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DEVELOPMENT OF A MICRO PCR REACTOR FOR LAB-ON-A-CHIP DEVICES

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ABSTRACT

The process of performing a Polymerase Chain Reaction (PCR) on a lab-on-a-chip (LOC) device is difficult to achieve. When treating a patient, a timely and accurate result is needed, but obtaining those results is challenging when using a conventional PCR machine. However, if the ability to detect molecules such as DNA during the PCR process happening inside the LOC device was accomplished, then the desired results would be obtained. One potential solution is the use of Real Time-Polymerase Chain Reaction (RT-PCR). Using SYBR Green as the principle dye for the RT-PCR process, this paper discusses the use of acrylic chips and RT-PCR tubes, exposed to different experimental parameters, being analyzed with the RT-PCR technique. According to our studies, better signals can be obtained from a RT-PCR melting curve with the use of FTA membranes than with the use of other membranes such as alumina and Porex® membranes. Good results were obtained from samples taken from chips that have experienced a PCR process with and without FTA membranes. Also, the acrylic plastic chip did not show signs of leakage or any kind of plastic damage while using normal PCR program settings. More importantly, RT-PCR tubes and chips were exposed to the different wash and incubation steps that would be performed on a LOC. Through the RT-PCR machine, good melting curves results were obtained from the vials but for the chips, further research is needed.

Keywords: Polymerase Chain Reaction (PCR), lab-on-a-chip (LOC), Real Time Polymerase Chain Reaction (RT-PCR), FTA membrane

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1. INTRODUCTION

In developing countries, a diseased patient may not receive proper medical care because of the unavailability of specialized equipment and lack of skilled personnel. [1,3] Countries with advanced technologies face other challenges, for example, late diagnosis of a disease like OSCC (Oral Squamous Cell Carcinoma) which leads to head and neck cancer. [2] A microfluidic, miniaturized laboratory system (Lab-on-a-chip or LOC) is being developed to address these challenges. LOC is one of many POC (Point-of-care) devices which accurately and rapidly analyze small volumes of samples, without the need for skilled professionals. [1]

Polymerase Chain Reaction (PCR) is an important part for the analysis of a clinical sample [4,5] and needs to take place on the POC device. Lab-on-a-chip devices have been shown to have promise. However, the difficulty of realizing such analysis in the field, with only a self contained, miniaturized chip has not been successfully resolved. [6]

2. BACKGROUND

2.1 PCR and LOC

LOC (Lab-on-a-chip) devices are composed of multiple microchannels and chambers where different stages of DNA analysis take place. [6] The PCR process can take place in one chamber or in multiple chambers. [5] PCR consists of the amplification of DNA by the creation of copies through a series of cycles where multiple reactions occur. Many diagnostic, forensic, agricultural and biologically-related applications utilize PCR technology. One application involves detecting DNA molecules from a diversity of pathogens within a solution [4], an application commonly found in a lab-on-chip device. For a complete analysis of a patient's disease, DNA must be traced and analyzed after it has been amplified through the PCR process; depending on the DNA result, a person can have a clear diagnosis of whether or not they have a disease. A simple LOC system is shown below in Figure 1.

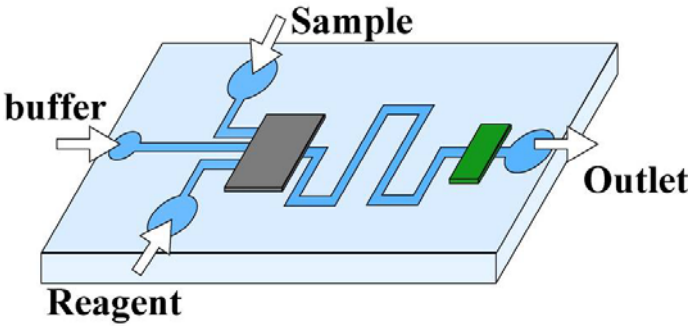

Figure 1: LOC system (Nanospad, 2007).

2.2 Polymerase Chain Reaction (PCR)

2.2.1 History

In 1971, Kleppe and his research group were the first ones to introduce DNA amplification into the science field; yet, this theoretical concept was ignored by most biologists due to inaccessibility of specialized instruments. In 1985, Saiky and colleagues gave the first demonstration of a PCR process. In 1988, the same group described a

thermostable polymerase which was the key component of the amplification process and the model used for PCR machines of today. [4]
Chunsun Zhang and colleagues [5] report that around 30 years ago, an attempt to fabricate PCR microfluidics devices was demostrated by a research group from Stanford University. In 1993, Northrup and colleagues described the first silicon-based stationary PCR chip. Zhang also mentions that in 1998, stationary chamber PCR microfluidics was introduced. Since then, many have shown great interest in the development of these systems to be used in, for example, LOC devices.

2.2.2 PCR: How does it work?

The DNA amplification process consists of three stages:

- ✓ *Denaturation*: The reaction mixture is heated to 94-98°C for 20-30 seconds; this causes the hydrogen bonds between the strands of a DNA sample to break, producing single strands of DNA.
- ✓ *Annealing:* Temperature is reduced to 50-65°C for 20-40 seconds; the primers (single strands of synthetically synthesized DNA [7]) form hydrogen bonds for the attachment with their matching sequences meaning they will pair with their corresponded bases: adenine (A) with thymine (T), and cytosine (C) with guanine (G). [8]
- ✓ *Extension:* Temperature is raised to 72°C for 30-50 seconds; here, the primers unite with the single-stranded DNA and thus, new DNA is created; the temperature may change depending on the primer used.

The process described above comprises a cycle and is illustrated below in Figure 2. Such cycles can be repeated 25-30 times to obtain millions of copies of the DNA under study. For a successful amplification cycle, the sample must be maintained accurately at the desired temperature. Also, if the time between temperature changes is small, the possibilities of having any undesired product, such as primer-dimers, are reduced. Primer-dimers are present when one primer combines with another primer instead of to the single-stranded DNA.

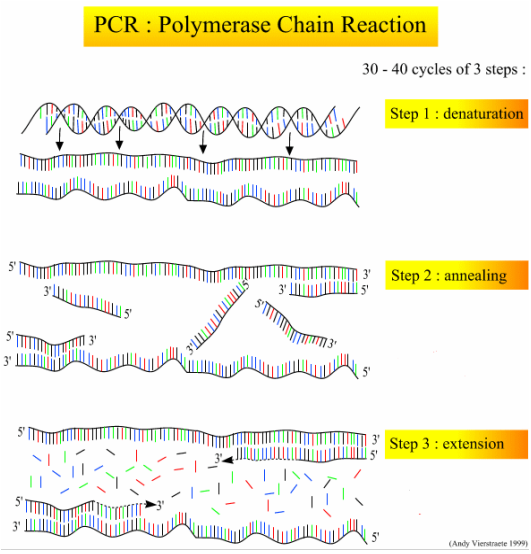

Figure 2: The PCR steps (Vierstrate, 1999).

Amplified DNA needs to be measured so it can be analyzed. PCR dyes are some of the tools used for the identification of DNA. Two principle types of dyes exist:

- ✓ *DNA fluorescent-binding:*

The dye shows low fluorescence in the unbound state. But when it binds to a double stranded (ds) DNA molecule, fluorescence increases, thus indicating the presence of the molecule. The signal from the dye is proportional to the quantity of nucleic acid. SYBR Green and Ethidium bromide are common dyes used for this purpose. In research, this kind of dye is used for direct analysis and quantification of DNA. [5,7,11,12]

- ✓ *Labeled nucleic acid:*

The dye contains fluorophore-coupled elements which are materials composed of fluorescent dye molecules, or the dye itself [12]. Either of these elements interacts with the DNA, allowing quantification of the nucleic acid. This method uses the phenomenon of Fluorescent Resonance Energy Transfer (FRET) where at least two fluorophores, called the donor and acceptor, must be near each other on a DNA strand. The donor fluorophore is excited by an external light source and transmits light to the acceptor fluorophore, exciting the acceptor. When both molecules are at the same level of excitation, the emitted energy (light) of the donor travels around its space at different distances and orientations.[12] Changes in the fluorescent intensity of a DNA molecule resulting from such variations are what permits the tracking of the genetic material throughout the amplification. This phenomenon permits the dye to be very specific, targeting a portion of the dsDNA. A common dye used for this technique is TaqMAN. [11]

2.2.3 Difference between RT-PCR and conventional PCR

A PCR machine must have a thermal engine. The engine's function, as its name indicates, is to produce heat which is utilized during the amplification process. This process may be analyzed during its development depending on the PCR platform used. There are two types of platforms:

- ✓ *Conventional PCR machines:*

The PCR progress proceeds in a tube inside the machine. After the cycling process is complete, the amplified DNA is analyzed by agarose gel electrophoresis. Agarose is a purified, gelatinous substance obtained from algae. [7] Gel electrophoresis is a technique used for the separation of DNA in a gel matrix depending on the size of the DNA molecule. The negatively charged DNA molecule moves through the gel via an electric field produced by an external power source. [7] The most common dye used for DNA tracking is Ethidium Bromide (EtBr). The dye reacts with the DNA in the gel and fluoresces under ultraviolet light, creating different band sizes that represent different DNA sizes as shown in Figure 3. Yet, EtBr is a known carcinogen, so safer dyes such as SYBR Green can be used.

The time required for the PCR process (2-3 hours) plus the extra time required for gel analysis makes this approach impractical for field applications. More importantly, this analysis is not efficacious since the initial status of the nucleic acid or the number of copies obtained is unknown. [9]

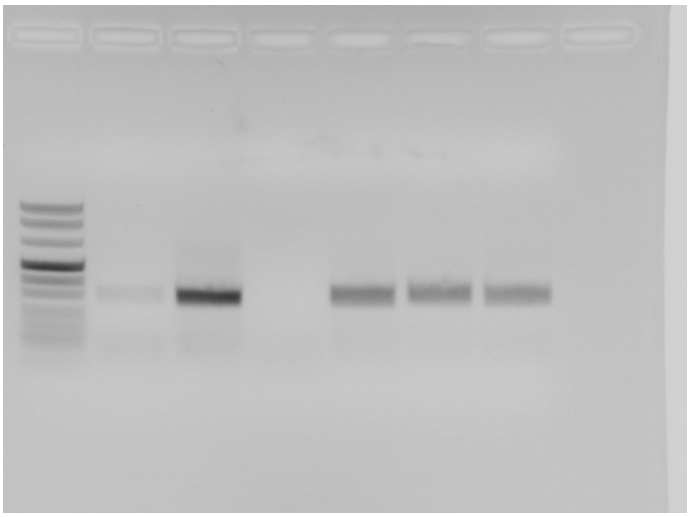

Figure 3: Electrophoresis gel from membrane test (more details in Sec.:4.0)

- ✓ *RT-PCR (Real Time-Polymerase Chain Reaction):*

The PCR progress proceeds inside the RT-PCR machine, and DNA amplification is monitored by a fluorometer. The machine emits light which interacts with the DNA copies. The light emitted by the copies is then detected by the optic component of the machine and analyzed. This way, the instrument measures the amount of florescence emitted by a dye bound to DNA molecules during the amplification. [10]

The use of gel electrophoresis is not needed in RT-PCR, thereby reducing the time the analysis takes. Also, the real time dye measurement gives the platform knowledge of the status of the initial DNA during the PCR process. The quantity of copies produced, and the detection of any mutation or pathogen present during the process are some of the results the machine provides. Researchers have developed miniaturized versions of such technology. [5] A disadvantage of this platform is that the light received by the optic instrument from the sample can be limited, thus reducing the sensitivity of detection.

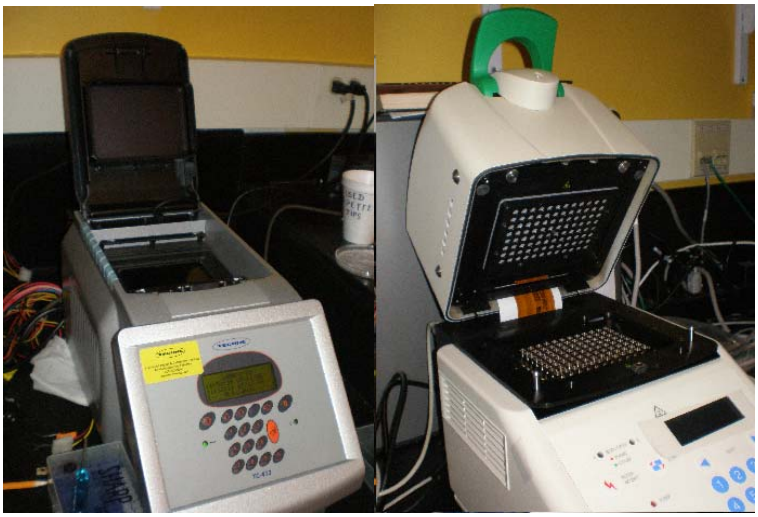

Figure 4: Conventional PCR (left) and RT-PCR machines (right)

3. PCR CHIP: DESIGN, FABRICATION AND TESTING

To simulate a chamber from an LOC device, a chip with one chamber was created. The chamber was designed using SOLIDWORKS software (as seen in Figure 5) and fabricated with a UNIVERSAL LASER laser cutter. The chip was composed of three layers which were bonded with a MMA/methanol solution or double-sided tape. The simple 25~50 µl (various chips were used in the experiments) chamber was located in the middle layer where it contained two microchannels for liquid flow. The plastic used for the fabrication of the chip was acrylic. Another option would be the use of polycarbonate, but light transmission through acrylic is higher than the former which makes it a better option for microscope analysis and for RT-PCR analysis [13, 14].

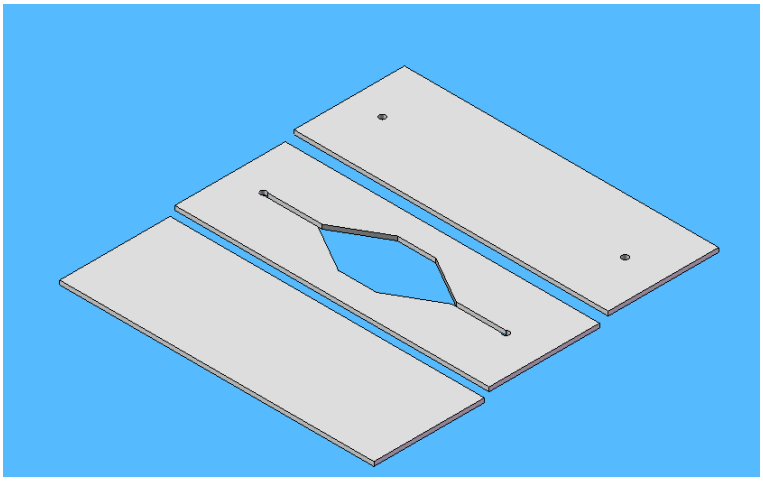

Figure 5: Top, middle, and bottom of PCR acrylic chip assembly

The PCR chip must resist high temperatures during the amplification process inside the PCR machine and most not lose any liquid to be efficient.

For this, some chips were loaded with *Water, Deionized Ultra Filtered (D.I.U.F.)* and then the inlet and outlet holes were sealed with aluminum-foil tape. Others were sealed with a piece of acrylic plastic (6 by 4 mm), which was bonded using MMA/methanol solution. The chips were placed on a hot plate for 10 minutes with a constant temperature of 95-100 ºC. After the 10 minutes passed, the results were that neither the foil-tape chip nor the solvent-bonded (MMA/methanol solution) chip showed leakage. Yet, after several tests, it was decided that the better solution to prevent leaks was to use the aluminum foil tape.

The next step was to test the PCR chips in the RT-PCR machine. An acrylic chip containing a thermocouple was placed inside the machine to measure the chip's temperature (see Figure 6), and compare it to the temperature of the PCR's cavity as indicated by the RT-PCR program. After several runs where the lid and plate temperatures were also measured, it was found that the chip was not at the desired temperature. Nevertheless, when the chip was tested in a conventional PCR machine (*Techne TC-912)*, there was no kind of damage to the PCR chip or liquid loss. During the experiments, a piece of rubber was placed on top of the chip, and later the lid was closed

tightly. The rubber would make the chips have a good contact with the machine's block, thus creating a good heat transfer between the block and the chip. The pressure made by the rubber did not affect the amplification process occurring inside the chip (this will be discussed in Sec.: 4.0)

Figure 6: Thermometer (R); thermocouple (L). With the thermocouple attached to a PCR chip, the temperature inside the RT-PCR machine was measured.

In other tests, the PCR chips were loaded with water, sealed and placed inside the RT-PCR machine. The RT-PCR program was set to perform a melting curve analysis in which the sample, after the PCR process is completed, is heated and the change in fluorescence is detected from the resulting single-stranded DNA [15]. In this case, there should be no signal since there was not any DNA present inside the chips. Yet, the signal obtained was not clear (this will be discussed in Sec. 5.0).

It was concluded that for further studies of the DNA amplification process in the chip to be done, a normal PCR machine must be used. After the process is completed, a melting curve analysis, and microscope and electrophoresis gel analysis can be performed to confirm that the PCR process was successful.

4. PCR CHIP AND RT-PCR

4.1 Membranes

In a PCR process, the membrane's function is to recover DNA molecules that have been liberated after, for example, a lysis process has taken place. However, it is not well known if the membrane has any chemicals that could interfere with data obtained by the RT-PCR machine and thus, affect the resulting melting curve.

In the next tests, several white PCR vials were loaded with different membranes to see if a signal could be obtained from inside the vials (white vials must be used for better results).
If clear vials are used, the light coming from the optical instrument would trespass them and no information could be gained. Different membranes were used such as Flinders Technology Associates (FTA) membrane [16], alumina membrane and Porex® membrane. Also, the use of a shredded FTA membrane was examined.

The FTA membrane is a cellulose membrane that contains different kinds of lyophilized chemicals and proteins for the lysis process of microorganisms and the capturing of their DNA. [16] Alumina has strong atomic bonding which makes it a widely used material in engineering ceramics. [17] However, when this material is combined with a salt solution and microorganism, a lysis process occurs. [18] During the reaction, the interaction between the DNA molecules and the alumina surface gets stronger, ending in the attachment of the DNA molecules to the alumina. The Porex membrane is a porous, polymeric membrane which works as a filter that separates molecules depending on their size and the membrane's overall porosity. Two possibilities for molecules to pass through the porous structure is by temperature gradient or concentration. [19] Some polymers used to fabricate the membrane are polytetrafluoroethylene, polyurethane and polyacrylate. [20]

First, FTA and alumina membranes were tested. Some tubes could contain one or two FTA membranes or alumina membranes; others could contain FTA membranes not washed or washed once or twice with *Whatman FTA purification reagent* buffer and TE (10 mM Tris, 1mM E, Ph= 7.5) buffer. Each vial contained 4 µl of *template DNA 10 ng/µl* and *lambda primer mixture-A 20 pmol/µl*; 25 µl of *SYBR Green Super Mix* and *Cellgro, molecular grade water* for a total volume of 50 µl. These were placed in the RT-PCR machine and with them, two positive controls (vials with no membranes). A PCR program for lambda DNA (found in the Appendix) was used and after 35 cycles, a melting curve was obtained as shown in Figure 7. Later, the results were confirmed with an electrophoresis gel analysis and fluorescent microscope analysis.

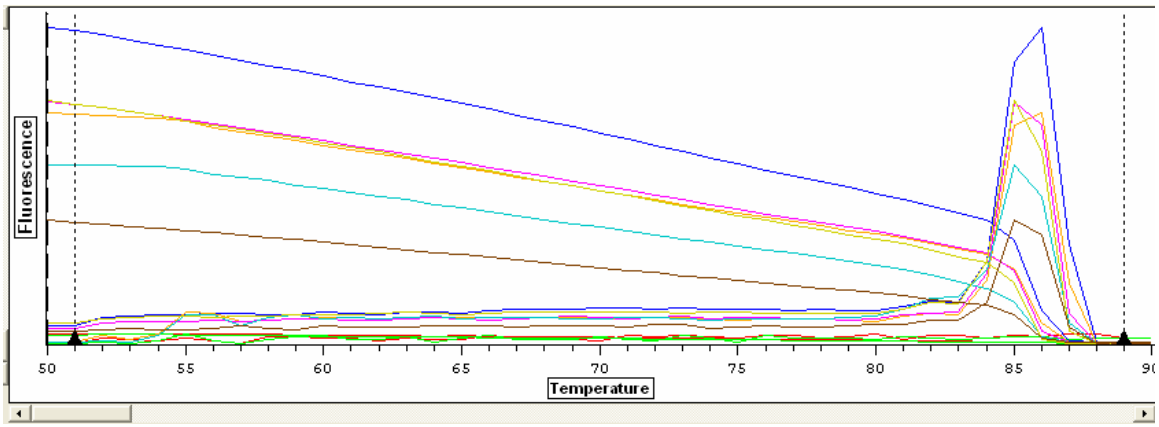

Figure 7: Melting curve from membranes within tubes test.

In chips, to verify that amplification had occurred, a melting curve analysis was performed. PCR chips were loaded with 60 µl of the following solution: 90 µl of *SYBR Green Super Mix*; 10 µl *template DNA 10 ng/µl*; 3 µl of *lambda primer mixture-A 20 pmol/µl* + 77 µl of water. Each chip was placed in a normal PCR machine and to ensure good heat transfer, a piece of rubber was placed on top of them. A lambda DNA program was run (35 cycles) and, then a microscope and electrophoresis gel study was completed.

After several tests, normal FTA membranes as well as shredded FTA membranes were placed inside the chips.

- ✓ *If cells were used:*

Unwashed membranes were placed inside the chambers and, then diluted cells with a concentration of 10^6 cells/ml filled the chip. After twenty minutes, the remaining cells were removed and 80 µl of PCR solution (160 µl of *SYBR Green Super Mix;* 4.0 µl of *B-cereus cells primers;* 2.0 µl of *TAQ-DNA Polymerase-Native*; 150 µl of water) was added to each *50 µl chamber. The chips were placed in the conventional PCR machine and then, a lambda DNA program was run. Later on, a melting curve and an electrophoresis gel and microscope analysis were completed.

- ✓ *If lambda template DNA was used:*

Membranes were washed with FTA buffer and TE buffer and, then placed inside the chips. Each chamber was loaded with 25 µl of DNA mix (3.5 µl of *lambda primer mixture-A 20 pmol;* 10 µl *lambda template DNA* + 75 µl of water) and 25 µl of *SYBR Green Super Mix.* After they were placed in the normal PCR, a lambda DNA program was run and afterwards, microscope and electrophoresis gel analyses were made. A fluorescent microscope image of the FTA membrane inside the chip after experiencing the PCR process is shown in Figure 8.

*****Different chip sizes were used.

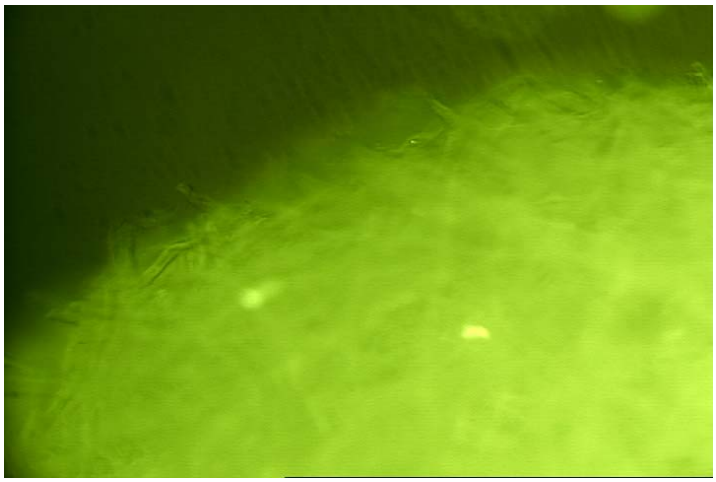

Figure 8: Fluorescing FTA membrane inside a PCR chip.

4.2 Solidification process

Next, membranes were tested in a paraffin environment with the idea of completing lysis, washing, and PCR in a single chamber. This simplified process would make the design more efficient since no extra chamber would be needed. Before studying this idea, one test was done to ensure that paraffin did not interfere with the amplification process. Three PCR vials containing solidified PCR reagents (*GE Healthcare PuReTaq™Ready-To-Go™PCR bead; SYBR Green Super Mix; lambda primer mixture-A 20 pmol/µl*) were loaded with different components. One vial was a positive control (solidified PCR reagents only); one vial had paraffin, and last vial had reagents plus one FTA membrane. A melting curve was obtained and gel electrophoresis was run.

After this test, various PCR vials were loaded and left to dry overnight with 20 µl of the following solution: 8 *GE Healthcare PuReTaq™Ready-To-Go™PCR bead;* 108 µl of *SYBR Green Super Mix;* 4.0 µl of *B-Cereus cell primers* if *B-Cereus* cells are used and 3.5 µl of *lambda primer mixture-A 20 pmol/µl* if *template DNA 10 ng/µl* is used; 3.0 µl *Invitrogen TAQ-DNA Polymerase, Native* (this reagent is optional). In the morning, 1/3 of a *Ampliwax® PCR Gem 50* was loaded to each tube and heated to 75 ºC for 50 seconds.

- ✓ *If FTA or shredded FTA membranes and cells were used*:

Unwashed membranes and diluted cells with a concentration of 10^8 cells/ml were added. After twenty minutes, the remaining cells were discarded; the membranes were washed once or twice with 25 µl of FTA buffer and TE buffer; finally, 40 µl of water were added to each vial. PCR B-cereus cells program was run on the RT-PCR machine and, later an electrophoresis gel was performed (program found in the Appendix section).

- ✓ *If alumina or the porous membranes and cells were used:*

Unwashed membranes and 50 µl of lysis buffer with cells (200 µl of *Buffer AL Lysis buffer 12 ml* + 20 µl of *QIAGEN Proteinase K 10 ml*; 590 µl of water; 40 µl of diluted cells with a concentration of 10^8 cells/ml) were added to each vial. After twenty minutes, the remaining solution was removed; the membranes were washed once or twice with *200mM NaCl* and water (sometimes, the alumina membrane was washed with TE buffer). Finally, 40 µl of PCR reagents (100 µl of *SYBR Green Super Mix*; 3.5 µl of *B-cereus cells primer*; 1.0 µl of *TAQ-DNA Polymerase-Native*; 102 µl of water) were added. A PCR B-cereus cells program was run on the RT-PCR machine and, later an electrophoresis gel was performed.

- ✓ *If lambda template DNA was used:*

Any membrane used was washed first; then it was loaded into the vial with a solution of *template DNA 10 ng/µl* and water; a lambda DNA program was run on the RT-PCR machine and later, an electrophoresis gel was performed.

The dry storage process on a PCR chip was also tested. A number of PCR chips were loaded and left to dry overnight with 6 to 8 µl of PCR solution (4 *GE Healthcare PuReTaq™Ready-To-Go™PCR bead;* 4.8µl of *B-cereus cells primers* or 2.0 µl of *lambda primer mixture-A 20 pmol/µl; Biomatrica™ tubes;* 20 µl of *SYBR Green Super Mix).*In the morning, small amounts of wax were added on top of the PCR reagents and heated up to 75º C (the PCR reagents must be completely covered). Unwashed FTA and shredded FTA membranes with diluted cells (conc. 10^8 cells/ml) were added to the chambers. Twenty minutes later, the remaining cells were removed and left to dry for one to two hours. The membranes were washed once with FTA buffer and TE buffer and, half an hour later, they were loaded with water. After placing a piece of rubber on top of the chips and securing the lid, a PCR B-cereus cells program was run on the normal PCR and later, a melting curve and an electrophoresis gel and microscope analysis were performed. A chip following the PCR process is shown in Figure 9.

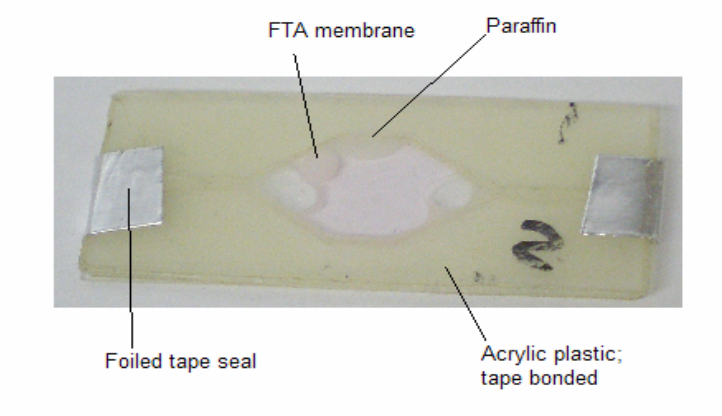

Figure 9: Chip after PCR process

To melt the wax, the heat from the RT-PCR and PCR machines was used. For the vials, the RT-PCR was programmed to maintain a constant temperature of 75° C. For the chips, the same program was used with the only difference being the application of a metal plate into the RT-PCR block. The chips were placed on top of the plate until the paraffin melted. The same process could be done on a normal PCR machine.

5. DISCUSSION AND CONCLUSIONS

Results showed that the foil tape seal protection on the inlet and outlet holes was as good as the acrylic solvent bonded seal. However, the foil tape is easier to handle and faster to apply to the chip.

The material can resist the temperature changes of the PCR process, thus no loss of sample is seen. Also, the acrylic plastic shows no signs of stress after the PCR process unless too much pressure is applied, in which case the material expands.

One possibility for the difference in temperatures readings between the thermocouple and the RT-PCR software could be the finite distance that exists between the lid of the machine and the chip. For a successful PCR process, there must be a good heat transfer between the chip and the plates of the machine.

Some reasons for the unclear melting curve obtained from the chip on the RT-PCR machine (as showed in figure 10) could be the depth of the PCR chip, the unsuitable background were the chip is placed (black background) and again, the distance between the chip and the lid of the machine. If a good signal is to be acquired, there must be a minimum amount of time were the light coming from the optical instrument can interact with the sample's molecules so the sufficient information can be received. If the depth is reduced, the volume is reduced, thus the time must increase if any valuable data is hoped to be collected. Also, a suitable background is needed because of the machine's sensitive optical instrument.

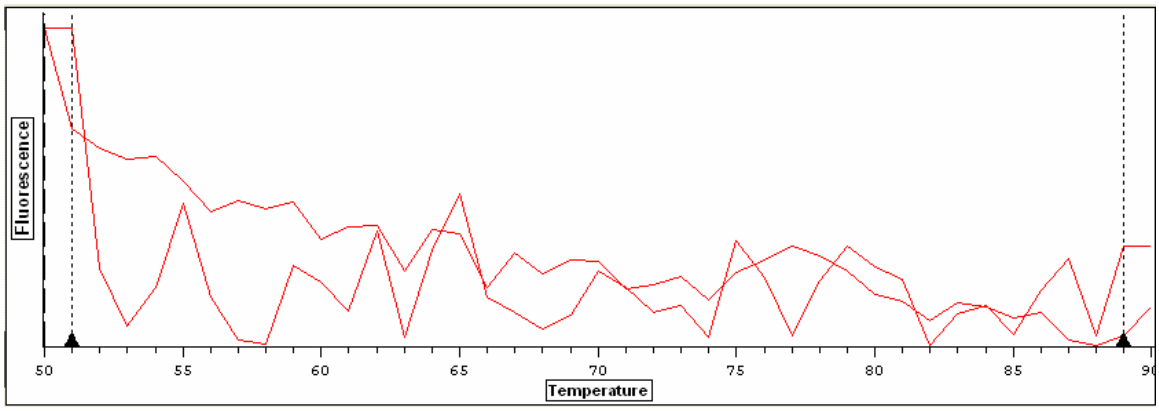

Figure 10: Unclear melting curve obtained from a chip.

Paraffin is a promising encapsulation method that can be used for the PCR process and as part of the LOC system. Studies have shown that paraffin can conserve the PCR reagent during the washing of any membrane used in the experiments. The lysis and purification process of cells is not affected by the paraffin. Results from melting curve analysis also show that paraffin does not affect the resulting DNA copies. Some melting curve results obtained from chips demonstrate that DNA amplification can take place inside the chips. Amplification can be confirmed with microscope and electrophoresis results. In addition, in past experiments from colleagues, the solidification of PCR reagents and the use of wax in the chip have been shown to work. However, the results from experiments made in summer 2008 are not consistent.

According to our studies, FTA membranes work best on tubes and on chips in the solidification process. Excellent results have been obtained from melting curve analysis and from microscope and electrophoresis gel analyses. From melting curve results, fluorescence coming from a sample containing an FTA membrane can be as much as 50% stronger than a signal coming from a sample containing an alumina membrane.

However, samples containing FTA membrane are 25% to 30% or even less strong than samples containing the porous membrane or shredded FTA membranes. These results bring high hopes for the porous membrane to be used on the chips and thus, the LOC system. The use of the shredded FTA membrane will be a good choice too. The studies demonstrate that shredding does not cause the FTA membrane to lose its properties. Being shredded gives it the advantage of covering more surface area, thus “capturing” more DNA.

Nevertheless, the alumina membrane worked best in tubes where no wax was added. Melting curve results show that signals coming from samples containing alumina membranes could be as strong or stronger than signals coming from samples containing FTA membranes. These results show that the alumina membrane can work in conditions were no other materials, like wax, can interfere with it. Because alumina is a brittle material it is possible that during the PCR process, in tubes where wax was used, the alumina cracked and interfered with the RT-PCR optical analysis.

In chips where no paraffin was placed, results show that the FTA membrane is not affected by the pressure the chip experiences in the PCR machine. Melting curve results show strong bands from the chip's sample. This can be confirmed with the strong fluorescence seen under the microscope and the strong, clear bands obtained from the electrophoresis gel.

Yet, our studies show that the FTA membrane can fluoresce on its own. This detail must be kept in mind when a melting curve or other type of analysis is made based on the RT-PCR results. Also, another detail is that washing the membranes once or twice does not significantly impact the results.

6. RECOMMENDATIONS

DNA amplification inside a chip inside an RT-PCR machine is challenging. The tape bonded method is not recommended for RT-PCR analysis because of the machine's sensitive optical instruments. The tape's fluorescence is stronger than the dye normally used, thus the information gathered by the machine would not be correct. Another kind of bonding method must be used. In addition, since acrylic can auto fluoresce, a good signal might not be obtained from the RT-PCR. Another matter that must be resolved is the heat transfer problem. This fact brings another problem which is the depth of the chip as compared to the depth of the PCR vials. The RT-PCR machine was designed to realize vial analysis and not chip analysis.

The solidification process using paraffin in a chip must be further investigated if good results are to be obtained from the RT-PCR device. The Porex membrane and the shredded FTA membrane must also be further investigated.

7. ACKNOWLEDGEMENTS

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9. APPENDIX

A. PCR B-cereus program specifications in RT-PCR:

Cycles: 35

-Lid temperature: Constant, 99º C

-Starting temperature: 95º C for 3:00 min.

-Cycle:

95º C for 30 sec.,
55º C for 30 sec.,
68º C for 40 sec.
-Plate read after each cycle
-Melting Curve: 50º C to 90º C; when the temperature raises 1º C, there is a plate read which takes 10 sec.

B. PCR lambda DNA program specifications in RT-PCR:
Cycles: 35
-Lid temperature: Constant, 99º C
-Starting temperature: 94º C for 1:00 min.
-Cycle:
94º C for 30 sec.,
60º C for 30 sec.,
72º C for 1:00 sec.
-Plate read after each cycle
-Melting Curve: 50º C to 90º C; when the temperature raises 1º C, there is a plate read which takes 10 sec.

C. PCR B-cereus program specification in a normal PCR:
Cycles: 30
-Lid temperature: off
-Starting temperature: 94.5º C for 3:00 min.
-Cycle:
94.5º C for 15 sec.,
54º C for 15 sec.,
68º C for 30 sec.
Holding temperature: 10º C forever
-Melting Curve: 50º C to 90º C; when the temperature raises 1º C, there is a plate read which takes 10 sec.

**The melting curve for the chip's sample is the same as that used in the other programs.