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Assured bacterial detection towards paper-based microfluidic chip for resource-limited areas

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ENTITLED

ASSURED BACTERIAL DETECTION TOWARD PAPER-BASED MICROFLUIDIC CHIP FOR RESOURCE-LIMITED AREAS

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By

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ABSTRACT

There is a significant and urgent need for affordable, fast, and accurate pathogen detection methods in resource-limited settings. Currently, accurate pathogen detection methods are dependent upon special equipment or reagents, specialized training to operate such equipment, electricity or cold storage, or sterile environments not feasible outside of the laboratory. Here we present a functionalized cellulose paper device towards an autonomous 3-dimensional microfluidics chip to detect bacterial pathogens. The microfluidic device utilizes a nucleic acid sandwich assay that detects the presence of bacterial RNA through complementary strand binding. An oligonucleotide "capture strand" immobilizes the targeted RNA sequence to the device, while a "detection strand" produces a visible colorimetric change due to gold nanoparticle conglomeration. A smart phone application and camera quantifies the concentration of the bacterial RNA present in the sample. This detection method can determine concentrations in the lower limits of the femtomolar range. The microfluidic device was fabricated using wax printing on cellulose filter paper, which was then folded into a final 3-D configuration. The simplicity and specificity of this paper-based assay was verified by the detection of *E. Coli* target oligonucleotide.

Keywords: 3-Dimensional microfluidic chip, Lab-on-a-Chip, wax printing, oligonucleotides.

ACKNOWLEDGEMENTS

We dedicate our thesis to the Santa Clara University bioengineers who will continue this project and improve upon our designs for years to come. May you remain motivated by those who need this technology the most.

We give the greatest thanks to Dr. Ashley Kim for her unwavering support from the moment we began our project in winter 2014. Her insight and critical eye have culminated in what we report in this thesis.

We also thank Dr. Korin Wheeler for her helpful conversations and lab materials that allowed us to more deeply understand the biochemical nature of our project.

Lastly, we thank our friends and family who have joined us in our successes and shortcomings over the past year.

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LIST OF ABBREVIATIONS

ASSURED – Affordable, Sensitive, Specific, User-friendly, Reliable, Equipment Free, Deliverable to end-user

WHO – World Health Organization

EDTA – Ethylenediaminetetraacetic Acid

AuNP – Gold Nanoparticle

DNA – Deoxyribonucleic Acid

RNA – Ribonucleic Acid

DTT – Dithiothreitol

CHAPTER 1: INTRODUCTION

I. Problem Statement: The Need for ASSURED Bacterial Detection

There is a real and urgent need for better detection methods for bacterial pathogens. In 2008, the World Health Organization (WHO) published a report stating that a tenth of the global health burden is caused by unsanitary water [1]. Included in this statistic are the estimated 3.4 million deaths that occur every year due to waterborne illnesses [1]. Too often, these deaths arise from inadequate preventative detection methods for bacterial pathogens. While many methods currently exist to detect these pathogens, they all fail to meet the WHO's guidelines for low-cost and deliverable technologies that can be used on-site in remote locations. These criteria, abbreviated ASSURED and listed in Table 1, outline the necessary characteristics of a feasible technology for use in resource-limited areas [2]. Those who live in resource-limited areas with minimal sanitation have the greatest need for an improved detection method, so any device that does not meet all of criteria outlined by the WHO is futile in this setting. We wish to address the alarming health concern of bacterial illnesses globally by developing an ASSURED device that can detect bacterial pathogens at the point of care in even the most remote locations. Those living in at-risk areas will benefit from quicker and affordable detection of bacteria in water, food, or infections while World Health workers benefit from easier diagnosis and reduced costs for these tests.

Table 1: ASSURED Criteria. World Health Organization's ASSURED criteria for point of care devices in resource-limited areas.

A	S	S	U	R	E	D
Affordable	Sensitive	Specific	User -Friendly	Rapid and Robust	Equipment-Free	Deliverable to End Users

CHAPTER 2: LIMITATIONS OF EXISTING TECHNOLOGIES

I. Reliance upon External Equipment

Currently, accurate pathogen detection methods are dependent upon special equipment or reagents, electricity or cold storage, or sterile environments not feasible outside of the laboratory. To address these limitations, devices using nucleic acids to detect specific gene targets have been implemented with great success; they are valid in a wide variety of applications, such as food and environmental monitoring and medical diagnostics. Methods of hybridizing target nucleic acids to complementary oligonucleotides have been studied extensively over the past several decades [3,4]. Nucleic acids are favorable biomarkers because they are specific and sensitive [5], stable over a wide range of temperatures [6], durable over time [6], and non-toxic. The advantages of nucleic acid biomarkers address many of the WHO's ASSURED criteria including sensitivity, specificity, robustness, and deliverability to end users. Nucleic acids only bind to complementary strands of oligonucleotides, making the assay incredibly sensitive and specific for the detection of a chosen bacterial pathogen. A device intended for use in remote locations must be durable enough to withstand travel to the location without losing functionality along the way. Because nucleic acids are stable over a wide range of temperatures and durable over time, they are the ideal sensing platform to be used in a point-of-care device.

II. Unsuitable Assay Substrates

Oligonucleotide sandwich assays have been performed on numerous substrates such as glass [7], hydrogels [8], quantum dots [9], and carbon nanotubes [10]. While each biomaterial has its own advantages, toxicity and decreased specificity due to small surface area [11] hinder the implementation of these substrates in point-of-care devices. For this reason, we propose a paper-based device because cellulose has a high surface area and is inherently non-toxic [12]. Cellulose is comprised of numerous fibers, which allow oligonucleotides to bind to the cellulose in 3-dimensions, as opposed to only the 2-

dimensional surface of other substrates [11]. The increased surface area enhances the overall sensitivity of the assay. Cellulose is fundamentally inert, but can be functionalized with zirconia, which has been shown to covalently link cellulose and oligonucleotides [11]. Gold nanoparticles (AuNPs) used in sandwich assays performed on zirconia-coated cellulose can still produce colorimetric results with high sensitivity [7, 13-14]. Additionally, cellulose is the most abundant natural polymer on earth [15], making it one of the cheapest biomaterials available for use in a medical device. This therefore meets the affordability criteria outlined by the WHO for a point-of-care device.

III. Inadequate Analysis Methods

Electrochemical [16], optical [17], and mass-sensitive methods [18] have been used to detect a binding event between oligonucleotides and the target sequence. While these methods are accurate and well established, they require additional equipment and training to operate. Such devices are not practical in resource-limited settings because laboratories are scarce, and the end-users who need a bacterial detection device may not know how to use them. Therefore, we move toward an autonomous microfluidic chip that uses a smartphone and application for analysis. Smart phones are becoming more and more prevalent in the developing world, and because of this scientists are looking to bridge the gap between patients and doctors by using smart phones as diagnostic tools [19]. The cell phone camera takes a picture of the colorimetrically altered substrate; the picture is then analyzed by a mobile application that can produce quantitative measurements that cannot be deduced by the naked eye alone [19]. The use of smart phones in medical diagnostics meet the equipment-free and user-friendly criteria given by the WHO. Because mobile phones are common and simple to use, patients can easily take charge of their own health, even in remote locations with limited access to trained professionals and laboratories.

CHAPTER 3: NOVEL DESIGN PROPOSAL

I. Sandwich Assay Platform

Our method of bacterial detection employs a sandwich assay using customized, single-stranded oligonucleotide sequences that are mimic to bacterial RNA sequences. Our "Capture" strand, named as such because it will "capture" the bacterial RNA sequence, is immobilized on cellulose functionalized with a thin, zirconia coating. The Capture sequence is complementary to the known "Target" sequence of pathogen RNA. Therefore, the Target strand represents the specific bacterial RNA sequence of the pathogen we are detecting. For the purposes of this device, we are using a known sequence unique to *E. coli* as our Target strand. When the Target bacterial strand binds to the Capture strand, it is immobilized on the paper substrate. To detect a binding event between the Capture strand and the Target strand, a third oligonucleotide sequence, called the "Detection" strand, is then introduced to complete the sandwich assay. The Detection DNA strand is conjugated with 20nm gold nanoparticles on the 3' end of the sequence. When the detection strand binds to the immobilized Capture+Target complex, the conglomeration of gold nanoparticles produces a red color on the paper. This colorimetric change can be difficult distinguish, as the red color is faint. Therefore, silver enhancement solution is then used to amplify the color change. In the presence of gold nanoparticles, silver enhancement solution will deposit solid, silver nanoparticles onto the gold nanoparticles. The papers then turn gray in color, with darkness varying upon the concentration of the Target strand. A schematic of this assay can be found in Figure 1. This assay is both sensitive and specific, detecting target concentrations well into the femtomolar range.

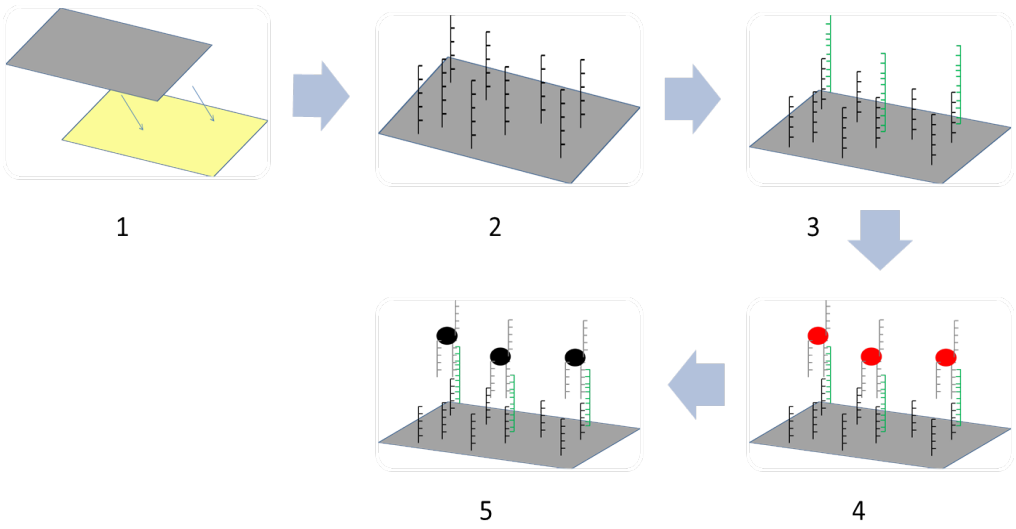

Figure 1: Sandwich Assay Design Schematic (1) Cellulose is coated with a thin film of zirconia. **(2)** Zirconia-coated cellulose immobilizes Capture DNA. **(3)** Target DNA binds to Capture DNA. **(4)** AuNP/Detection probe completes sandwich assay and **(5)** is enhanced with silver solution, producing colorimetric response.

II. Integrated Platform in Microfluidic Chip

To create a WHO ASSURED device, the sandwich assay platform must be integrated into an autonomous, user-friendly microfluidic chip. On the lab bench, the different reagents are added sequentially by hand to produce the desired detection signal. However, this device is not intended for use in a lab with specialized lab equipment to carry out the reaction. Therefore, we must engineer a device to automatically carry out all steps of the sandwich assay so that the device is user friendly, even for an untrained end user.

Our proposed microfluidic chip will use the fundamentals of paper origami to construct a 3-dimensional (3D) chip. As presented by Crooks et. al., this paper-folding technique to fabricate 3D microfluidic chips minimizes costs, and allows for simple and rapid assembly and unfolding for analysis [20]. The device will be fabricated onto a single piece of cellulose filter paper, and then folded into the final 3D shape. When a fluid is introduced to the device, it will flow horizontally due to capillary action, and vertically due to gravitational force. To guide the fluid movement, hydrophobic regions and hydrophilic channels will be created. Before folding, we will be able to deposit and dry the various reagents of the sandwich assay into the channels. We intend to carry out

each step of the sandwich assay as the fluid moves from channel to channel, rehydrating the required assay reagents along the way. A schematic of our proposed chip can be found in Figure 2.

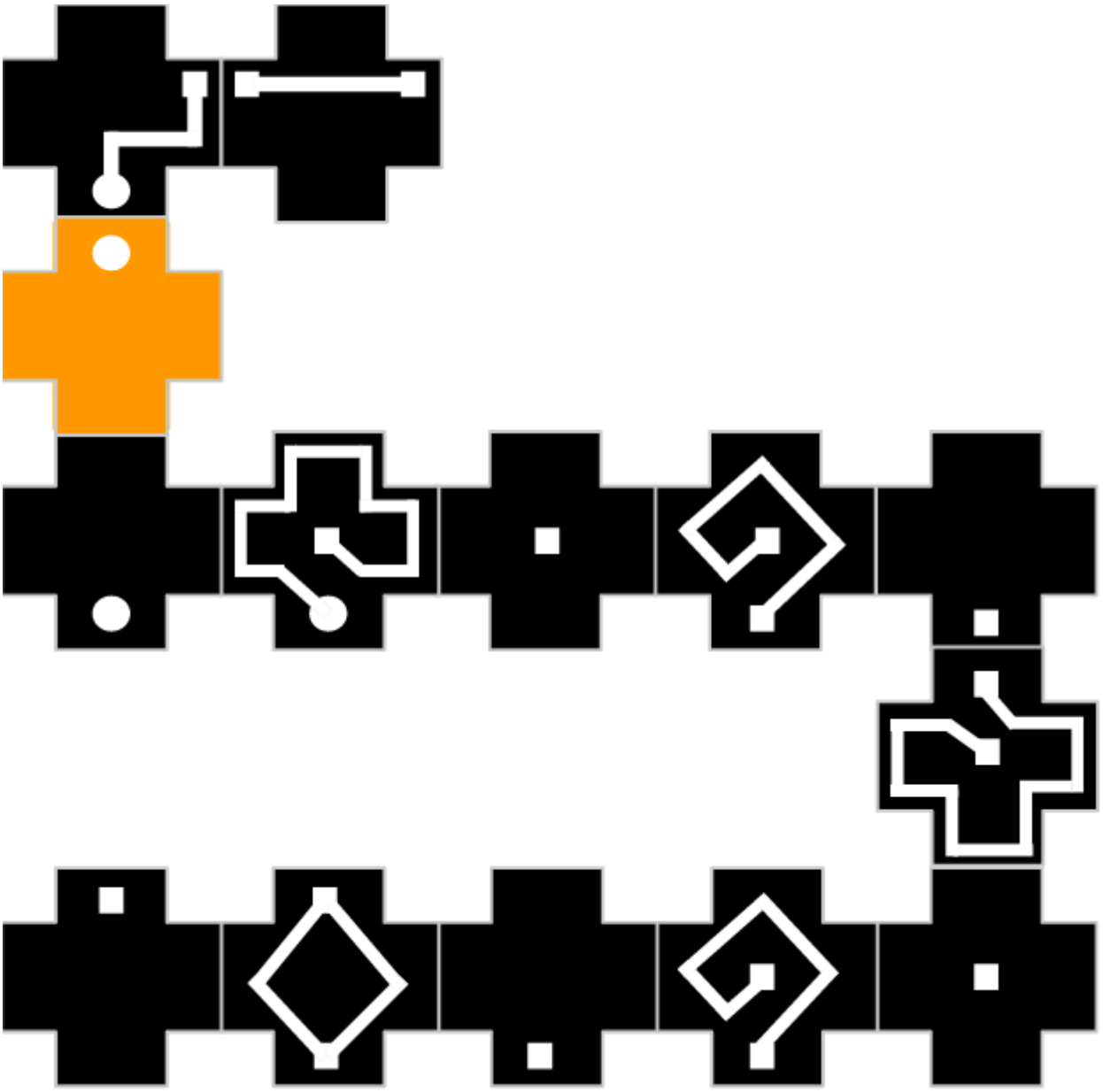

Figure 2: 2-Dimensional View of Device. The orange layer represents the layer upon which the assay takes place. This layer is predeposited with zirconia+Capture oligonucleotide and will change color in the presence of the target strand. The 2 layers above the zirconia piece will contain the Detection strand that will anneal to the introduced Target strand and then be introduced to the zirconia paper. The layers below the orange piece will contain the reagents required to carry out silver enhancement to amplify the biosignal given off by the Detection strand.

To fabricate our 3D chip, the proposed design of Figure 2 will be wax-printed onto cellulose filter paper. Wax printing is analogous to inkjet printing except that it utilizes solid ink blocks as opposed to liquid ink cartridges. The solid wax ink is hydrophobic, and will not smear when exposed to fluids. Using the ink's innate hydrophobicity, we will be able to create hydrophilic channels in which the sample fluid will flow. As explored by Renault et. al., wax printing has several advantages over other fabrication methods; wax printing is incredibly fast, allowing hundreds of devices to be made in a short amount of time, therefore increasing the manufacturability of these devices [21]. Additionally, wax printing minimizes contamination of the channels through which fluid will flow, ultimately resulting in a more accurate and reliable device [21].

The final component of our device will be a case to house the 3D paper substrate. Using Solidworks, a drawing will be created to be 3D-printed using the standard Makerbot plastic extruders. The layer upon which the full assay takes place is the third layer of our proposed design. Therefore, we must design a housing component that will not have to be taken apart entirely so that the paper can be unfolded to reveal the results on the third layer. To this end, we will design the third layer to land on a removable part so that it can be extracted for analysis without disturbing the entire column of layers.

III. Analysis by Mobile Phone Application

Once the assay is completed and the critical zirconia layer has been removed from the vertical column, the colorimetric change must be analyzed. We are using a cell phone camera and mobile application to carry out the analysis because smart phones are becoming increasingly common in the developing world. The amount of bacteria in a sample will be quantified by taking a picture of the colorimetrically altered paper. The mobile phone will analyze the red, green, and blue (RGB) values of each pixel. This pixel analysis will ultimately produce a quantitative value related to the concentration of bacteria present in the sample. This mobile phone application gives the end-user the unique opportunity to take charge of their own health, even when professional medical resources are limited. This preventative care device has the potential to greatly reduce the disease states caused by unsanitary water.

CHAPTER 4: PROJECT PLANNING

I. Team Management

To bring this project to fruition, we will be working closely together to ensure that the work is done accurately and in a timely manner. Each team member will spend around 6 hours in the lab per week; this amount of time will be required to meet our proposed goals. During the lab time, both partners will work on both the bench top assay and integration into the microfluidic device. Both partners do not have to be in the lab at the same time, but will split the work evenly. For difficult or tricky procedures, both partners will work together to ensure each protocol is carried out accurately.

Our team will be meeting weekly with our project advisor, Dr. Ashley Kim. During these meetings, results obtained on a weekly basis will be presented for review. This time will be used to make appropriate modifications and to answer any questions that may arise. Outside of our weekly meetings, we will communicate efficiently, ensuring that Dr. Kim is up to date on our progress. Additionally, meetings will be scheduled with Dr. Kim and other Santa Clara University faculty members who have expertise in a given topic. By utilizing these resources, we are more likely to create a viable and sustainable product.

II. Proposed Timeline

Table 2: Quarterly Goals. List of goals to complete on a quarterly basis. Note that work done in fall 2014 must be validated before the winter and spring goals can be achieved.

Quarter	Goals
FALL 2014	Improve AuNP-Detection conjugation methods
	Determine viability of silver enhancement solution
	Test gold enhancement technique
	Research microfluidic chip fabrication methods
WINTER 2015	Design microfluidic chip
	Begin wax printing devices
	Test fluid movement through wax-printed devices
SPRING 2015	Validate DNA movement through device
	Dry assay reagents into channels to integrate sandwich assay into the device
	Test enhancement step on device
	Present at Senior Design Conference
	Complete thesis.

III. Budget

Table 3: Budget. Outline of the expenses of this project. The source of funding is the Roelandt's Grant, totally $3900.00

Material	Cost
Oligonucleotide Sequences	- $2000.00
Xerox wax printer	- $630.00
Wax ink cartridges	- $175.00
Zirconium IV Butoxide	- $300.00
Gold Nanoparticles	- $200.00
Cellulose filter paper	- $50.00
Denhardt's Solution	- $130.00
Conference Registration	- $415.00
Total Expenses	**- $3900.00**
Roelandt's Grant	**+ 3900.00**

CHAPTER 5: PRELIMINARY RESULTS AND PROTOTYPING

I. Benchtop Sandwich Assay Analyzed by Mobile Application

Before the final microfluidic prototype is made, the oligonucleotide sandwich assay needed to be validated on the benchtop to demonstrate the specificity and sensitivity of the detection platform. To this end, we performed the assay manually (methods described in chapter 8); in the final design, the manual steps will be integrated into the chip design so the end-user does not have to carry out any difficult protocols to produce the results. Ultimately, higher concentrations of the target strand (the complimentary bacterial DNA strand) will produce the darkest colorimetric change, while lower concentrations will produce a lighter colorimetric change. To test this theory, we introduced 1µM-10fM (5 100-fold dilutions and a control) of Target oligonucleotide to the immobilized zirconia+Capture complex, followed by incubation in the AuNP-conjugated Detection strand. Therefore, a gradient of darkness should be visible to the naked eye, with 1µM paper being the darkest, 1fM paper being the lightest, and the control showing no color change. The color gradient produced by the assay can be found in Figure 3.

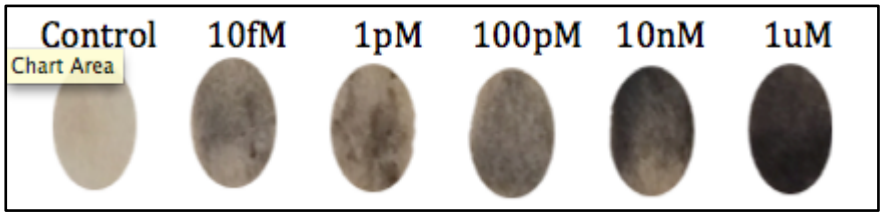

Figure 3: Camera Phone Images of Completed Sandwich Assay. Images demonstrate the color gradient produced by varying concentrations of target oligonucleotide.

As seen in Figure 3, the color gradient was visible to the naked eye. However, a more quantitative analysis of the color change can be garnered using the mobile phone application. The mobile application is a replacement for a lab microscope, which can easily analyze colorimetric changes. We intend that this device will be used in a resource-limited settings where traditional microscopes are unavailable. Therefore, we tested the validity of the mobile application in comparison to a lab microscope.

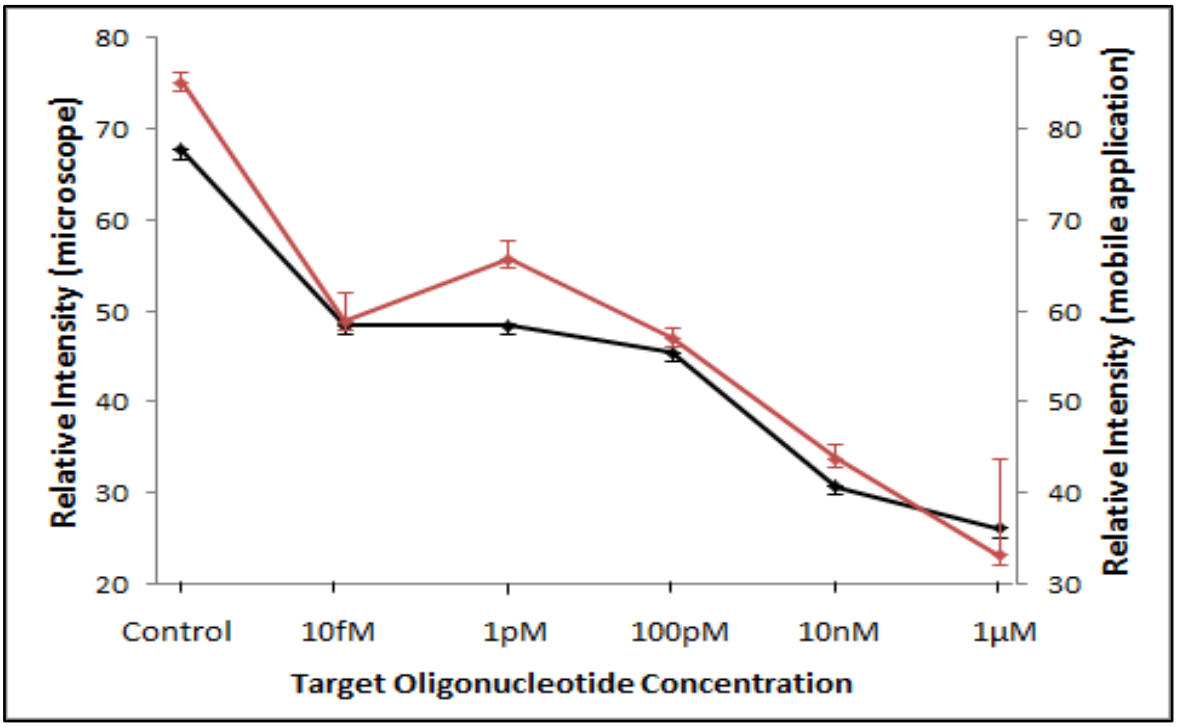

Figure 4: Microscope and Mobile Image Analysis. Microscope results shown in black and mobile app results shown in red. Both instruments produce comparable trend line.

From the data shown in Figure 4, the mobile phone application will serve as a reasonable analysis device for use in resource-limited settings. Relative intensity is a measure of how much light is present in a given image. Therefore, the lightest paper (the control) has the highest intensity and so on down to the 1µM concentration. The computer engineering team will continue to improve the mobile phone application so the trend line of the mobile phone more closely mimics that of the lab microscope. Given the results of the benchtop sandwich assay in conjugation with the mobile phone application analysis, we believe this platform can be successfully integrated into a 3D microfluidic chip.

II. Microfluidic Chip Fabrication

Upon completion of the sandwich assay validation, we gathered the parts required to actually fabricate our device. We purchased a wax printer, and have been able to print our microfluidic chip. We have printed our device onto an 18cm diameter cellulose filter paper, cut out the unnecessary components, and folded the device into its 3D shape. We

have validated the guided fluid movement through the hydrophilic channels by using dyed water samples. The wax printer ink has proven its hydrophobicity as no traces of water can be seen in the hydrophobic spaces.

CHAPTER 6: EXPECTED RESULTS

I. Sequential Assay Performed on Self-Contained Microfluidic Chip

Upon validation of the assay platform and directed fluid movement through the 3D wax printed microfluidic chip, we move to integrate the platform onto the chip itself. The platform can be split up into two distinct phases; the first phase contains the immobilization of the zirconia+Capture+Target+Detection complex. To do so, we will dry the various reagents of the assay onto the chip. Introduction of the fluid sample will rehydrate the reagents so the full assay can take place. In our final fabricated device, we intend to pre-load the critical zirconia paper with the Capture strand already immobilized, which will ultimately reduce the time required to complete the assay. Therefore, the Detection strand will be dried onto a channel, and then will be rehydrated upon introduction of the Target oligonucleotide. Once rehydrated, we anticipate that the Detection strand will be able to anneal freely to the target strand and the preloaded zirconia+Capture complex. Once immobilized onto the zirconia layer, the complex will be stable for the signal enhancement step.

II. Signal Production and Enhancement on Microfluidic Chip

Once the zirconia+Capture+Target+Detection complex is immobilized, the biosignal caused by the gold nanoparticle conjugated to the Detection strand must be amplified so that analysis can be done using the smart phone application. The silver enhancement step (see Chapter 7 for methods) includes the mixing of two solutions before addition to the immobilized complex. Therefore, we will be drying the reagents of the silver enhancement procedure into the chip channels. We anticipate that these solutions will mix upon rehydration, and the guided channels will introduce the silver solution to the immobilized complex from the assay completed in phase one. We expect that the papers will have a colorimetric change based upon the concentration of the target strand introduced in phase one comparable to the results seen when the procedure is carried out on the benchtop (see Figure 3).

CHAPTER 7: BACKUP PLANS

I. Silver Enhancement

In our early work benchtop work with the platform assay, we quickly learned that the silver enhancement step was not just a simple mixing to two solutions. The silver enhancing solutions are incredibly reactive, so the reaction step must be carried out with great precision. Although preventative measures are taken to limit false-positive results, we found that silver enhancing solution reacts with simple cellulose, and not gold nanoparticles exclusively. This is of grave concern for our device because it is entirely cellulose-based. Although we were able to produce clear results with this method, we had to carefully monitor the reaction time to gauge how long the papers should be exposed to the silver solution. However, an enhancement step that requires vigilant monitoring and some extent of scientific discretion may not suitable for a device intended for use by untrained user. Fu et. al. also experienced the inconsistencies of silver enhancement in his work with a 2D paper microfluidic chip. As an alternative, his research group utilized a gold enhancement solution for signal amplification [22]. Instead of the solution causing silver nanoparticles to precipitate onto gold nanoparticle probes, the gold enhancing solution causes gold nanoparticles to precipitate onto gold nanoparticle probes. Fu reports that the gold enhancement solution is much more sensitive and greatly reduces false positives. If we continue to experience complications with the silver enhancing solution, we could possible substitute it for gold enhancement solution.

II. Nanoparticle Conjugation

When the Detection oligonucleotide is synthesized, it is made with a 3' thiol modification. Once the thiol is reduced, the gold nanoparticles can be conjugated to the strand for use in the final component of the sandwich assay. This step has proven difficult because the 3' thiol may be very reactive. Therefore, once reduced, the thiol may oxidize quickly with any exposure to oxygen. In our early research, we experienced many difficulties trying to conjugate the nanoparticles to the Detection strand, which could likely be caused by inert thiol due to oxidization. The biosignal-emitting Detection strand is integral to the completion of our device as we can do no testing or analysis without it.

Therefore, if we cannot successfully conjugate the nanoparticles to the Detection strand, we can have the Detection strand synthesized with a 3' C3 fluorescein modification. This strand would be analogous to the nanoparticle-conjugated strand, but the biosignal can only be seen under a microscope and cannot be enhanced by silver or gold enhancing solutions. Although this is not an ideal substitution, it would allow us to track the movement of the various assay components through the microfluidic chip. Ideally, the fluorescein labeled Detection strand could move through the chip and ultimately immobilize on the zirconia layer; therefore, if the immobilization occurs, the zirconia layer should glow under the microscope. A device that requires a lab microscope for analysis is not suitable for use in a resource-limited setting, but it could at least validate that the assay itself will work on a microfluidic chip.

III. On-Chip Enhancement - Proof of Concept

If the detection strand cannot be successfully conjugated with gold nanoparticles consistently, we will be unable to determine if silver or gold enhancement can occur on a paper microfluidic chip; the enhancing solution is unable to deposit silver precipitates onto a C3 fluorescein modification. Until the conjugation step is perfected, we could still test the viability of on-chip signal enhancement. In this case, we will spot nanoparticles onto a zirconia-coated cellulose paper and let it dry. We would then deposit and dry the various solutions required for the enhancement into the channels of the microfluidic chip. Upon rehydration with water, we expect the reagents will all mix appropriately to precipitate the silver nanoparticles onto the dried gold nanoparticles. We would vary the concentrations of gold nanoparticles dried onto the zirconia layer in hopes of seeing a clear grayscale gradient much like the results we saw in the benchtop assay (see figure 3). The enhancement step is vital to produce a signal that can be analyzed by the mobile application; this step can still be validated by these methods even if we cannot successfully conjugate gold nanoparticles to the detection oligonucleotide.

CHAPTER 8: MATERIALS AND METHODS

I. Materials

Oligonucleotides used in the testing and validation of the sandwich assay were obtained from Elim Biopharmaceuticals Inc (Hayward, CA). Gold nanoparticles that were later conjugated to the Detection oligonucleotides were purchased from Sigma-Aldrich, Inc (St. Louis, MI). The zirconia utilized to functionalize the cellulose paper for DNA binding was obtained from Sigma-Aldrich, Inc (St. Louis, MI). Denhardt's Solution, a component of the non-specific binding buffer, was also obtained from Invitrogen (Grand Island, NY). Dithiothreitol (DTT), which was used to reduce the thiol on the 3' end of the Detection oligonucleotide, was purchased from Sigma Aldrich, Inc (St. Louis, MI). The silver enhancement kit used to amplify the AuNP biosignal was obtained from Sigma-Aldrich, Inc (Saint Louis, MI). Salmon sperm DNA, another component of the non-specific binding buffer, was obtained from Thermo Fisher Scientific (Santa Clara, CA). The gold enhancement kit, used to amplify the AuNP biosignal, was obtained from Nanoprobes Inc (Yaphank, NY). Concentrations of oligonucleotide solutions were determined using the NanoDrop UV-Vis Spectroscopy (Wilmington, DE). Fluorescent imaging was conducted using the Olympus BX51WI epifluorescent microscope, and QCapture Pro6.0 software was used to characterize the fluorescence of the samples.

II. Benchtop Sandwich Assay Validation

a. Deposition of Zirconia

Zirconia was cyclically deposited onto cellulose filter paper as described by Xioa and Huang [23]. In summary, filter paper was placed into a pump-powered filtration apparatus. To begin the first cycle, the filter paper was washed thoroughly with ethanol. Then, 10mL of zirconia were added to the filtration funnel. The zirconia was left standing for 5 minutes, and then pulled through the filter for 3 minutes. The paper was then washed and filtered with sufficient water, which washes away any unreacted zirconia. To

complete the cycle, the paper was washed thoroughly with ethanol. This 5-step cycle was repeated 10 times to obtain a thin but visible layer of zirconia on the filter paper.

b. Immobilization of Capture Oligonucleotide

The Capture oligonucleotide was then bound to the cellulose+zirconia substrate at the 5' terminus through a 24-hour submersion of the cellulose+zirconia substrate in the oligonucleotide. Capture strands tagged with C3-Fluorescein at the 3' terminus were synthesized with a fluorophore modification. The binding of the Capture+tag strands to the substrate was then visualized using fluorescent microscopy to determine the optimal concentration of capture bound to the paper. Zirconia+cellulose substrates were then incubated with untagged, optimal concentration capture strands for 24 hours, washed with water, and then air dried. To prevent nonspecific binding to the cellulose+zirconia substrate, the cellulose+zirconia+Capture complex was bathed in a pre-hybridization solution (Denhardt's solution, 0.1M salmon sperm DNA). The substrates were then washed in 10mM, 1mM Tris-EDTA buffer and air dried [23].

c. Immobilization of Target Oligonucleotide onto Zirconia+Capture Complex

Target oligonucleotides were introduced at varying concentrations to the cellulose+zirconia+Capture substrates. Target sequences tagged with a CAL Flour Red 610 modification at the 5' terminus were imaged to verify the binding to the substrate using fluorescent microscopy. The lower limit of detection of the target was determined [23]. Once the limit of detection was determined, untagged Target oligonucleotide was introduced to zirconia+Capture complex in varying concentrations ranging from 1μM to 10fM. The zirconia+Capture complex was incubated with the target strand for 24 hours and then washed with water and air dried.

d. Detection Nanoparticle Conjugation

The Detection oligonucleotide was synthesized with 3' thiol modification to allow for gold nanoparticle conjugation. The dried Detection probe was resuspended in at 100mM DTT solution for 1 hour. The Detection strand was then purified through G25 columns. Gold nanoparticles were prepared by centrifugation at 13,200 rpm for twenty

minutes. The supernatant fluid was removed, and the nanoparticles were incubated with dATP in a ratio of 1000:1 dATP:AuNP for 15 minutes. The purified detection was then added to the dATP/AuNP mixture in a ratio of 1:500 AuNP/dATP:detection oligonucleotide. The probes were then left on a shaker at 60℃ for three hours, and then left to incubate on the benchtop for 2 hours at room temperature. After incubation, the probes were centrifuged and washed with 100mM phosphate buffer at pH 8.5. This wash step was repeated 3 times, and the probe was stored in 100µL of 0.3M NaCl, 10mM PBS with pH 8.0 at 4℃ [24].

e. Introduction of Detection Sequence

The gold nanoparticle conjugated Detection probe was then incubated with the zirconia+Capture+Target complex at 2µM for 90 minutes. The papers were then washed with water and bathed in 0.5M EDTA at pH 4.5 to prevent nonspecific signal enhancement. They were then washed with water again and left out to dry for 30 minutes.

f. Signal Enhancement

The silver enhancement solution was prepared by mixing equal volumes of Solution A and Solution B. The papers with the zirconia+Capture+Target+Detection complex were then incubated in the mixed solution for approximately 8 minutes followed immediate by a bath in Silver Stabilizer Solution. Finally, the papers were washed with water and dried for analysis.

g. Assay Analysis under Microscope and Mobile Application

To quantitatively analyze the colorimetric signal produced in the enhancement step, papers were visualized under a fluorescent microscopy. Using ImageJ software, the relative intensity of the color was measured. A smart phone with an attachable microscope piece took pictures of each paper, and also produced the relative color intensity. The values given by the software and mobile phone application were compared to validate the mobile phone application.

III. Microfluidic Chip Fabrication

a. Chip Design, Printing, and Heating

The microfluidic chip was designed using Microsoft Powerpoint. Once the image was made, it was wax printed onto 18 cm filter paper. The filter paper was then heated on a hot plate at 100℃ for approximately 8 minutes to allow the wax to melt into the cellulose. The unnecessary filter paper was cut away and the device was folded into its 3D shape.

IV. Integrating Sandwich Assay with Microfluidic Chip

a. Determining Time Constraints and Volume Requirements

To transition the sandwich assay into the device itself, the time required for incubation and the volume of fluid required to run through the device must be determined. To this end, various volumes of colored fluid were introduced to the device to determine the minimum volume needed to reach the inner detection layer of the device. After this volume was determined, the time it took for the minimum volume to reach the detection layer was recorded.

b. Validation of DNA Movement through the Device

For the proposed device design to function properly, the oligonucleotides must be able to move through the device. The oligonucleotides are carried in the fluid horizontally due to capillary action, and vertically through gravitational force. A small zirconia-coated cellulose strip (~4mm x 4mm) with immobilized Capture oligonucleotide (see chapter 7.II.B for methods) was placed onto the detection pad. The required layers of the device were folded on top of the critical detection layer. Small weights that did not interfere with the device channels were laid atop the 3D device to compress the various layers of the device and to stabilize the zirconia+Capture substrate in its position. Following stabilization, 40µL of varying Target+tag oligonucleotide sequences were pipetted into the first channel of the device. The devices were left to incubate overnight (approximately 17 hours). After incubation, the zirconia+Capture+Target+tag papers were washed thoroughly with water and left to dry. Once the papers were dry, they were

analyzed using the fluorescent microscopy and ImageJ software. The device itself was also viewed under fluorescent microscopy to further view how the fluorescent oligonucleotides traveled through the device layers.

Once the oligonucleotide movement through the device was validated using fluorescently tagged oligonucleotides, the above procedure was repeated. However, untagged Target oligonucleotides were introduced to the first channel as opposed to the Target+tag sequences. After incubation, the papers were washed with sufficient water and bathed in EDTA to prevent non-specific binding. The papers were then incubated in 2μM Detection oligonucleotide for 90 minutes. Following incubation, the papers were washed with water and placed into gold enhancement solution for 2 hours. After the enhancement step, the papers were washed with water and left to dry. When papers were dry, the colorimetric change was analyzed using fluorescent microscopy and ImageJ software.

CHAPTER 9: RESULTS

I. Capture+Tag Verification

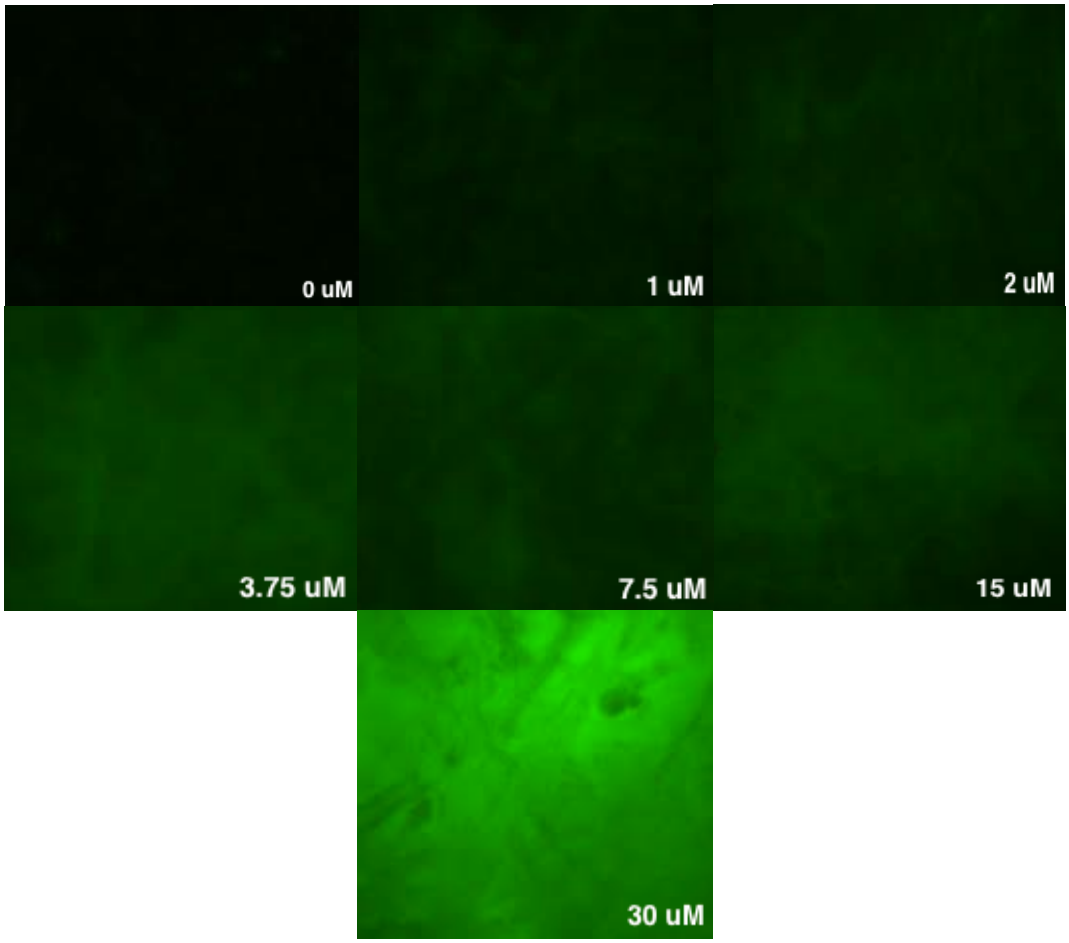

Figure 5: Capture+Tag Fluorescent Images. Fluorescent images taken of cellulose+zirconia papers with immobilized Capture+Tag oligonucleotides. Based on qualitative analysis, the brightness of the fluorescence increases as the Capture oligonucleotide concentration increases.

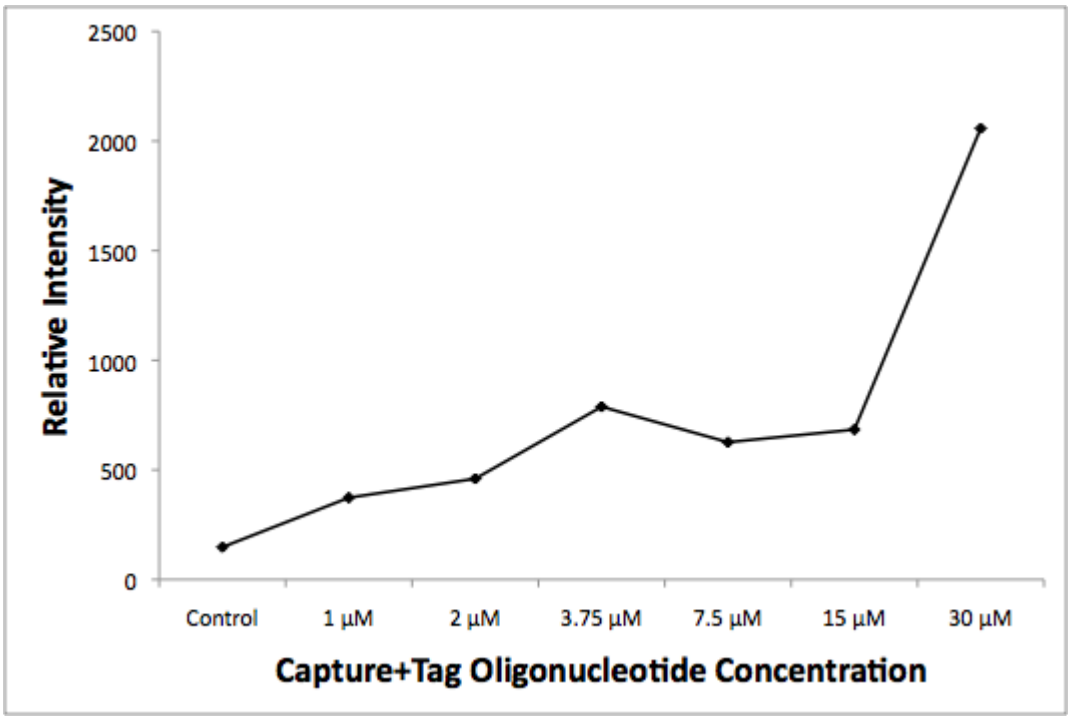

Figure 6: **Capture+Tag Image Analysis**. Numerical analysis of fluorescence brightness garnered from the fluorescent microscope and ImageJ software. This quantitative analysis demonstrates that the fluorescent intensity increases with increasing Capture+Tag oligonucleotide concentration. The 7.5µM and 15µM concentrations have similar intensities, suggesting the papers become saturated with oligonucleotide in this region.

II. Target+Tag Verification

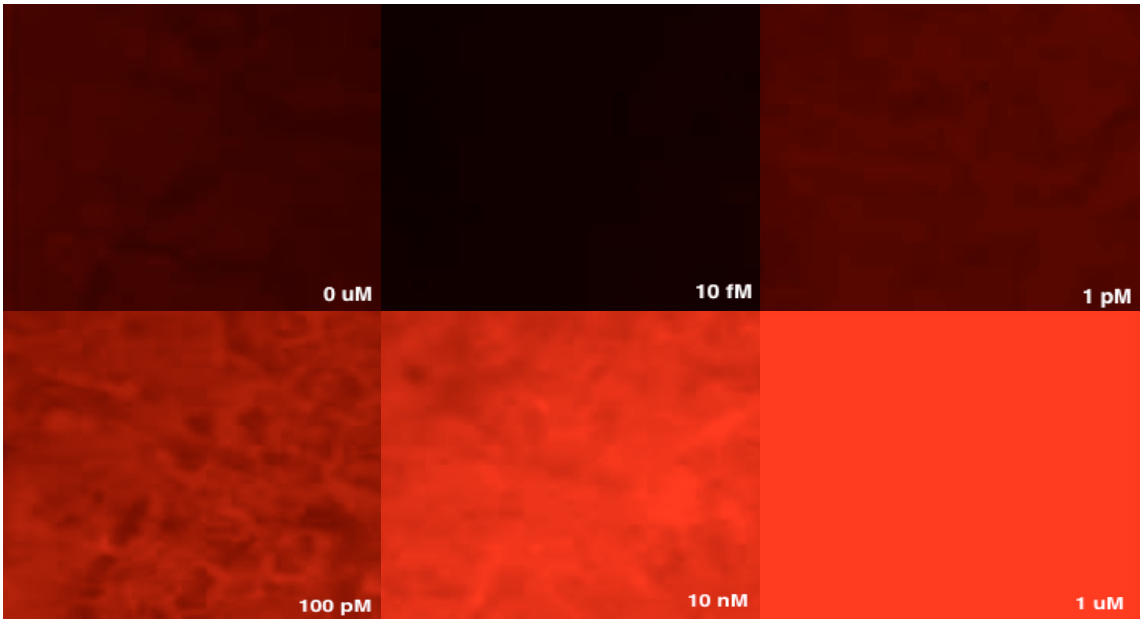

Figure 7: Target+Tag Fluorescent Images. Oligonucleotides were added at varying concentrations to zirconia+Capture strands. From qualitative observation, the brightness of the fluorescence increases with increasing Target+Tag oligonucleotide concentration.

III. Gold Enhancement Verification

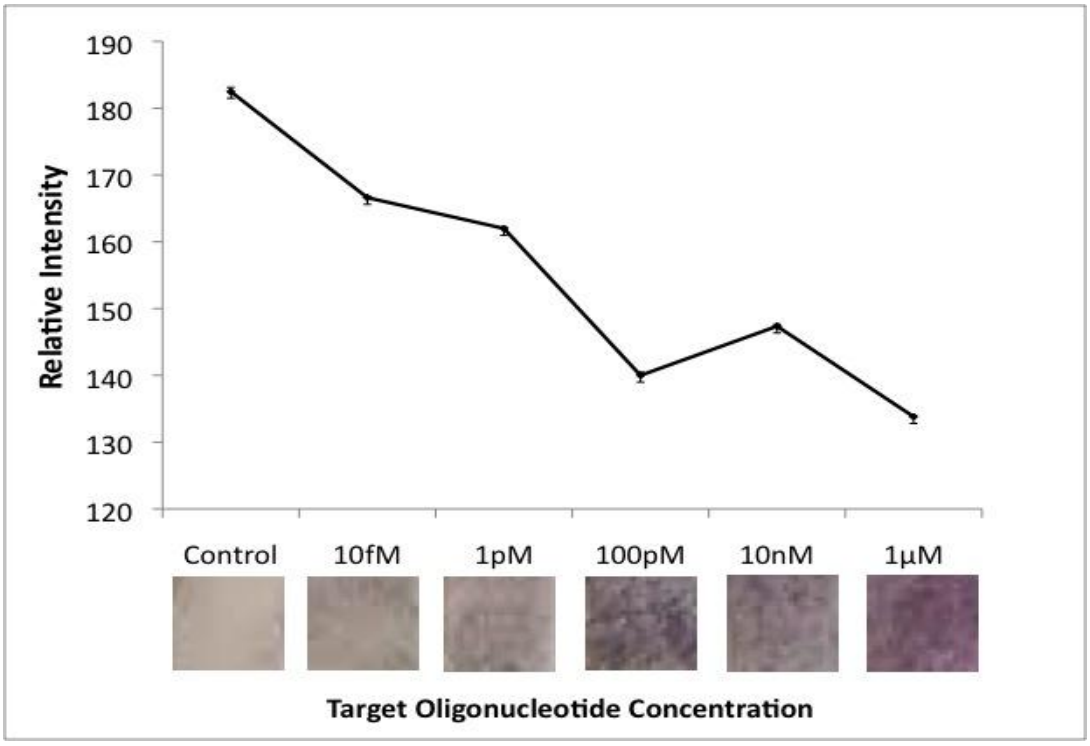

Figure 8: Completed Sandwich Assay. Quantitative measure of the darkness of zirconia+Capture+Target+Detection paper substrates after soaking in gold enhancement solution. Gold enhancement solution increases the effective radius of the gold nanoparticles conjugated to the Detection strand, changing the paper color from red to purple. As the Target oligonucleotide concentration increases, the papers become darker, therefore decreasing the relative intensity.

IV. Target+Tag Movement through Device Demonstration

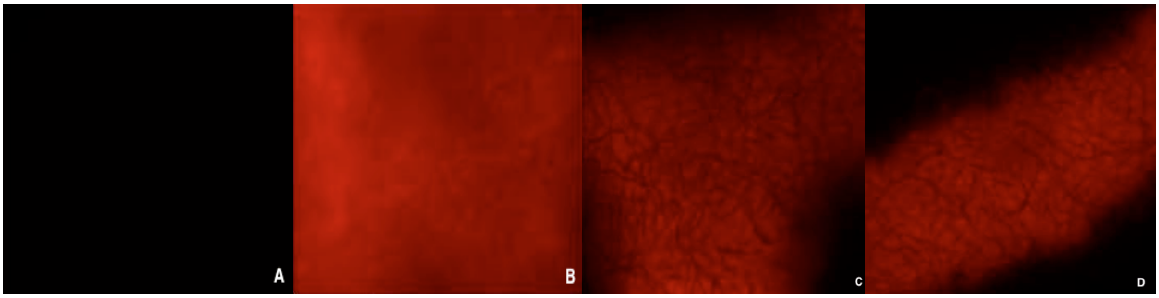

Figure 9: DNA Flow through Device. Fluorescent Target+Tag DNA was passed through the device to land on zirconia+capture paper substrates. **A)** Fluorescent image of zirconia+Capture substrate to which no Target+Tag DNA was introduced through the device, therefore yielding minimal fluorescence. **B)** Fluorescent image of zirconia+Capture substrate to which 5µM Target+Tag DNA was introduced through the device, ultimately producing a fluorescent signal. **C)** Image of one channel of the microfluidic device, showing that the fluorescent DNA moves through the device channels. **D)** Image of a second channel of the device, also demonstrating the movement of DNA through the device.

V. Target Movement through device Followed by Detection-AuNP incubation and Gold Enhancement

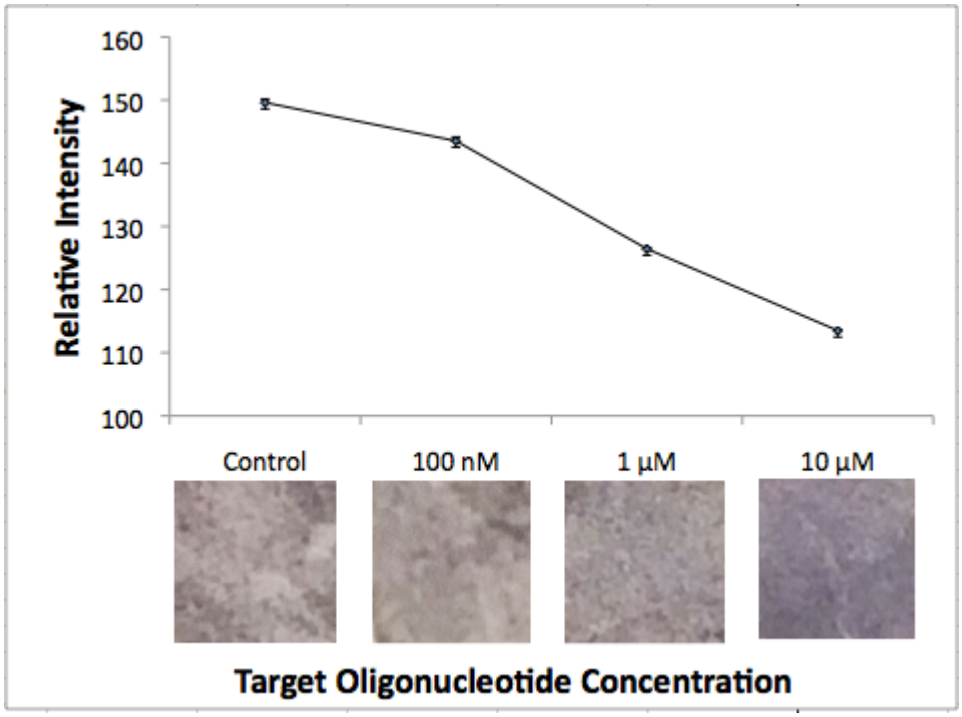

Figure 10: Completed Sandwich Assay after DNA Flow through Device Images and quantitative analysis of Target DNA ran through the device to land on zirconia+Capture paper substrate. Following incubation, the papers were washed and incubated with the Detection probe. The papers were then washing and bathed in gold enhancement solution. The images and numerical data show that darkness of the paper increases with increasing Target oligonucleotide concentration.

CHAPTER 10: DISCUSSION

I. Assay Platform

a. Capture+Tag Validation

In our final device proposal, we intended to manufacture the zirconia-coated detection layer with the Capture strand immobilized onto it. With this initial step done in the manufacturing process, the Capture strand is prepared to anneal to the target bacterial RNA as soon as it is introduced. Therefore, we needed to determine an optimal concentration of Capture oligonucleotide to immobilize onto the zirconia-coated cellulose. To determine this concentration, we incubated the zirconia-coated papers with varying concentrations of Capture+Tag oligonucleotides. The 5' phosphate groups covalently bonded to the positively charged zirconia surface. The 3' end was modified with C3 Fluorescein, which fluoresces a bright green color. In these trials, the 3' end is not bound to any other substrates so the fluorescence can be easily analyzed.

In Figure 6, the images appear to fluoresce with more intensity as the concentration of Capture+Tag increases. Because the papers have a limited surface area, there is a limited concentration of Capture that can be bound to them. We aimed to determine an ideal Capture concentration that was larger that the upper limit of the RNA we would be testing in the second assay step (1μM), but was small enough to produce consistent results without wasting materials in manufacturing. In Figure 6, the fluorescence of the 7.5 μM and 15 μM Capture concentrations were comparable. Therefore, we determined that we would load the zirconia-coated cellulose papers with 10 μM of Capture DNA for all future testing. 10 μM is well above our upper limit of detection, meaning there is enough Capture strands bound to the paper to immobilize the largest concentration of Target oligonucleotide we planned to test in the second step of the assay.

b. Target+Tag Validation

To validate the second step of the sandwich assay, the Target oligonucleotide strands were synthesized with a 5' CAL RED 610 modification. We hypothesized that the 3' end of the oligonucleotide would anneal to the complementary Capture strand already

immobilized on the zirconia-coated cellulose substrate. Therefore, the 3' end containing the fluorescent modification would be unbound to any other substrates, allowing us to analyze the fluorescence accurately. We anticipated that the brightness of the fluorescent images would increase with increasing concentration of Target oligonucleotide. As seen in Figure 6, the 10 fM concentration fluoresces the least, and the brightness intensifies leading up to the 1 μM concentration.

To quantitatively analyze the fluorescent intensity, the fluorescent microscope and ImageJ software was used to produce numerical values of the intensity. Our qualitative analysis was affirmed with the quantitative data seen in Figure 7; the relative intensity, which is the measure of brightness in an image, increases with increasing concentrations of Target oligonucleotide.

Demonstrating a fluorescent gradient was a critical step in our research. Ultimately, we not only wanted our device to detect the presence of bacterial RNA in a sample, but we wanted to determine to what degree the bacteria is present. This step demonstrated that the assay was sensitive enough to distinguish one Target strand concentration from another.

c. Gold Enhancement Validation

In a resource-limited settings, fluorescent microscopes are unavailable to use for the detection of bacterial pathogens. Therefore, the third strand of the sandwich assay was synthesized to create an optical signal when a binding event occurred between the Capture and Target strands. The Detection strand binds to the Capture+Target complex on its 5' end, leaving the 3' end conjugated with gold nanoparticle free. The signal produced by the nanoparticles alone is not strong enough to been seen and analyzed by the naked eye. Therefore, the papers are soaked in gold enhancement solution to darken the original signal. We anticipated that the gold enhancement step would produce a gradient similar to the gradient obtained by the fluorescent microscopy. As seen in Figure 8, the papers become darker with increasing concentration of Target oligonucleotide. This therefore confirms that the Detection strand conjugated with gold nanoparticles can accurately detect varying concentrations of Target oligonucleotide present in a sample.

In our original design proposal and preliminary results, we were using silver enhancement solution as opposed to gold enhancement solution. We quickly learned that the silver enhancement solution is highly reactive. The silver did not react with gold nanoparticles exclusively, as the silver would precipitate onto control groups that contained no gold nanoparticles. After producing many false-positives, we switched our signal amplification method to gold enhancement. Gold enhancement works similarly to silver enhancement; instead of precipitating silver particles onto the gold nanoparticles conjugated to the Detection strand, the solution precipitates gold nanoparticles. The gold nanoparticles we conjugate to the detection strand have a 20 nm diameter and are red in color. As the radius of the gold nanoparticles increases, the color changes to purple and to blue. When the gold enhancement solution precipitates gold nanoparticles onto the existing gold nanoparticles, the effective radius of the original probes increases. Therefore, the darkest colorimetric change caused by high Target oligonucleotide concentrations appears purple. In our testing, we have seen that gold enhancement solution significantly decreases the chance of producing false positives and the solutions are more selectively reactive.

II. Chip Fabrication

Fabricating our device using wax printing is incredibly simple, therefore increasing the manufacturability of our product. The cellulose filter paper can be fed into the printer directly, allowing us to make dozens of devices within an hour. The printer only patterns the design onto one side of the filter paper, so the wax must be melted through the paper and on to the other side. To do this, we placed the patterned filter paper onto a hot plate, face up. The melting process must be monitored vigilantly because the wax does run when heated. Therefore, we saw that the widith of the channels would decrease, and sometimes completely close off if the paper is left on the hot plate too long. After fabricating many devices, the average device takes about 8-10 minutes to melt the wax onto the reverse side of the filter paper at 100^{o}C without closing the channels.

III. On-Chip Assay and Enhancement

a. Target+Tag Movement through Device

The assay steps involving fluorescent analysis and gold enhancement have all been done on the bench top in our lab. However, this device is intended for use in the developing world, so the assay must be integrated into the device itself in order for our methods to be valid in a resource limited setting. The first step to integrating the assay was to demonstrate that the oligonucleotide strands could travel through the device layers to land on the detection layer containing the Capture strand immobilized on the zirconia-coated cellulose. To show the movement of DNA through the device, we ran the Target+Tag oligonucleotide through two channels leading to a small piece of zirconia+Capture paper placed into the detection layer. Because this step was a proof of concept, we first ran fluid containing no Target DNA through the device as seen in Figure 9A. We then ran a 40μL of 5 μM Target oligonucleotide through the device as seen in Figure 9B. After the incubation time, the paper was washed with water, dried, and viewed under the fluorescent microscope. By comparing Figure 9A and 9B, we have shown that oligonucleotides will move travel both horizontally through channels and vertically onto subsequent layers.

Figure 9C and 9D show the two channels the Target+Tag strand traveled through. Both of these images show some degree of fluorescence, indicating that there is Target+Tag DNA remaining in the channels. The channels were not washed before imaging so that we could verify that the DNA did in fact travel through every portion of each channel.

b. Target Movement through Device Followed by Benchtop Detection Incubation and Gold Enhancement

After demonstrating the movement of DNA through the device, we aimed to produce a colorimetric gradient visible to the naked eye using varying concentrations of Target oligonucleotide to run through the device. Following incubation with the Target oligonucleotides, the zirconia+Capture+Target paper substrates were removed from the detection layer. They were incubated with the third Detection sequence, and were then incubated in gold enhancement solution. As seen in Figure 10, a gradient can be seen qualitatively and is affirmed by the quantitative analysis by the microscope. This step further verified that the assay is sensitive enough to detect varying concentrations of the Target DNA even when it is run through the device as opposed to incubating on the

benchtop. We did notice that the gold enhancement reaction took a great while to produce gold precipitates to change the color of the papers. Typically, this reaction takes minutes but this step took about 2 hours to complete. The control in this trial was not entirely white, suggesting that the enhancement solution has non-specifically precipitated gold nanoparticles that were not removed in the wash steps. Even though the control turned color slightly, it was still lighter than the lowest concentration we tested for (Figure 10).

In this step, we chose to use higher concentrations of Target DNA than usual because we were uncertain of how much Target DNA was going to be lost while moving through the channels. On the benchtop, we have shown that the assay can detect into the femtomolar range. Future work on this device will work to achieve the same sensitivity through the device as there is on the benchtop.

CHAPTER 11: PROJECT SUMMARY

I. Objectives and Milestones

a. Project Scope

Our research demonstrates important advances in the long-term scope of this project. Our role focused on validating the nucleic acid assay, improving the enhancement steps, and developing a prototype for the microfluidic device.

We began working on this project in 2014. At this time, nobody had been working on this research at Santa Clara for about a year. Therefore, our initial stages of research involved restarting the research. Table 4 shows the overall scope of the project and our role in it (shown in bold).

Table 4: Project Timeline and Scope. Our contributions are bolded.

Project Task	Timeline
Project Inception	2012
Preliminary Research	2012
Initial Assay Testing & Validation	2012 & **2014**
Assay Refinement	**2014**
Device Fabrication	**2015**
Device Integration	**2015**-Future
Field Testing	Future
Project Completion	Future

b. Objectives

Our initial objective for this project was to have a functioning prototype of the microfluidic device. Quickly, we realized that this projection was overly ambitious, and we adjusted our objectives to reach attainable milestones. Table 5 shows our completed and uncompleted milestones in this project.

Table 5: Project Milestones

Milestone	Date Completed
Verify Capture Binding	July 2014
Verify Target Binding	July 2014
Conjugate DNA with AuNP	August 2014*
Verify completed sandwich assay	August 2014*
Design microfluidic device	January 2015
Test fluid flow on microfluidic device	April 2015
Verify DNA flow through device layers	May 2015
Test dried reagents on device	May 2015
Develop on-device lysis method	Future
Test device with bacterial cells	Future
Field test device for constraints	Future

* DNA/AuNP conjugation was not reproducible until February 2015

II. Design Evaluation

a. Positives

The microfluidic device that we have produced has numerous positive attributes that make it a promising avenue for future work. It is design to detect bacterial pathogens, and we have demonstrated that it can accomplish this task. Through both fluorescent imaging and colorimetric change, we have verified sensitive nucleic acid binding that corresponds to known bacterial RNA sequences.

Not only does the design detect bacterial pathogens, it does so while meeting most of the ASSURED criteria for a diagnostic device. Our current prototype costs around $3 or $4 dollars, making it affordable. The nucleic acid assay is both sensitive and specific. The design of the device is user-friendly because it requires minimal sample-loading and easy to interpret results. The device is also equipment free, as it can be used with or without a mobile phone. All these aspects of the device make it deliverable to the end user.

b. Negatives

One challenge that has been associated with this device has been producing consistently reliable results. Occasionally, the gold nanoparticles will color the control paper, confounding the results. This issue with the design has been mitigated with control steps, such as rinsing the papers with EDTA to further prevent non-specific

binding. Gold enhancement has also improved the consistency of the results, as opposed to previous methods which used silver enhancement instead. This issue of reliability is a challenge in the design, but one that can be addressed with further testing and optimization of reagent concentrations.

III. Future Work

a. Assay Reproducibility

The sandwich assay is the fundamental platform of the device, and therefore must produce accurate results from trial to trial. In the early stages of our research, we had difficulty reproducing results from trial to trial. Through fluorescent microscopy, we were able to see that the Capture and Target strands were binding to their respective locations properly. The issues usually arose in the conjugation of gold nanoparticles to the Detection oligonucleotide. Without properly conjugated nanoparticles, the Detection strand cannot accurately detect the binding event between the Capture and Target strands. After much discussion with Dr. Korin Wheeler of the biochemistry department, we pinpointed the error we were making in the conjugate synthesis. The thiol on the 3' end of the Detection strand will oxidize quickly after the initial reduction protocol. Therefore, the nanoparticles must be added to the reduced Detection strand immediately to force the reduced thiol to bind to the nanoparticles as opposed to oxidizing again. Towards the end of our project, we were able to make suitable AuNP-Detection conjugates that accurately detected the binding event between the Capture and Target sequences. The next team of researchers who picks up this project will need to verify our results by synthesizing more conjugates that produce a clear, colorimetric gradient when varying concentrations of Target oligonucleotide are added to the zirconia+Capture paper substrates.

b. Dried DNA/AuNP on device

Because we have been able to demonstrate that DNA will move through the device, the next step in integrating the assay into the device is to dry the AuNP-Detection conjugates into the device channels. The AuNP-Detection strand can be dried in the channel right before the detection layer. When the Target oligonucleotide is introduced to the device, we anticipate that the fluid will rehydrate the dried Detection strand. Ideally, the AuNP-Detection strand will anneal to the Target oligonucleotide within this device

channels, and ultimately travel to the detection layer containing the zirconia+Capture paper substrate.

c. Bacterial Lysis on Device

The focus of our research was on the development of the nucleic acid assay and device manufacturing. This focus meant that we wanted to be as exact as possible in verifying the binding events for each stage of the assay. Therefore, instead of using RNA from lysed bacterial cells, we used synthesized oligonucleotide sequences that were mimetic of the *in vivo* RNA. Approaching the project in this way meant better control over our design experiments, but also meant that our research did not exactly simulate the conditions in which the device will be used.

Since we used synthesized sequences for the Target bacterial strand, we did not need to use bacterial cells during our research, which by extension meant that we did not need to lyse bacterial cells. In future work, bacterial cell lysis will be an important step in device use. We anticipate that a lysis step will be integrated into our device, and will probably be the first stage of the device reactions. Current literature in cell lysis suggests that chemical methods may be a viable solution. Depositing detergents, such as SDS, on the device during production may be able to lyse the samples sufficiently to expose the RNA to the external environment. Further research will be required to determine which chemicals to use, how much, and how they may affect subsequent reactions and DNA binding.

d. Gold Enhancement on Device

We have demonstrated that gold enhancement of gold nanoparticles is a reliable method of signal clarification for our device. This method requires the combination of four different solutions – two combined for five minutes, followed by subsequent serial addition of the following two solutions – in equal volumes. While this is simple to perform in a lab, the conversion of such protocol to a device requires more attention. Tests need to be performed that the reagents can be dried on the paper and successfully rehydrated while retaining functionality. Furthermore, the concentrations of the solutions may need adjustment to account for material lost while the volumes run through the device; earlier reactions steps need to travel farther than later steps, so higher concentrations will likely be needed to account for the loss. Our current design includes

prototypical designs for the fluid flow of the gold enhancement solutions, but further testing will be required to optimize the reaction timing and concentration balances.

e. Assay Validation with Bacterial RNA

As mentioned in the "Bacterial Lysis on Device" section, we have been using synthesized DNA sequences to emulate the bacterial RNA which we will actually be testing for in real life applications. Therefore, future work will require that bacteria cells be cultured and RNA be extracted. Once the RNA is isolated from the bacterial cells, the sandwich assay will need to be tested using this RNA, as opposed to the sequenced Target strands which we have used thus far.

f. Field Testing

Once all the reaction steps have been transferred to the device and reliable results have been generated, field testing will be required to determine the practicality and constraints to the device. Important factors, such as the shelf life, temperature range, humidity conditions, and numerous other environmental factors must be tested before the devices can be considered practical for field use. Since an important aspect of our device design is to make it meet ASSURED criteria, comprehensive field testing will be vital to optimize the device.

IV. Design Validity

The principles upon which our design is based are well-established. Paper-based microfluidics is an established field. Nucleic acid reactions are thoroughly categorized and validated as sensitive and specific detection methods. Our design repurposes these well-defined fields in a novel way that promises to be a valid approach to a serious global health issue. At this stage in our research, we have no reason to believe that the design is invalid.

V. Design Constraints

Our project has many strengths in the design that make it adaptable and applicable in numerous settings; however, there are certain limiting factors that are associated with the inherent characteristics of our device design.

a. Liquid Phase

This project uses microfluidics to carry out the biological reactions on the device. Inherently, this approach requires that the samples being tested be in a liquid phase. Therefore, solid or gaseous samples cannot be analyzed using our device. These phases cannot pass through the layers of the device, and the dried reagents on the layers will not be functionalized unless rehydrated with a liquid of near-neutral pH. A possible solution to this issue is to dissolve samples in a simple liquid, such as water, and then add that solution to the device. However, it is possible that with some samples this would be very difficult or impossible. In such situations, our device would not be an appropriate solution for bacterial detection.

b. Simple solutions

A key step in our microfluidic device is the fluid flow throughout the device. If the sample being tested is very complex, viscous, highly acidic or alkaline, or at extreme temperatures, then the functionality of our device may be constrained. The fluid sample needs to be hydrophilic in order to flow through the cellulose channels, and it needs to be of a low viscosity in order to ensure fluid flow. Complex solutions may hinder bacterial lysis and interfere with the reaction steps later in the device. Extreme pH, salt balances, or temperature may affect the molecular structure of the DNA sequences and impair the functionality of the device. The constraints that these factors place on the device may be mitigated since such extreme conditions may make bacterial survival unlikely as well.

CHAPTER 12: ETHICAL CONSIDERATIONS

I. Ethical Considerations

The motto for Santa Clara's School of Engineering is "Engineering with a mission", which emphasizes the importance of using technical skills to benefit society. This focus demands a deep understanding of the ethical implications that accompany our project. The goal of our work is to address the unequal access to diagnostic medical devices between resource-rich and resource-limited areas. By developing a low-cost microfluidic device that detects bacterial pathogens, we aim to breach the gap between life-saving technology and those who need it most. Through the lens of "A Framework for Thinking Ethically" provided by Santa Clara's Markkula Center for Applied Ethics, we will analyze the ethical justification, embedded impact, and decision-making process involved in our project.

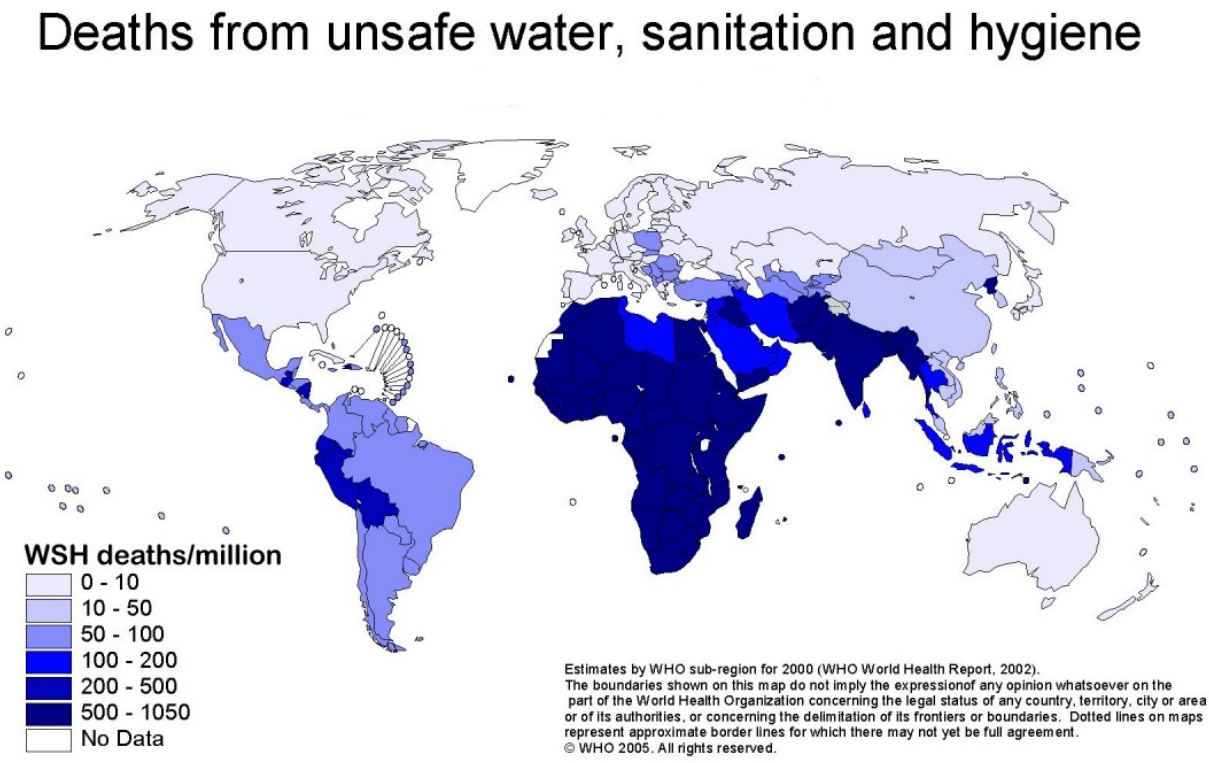

Figure 11: Deaths Caused by Contaminated Water. World Health Organization's 2002 report mapping the numbers of deaths worldwide caused by water/sanitation/hygiene (WSH) related diseases.[25]

II. Ethical Justification

a. Utilitarian Justification

In 2014, approximately 2,300 men, women, and children died every day as a result of unsanitary conditions.[26] Similar deaths and illnesses could be avoided by rapid and reliable diagnostic tests to determine the safety of water, food, or other possible contaminated sources. Quicker identification prevents prolonged exposure to bacterial pathogens and improved overall health for the individuals and communities at risk. Therefore, better diagnostic tools lead to more efficient health care, which saves lives and lowers medical costs for patients and professional health care services.

b. Rights & Responsibilities Justification

The United Nations' Universal Declaration of Human Rights states in Article 3 that "everyone has the right to life, liberty, and security of the person." Yet, the rights to life and basic health care are not being adequately met on a global scale. Millions of people are made sick by contaminated water every year, and they often do not know the cause of the illness until it is too late. By providing quality diagnostic devices to communities in need, we can prevent pathogenic illness and save lives. In doing this, we uphold our ethical responsibility to preserve the dignity of others and respect their rights to life and health.

c. Justice & Fairness Justification

750 million people are exposed to unsafe drinking water worldwide, with a vast majority of these people living in Africa and Asia.[27] This limited accessibility leads to improper hygiene, ultimately creating a heightened risk of bacterial infection. The unequal distribution of resources and basic health care disproportionately harms impoverished communities. Our device can quickly detect the presence, identity, and quantity of pathogens, therefore contributing to medical care and illness prevention. We aim to serve justice by providing fair and equal access to our device by making it low cost. Despite income inequalities and disparities in access to resources, our device promises high-quality diagnostics that are affordable to resource-limited areas.

d. Common Good Justification

The old expression "an ounce of prevention is worth a pound of cure" holds especially true when dealing with health care. Healthier people cost healthcare systems less and contribute to society more. Therefore, society benefits as a whole when disease is reduced. The targeted development of our device towards the marginalized not only helps the patients themselves, but also contributes to the common good of the community and society as a whole. As referenced in the Santa Clara Engineering Handbook, bioethics expert Daniel Callahan emphasizes "replacing the current 'ethic of individual rights' with 'an ethic of the common good'" in order to address the health care concerns in our modern world. Our device serves the common good because the whole community benefits from the knowledge of what water, food, or environmental hazards to avoid.

e. Virtue Justification

The Biomedical Engineering Society sets forth a Code of Ethics that guides us to be good and just biomedical engineers. It obligates us to use our knowledge to "enhance the safety, health, and welfare of public" as well as "strive by action, example, and influence to increase the competence, prestige, and honor of the biomedical engineering profession." This two-fold obligation not only compels us to be competent engineers, but also to form our character as leaders for good. Adopting these virtues has compelled us to take on a project that improves the quality of life for the users of our device.

III. Embedded Concerns & Decision-Making

a. Utilitarian Embedded Concerns & Decision-Making

To maximize the positive outcomes of this research, proper prioritization is necessary when allocating funds, time, and energy. Optimizing the balance of these resources is crucial to accelerating the project and bringing it closer to the end user. Additionally, this project must be the best option for bacterial diagnostics in resource-limited areas to be ethically justified. If at any point a better technology arrives that outperforms this, then we must reevaluate the project and redirect it to maximally provide good.

b. Rights & Responsibilities Embedded Concerns & Decision-Making

In addition to our responsibility to protect the basic human rights, we have responsibilities to address these concerns in the right way. This includes our responsibility to maintain proper Health and Safety standards while employing environmentally conscious and sustainable practices. Our device uses non-toxic chemicals, so it is safe to manufacture and produce for those involved in the fabrication process. Furthermore, our device uses cellulose as the substrate, which is environmentally friendly, especially when compared to alternative plastic materials that often involve production methods hazardous to both workers and the environment. After use, our devices can be cleanly burned, which eliminates waste without contributing to pollution.

Ensuring proper and reliable use is another important aspect of our project. We must be sure to properly test and validate the device to eliminate possible false-negatives and false-positives, as inaccurate readings may result in the end user being at risk. Accompanying this concern is the matter of usability. If this device can practically be used by communities globally, it must be simple and durable to serve real-world applications. Keeping these responsibilities in mind, we have taken the extra time to validate every step and develop the device before using it outside of the lab.

c. Fairness and Justice Embedded Concerns & Decision-Making

If the device is used fairly and justly, it can help marginalized groups have better control over their health and safety. However, a concern is the proper distribution of this device to ensure that those in need have access to it. By partnering with global non-profit organizations, such as the WHO, we aim to avoid the commoditization of the technology. At the same time, economic considerations must be taken to ensure continued production of the device and proper wages for the workers involved in this process. Striking the balance between keeping the device affordable and respecting workers and the environment is a fine line, and partnering with the WHO or UN could help facilitate this balance.

d. The Common Good Embedded Concerns & Decision-Making

Serving the common good means contributing to the interlocking relationships of society. As mentioned earlier, the diagnostic device must be made equally accessible for

the sake of justice, but the common good depends on that distribution as well. Foreigners bringing in new technologies to communities may cause division within communities or complete rejection of the technology if the interaction is not conducted with respect. Also, it is possible some members of communities may seek to leverage access to this device for power or money, which would harm the common good even more. Therefore, we must develop a model of distribution that respects local customs and values while still effectively reaching the targeted users. This may come in the form of working with local health professionals who have both medical training and cultural ties to the communities.

e. Virtue Approach Embedded Concerns & Decision-Making

Charles Harris' article "The Good Engineer: Giving Virtue Its Due in Engineering Ethics" addresses certain virtues that good engineers must have to address concerns in the field. These include techno-social sensitivity, respect for nature, commitment to the public good, teamwork, and courage. It is not enough for an engineer to create one ethical project, if he or she will then abandon those ethics in the sake of serving self-interest. Rather, a virtuous engineer is informed by society's needs and acts in a way that serves the larger public good. Additionally, any project undertaken will take into account the impact it will have on nature. While these decisions may not always be easy, a virtuous engineer will never hesitate to take the hard road if it is the right road.

IV. Conclusion

We aim to address the global issue of illnesses caused by bacterial contamination through the development of a low-cost diagnostic device that could be used in resource-limited areas globally (summarized in Table 6). Identifying and quantifying contaminated sources can improve medical professional's ability to treat illnesses, as well as prevent them. As a result, human dignity and right to life are upheld. By producing the device at a low cost we preserve justice because all people will have equal access. Proper manufacturing methods will protect the environment and respect the rights of workers. As the device is used, it will gradually lead to healthier communities, contributing to the common good. If all these steps are taken with care and ethical leadership, then the greatest good will be done for the greatest number of people.

Table 6: Ethical Analysis. Breakdown of Ethical Framework for low-cost bacterial diagnostic device

	Justification	Embedded Concerns	Decision-Making
Utilitarianism	Better diagnostic tools contribute to preventing disease	Alternative technologies may solve the issue better	Stay informed about other research and technology to ensure that our device develops in a way that best meets healthcare needs
Rights & Responsibilities	We have responsibilities as engineers to protect human dignity and life	1. Environmental Responsibility in manufacturing and disposal of device. 2. Responsibility to create safe work environment for those who assemble the device	1. Use cellulose as substrate because it is cost-effective, safe, and environmentally friendly. 2. Construct ethical business model that respects rights of workers
Justice & Fairness	Our devices are affordable and therefore accessible to resource-limited communities	Proper distribution and delivery of the device in resource-limited settings	Partner with global health organizations, such as the WHO or UN, to use their expertise in delivering the technology to those in need
Common Good	Healthy communities are more prosperous, and provide a better quality of life	Proper use of device is required to actually help the end-users	Design user-friendly device that is intuitive and practical for real-world settings across cultures
Virtue	Good biomedical engineers demonstrate values that serve society, such as integrity and compassion	Staying informed about society's needs and consistently acting in an upright and ethical manner	Collaborate with respected colleagues to further develop ethical character and decision-making skills

CHAPTER 13: CONCLUSION

The research presented in this report is foundational for the future growth of this device. We were posed with the challenge of creating a bacterial detection device that could be used in the developing world because current methods fail to meet the WHO's ASSURED criteria. With these criteria in mind, we have developed a platform to detect bacterial pathogens in solution. We demonstrated that an oligonucleotide sandwich assay is specific and sensitive enough to detect varying concentrations of Target oligonucleotide. We have fabricated a microfluidic chip that carefully controls the movement of fluids, allowing for future integration of the detection assay onto the device. The direction of this research is promising, as our current data has validated the device components to this point. Future work built on the research we have complied thus far promises to achieve the goal of an ASSURED device for bacterial pathogen detection.

Table 7: ASSURED Components of Device. Evaluation of our bacterial detection method against the WHO's ASSURED criteria.

A	S	S	U	R	E	D
Affordable	Sensitive	Specific	User-Friendly	Rapid and Robust	Equipment Free	Deliverable to End Users
Paper-based	Can determine varying concentra-tions of Target	Oligonuc-leotide sandwich assay to test for specific bacterial pathogens	Autono-mous microfluidic device	Device materials	Smart phone and applicati-on used for analysis	Durable, manufactu-rabl device.

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APPENDICES

Oligonucleotide Sequences

Sequence Name	Nucleotide Sequence 5'-3'	Modifications
Capture	TTT GTC AAT GAG CAA AGG T	5' Phosphate Addition
Capture+Tag	TTT GTC AAT GAG CAA AGG T	5' Phosphate Addition, 3' C3 Fluorescein
Target	GGG GAG GAA GGG AGT AAA GTT AAT ACC TTT GCT CAT TGA C	None
Target+Tag	GGG GAG GAA GGG AGT AAA GTT AAT ACC TTT GCT CAT TGA C	5' CAL Flour Red 610
Detection	TAC TCC CTT CCT CCC CTT TTT TTT TT	3' Thiol Addition

Cost Calculations: Material costs based on purchased

Materials	Cost per Device
Cellulose	$0.001
Zirconia	$1.00
Capture DNA	$0.60
Detection DNA	$0.50
Gold Nanoparticles	$1.40
Wax	$0.01
Gold Enhancement	$1.25
ESTIMATED COST per DEVICE	$4.76

Materials

Material	Item Number	Vendor	Location
Gold Nanoparticles	741965	Sigma Aldrich	St. Louis, MI
Zirconia	771600	Sigma Aldrich	St. Louis, MI
Denhardt's Solution	750018	Invitrogen	Grand Island, NY
DTT	D9779	Sigma Aldrich	St. Louis, MI
Silver Enhancement (Solutions A & B)	A: S5020 B: S5145	Sigma Aldrich	St. Louis, MI
Salmon Sperm DNA	15632011	Invitrogen	Grand Island, NY
Gold Enhancement Kit	2115	Nanoprobes	Yaphank, NY