μm)	Dose (mj/cm^2)
10	210	210	810	410	1410	610	2010
20	240	220	840	420	1440	620	2040
30	270	230	870	430	1470	630	2070
40	300	240	900	440	1500	640	2100
50	330	250	930	450	1530	650	2130
60	360	260	960	460	1560	660	2160
70	390	270	990	470	1590	670	2190
80	420	280	1020	480	1620	680	2220
90	450	290	1050	490	1650	690	2250
100	480	300	1080	500	1680	700	2280
110	510	310	1110	510	1710	710	2310
120	540	320	1140	520	1740	720	2340
130	570	330	1170	530	1770	730	2370
140	600	340	1200	540	1800	740	2400
150	630	350	1230	550	1830	750	2430
160	660	360	1260	560	1860	760	2460
170	690	370	1290	570	1890	770	2490
180	720	380	1320	580	1920	780	2520
190	750	390	1350	590	1950	790	2550
200	780	400	1380	600	1980	800	2580

APPENDIX C: LIGA PROCESS

Figure A3: Schematic of the LIGA

X-RAY MASK FABRICATION

Graphite wafers were purchased from Poco [Poco, Decatur, TX, DFP-3] in 4 inch disks that were 200 μ m thick. They were polished and cleaned thoroughly using acetone, IPA, and DI water to remove all unwanted particles. Approximately 5 ml of SU-8 50 (MicroChem Corp., Newton, MA) negative photoresist were placed on the graphite disk and spun at 2500 rpm for 20 seconds to give a uniform coat. All bubbles

have to be removed from SU-8 layer using a small pin. The layer is then allowed to relax to lessen any internal stresses for 5 minutes, while being covered.

The SU-8 coat was pre-baked in a convection oven, starting at a temperature of 65°C, then ramped up to 95°C at a rate of 4°C/min and maintained at this temperature for about 1 hour, then slowly cooled down to 30°C at 2°C/min in the oven. Final cooling from 30°C to room temperature (~23°C), took place outside the oven. The slow cooling was necessary to reduce the internal stresses generated in the SU-8 layer during solidification.

For 50 µm thick layer of SU-8 the required dosage should be 350 mJ/cm² of UV light. The SU-8 coated wafers were exposed to ultraviolet light through the clear field optical mask for 18.3 seconds. After exposure the disks were post-baked in a convection oven, starting at a temperature of 65°C, ramped up to 95°C at a rate of 4°C/min and maintained there for 15 minutes, after which they were cooled down to 70°C at 1°C/min. Cooling to room temperature was done in ambient air. The post-baked, patterned disks were developed in the SU-8 developer (MicroChem Corp., Newton, MA) for a total of 6 minutes, moving back and forth from a cleaning bath to a rinse in IPA in 3 steps. The appearance of a white residue indicated incomplete development of SU-8. Whenever there was any white residue the disk was re-immersed in the solution for more development. After complete development it was rinsed and dried with compressed air.

Gold sulfite solution (Technic Inc, Irving TX), containing 1 troy oz gold per gallon was used for gold plating. A current density of 1.5 mA/cm² was used over the total area for the micromixers of 81 cm² for five hours to get a 20-25 µm thick layer of

gold. The plating rate was approximately 4-5 μm per hour. A platinized titanium mesh was used as the anode, with the bath temperature at 55°C under constant stirring. For future gold plating runs a more concentrated commercially available neutral gold sulfite solution (Technic Inc, Irving TX), containing 1 troy oz gold per 500ml was used to make up the solution.

After electroplating the graphite wafer was bonded to a standard NIST (National Institute of Standards and Technology) aluminum adapter ring to avoid sagging of the graphite wafer under its unsupported weight plus the weight of the resist. This was done using using super glue (Loctite 4210, Loctite Corporation, Newington, CT). The schematic for the process is shown in Figure A4.

PREPARATION OF STAINLESS STEEL MOLD INSERT SUBSTRATE

The stainless steel was cut and machined into 5-inch diameter base plates and $\frac{1}{4}$ mm thick. Six counter sink screw holes were placed around the edges for attachment to the hot embossing machine. All stainless steel bases were measured on the profilometer to characterize flatness. The profilometer stylus was moved across the surface along a diameter line then the disk turned 180 degrees to repeat the profile in the orthogonal direction. This was done on both sides of the disk. If the profile varied by 2-7 μm the disk was lapped using diamond slurries with an ENGIS lapping machine (Model 15LM11V Engis Wheeling, IL) until the required profile was obtained. The stainless steel was then sandblasted to create a roughened surface with an rms value of approximately 2 μm . It is then activated in C-12 activator (Puma Chemical, Warne, NC) with a current density of (30 mA/cm^2) at 55°C, for 5 minutes, to clean the surface. The C-12 bath was composed of 7 parts DI water with one part C-12 activator with 2% HCL to reach a pH of 1.5.

Figure A4: Schematic of the fabrication procedures for graphite X-ray mask.

The substrate was then treated with Wood's strike. The Wood's strike solution composed of 240g of nickel chloride ($\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$), 80g of hydrochloric acid (HCl) and 1 liter of distilled water at a pH of 1.5 was used. This was done at room temperature with reverse plating for two minutes at a current density of 30 mA/cm^2 to etch the surface of the steel then switched to forward plating for six minutes at 30 mA/cm^2 to deposit a thin layer ($\sim 1 \mu\text{m}$) of nickel.

PMMA BONDING

PMMA resist, 3000 μm thick sheet stock (CQ grade, Vista Optics, UK) was cut into 4 in disks using a CNC mill and bonded to the stainless steel base with an MMA glue. After cutting the disks were annealed at 80°C for 3 hours and cooled to room temperature. This reduced any internal stresses. A solvent bonding process, using a MMA bonding solution was used to achieve the bond. The constituents of the bonding solution are shown in Table 3.3.5.7 and added to the mixture in that same order. Powdered PMMA was dissolved in the MMA over a period of 24 hours with constant stirring. Some of the MMA may evaporate hence the weight of the container was recorded before and after so that the correct amount of MMA could be added to maintain the correct proportions. DMA acted as a starter for polymerization and BPO was used as the hardener. The BPO was added, then DMA, then MEMO. After each stage the container in placed under vacuum to remove any bubbles.

The solution is then poured in the center surface of the cleaned stainless steel. The 3000 μm thick PMMA resist was pressed onto the substrate so that no bubbles are made in the glue. Once this was done the resist should not be moved. The sample was then placed in a pneumatic press overnight under a pressure of 42 psi at room

Table A2: Composition by mass of MMA bonding glue

MMA (Methyl methacrylate) + Powdered PMMA	8.5 gm + 1.5 gm
BPO (Benzoyl peroxide)	0.15g
DMA (Dimethyl aniline)	0.1g
MEMO (Methacryloxpropylltrinethoxysilane)	0.1g

temperature. After bonding the PMMA was fly cut to the final desired height of the microstructures, plus 50 μ m for surface grinding and polishing. The thickness of the PMMA resist needed to be known to calculate X-ray exposure dosage.

X-RAY EXPOSURE OF PMMA

X-ray exposures were performed on the XRLM-1 beamline at CAMD. A 125- μ m Beryllium filter was installed in the beamline to act as an initial filter for low energy X-rays. The exposure dose was carefully calculated using DOSE SIM [<http://www.camd.lsu.edu>, 2000]. For full exposure of PMMA, the bottom surface of the resist must receive at least 4000 mJ/cm³ of X-ray energy (www.camd.lsu.edu).

PMMA RESIST DEVELOPMENT

Table A2 shows the composition by volume of the GG developer and GG rinse used to dissolve the exposed PMMA.

Table A3: Composition by volume of the GG Developer formulated at the Institute for Microstructure Technology at the Forschungszentrum, Karlsruhe.

Developer:	(ml)
2-(2-Butoxyethoxy) ethanol / (diethylene glycol butyl ether)	900
Morpholine	300
2-aminoethanol	75
Water	225
Total:	1500
Rinse	
2-(2-Butoxyethoxy) ethanol / (diethylene glycol butyl ether)	
Water	1200
Total:	300
	1500

For every 200µm of PMMA thickness, the wafer was run through a development cycle of 20 min in the GG developer and a rinse cycle of 40 min in the GG rinse at room temperature for three repetitions. The GG developer was filtered continuously. After development the microstructures were dipped in DI water and dried with compressed air before electrodeposition.

NICKEL ELECTRODEPOSITION

A nickel sulfamate electroplating solution (Alfa Aesar, Ward Hill, MA), mixed with other reagents as shown in Table A3, was used to electrodeposit nickel into developed PMMA pattern. Wood's strike was done again before nickel was electrodeposited. The substrate was then placed directly into the nickel-plating bath without rinsing.

Six liters of nickel sulfamate electroplating solution was prepared for the bath.

The manufacturer of the nickel solution specified a pH in the range of 3.6-4.3. After mixing the pH was below this value so sodium hydroxide was used to bring this value up to the required range. Boric acid was used as a buffering agent to minimize the pH rise of the solution while electroplating. Lauryl sulfate served as a wetting agent

Table A4: Composition by mass of the nickel sulfamate plating solution

Component	Chemical formula	Amount (g)/liter
Nickel sulfamate	$\text{Ni}(\text{SO}_3\text{NH}_2)_2 \cdot 4\text{H}_2\text{O}$	450
Boric acid	H_3BO_3	37.5
Sodium lauryl sulfate	$\text{C}_{12}\text{H}_{25}\text{O}_4\text{SNa}$	1.0

reducing the surface tension of the solution. Plating current density was 15 mA/cm² at 57°C with constant stirring and filtering of the solution.

LAPPING AND POLISHING

Diamond slurries (15µm, 6µm, 1µm) were used with an ENGIS lapping machine (Model 15LM11V Engis Wheeling, IL) to lap the microstructures to the final height and then polished for a smooth finish. Lapping speed was 55 rpm and the wheel conditioned for 2 minutes for every 20 minutes of lapping. The PMMA was flood exposed at CAMD and developed in the GG developer to remove the remaining PMMA.

APPENDIX D: ANSYS CODES FOR SIMULATION

```
! simulation LDR layout
/PREP7
SMRT,OFF
HT=25
HL=1000E-6
!BUILDING MODEL
BLOCK,0,10e-3,0,4.5e-3,0,HL
BLOCK,0,20.5E-3,4.5E-3,14.5E-3,0,HL
BLOCK,11E-3,15.5E-3,3.5E-3,4.5E-3,0,HL
BLOCK,11E-3,15.5E-3,0,3.5E-3,0,HL
BLOCK,16.5E-3,20.5E-3,3.5E-3,4.5E-3,0,HL
BLOCK,16.5E-3,20.5E-3,0E-3,3.5E-3,0,HL
VGLUE,ALL,ALL
!MATERIAL pc
MPTEMP,,,,,,,,
MP,DENS,1,1200
MP,C,1,1200
MP,KXX,1,0.2
! MESH THE VOLUME
ET,1,70
MAT,1
SMRT,4
MSHAPE,1,3D
VMESH,ALL
!=====CONVECTIVE BOUNDRY CONDITION
SFA,1,,CONV,HT,300
SFA,2,,CONV,HT,300
SFA,5,,CONV,HT,300
SFA,6,,CONV,HT,300
SFA,10,,CONV,HT,300
SFA,12,,CONV,HT,300
SFA,13,,CONV,HT,300
SFA,14,,CONV,HT,300
SFA,17,,CONV,HT,300
SFA,18,,CONV,HT,300
SFA,29,,CONV,HT,300
SFA,39,,CONV,HT,300
SFA,40,,CONV,HT,300
SFA,41,,CONV,HT,300
SFA,43,,CONV,HT,300
SFA,44,,CONV,HT,300
SFA,47,,CONV,HT,300
SFA,48,,CONV,HT,300
SFA,49,,CONV,HT,300
SFA,50,,CONV,HT,300
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SFA,51,,CONV,HT,300
SFA,52,,CONV,HT,300
SFA,53,,CONV,HT,300
alls
!HEAT FLUX
SFA,37,,HFLUX,1400,
SFA,45,,HFLUX,-600,
alls
!=====GRAHPICAL SOLUTION
/solu
solve
finish
/POST1
/TITLE,TEMPERATURE PROFILE
PLNSOL,TEMP
FINISH
/POST1
PATH,TEMP,2,,48          ! DEFINE PATH WITH NAME = "PIPE"
PPATH,1,,0,0,250e-6      ! DEFINE PATH POINTS BY LOCATION
PPATH,2,,20.5e-3,0,250e-6
PDEF,TEMP,TEMP           ! MAP TEMP TO PATH
!PLPATH,TEMP!,,AUG
/TITLE,OUTLET TEMPERATURE PROFILE
/GRID,1
/YRANGE,380,270
/XRANGE,0,20.5e-3
/GROPT,DIVX,20
PLPATH,TEMP!,,AUG
! simulation LDR layout
/PREP7
SMRT,OFF

```

VITA

Dwhyte Barrett was first born on February 23, 1980, in Trelawny, Jamaica, to Villair and Sytre Barrett. He came to Louisiana State University, Baton Rouge, Louisiana, USA, in 1998 on an athletic scholarship and became an All-American in track and field in 2000. He received his Bachelor of Science in Mechanical Engineering degree in May of 2001 from Louisiana State University.

He joined the graduate program in Mechanical Engineering at Louisiana State University in 2001 as a member of the Microsystems Engineering Team with the Mechanical Engineering Department and started working on this project in 2001. He expects to receive a Master of Science in Mechanical Engineering degree in December 2004 and to begin his working career with Exelon Corp. in Kennett Square, Pennsylvania.