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Automation of nucleic acid amplification-based diagnostic assays for point-of-care use

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Automation of nucleic acid amplification-based diagnostic assays for point-of-care use

A thesis submitted in partial satisfaction

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in Bioengineering

by

Milad Azimi

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ABSTRACT OF THE THESIS

Automation of nucleic acid amplification-based diagnostic assays for point-of-care use

by

Milad Azimi

Master of Science in Bioengineering

University of California, Los Angeles, 2020

Professor Daniel T. Kamei, Chair

Every year infectious diseases are among the top major causes of death worldwide. Infectious diseases disproportionately impact resource-poor areas. While most developed countries have the ability to screen and quickly detect infectious diseases in humans, animals, and plants, developing countries lack this ability. In such areas, access to medical treatment and testing facilities is severely limited. Additionally, developing countries are more densely populated, which

makes infectious disease surveillance exceedingly more difficult. As a result of this, infectious diseases tend to spread more quickly and outbreaks tend to occur more often in developing countries. Additionally, the increasing rate of antimicrobial resistant infections and emergence of novel infectious diseases increase the likelihood of global pandemic occurrence.

It is evident that developing novel devices for detection of infectious diseases would be greatly beneficial to global health. It is important to note that developed countries would benefit equally from such a device. Pandemics tend to burden the economy and put strain on resources even in developed countries. The impact of such a device is determined by a set of criteria put forth by the World Health Organization (WHO). Based on the ASSURED criteria, an ideal device for point-of-care (POC) diagnostics must be affordable, sensitive, specific; and yet it must also be user-friendly, rapid and robust, equipment free, and deliverable to end-users. Additionally, this device must be easily adaptable for detection of novel pathogens. The nucleic acid amplification test (NAAT) meets many of these criteria. However, NAAT's current workflow involves many user liquid handling steps as well as a thermocycler.

This thesis aims to address these issues by first automating isothermal nucleic acid amplification, followed by coupling the amplification step with the lateral-flow immunoassay (LFA), which is a paper-based diagnostic device. Thus, taking advantage of the affordability and POC features of the LFA. The aim of these efforts is to work toward the ultimate goal of developing a device that receives the patient sample, then carries out all the steps in NAAT in an automated manner and produces results with just a push of a single button.

The thesis of Milad Azimi is approved.

Benjamin M. Wu

Zhen Gu

Daniel T. Kamei, Committee Chair

University of California, Los Angeles

2020

This work is dedicated to my sisters and my parents,

without whom none of this would be possible.

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Acknowledgments

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Chapter 1: Motivation and Background

1.1 Introduction

On a regular basis, infectious diseases are among the top ten global causes of death. The World Health Organization (WHO) has attributed an approximate 29% of global deaths to communicable diseases. However, the majority of these deaths occur in underdeveloped regions.[1] Many of the communicable diseases, such as tuberculosis and malaria, ranked high among causes of death in low-income countries. Although effective treatments and preventative measures are available for these diseases, they still cause a high number of deaths in these regions.[1,2] The situation is even more dire during outbreaks of infectious diseases caused by novel pathogens. Unsanitary living environments, lack of access to clean water, and reliable healthcare infrastructure in low-income countries often provides an environment that allows pathogens to spread and cause outbreaks.[3] Since the beginning of the 21st century, we have witnessed the occurrence of several epidemics including the two largest outbreaks of Ebola, not to mention outbreaks of SARS, MERS, Nipah, Influenza A subtype H5N1, yellow fever, Zika, and the most recent outbreak of SARS-CoV-2 in 2019.[4–11] Phenomena, such as global warming, emergence of drug resistant pathogens, increase in rate of global travel, and an explosion in the population, have made it increasingly easier for outbreaks that were once limited by geographical barriers and relatively sparse populations to now quickly become worldwide epidemics.[12,13] When faced with the outbreak of any infectious disease, the first step in limiting the spread of the disease is to quickly and accurately identify the infected individuals.[14] Such accuracy and corresponding quick response will not be possible if we remain reliant on visual symptoms and bodily signs of sickness to identify the infected individuals. Many infectious diseases have an incubation period, during

which their host exhibits either mild symptoms or in some cases no symptoms at all. However, despite the lack of visible signs and symptoms, the host is infectious and continually spreads the disease to those they come in contact with. Therefore, there exists an essential need for a device that allows for the rapid detection of the infectious agent even at the very early stages of a disease. The early detection of an infectious disease would thus allow for quick advancement to the next stages in containment of the disease and prevention of a large-scale pandemic.

1.2 Infectious agents

Infectious diseases could be caused by microbial, viral, fungal, or parasitic pathogens.[15] Most pandemics are caused by viral pathogens and often bacterial pathogens cause secondary infections.[16] However, with the emergence of antimicrobial resistant strains of bacteria, we may see a resurgence of large-scale bacterial infectious diseases. Perhaps one of the greatest challenges we face in our fight against infectious diseases is to identify and respond to pathogens that are currently unknown.[13] Although the origins of a few viruses that infect humans are still unknown, most human viral infectious diseases have been traced to zoonotic roots. The genetic gap between us and many animals prevents most animal pathogens to infect us directly. However, certain events, whether natural or man-made, present viruses with an opportunity to transmit to new host species that have adequate genetic similarity to the original host.[17] The rate of occurrence of such events have been rising and will continue to rise throughout the course of the 21st century due to global warming, illegal poaching, and destruction of natural wildlife habitats. These events often force different animal species and humans to co-mingle for prolonged periods of time, which in turn promotes intraspecies transmission of virus until it inevitably reaches a human host.

On the other hand, transmission and infectivity of bacterial pathogens is less dependent on genetic makeup of the host but rather the bacteria itself. After the discovery of antibiotics in the early 20th century, rate of mortality due to bacterial infections dramatically decreased. Deadly diseases such as pneumonia and tuberculosis were now treatable.[18] Prior to that, bacterial infectious disease had a higher mortality and occurrence rate. For instance, there were six global outbreaks of cholera, which is caused by the bacteria *Vibrio cholerae*, throughout the 19th century.[19] Unfortunately, that is not to say bacterial infectious diseases are relics of the past. The most recent outbreak of cholera, which began in 1991, is still ongoing exclusively in developing countries with 1.3 to 4.0 million cases and 21 to 140 thousand reported deaths every year.[20] Since the early 21st century, antimicrobial resistance has also become more prevalent.[21] Through evolutionary pathways, bacteria are capable of not only acquiring new antibiotic resistance genes, but also regulatory pathways to adjust their level of resistance as a response to increasing dosages of antimicrobial drugs. Overuse of antibiotics in animal farms and improper use of these drugs by humans has enhanced this ability of bacterial pathogens to develop drug resistance.[22] Until now, we combatted drug resistant bacterial strains by discovering new antibiotic drugs. However, the rate of discovery of new antibiotics has been gradually decreasing.[18] A current estimate for the number of deaths attributed to antimicrobial resistance pathogens is about 700,000 a year. This number is expected to increase to 10 million by 2050.[21] The emergence of new infectious pathogens is inevitable since we are incapable of stopping bacteria and viruses from evolving. Therefore, early detection and containment of novel infectious diseases is the first line of defense, and there is a clear demand for a device that can be quickly adapted for detection of novel pathogens. The aim of this thesis is to make significant progress towards developing such a device.

1.3 Nucleic acid amplification test (NAAT)

Microscopy, antibody-based assays such as enzyme linked immunosorbent assay (ELISA), and nucleic acid amplification tests (NAATs) are the current gold-standard methods for detection of infectious diseases in laboratory settings. Laboratory diagnostic tests generally require trained lab personnel, expensive laboratory equipment, and power.[23] Among these laboratory tests, NAATs provide the best balance between time-to-result and accuracy. Additionally, NAATs are capable of detection of the target at very low concentrations.[24] There are three general steps in a conventional NAAT workflow, which are demonstrated in the *Figure 1-1*. First, during the sample preparation step, genomic material is extracted and purified using commercial kits. The target nucleic acid sequence is subsequently amplified by polymerase chain reaction (PCR) in the presence of target specific primers. During the final step, typically target amplicons are detected by fluorescence or gel electrophoresis.

Figure 1-1: *Schematic of the workflow of the NAAT.*

Gel electrophoresis lacks specificity since it is reliant on the appearance of a band that matches the size of the amplicon. To achieve higher specificity, the product of the amplification step must be sequenced to ensure it matches with the target sequence.[25] Gel electrophoresis also has equipment and training requirements. If fluorescence is used for detection, training could be reduced, but expensive equipment is still required. In addition, commercial nucleic acid extraction

kits often require several user steps, which must be carried out by trained personnel. Moreover, PCR is dependent on thermocyclers, which are bulky and power inefficient.[26,27] Therefore, as it stands, the current workflow of NAAT is unsuitable for use in point-of-care (POC) settings, and there is a critical need to adapt NAAT for detection of infectious diseases in-field by minimally trained personnel.[28]

1.3.1 Aqueous Two-Phase Systems (ATPSs)

The sample preparation step of NAAT is carried out in two stages, nucleic acid extraction followed by purification. The nucleic acid extraction step in the conventional NAAT is often carried out by either mechanical means, such as homogenizers, or chemical means, such as detergents for lysing the cells.[29] The extracted genomic material is subsequently purified using one of the commercially available kits. As it stands, the sample preparation step of NAAT requires multiple liquid handling steps, making it unsuitable for use in a device aimed at POC settings. Therefore, an automated, lightweight, and cost effective NAAT device must take advantage of a simple and equipment free sample preparation step.

To simplify the sample preparation step for the automated NAAT device, our research group previously incorporated a TX-100 micellar ATPS into the NAAT workflow.[26] An ATPS is a liquid-liquid extraction unit operation that exploits its two immiscible phases to partition a target molecule extremely into one of the phases. Conventional organic solvent-water systems, which either denature biomolecules or will not dissolve them, are not suitable for biomolecule separation. Predominantly made up of water in both phases, ATPSs provide a mild environment for biomolecules.[30] In an ATPS, biomolecules partition to a particular phase of the system due to an

assortment of physical and chemical properties, such as their size, hydrophobicity, and surface charge[31–33]. Furthermore, their scalability, affordability, and lightweight nature make ATPSs an ideal candidate for integration with POC diagnostics.[34]

Our lab has previously demonstrated the compatibility of ATPSs with the lateral-flow immunoassay (LFA) by using the ATPS to concentrate a target biomolecule prior to running an LFA.[35] Moreover, our lab also directly applied ATPSs to paper membranes to significantly facilitate macroscopic phase separation, which allowed for simultaneous phase separation and detection of the target biomolecule.[36] Using this pre-concentration step, the detection limit of the LFA was improved by 10-fold and the assay time was reduced to minutes from hours. More recently, ATPSs were utilized in our lab for automation of the sequential delivery of enhancement reagents while also preconcentrating the target biomolecule.[37]

1.3.2 Isothermal Amplification

Targeting genomic materials for detection of pathogens allows for specificity.[24] However, nucleic acids are present at such low concentrations that without amplification their detection would be very difficult.[28] One constraint on the use of NAATs in POC settings is the dependence of PCR on a thermocycler to precisely control the sequence of temperatures necessary for each cycle of amplification by PCR. Although open-source thermocyclers are now available that attempt to reduce the price, PCR still remains unsuitable for use in the field and where there is a need for a portable solution.[27,38] Many isothermal amplification methods have emerged in recent years, such as loop-mediated isothermal amplification (LAMP), helicase-dependent amplification (HDA), exponential rolling circle amplification (E-RCA), and recombinase polymerase

amplification (RPA).[38] Amongst these techniques, RPA has proven to be the most suitable for POC diagnostics for several reasons.[39] RPA operates at a much lower temperature compared to other isothermal methods, while still exhibiting comparable or better performance. It requires fewer primers compared to most other methods. It also has one of the shortest amplification times and the best limit of detection.[40,41] Therefore, RPA would be the most suitable isothermal amplification method for POC applications.

1.3.3 Lateral-Flow Immunoassay

The LFA is a well-stablished and widely used paper-based diagnostic device. The LFA is ideal for POC settings because it is rapid, user-friendly, and low-cost. Additionally, the LFA meets many criteria set forth by the WHO's ASSURED criteria, and it is best known for its use as an over-the-counter pregnancy test. The LFA (*see Figure 1-2*) is usually comprised of a sample pad, conjugate pad, a detection zone, and an absorbent pad. Each section of the LFA is made up of different paper-based materials, such as cellulose or fiberglass.[42] Often, the sample pad and the membrane containing the detection zone can be pretreated with blocking agents to promote flow through the LFA strip, as well as reduce any nonspecific interactions between sample components and the paper material. The sample pad can also be used as a filter in order to remove large contaminants in the sample. The next component after the sample pad is the conjugate pad, which contains the dehydrated detection probes. Nanoparticles, often gold, are decorated with antibodies or other molecules capable of specifically binding to the target molecule. In the case of detection probes made of gold nanoparticles, the probes also act as colorimetric indicators due to their red hue. Following the conjugate pad is the detection zone, which is typically made of nitrocellulose membrane, to permit immobilization of different molecules involved in the detection. Finally,

cellulose paper with high absorption capacity is generally used as the absorbent pad material at the end of the strip to wick excess solution and act as a sink for fluid flow. To run a test, the LFA strip could either be dipped into the sample solution or horizontally laid with the detection zone facing up and the sample solution dispensed on the sample pad.[42–44]

As the sample solution travels through the strip due to capillary flow and rehydrates the gold nanoprobes on the conjugate pad, the target molecules form complexes with the antibodies on the probes in the case of a positive test. Subsequently, the liquid will travel to the detection zone, where a test line is printed with primary antibodies specific to the target antigen. As the complexes reach the detection zone, the test-line antibodies bind to the target, thus sandwiching the target between the test-line antibody and the antibody on the gold. Further downstream, a control line is printed with secondary antibodies that will nonspecifically bind to the primary antibodies on the probe. Therefore, a positive test is indicated by appearance of two lines. However, in the case of a negative test, since the target molecule is not present, there are no complexes and the probes pass the test line without binding. The control line still nonspecifically binds the to the probes. Therefore, in case of a negative test, only one line will appear.[45,46]

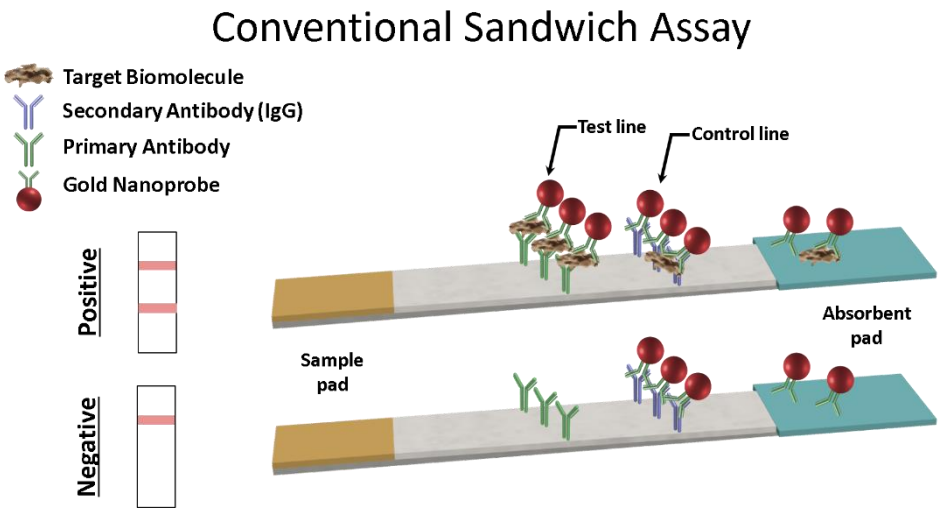

Figure 1-2: *Illustration of a positive and negative sandwich LFA.*

Chapter 2: An automated platform for isothermal nucleic acid amplification and detection

2.1 Introduction

In this study, we experimented with several materials for our liquid handling steps, mechanisms for dispensing and transferring liquids, and controlled heating for the extraction and amplification steps. Design parameters were chosen with the aim to engineer and develop a versatile and automated platform for early stage infectious disease detection. This platform was based on the NAAT. The first stage of the device will utilize a Triton X-114 (TX-114) micellar ATPS to lyse bacteria, extract genomic materials, and concentrate the DNA. All aforementioned steps will take place in an ATPS chamber, which is one of the two liquid-handling chambers contained in the device. Specifically, the chamber containing the sample and TX-114 ATPS will be heated to lyse the bacteria and concentrate the extracted DNA. In the case of the TX-114 ATPS used in this study, the DNA partitions extremely into the top phase of the ATPS. As such, using a linear actuator, the device will move the ATPS chamber over a waste pad where the bottom phase can be dispensed and disposed of. A lightweight, low-cost, and energy efficient precision pump was designed to dispense liquids from the chambers drop-wise in a controlled manner. Next, the top phase of the ATPS phase containing the concentrated DNA will be dispensed over a reagent pad where isothermal amplification will take place. RPA reagents will be dehydrated onto a paper membrane. The amplification step is integrated here with the detection step by combining the RPA reagent pad with an LFA. During the RPA reaction the amplicons will be dual-labeled, with a FAM marker on the 5’ end and a biotin label on the 3’ end. The streptavidin immobilized on the LFA membrane will then bind to the biotin on the amplicon. Gold nanoparticles decorated with

anti-FAM antibodies will bind to the FAM marker on the amplicons and act as colorimetric indicators on the LFA.

2.2 Materials and Methods

2.2.1 Fabrication of the heat-block

A 2"x2" block of aluminum was cut into 3 pieces using a miter saw. The heating element used was an E3D ceramic heater cartridge 12 V, 40 W (E3D Online, Oxfordshire, United Kingdom). To encase the resistive heating elements in the block, four 0.8-inch deep holes, two holes on two opposing sides, had to be drilled into the block. Holes were drilled using a drill press and a 5.9 drill bit. Subsequently, a hole was drilled in the enter of the piece using a 2 mm drill bit to a depth of 0.92 cm to house the thermistor for sensing the temperature at the center of the block. Four FQP30N06L (ON Semiconductor, Phoenix, Arizona) n-channel logic-level MOSFETs were used to switch the heating elements on and off to control the temperature. The MOSFETs were assembled onto a prototyping circuit board. The logic pin of each MOSFET was attached to the pulse width modulation (PWM) enabled pin of an Arduino Uno (revision 3). Subsequently, a proportional–integral–derivative controller (PID) script was implemented using the Arduino AutoPID library for precise control of the heat-block temperature.

2.2.2 Fabrication of the PDMS chambers

The chamber molds were designed in Fusion 360 (Autodesk, San Rafael, California). The molds were then 3D printed on an Ultimaker 3 printer (Ultimaker, Geldermalsen, Netherlands) , using Ultimaker co-polyester (CPE) thermoplastic. Each mold consisted of an inner mold and an outer mold. The outer mold is the negative of the inner mold with a 2 mm offset. Therefore, when

the inner and outer mold are assembled, a 2 mm gap will remain between the two halves of the mold, which will essentially form the walls of the chamber. To fabricate the chambers, polydimethylsiloxane (PDMS) (13:1 w/w ratio of prepolymer to curing agent; Sylgard 184; Dow Corning, Midland, MI) was poured in the bottom half of the mold until the mold was full to the brim. The mold was then placed in a vacuum chamber for two hours to remove air bubbles trapped in the mold. The top half of the mold was then pressed into the PDMS, overflowing the excess. The molds where then placed in a 55° C oven for 5 h. After the 5 h-mark, the mold was removed from the oven. Finally, the molds were taken apart to release the PDMS. To close off the opening of the chambers, a thin cubic mold was designed and printed following an identical process. Subsequently, the mold was filled with PDMS and baked to form a 2 mm layer of PDMS. The two pieces were then bonded together using PDMS mixed with curing agent at a 10:1 ratio and baked in the oven at 55°C for 5 h.

2.2.3 Fabrication of a precision pump

The pump apparatus was designed in Fusion 360 and 3D printed on the Ultimaker 3. A 150 mm 303 stainless steel precision leadscrew with a 1.25 mm thread pitch (McMaster-Carr, Elmhurst, Illinois) was used in combination with a precision leadscrew flange nut (McMaster-Carr, Elmhurst, Illinois) to achieve high precision linear movements. The flange nut was fixed into the plunger handle. One end of the leadscrew was attached to a stepper motor piece that was attached to a 35L bi-polar stepper (Portescap,West Chester, Pennsylvania). On the other side, the pump was attached to a deep groove bearing to allow smooth rotation of the leadscrew, and the bearing was fixed into the pump apparatus. To stabilize the movement of the plunger handle, two smooth rods were added to each side of the leadscrew. Linear ball bearings were attached to each

side of the plunger handle. These linear ball bearings on each side of the plunger handle served to distribute the weight and reduce friction.

2.2.4 Preparation of the Triton X-114 ATPS

A 500 µL Triton X-114 micellar ATPS with a volume ratio (top phase:bottom phase) of 1:1 was utilized. Since magnesium acetate (MgAc) is required to initiate the RPA reaction, its concentration was maintained at 14 mM in the ATPS. After the addition of the salt, the concentration of Triton X-114 (MP Biomedicals, Santa Ana, CA) was adjusted from 8% w/w to 10% w/w to obtain the desired equilibrium volume ratio of 1:1 at 39 °C. Finally, Nuclease Free Water was added to a final volume of 500 µL.

To visualize the phase separation of the ATPS and distinguish the two phases in the PDMS chambers, 5 µL of 2% Brilliant Blue Dye FCF (The Kroger Co., Cincinnati, OH) were added to the mixed ATPS solution and vortexed thoroughly. The bright blue dye highlighted the micelle-rich bottom phase, while the micelle-poor top phase remained light blue. All images of the chambers and dispensed ATPS were taken with a Nikon D3400 digital camera (Nikon, Tokyo, Japan) in a light-box.

2.3 Results and Discussion

2.3.1 Testing the fabricated heating block

The type of heater embedded in the heat-block is often used to heat 3D printer hotends for melting the thermoplastic used in printing. Each of the ceramic heater cartridges has the capacity to reach temperatures up to 300 °C. However, to ensure RPA reagent pads are heated evenly all across, the aluminum heat block must be able to maintain even heating across its surface. The temperature of the heat block was controlled by a PID script loaded onto the microcontroller board. Additionally, each of the heater cartridges was controlled by a separate MOSFET. The heat block setup and temperature measurements recorded can be seen in *Figure 2-1*. The plot in *Figure 2-1D* proves that the heat block is capable of maintaining a single temperature for prolonged periods of time with little to no deviation. Therefore, these results demonstrate that our engineered heat block is capable of heating and maintaining temperatures suitable for the RPA for the duration of time necessary for completion of the RPA reaction. Additionally, this experiment also proves that the temperature control circuits worked as intended and thus can be used with any heating mechanism.

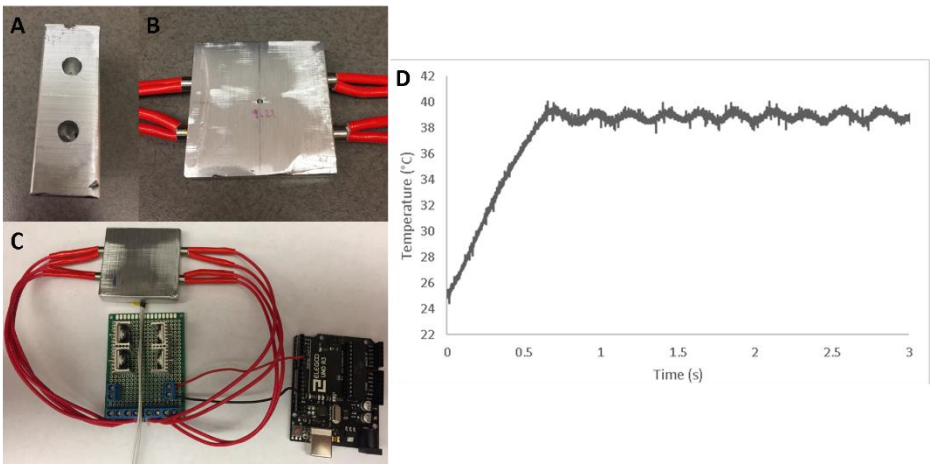

Figure 02-1: A)*Two drilled holes on the side of the aluminum heat block.* ***B)*** *The hole made in the center of the heat-block to embed the thermistor.* ***C)*** *The heat block and temperature control circuitry.* ***D)*** *Plot of temperature as a function of time reported by the microcontroller.*

2.3.2 Fabrication of the PDMS chambers and precision pump

The fabricated chambers can be found in *Figure 2-2*. As indicated in the figure, this approach yielded chambers of appropriate size, durability, and flexibility. The fabricated pump with high precision linear movements is shown in *Figure 2-3*.

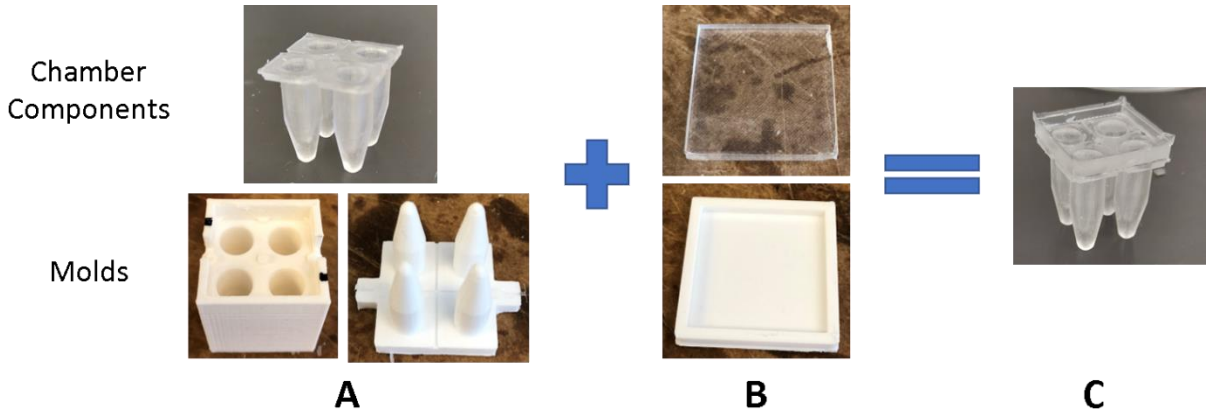

Figure 2-2: *The process of fabricating the PDMS chambers from the positive and negative molds.* ***A)*** *The positive and negative molds and the resultant PDMS chambers.* ***B)*** *2 mm thick, cubic PDMS layer.* ***C)*** *Assembled and sealed PDMS chambers.*

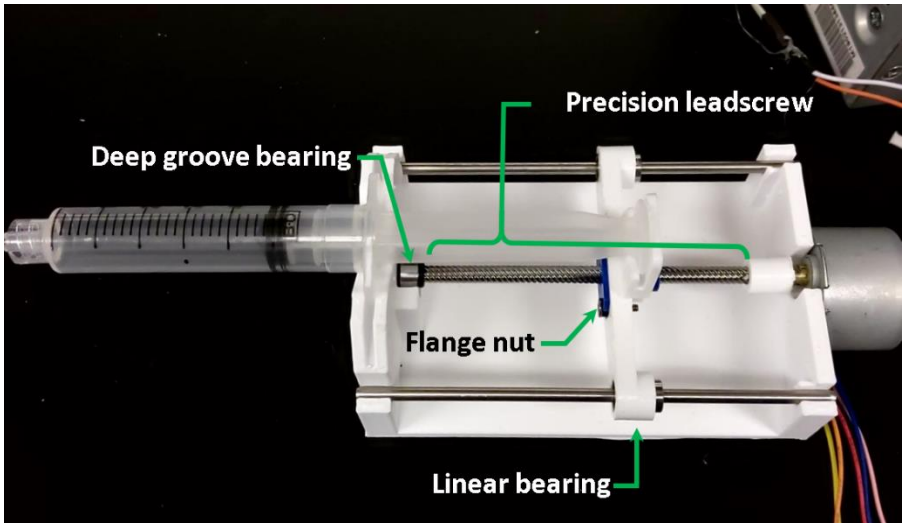

Figure 2-3: *Fabricated precision pump.*

2.3.3 Dispensing ATPS drop-wise

The purpose of this study was to determine whether it was possible to dispense the top and bottom phases of the ATPS separately from the chamber using the precision pump. We hoped to accomplish this in a drop-wise manner without extensive mixing of the two phases. In the time lapse images shown in *Figure 2-4*, the two phases are clearly distinguishable from one another. The bright blue drops in the top images correspond to the bottom micelle-rich phase, where the blue dye preferentially partitions. In the first three images in the bottom row, the light blue drops correspond to the top micelle-poor phase. In the last image, there is again a bright blue drop, which can be attributed to some of the bottom phase remaining on the walls of the chamber that were being dispensed after the entire top phase was expelled.

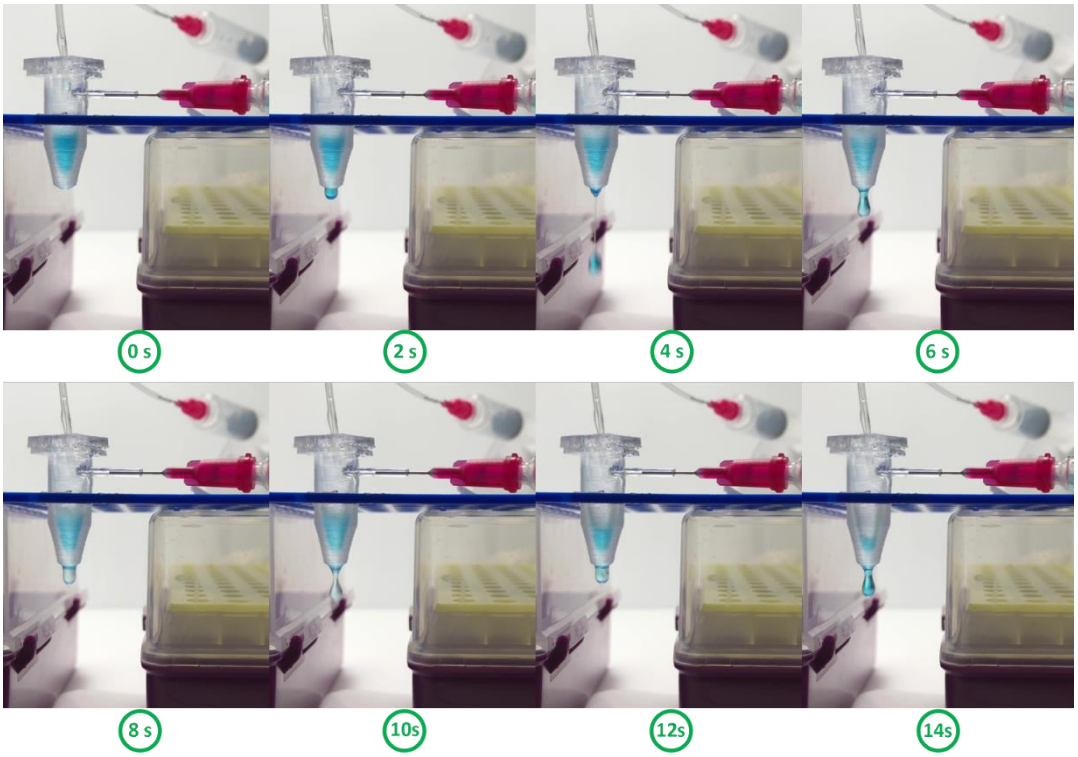

Figure 2-4: *Time lapse images of both ATPS phases being dispensed dropwise.*

2.4 Conclusion

This research aimed to lay the groundwork for the development of a simple battery operated, portable, and fully automated NAAT device. The results of this study demonstrated that the individual steps in the NAAT workflow can be automated. The next step would be to combine these individual automated steps to develop an all-in-one device capable of performing all steps of NAAT automatically.

Affordability is one of the most essential requirements for developing a POC diagnostic device, and these studies demonstrated that the development of an automated NAAT device in a cost-effective manner is possible. Rough calculations estimate the cost of the device to be $103, which would further decrease with large-scale manufacturing. Additionally, the cost of performing each test using this device is inexpensive as LFA-based cartridges will be used, and rough calculations estimate the cost of each cartridge to be $5 which also can drop further with large-scale production. Overall, this thesis has made significant progress toward realizing the goal of delivering a device that takes in patient samples, performs the steps of NAAT automatically, and produces results all with one push of a button.

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