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## Rapid, Low-Cost Biosensor for Water-Borne Pathogens in Low-Resource Environments

Kimberly Cribbs

*University of Arkansas, Fayetteville*

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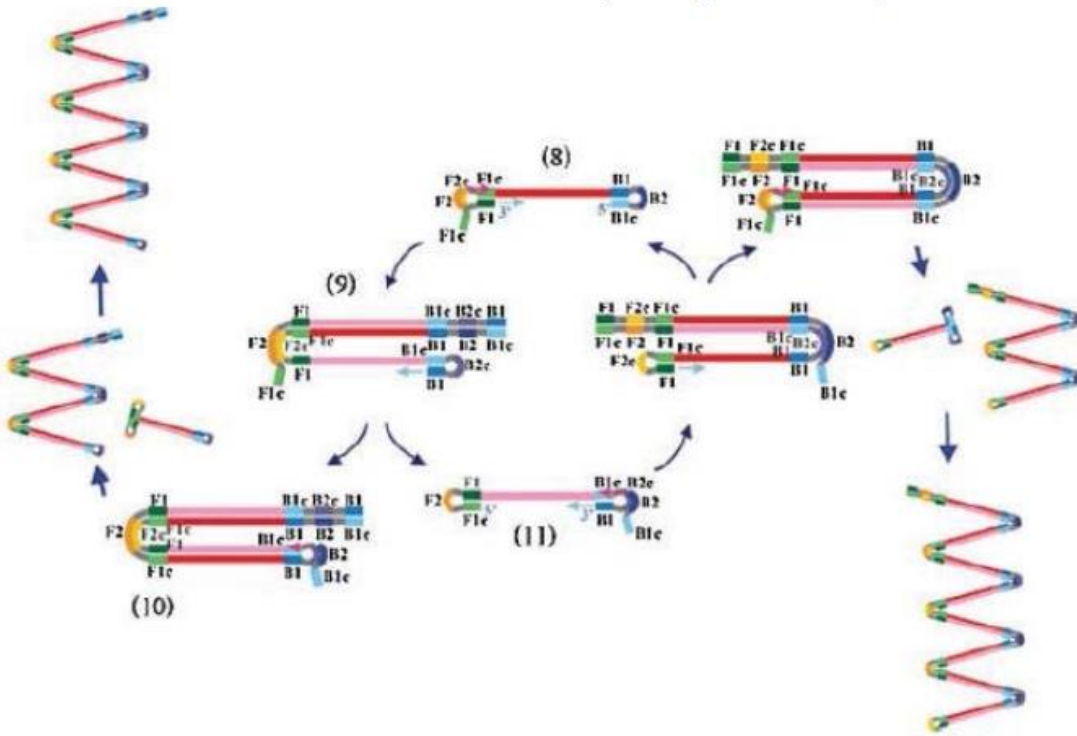
## A. Summary of Phase I Results

### 1. Background and Problem Definition

When testing water quality, “microbiological results typically require a minimum of 24 hours to complete”<sup>1</sup> due to reliance on the traditional and inexpensive cultivation method. For example, the Hach PathoScreen Medium Presence/Absence Powder Pillows, a simple presence-absence test to detect waterborne pathogens without the need for an incubator in warm climates, is approximately \$1.17 per test and requires 24-48 hours to complete.<sup>2</sup> Advancements in molecular biology in the past four decades has led to the development of more rapid methods of microbiological assessments. In the last two decades of the 20<sup>th</sup> century, polymerase chain reaction (PCR), a DNA replication method that is rapid (requiring a few hours to complete), sensitive, and specific, was invented.<sup>1</sup> PCR requires the use of a thermocycler, a traditionally expensive machine costing \$650-\$8,000, to alternate the temperature during the reaction. In 2000, an isothermal DNA amplification method termed loop-mediated isothermal amplification (LAMP) was invented allowing for microbiological assessments to be performed in less than one hour without the need for an expensive thermocycler.<sup>3</sup> In 2013, the World Health Organization reported LAMP as an “attractive diagnostic platform for resource-poor settings: it is fast [15-40 min], isothermal [requiring only a heat block], robust to inhibitors and reaction conditions that usually adversely affect PCR methods, and it generates a result that can be detected with the naked eye.”<sup>4</sup> Typically an electric heat block, costing \$80-\$350, is used to maintain an isothermal temperature during LAMP reactions. The WHO report notes the need to consider operational issues such as a lack of continuous electricity supply, limited storage and bench space, and storage settings where temperatures are above the manufacturer’s recommendation when working in low-resource environments. As commercial LAMP assays begin to enter the market,<sup>4</sup> there is need for a low-cost, compact, and LAMP-specific heating block that can be used in resource-poor environments. Furthermore, there is a need to utilize LAMP and the heating block for resource-poor environments to decrease the time needed to test drinking water for microbes at a price that competes with the traditional cultivation method. The use of LAMP for detecting microbes in water along with a heating block for resource-poor environments has the potential to advance people, prosperity, and the planet in developed and developing countries.

### Background on LAMP

LAMP is best understood by watching a short video located at <https://www.youtube.com/watch?v=ZXq756u1msE>. The basic tenet of LAMP relies on the unique capability of Bst polymerase to provide complementary strand synthesis while causing strand displacement, eliminating the requirement of changing temperatures for denaturation and annealing, as is necessary for conventional PCR<sup>5</sup>. LAMP requires six DNA primers that are easily designed based on complementary requirements. As LAMP proceeds, a single strand structure is formed that is self-complementary owing to two loop structures that are formed at the 5’ and 3’ end of the DNA molecule. Continued cycles generate a spectrum of sizes, not a single size as in conventional PCR, that contain multiple copies of the target being amplified. Within half an hour, the cycling reaction can amplify the target sequence up to 10<sup>9</sup> copies. The final products are stem-loop DNAs containing inverted repeats, and cauliflower-like shapes of alternating inverted repeats (Figure 1).



**Figure 1.** Summary of LAMP

Structure (8) is the self-complementary loop that forms the basis of LAMP. Subsequent rounds produce DNA of different lengths comprised of multimers of the sequence of interest.

### Background on chosen water-borne pathogen

Water pollution caused by fecal contamination is a serious problem due to the potential for contracting diseases from water-borne pathogens. Due to the wide range of water-borne pathogens, it is difficult and expensive to conduct a complete analysis for every type of pathogen.<sup>6</sup> As a result, the presence of pathogens is determined with indirect evidence by testing for an “indicator” organism. The presence of an “indicator” organism “does not guarantee that the water will cause an illness. Rather, their presence indicates that a contamination pathway exists between a source of bacteria (surface water, septic system, animal waste, etc.) and the water supply.”<sup>7</sup> Currently, “*E.coli* is considered... the best indicator of fecal pollution and the possible presence of pathogens”.<sup>8</sup> This P3 project investigated the utility of LAMP in water quality assessment by testing for the *ipaH* gene of enteroinvasive *E. coli* and *Shigella flexneri*, pathogenic bacteria that cause profuse diarrhea and high fever. In 1999, the WHO reported that “the annual number of *Shigella* episodes throughout the world was estimated to be 164.8 million, of which 163.2 million were in developing countries.” It is estimated that *Shigella flexneri* was responsible for 60% and 16%, respectively, of *Shigella* episodes in developing and developed countries.<sup>9</sup>

## **Relationship to People, Prosperity and the Planet**

### **Benefit to people**

Each year more than 200 million people in developed and developing countries are affected by floods, earthquakes, and other natural disasters that often interrupt water and electricity services for extended periods.<sup>10</sup> Utilizing LAMP to detect *E. coli* and *Shigella* in a resource-poor environments has the potential to improve decision making regarding drinking water resources in disaster situations, resulting in better service to those affected.

### **Promote prosperity**

As LAMP-based detection kits for endemic diseases common in developing countries enter the market<sup>1</sup>, a low-cost heating block for resource-poor environments has the potential to make the kits more appropriate for developing countries. Increasing access to LAMP-based detection in low-resource medical clinics could improve the quality of health care by allowing for same day diagnosis. Overall, utilizing LAMP in resource-poor environments to detect endemic diseases and assess water quality will help prevent the spread of diseases. Maintaining a high quality of public health promotes prosperity in communities.

### **Protect the planet**

By 2050, the United Nations predicts that the population will increase by 2.4 billion people and that 85% of the global population will live in developing regions.<sup>11</sup> Currently, an estimated 1.8 billion people (25% of the population) lack adequate sanitation.<sup>12</sup> If substantial progress is not made in the sanitation sector, it is likely that water quality will continue to decline as the population density increases. The ability to cost-effectively monitor water quality in developing regions can help investigators determine when a water source becomes polluted. Awareness of pollution is the first step to developing an action plan to protect our planet's water resources.

### **Implementation of the P3 project as an educational tool**

Our P3 team contains five undergraduates, a biology major and four chemical engineering majors, who are supervised by a Ph.D. cell and molecular biology student and two (bio)chemical engineering faculty. The project forms the basis of honors thesis work for three of the students. Through working on the project, the students have applied knowledge and skills from a variety of fields, including chemical engineering, electrical engineering, and biology. Designing and building the prototype required knowledge of concepts such as heat transfer, heat capacity, materials, polymerase chain reaction, DNA-binding dyes, and the design of an electric heating component. All the students were involved in research, design, construction, and testing, but were divided into groups that focused on part of the overall design. This strategy allowed students to use their individual strengths. One group focused on the design and heating of the device, another found dyes and contributed designs for 3D printing using Solid Works, and a third group focused on designing and testing the PCR reaction. The groups met regularly to share progress and discuss ideas, and worked as a team to finish and test the prototypes. The team also collaborated with a local biotechnology company, NowDiagnostics, which gave students valuable experience in meeting and collaborating with professionals.

In designing the prototype, students had to balance multiple considerations including ease of use, accuracy, cost, and sustainability. Through the process of designing, testing, and redesigning the device, students were guided by principles of sustainability, such as reusability and environmental impact. In order to reduce waste and operating cost, the P3 team decided to construct a reusable prototype which demonstrates the heating principle for the LAMP device. Another consideration was the environmental impact of disposable components, which included choosing a dye that is non-mutagenic, non-toxic, and does not harm aquatic life.

In addition to its contribution to the education of the undergraduates involved, this project will be used as a tool to educate K12 students in engineering, sustainability, and science. The device will be used as an example of how engineering and sustainable design work, and demonstrate how engineering decisions are impacted by environmental concerns. Additionally, this device will be used to demonstrate multiple scientific concepts such as DNA replication, melting and freezing points, heat transfer, electricity, and dyes.

Finally, this project was also accepted as a 2016 Clinton Global Initiative University (CGI U) Commitment to Action in the focus area of public health. Three undergraduate members of the research team travelled to Berkeley, California to represent this Commitment to Action. While at Berkeley, the students participated in the CGI U Exchange, an exhibit where students and sponsors showcase their Commitments to Action and organizations to approximately 300 visitors. The students presented a poster of their EPA P3 research.

## **2. Purpose, Objectives, Scope**

The United Nations estimates that water-borne illness accounts for 80% of deaths in developing nations where nearly 1 in 6 do not have access to clean water.<sup>12</sup> Annually more than 200 million people in developed and developing countries are affected by floods, earthquakes, and other natural disasters that often interrupt water and electricity services for extended periods. As an example, consider the people of Louisiana and the impact of hurricane Katrina. During this emergency, at minimum 2.4 million people were without access to safe drinking water.<sup>15</sup> When testing water quality, “microbiological results typically require a minimum of 24 hours to complete”<sup>1</sup> due to reliance on the traditional and inexpensive cultivation method. The World Health Organization describes LAMP as an “attractive diagnostic platform for resource-poor settings: it is fast (15-40 min), isothermal (requiring only a heat block), robust to inhibitors and reaction conditions that usually adversely affect PCR methods, and it generates a result that can be detected with the naked eye.” In 2011, Eiken Chemical Co. released the first LAMP-based tuberculosis detection kit designed to be simple enough and inexpensive enough to implement in resource-limited settings.<sup>1,1</sup> Two important considerations provide motivation for our P3 effort:

- *Although LAMP reagent/assay kits have been developed for a number of infectious diseases, there has been limited focus on the development of LAMP-based diagnostic for water-borne pathogens, and*
- *LAMP-based diagnostic platforms traditionally rely on conventional-PCR devices which are bulky and not easily deployed in a resource-poor environment. A resource-poor environment is one that lacks access to electricity and lab equipment.*

To wit, the overall objective of Phase I was to develop a low-cost, LAMP-based diagnostic platform capable of detecting the presence of water-borne pathogens in resource-poor environments by individuals with a modicum of formal training with the device. The principal technical objectives of Phase I included:

### **Heating**

*A method for providing heat to the device had to be designed and assessed.*

### **Visualization**

*A dye that could provide a positive result when the water-borne agent is present had to be selected.*

### **LAMP**

*A primer set for the model water-borne agent had to be designed for the *ipaH* gene shared in enteroinvasive *E.coli* and *Shigella flexina*.*

While there were three technical objectives proposed in Phase I of the project, the overarching goal was to construct a prototype capable of demonstrating effective temperature control for demonstration of LAMP.

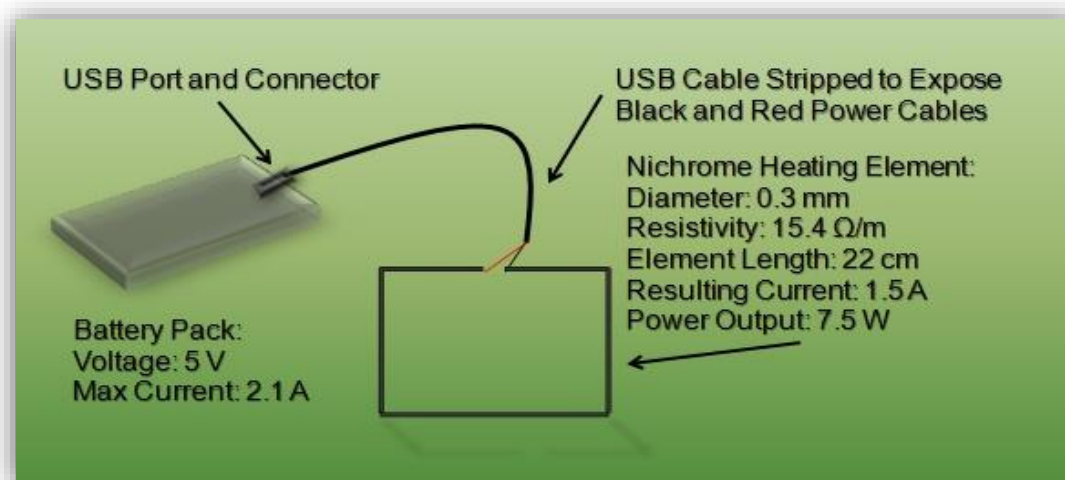
## **3. Data, Findings, Outputs/Outcomes**

### **Heating**

The objective of the device is to heat a sample of water containing LAMP reagents to 65°C such that DNA replication can be initiated and sustained for up to 45 minutes. Initially, three heating methods were considered: chemical heating, disposable microbatteries, and a rechargeable battery pack, the last method being chosen for this proposal. The case of the device is 3D-printed and contains a phase-change material, a heating wire, and a rack containing the water vials.

The rechargeable battery pack was chosen as an appropriate heating method because it minimizes waste, increases sustainability, and most importantly, may be recharged easily. In terms of waste and sustainability, while chemical heating methods like iron oxidation or forced crystallization are inexpensive and easily provide enough heat for a LAMP reaction, (i) the energy output cannot be precisely controlled, (ii) environmental factors like humidity and surrounding temperature cause these materials to fail, and most importantly (iii) require disposal when spent. Note that while microbatteries, like the ones that are used to power GPS trackers in fish, would provide improved control over power output, they do not provide nearly enough energy to sustain a LAMP reaction and still would require disposal of cadmium or similar metal.

Our final heating device consisted of a battery pack that provides current through a nichrome heating wire via a USB cable. The USB connector provides attachment to the battery, and as will be described in our Phase II proposal, is integral to the plans we have for the next phase of the prototype. At a diameter of 0.3 mm, nichrome has a resistivity of 15.4  $\Omega$ /m so that the resulting current through the heating element is 1.5 A for a wire length of 22 cm. Since USB devices operate at 5 V, the total power required of the device is a mere 7.5 W. Figure 2 is an illustration of the circuit as well as its relevant physical values.



**Figure 2.** Illustration of the device’s heating method

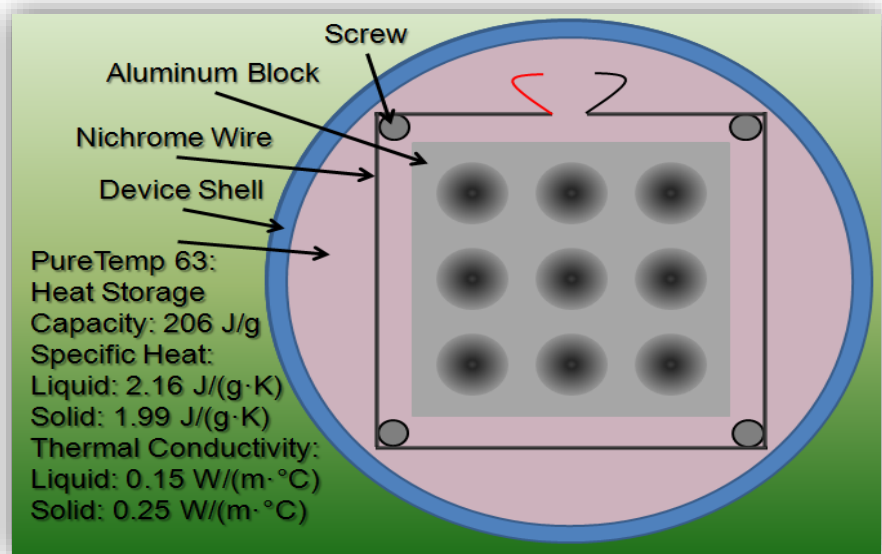
Once the heating method was chosen and constructed, the next phase of the development consisted of designing a housing capable of containing not only the nichrome element, but also a material that when used in the prototype virtually eliminates the need for thermocouple-based temperature control. As previously mentioned, virtually all useful LAMP-based detection methods still employ a PCR thermocycler. These devices, while capable of precise temperature control and cycling, use advanced process control and electronics to maintain temperature. In contrast, our prototype design uses a phase-change material to act as a thermal ballast. Phase-change materials are wax-like substances that upon initial melting, release heat as they are cooled back to a solid state. This solid / liquid transition provides excellent temperature control without the use of a thermocouple or ancillary electronics. In other words, the inclusion of phase-change material in the design greatly drives simplicity, which in turn keeps cost down and eliminates the use of electronics which eventually may end up in a landfill.

Several prototype devices were constructed via 3D printing and assessed for the following:

- *Reasonable start-time required to melt the phase-change material obtained from Entropy Solutions LLC.*
- *Ability to monitor the melt transition visually.*
- *Ability to maintain temperature at a value capable of supporting LAMP.*

With regards to the phase-change material, the company provided us with a material capable of maintaining 63°C, which is a reasonable temperature for LAMP.

Figure 3 is a diagram that provides a schematic of the first functional prototype. In addition to the phase-change material, prototype #1 included an aluminum heating block traditional to most PCR devices. The device was designed to hold nine small microcentrifuge tubes common to LAMP and conventional PCR.



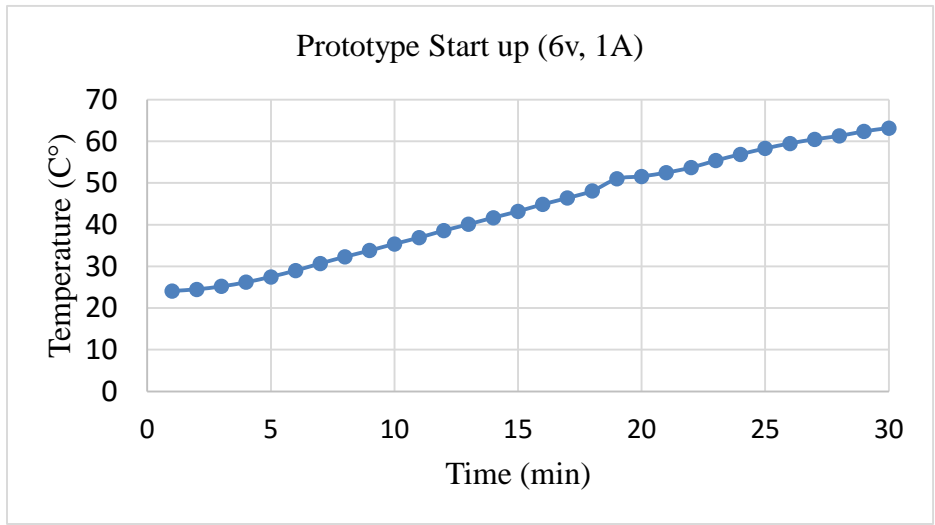
**Figure 3.** Functional Prototype #1

To examine the heating characteristics, the device temperature was raised to approximately 65 °C. Once the device reached this temperature, evident by the melting of the phase-change material, a 50  $\mu$ L Eppendorf tubes containing a water sample and thermocouple was placed in the aluminum block. During the test phase, the biosensor's temperature needed to be maintained between 63-67 °C, a reliable range wherein DNA replication can occur. Temperature control was achieved simply by turning the battery pack on and off and observing the state of the phase change material.

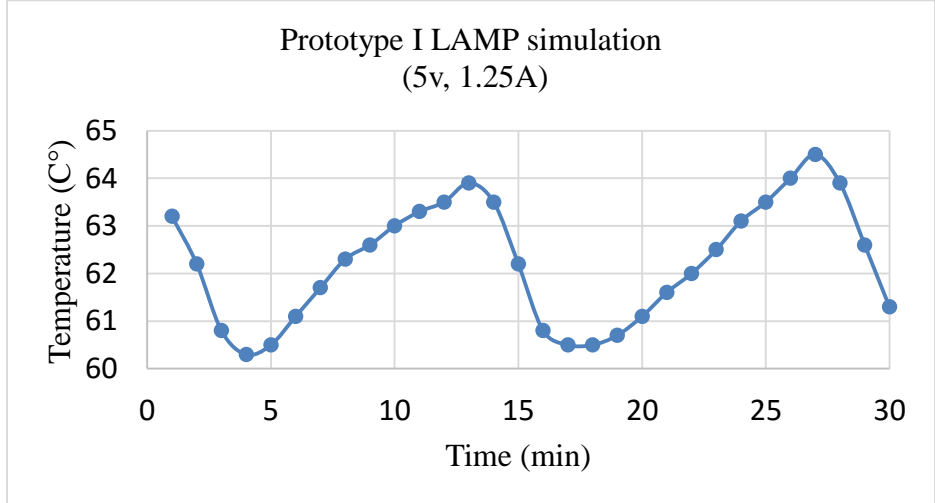
Examination of the temperature profiles indicate the success of the first prototype since a temperature of 62 °C +/- 2 °C was obtained. Figure 4 shows how the device required 30 minutes to heat up to approximately 63 °C. Figure 5 illustrates one simulated LAMP cycle for thirty minutes. The final functional prototype #1 (Figure 6) included the addition of a thermochromic material that changed from RED to CLEAR at 62°C, a small round footprint, and metallized polyethylene terephthalate lining to help reduce heat loss.

Our first functional prototype, “hockey puck”, was determined to lose heat to the surroundings via the aluminum that surrounded the tubes and the four windows that allowed for observation of the phase-change material. A second functional prototype was printed that simply had holes for the microcentrifuge tubes, eliminating the aluminum block, and a single window (Figure 5).

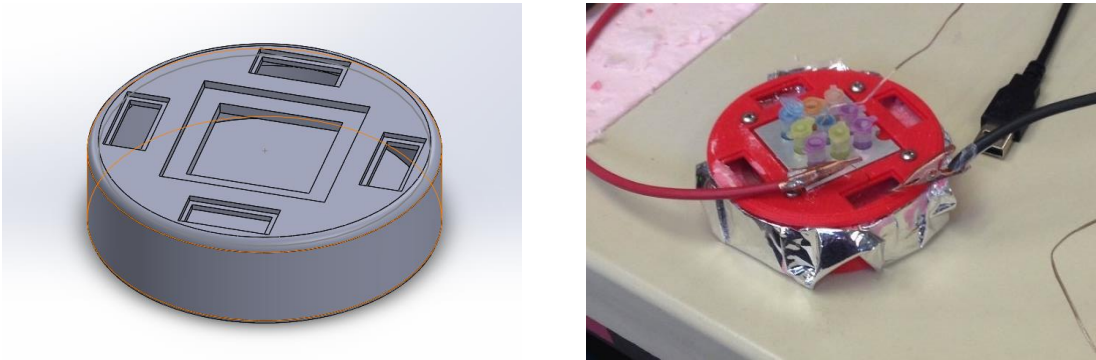




**Figure 4.** Temperature profile for the heating-up of prototype #1

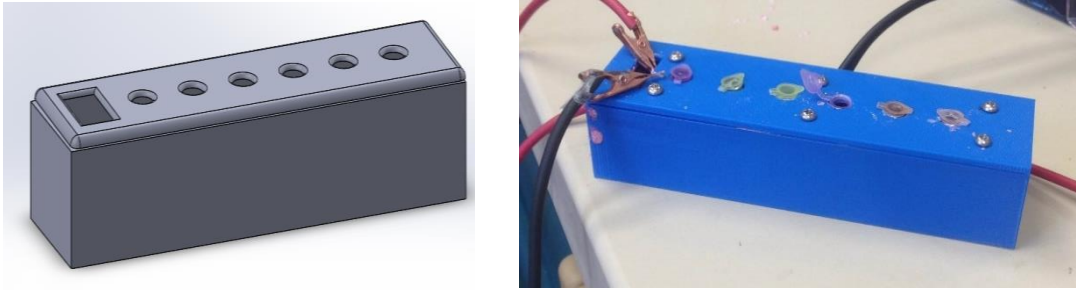


**Figure 5.** Temperature profile for the LAMP simulation on Prototype I



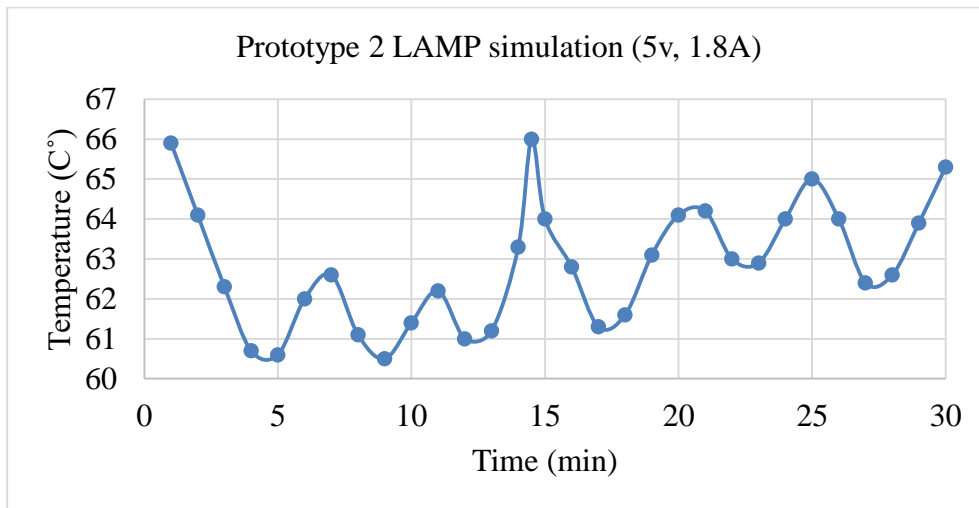
**Figure 6.** Hockey Puck functional prototype #1. Left panel is solid works rendering used to provide 3-D print instruction. Right panel is device.

The aluminum block was removed in prototype #2 (Figure 7) and the PCR tubes were put directly into the phase change material. In order to evenly heat, the holes were alighted and the device was made taller. This design required slightly more wire, with half of the wire as nichrome and the other half copper. The nichrome wire acted as a resistor and gave off heat, while the copper simply conducted the charge. This modification to the wire cut down on the energy required to heat the phase change material to its desired temperature: 63 °C. This change made a significant impact in the time it took to heat the phase change material during start up and PCR.



**Figure 7.** Stick functional prototype #2. Left panel is a Solid Works rendering used to provide 3-D print instruction. Right panel is the physical device.

Due to time constraints, a single temperature characterization was completed with prototype #2. Figure 8 demonstrates that this simple design maintained temperature, 63 °C +/- 2 °C.



**Figure 8.** Graph of temperature change over time during a 30-minute PCR using prototype #2.

## Visualization

Fluorescent dyes are the most common method of indicating the presence of DNA post-PCR. The function of these dyes is to bind to the sugar-phosphate backbone of DNA: the chemical structure of the dye (a positively charged compound) adheres to the sugar-phosphate backbone of DNA (a negatively charged compound). When exposed to a certain wavelength of light – its excitation wavelength – the dye will emit a specific wavelength of light. Therefore, a sample containing DNA will exhibit a fluorescence which can be visually observed. Originally, the project aimed to use SYBR Green, a well-known and widely-used dye for DNA quantification purposes. However, a characteristic of the majority of fluorescent dyes – including SYBR Green – is their inhibition of the DNA replication process as a result of their bond to the chemical structure of DNA. In order to simplify the device and minimize steps in the LAMP process, a new dye – EvaGreen – was chosen that is specifically designed for PCR applications. This allowed the dye to be added to the solution pre-PCR with no inhibition of DNA replication.

There are several unique characteristics of EvaGreen dye that make it useful for LAMP applications. The key property of EvaGreen is that it is less inhibitory toward PCR and less likely to cause nonspecific amplification. As a result, EvaGreen can be used at a much higher concentration, resulting in a more robust signal and eliminating dye redistribution problems. The excitation and emission wavelengths of EvaGreen are comparable to commonly-used dyes: both are within the visible spectrum of light, making a sample containing DNA visible to the human eye. Another beneficial property of the dye is its safety for use and disposal. EvaGreen is non-mutagenic and non-cytotoxic; it is also completely cell-impermeable, a factor responsible for its low toxicity. These features are significant because typical DNA dyes like SYBR Green are powerful mutation enhancers, inhibiting the natural DNA repairing mechanism in cells. The toxicity of SYBR Green is associated with its ability to enter cells rapidly. Finally, EvaGreen is classified as nonhazardous for drain disposal, and is classified as nonhazardous to aquatic life. As a result, the selected dye is favorable for the intended use and provides secondary environmental advantages over standard fluorescent dyes.

To prove that EvaGreen is able to be visually observed, a standard was prepared with known quantities of DNA and dye. Volumes of a known concentration of DNA, ranging from 20 $\mu$ L to 0.1 $\mu$ L, and a constant volume of 5 $\mu$ L of EvaGreen was added to every sample, totaling 25 $\mu$ L each sample. The standard shows that at relatively high concentrations of DNA, the visual signal is very strong and can be observed easily (**Figure 9**). At concentrations of DNA between 0.5 $\mu$ L and 0.1 $\mu$ L, the signal becomes visually unreadable. Thus, the target DNA range for PCR amplification is between 0.5 $\mu$ L and 1 $\mu$ L of DNA, which will make the sample easily observed.



**Figure 9.** EvaGreen standard.

## LAMP

Since the ipaH gene is common to enteroinvasive *E.coli* and *S. flexina*, it was chosen as a measure of water-borne pathogen contamination. LAMP requires six DNA primers to amplify the DNA target. Primer Explorer was used to design six primer sets for ipaH. One set is included in Table 1 as an example.

**Table 1.** Representative set of ipaH primers.

Primer Information												
1	ID:16	dimer(minimum)dG=-2.22		label	5'pos	3'pos	len	Tm	5'dG	3'dG	GCrate	Sequence
F3	73	92	20	60.36	-6.10	-6.24	0.50	GCTGGAAAACTCAGTGCCT				
B3	252	269	18	59.00	-5.02	-6.76	0.56	GGAACATTTCCCTGCCCA				
FIP			40					CATGTGAGCGCGACACGGTC-ACAGCAGTCTTTCGCTGTTG				
BIP			42					ATCTCCGGAAAAACCCTCCTGGT-AGCGCCGGTATCATTATCGA				
F2	106	125	20	60.53	-5.80	-4.66	0.50	ACAGCAGTCTTTCGCTGTTG				
F1c	152	171	20	65.57	-4.71	-6.18	0.65	CATGTGAGCGCGACACGGTC				
B2	222	241	20	60.74	-7.76	-4.17	0.50	AGCGCCGGTATCATTATCGA				
B1c	177	198	22	65.28	-4.59	-5.84	0.55	ATCTCCGGAAAAACCCTCCTGGT				

## 4. Discussion, Conclusions, Recommendations

Our Phase I project demonstrated that virtually all electronic components of a LAMP-based water borne pathogen detection system may be constructed based on the unique properties of phase-change materials. The 3-D printed prototypes, envisioned to be close to a final design, cost less than \$5.00 in material cost, making this system attractive as a means to provide this method of testing to both point-of-care and/or low resource settings. If selected for Phase II, the group plans to test for ipaH in control (DNA only) and actual water samples, refine the prototype and develop a simple set of instructional material for its use in both classroom and real-world environments, and finally make the device available to healthcare professionals and those responsible for water quality assessment.

## 5. Assurance statement

The University of Arkansas, through the Provost and Vice Chancellor for Academic Affairs, Academic Initiatives and Integrity strives to create a culture of honesty and personal and professional responsibility among its students, faculty and staff. As a community of scholars, we uphold academic integrity as foundational to appropriate conduct within the university setting. Academic Initiatives and Integrity manages outreach efforts for policy education and facilitates the University's process for alleged violations with the All University Academic Integrity Board. The Academic Integrity Policy was strictly followed throughout this project.

## B. Continued work

NowDiagnostics and the University of Arkansas will continue to develop the prototype into a portable LAMP device. P3 team member, Brandon Hart, will continue working on this project next year as a junior. This summer, he will be traveling to Ghana, Africa to do water

purification research. He has been accepted into the Southern University and A&M College International Research Experiences for Students Global Research Activates in Sustainable Water Purification Program. During the program, he will be conducting research on removal of naturally-occurring, excess fluoride from ground water. The goal of the research is to make the water safer for drinkable. He will be staying at the Kwame Nkrumah University of Science & Technology for four weeks before returning to the United States. During his stay, he will have the opportunity to perform a field test of our device. He will visit villages that do not have a reliable source of clean water. This opportunity will allow him to take water samples from the current water sources for testing our device. Field experiments of our device will be a natural extension of his research in Ghana.

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