# PATHOGEN DETECTION WITH LOOP MEDIATED ISOTHERMAL AMPLIFICATION

by

Raghav Khanna

B.S. in Electrical Engineering, University of Pittsburgh, 2007

Submitted to the Graduate Faculty of

Swanson School of Engineering in partial fulfillment

of the requirements for the degree of

Master of Science in Electrical Engineering

University of Pittsburgh

2010

UNIVERSITY OF PITTSBURGH

SWANSON SCHOOL OF ENGINEERING

This thesis was presented

by

Raghav Khanna

It was defended on

July 14th, 2010

and approved by

Alex Jones, PhD, Professor, Department of Electrical and Computer Engineering

Mahmoud El Nokali, PhD, Professor, Department of Electrical and Computer Engineering

Zhi-Hong Mao, PhD, Professor, Department of Electrical and Computer Engineering

Thesis Advisor: William Stanchina, PhD, Professor, Department of Electrical and Computer Engineering

**PATHOGEN DETECTION WITH LAMP**

Raghav Khanna, MS

University of Pittsburgh, 2010

Recently, a novel methodology termed Loop-mediated isothermal amplification (LAMP) was reported as the preferred technique for rapid diagnostic testing of pathogens. The advantages of LAMP over older techniques are its economic viability, rapidity and its obviation of complex instruments. Reported here is an interdisciplinary approach between medical practices and engineering to implement an affordable diagnostic device which employs LAMP for detection of pathogens. LAMP involves the optical excitation and detection of a pathogen sample mixed with a fluorescent dye as it is heated and amplified over an hour. The device reported here consists of readily available components which heat, optically excite and detect a LAMP sample. Finally, the integration will graphically display the amplification of the LAMP sample as a function of time on a palmtop computer by exploiting the ubiquitous 802.11 wireless standard. The diagnostic box implemented here and its supporting components accurately discriminated between positive (infected) and negative (not infected) LAMP samples of various pathogens in approximately one hour. These results were verified using the standard method of pathogen diagnosis termed electrophoresis. Due to the low cost and portability of the device reported here, it poses as a potential solution to the need for quality point of care diagnostic tools in developing countries.

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## PREFACE

The author would like to thank and acknowledge the following people for their support in the completion of this project: Dr. William Stanchina, Chairman of the ECE department at the University of Pittsburgh for advisement and direction on the project; Dr. Abhay Vats (MD) of Childrens Hosptial, University of Pittsburgh Medical Center, the principal investigator of the project. Additionally, the following people provided concrete support to the project: Dr. Alex Jones of the ECE department at the University of Pittsburgh; Dr. Di Gao of the Chemical Engineering department at the University of Pittsburgh; Dr. Mahmoud El Nokali, of the ECE department at the University of Pittsburgh; Dr. Zhi-Hong Mao of the ECE department at the University of Pittsburgh; Dr. Nilesh Dhamne (MD) of University of Pittsburgh Medical Center; Dr. Chandra Ishwad of UPMC Biological testing; Mr. Ashok Gurung of UPMC Biological testing; Mr. Ashish Yeri, PhD. candidate at the University of Pittsburgh in Chemical Engineering; Mr. Michael Rothfuss, PhD candidate at the University of Pittsburgh in ECE; Mr. Jim Lyle of the ECE staff; Mr. Bill Mcgahey of the ECE staff; and the staff in the University of Pittsburgh Engineering Machine Shop: Andy Holmes, Maxwell Pless, Micah Toll, Thorin Tobiassen.

Finally, the author would like to thank all of his family and friends for their support.

# 1.0 INTRODUCTION

## 1.1 PROBLEM STATEMENT AND GENERAL BACKGROUND

As technology and medical practices progress, it is incumbent in research to bridge the gap between the two widely disparate fields in order to apply pragmatic solutions to existing medical problems. For example, in developing countries there exists an emergent demand for the implementation of rapid, portable and affordable diagnostic tools. The purpose of this project is to satisfy the aforementioned emergent demand and design a diagnostic device that is economically viable, portable, time efficient and easy to use. Ultimately, the device would be utilized by physicians in clinical settings of developing countries as a routine point of care diagnostic tool. Even in technologically advanced countries like the U.S., such advances will contribute to potentially lowering the cost of health care.

The invention of DNA amplification has proven to be a plausible solution to the above described demand. [1] Various methods in DNA amplification have become vital world-wide, where they can be used in clinical settings to diagnose infectious diseases as well as genetic traits and disorders. However, older techniques in DNA amplification have proven to be expensive, non-portable and require a highly trained professional to operate. For example, the "Polymerase Chain Reaction" (PCR) was the original method used for diagnosis of pathogens with DNA amplification. [1] For the purposes of this project, PCR has a few shortcomings. One such

example is that only a highly trained professional can use PCR machines thereby limiting their use primarily to developed countries only. [1] Another drawback of these machines is the time required for a diagnosis to be made. These devices can take several hours up to and including a full day for a diagnosis to be made. [2] This would defeat the purpose of obtaining a diagnosis in a timely manner which is vital so that proper precautions can be taken promptly upon obtaining a positive (infected) result. Furthermore, PCR requires a second independent methodology, termed electrophoresis in order for the diagnosis to be obtained. The PCR machine is also large and cumbersome and is therefore not an easily portable device. Finally, the cost of PCR machines is approximately $25,000 which is not feasible for the intended widespread application. [2]

Recently, a novel technique in DNA amplification termed "Loop mediated isothermal Amplification" (LAMP) was reported as the means to obtain a rapid and simple diagnosis of pathogens. [3] If employed properly, LAMP can significantly alleviate some of the limitations associated with PCR. LAMP is a simple methodology involving the heating of a pathogen sample mixed with DNA amplifying reagents known as "primers" at a constant (isothermal) temperature. This obviates the need for the complex and cumbersome instrumentation utilized in PCR. Furthermore, the need for a highly trained professional is mitigated by LAMP's simplicity.[3]

The diagnosis of the pathogens is obtained by observing the amplification of positive (infected) LAMP products while also observing that the negative (un-infected) LAMP products remain relatively un-amplified. The term "amplify" here means to replicate or duplicate. When the LAMP products are heated to approximately 65 degrees Celsius, the primers will only replicate the DNA if the pathogen's DNA is present in the solution. Each pathogen has its own unique cocktail solution consisting of the primers for that pathogen. The presence of the specific

primers for an individual pathogen in the cocktail solution is what allows the infected DNA to amplify and for the uninfected DNA to remain unamplified. This amplification of infected DNA leads to the diagnosis. The reaction typically requires an hour to complete such that an accurate distinction between positive and negative samples is obtained. [3]

Traditionally, the diagnosis of LAMP products can be obtained in two different ways. First, the distinction between positive and negative samples can be visually discriminated when the amplified products are mixed with a DNA-binding fluorescent dye and transilluminated with ultra violet light. (a picture of a UV transilliuminator will be shown in a subsequent section) Here, a positive sample fluoresces at a greater intensity than a negative sample indicating larger sample amplification for the positive sample. [1][2]Unfortunately, the UV transilluminator is not portable which prevents its practical use as a routine and rapid point of care tool in developing countries. The second method of diagnosing a LAMP product is to run the reaction in a "Real Time PCR Machine." These machines have the ability to record and display the amplification level of the products over time. It would be expected that the amplification of the positive sample over time would significantly exceed that of the negative control sample. Thus by comparing the amplification plot of a sample with unknown contents with that of a negative control sample – a diagnosis is made. However, the "Real Time PCR" machines that are capable of performing these computations and diagnoses cost between $25,000 and $40,000 which is not manageable for the intended purpose of this project. [1][2] Thus the premise of this project is to implement LAMP in a fashion that is affordable, portable and easy to use.

## 1.2 SOLUTION TO PROBLEM STATEMENT

The implementation reported here takes the previously two described diagnostic methods of LAMP (UV excited fluorescence and amplification plotting) and incorporates them into one unique, portable, user-friendly and cost-effective solution. The DNA binding fluorescent dye is mixed with the LAMP sample before the reaction. The sample/dye cocktail solution is placed in a test-tube which is inserted into an aluminum block. This aluminum block is heated to 65 degrees Celsius (as required for the LAMP reaction) by sitting on top of a simple, customized and portable heater. Optical channels for excitation and detection traverse perpendicularly through the aluminum block such that an ultra violet light emitting diode can excite the fluorescent dye mixed with the cocktail solution. As the cocktail solution is heating, the UV LED excites the dye resulting in a fluorescence of the cocktail solution in the green spectrum. As the heating and UV excitation continue, the amplification and resulting fluorescence of the cocktail solution intensify according to the characteristic curve previously described. This fluorescence progression, or lack thereof, is detected using opto-electronic detection circuitry. The research results to be presented here show clearly distinguishable measured characteristics for positive versus negative detection of multiple varieties of pathogens. The output of the detection circuitry is routed into an analog to digital converter where the 802.11 wireless standard will be utilized to display the characteristic curve on a palm-top computer. A diagnosis is made by comparing this characterization of a LAMP sample (with unknown level of infection) against the same characterization of a known negative control sample.

For the purpose of demonstrating the beneficial use of this simple affordable design, the methods of PCR and LAMP will first be explained more in detail before describing this current

diagnostic tester design and performance. The general theory of fluorescence will also be briefly described.

# 2.0 PCR, ELECTROPHORESIS, LAMP & FLUORESCENCE

## 2.1 PCR

The Polymerase Chain Reaction (PCR) was invented by Kary Mullis in 1986. It is a method in DNA amplification that can be employed for diagnostic testing. It was initially used by a group in the Human Genetics Department at Cetus for the purpose of DNA amplification as well as prenatal diagnosis of sickle cell anemia.[4]

Since its invention, PCR has become a widely applied method. PCR is the combining of oligonucleotide primers, deoxynucleotide triphosphates in an appropriate buffer mixture which together are then heated and cooled for many hours until the desired amount of amplification is obtained. A "primer" is a strand of nucleic acid that is used as a basis for DNA replication.[4] PCR requires a repetitive number of cycles of temperature fluctuations, primer annealings as well as the expansion of annealed primers by DNA polymerase. The number of DNA copies approximately doubles at every cycle. Therefore, 20 cycles of PCR will lead to $2^{20}$ or approximately 1 million copies. [5]

A typical PCR is done in 50 to 100uL volumes. In addition to the sample DNA and the above mentioned primers, the following chemicals and their concentrations are often required: [5]

1. 50mM KCL
2. 10mM Tris. HCl
3. 1.5mM $MgCl_2$
4. 100 ug/ml gelatin

The amplification is then performed in a thermal cycler. A "Step-Cycle" type procedure is used. It entails the following: [6]

1. Denaturing at 94 degrees C for 20 seconds
2. Annealing at 55 degrees C for 20 seconds
3. Extending at 72 degrees C for 30 seconds

The above process is repeated for 30 cycles inside a "Real Time PCR Machine" or equivalently a thermal cycler. Apart from subjecting the samples to temperature fluctuations, the Real Time PCR machines have the ability to record and display the level of amplification of the products inside the machine over time. This attribute is vital and will be demonstrated as such in the subsequent section when performing LAMP in "Real Time PCR Machines" is discussed.[6]

The final PCR solution is then subjected to the DNA extraction technique of electrophoresis (to be discussed later). If different strands of DNA exist in the sample, the electrophoresis will disperse them. This is where the benefit of using PCR and subsequently electrophoresis for pathogen detection is revealed since an infected strand of DNA contains different properties from those of a non-infected strand. [7]

PCR has several attributes which make it an unattractive option for the purpose of this project. PCR is a relatively complicated and not a completely understood methodology. PCR is known to yield varying results from high quality to merely extraneous. This is due in part to the

dynamic kinetic interactions among the components which determine the quality of the products yielded.[7]

PCR requires a skilled person to perform these experiments. This diagnostic approach is not consistent with the purpose of this project. Also, the entire process can take many hours which is too long for a developing country where a medical professional must care for large numbers of people quickly. Finally, PCR is not easily portable and can cost as much $25, 000 which is too expensive for the goals of this project. [8]

## 2.2 ELECTROPHORESIS

When scientists study a material of biological origin it often becomes imperative to separate and examine the properties of the material. Such properties of interest can be the existing proteins and enzymes, the nucleic acids and the complex lipids and carbohydrates. For applications of PCR, it is the ability of electrophoresis to separate nucleic acids that is vital. This allows for the detection of infected DNA. After performing PCR on a sample, electrophoresis is used to determine if a pathogen is present in the sample.[9] Electrophoresis involves taking amplified PCR products and mixing them with an agorose gel. The resulting solution is placed in ladder wells and subjected to a potential gradient for approximately half an hour. The potential gradient extracts extraneous DNA sequences in the products leading to a diagnosis.

Electrophoresis describes the transition of a charged particle under the influence of an electric field. [9] When the particle has a constant velocity, the major force on the particle is the product of the effective charge on the particle and the potential gradient it is subject to. During electrophoresis, the movement of charged particles can occur through an agorose gel. [9]

Electrophoresis is its own process and isn't only performed after PCR. This means that using PCR and electrophoresis is a two-step process, both of which are time consuming. Furthermore, like PCR electrophoresis is a task that requires a highly trained professional. These observations lead to further limitations of PCR for timely and global diagnostic testing of pathogens.[8]

Electrophoresis can also be performed on amplified LAMP products in the same way. This allows for the reliable technique of electrophoresis to compare LAMP and PCR results of the same DNA. As will be discussed later, LAMP does not require electrophoresis for the diagnosis. *Figure 1* shows an electrophoresis result after LAMP was performed. In this assay, LAMP and electrophoresis were used to detect BKV virus. [2] As shown in *Figure 1* the sample containing BKV dispersed throughout the ladder-like network of the assembly indicating DNA extraction which would only happen for positive (infected) tests. This separation does not occur for the "NTC" (negative control).

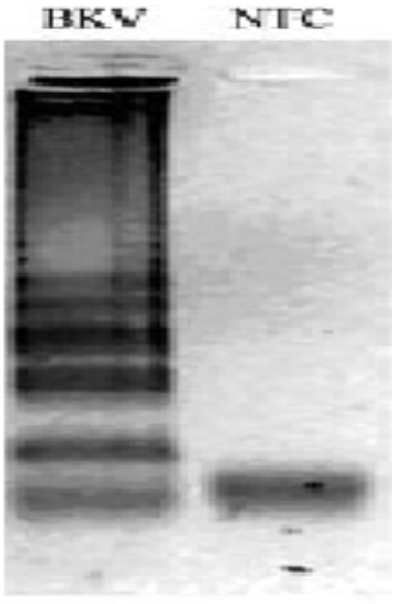


***Figure 1*: Detection of BKV virus on left hand side versus negative sample on right hand side. This is done by electrophoresis on LAMP products. Taken from Reference 2**

To summarize the shortcomings of PCR: It requires a precision instrument for amplification and also an elaborate method for the detection of the amplified products – specifically electrophoresis.[1][2] Additionally, the requirement for a high precision thermal cycler inhibits this method from being used globally such as in a clinic as a routine diagnostic tool. Also the potential for residual co-amplification of extraneous sequences limits the method for diagnostic use. [8] Finally, the equipment is too expensive, not easily portable and requires a highly trained expert to operate.

## 2.3 LAMP

To overcome the shortcomings of PCR, a novel method called LAMP was invented in 2000.[10] The technique was developed for rapid DNA amplification with high efficiency and specificity by Tsungunori Notomi at the Eiken Chemical Company in Japan. It can produce $10^9$ copies of DNA in less than one hour in contrast to PCR which can obtain one thousand times less copies in at least double the amount of time. [10] Additionally, the amplification can occur under isothermal conditions which alleviates the necessity for a thermal cycler.

LAMP can be done in a 25 microliter reaction mixture containing the following: [10]

1. 10mM KCl
2. 1 M beatine
3. 20 mM HCl
4. 10 mM $(NH_4)_2SO_4$
5. 4 mM $MgSO_4$

Hence the total volume for a LAMP reaction is less than PCR. The mixture is heated at approximately 65 degrees C for one hour. LAMP employs four primers initially and two more towards the end of the reaction. These primers can be used to simultaneously initiate DNA synthesis from the original unamplified DNA during the heating process.[11] This hastens the reaction for LAMP as opposed to PCR where the reaction is slow due to the thermal cycling.

LAMP has a higher sensitivity than PCR. In a test conducted by Notami and Okayama at the Eiken Chemical Company in Japan, six copies of Hepatitis were amplified to a detectable level.[11] The LAMP solution contained 100 ng of human genomic DNA. This did not

significantly inhibit the amplification. Conversely, the presence of 100 ng of human genomic DNA completely inhibited the PCR reaction performed under the same conditions as the LAMP reaction.[11] These findings demonstrate that LAMP is a more viable solution for diagnostic testing than PCR.

To summarize the advantages of LAMP: LAMP is a straight –forward methodology once the primers are prepared. LAMP can be done under isothermal conditions not requiring thermal cycling. LAMP can be done in far less time than PCR. LAMP is highly specific for the target sequence ensuring proper amplification of the correct product. This mitigates the general problem of extraneous product amplification associated with PCR. [1][2] Furthermore, the diagnosis of the sample does not require electrophoresis, but rather simply subjecting the sample to UV radiation when mixed with a fluorescent dye. This is the cost effective, qualitative approach. A more sophisticated approach is take the data recorded during the LAMP reaction and plot it as a function of time. This data is taken and displayed by Real Time PCR Machines. By comparing this graph with the corresponding graph of a negative control sample, a diagnosis is made. This is a more quantitative approach.

For a positive LAMP sample, the level of DNA amplification will be significantly greater than that of a negative LAMP sample. This is due to the presence of the primers in the reaction for any particular pathogen. For example, the LAMP "cocktail solution" for a pathogen like Ecoli requires a unique set of Ecoli primers in order for the amplification to take place. Therefore, if DNA is taken from a human being with Ecoli, and placed into the cocktail solution with Ecoli primers, the reaction will take place when heated and the DNA will amplify. Thus, after the reaction when the fluorescent dye is mixed in, it will have more DNA to bind to and will fluoresce intensely upon UV excitation. Conversely, when DNA from a completely healthy

human being is inserted into a LAMP cocktail solution with Ecoli primers, the DNA will not replicate when heated. As a result, there will be significantly less DNA for the fluorescent dye to bind to and the resulting fluorescence upon UV excitation after the reaction would be less intense. It is imperative to note that in order to test for any specific pathogen, the appropriate primers must be utilized. One cannot place a positively infected DNA sample of H1N1 virus into an Ecoli cocktail solution of Ecoli primers and obtain a positive result after running the LAMP reaction. Shown in *Figure 2* is the characteristic graph for positive LAMP samples as they are amplified over time.

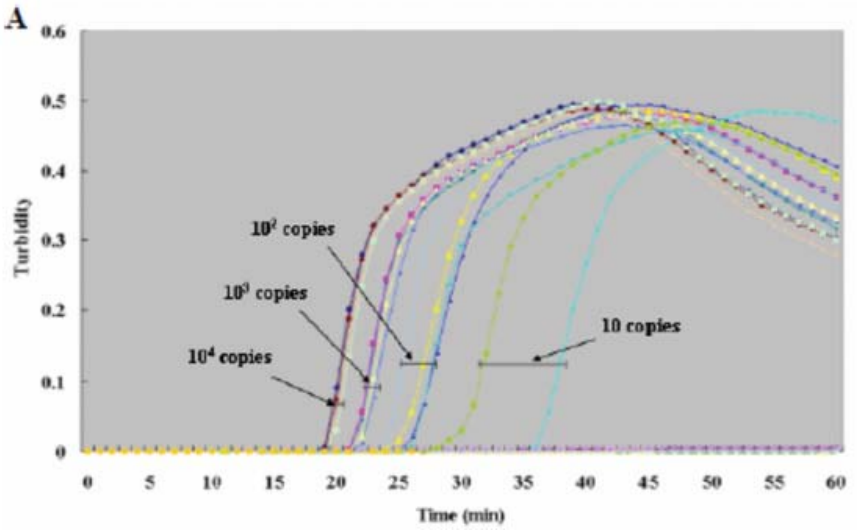


***Figure 2: Characteristic graph of a positive LAMP sample. This plots amplification over time. Taken from Reference 11. A negative sample is not shown, however it is expected that it would show insignificant amplification***

The characteristic curve of *Figure 2* and other LAMP curves are actually generated by Real Time PCR machines or thermal cyclers. As previously mentioned, these machines can cost $25, 000.[8] To obtain characteristic curves like *Figure 2*, LAMP is performed on a Real Time PCR Machine. The machine is set to oscillate between 63 and 67 degrees Celsius which is an adequate temperature range for the LAMP reaction to take place. Using this method for diagnosis of LAMP products is both sophisticated and expensive. [1][2]

The primary objective of this project would be to reproduce *Figure 2* by plotting the voltage output of a photodetector as it detects the fluorescence of a positive LAMP sample under UV excitation while also being heated at 65 degrees Celsius. This is feasible because the level of amplified products varies directly with the intensity of the fluorescence as described earlier. This would require one modification of the LAMP process which would be to add the fluorescent dye before the reaction as opposed to after the reaction. Thus, the goal here is to take extremely expensive technology and boil it down to an affordable point of care diagnostic tool. The total cost of the box reported here should cost no more than $300.

## 2.4 LAMP AT UPMC

In a test performed at the University of Pittsburgh Children's Hospital Research Center, Dr. Abhay Vats (principle investigator for this project) and his team used LAMP to detect E. coli in human urine in one hour. This test only required a heating block which negated the need for a thermal cycler.[1] Neither DNA extraction nor special processing were required for this test which alleviated the need for the time consuming process of electrophoresis.

The detection of a positive sample is made by identifying a color transition of the sample when a DNA binding dye is mixed after the reaction and is subjected to UV transillumination. [2] For infected DNA, the sample fluoresces a bright green under UV radiation. However for the non-infected DNA, the sample remains a dull green. This is shown in *Figure 3*.

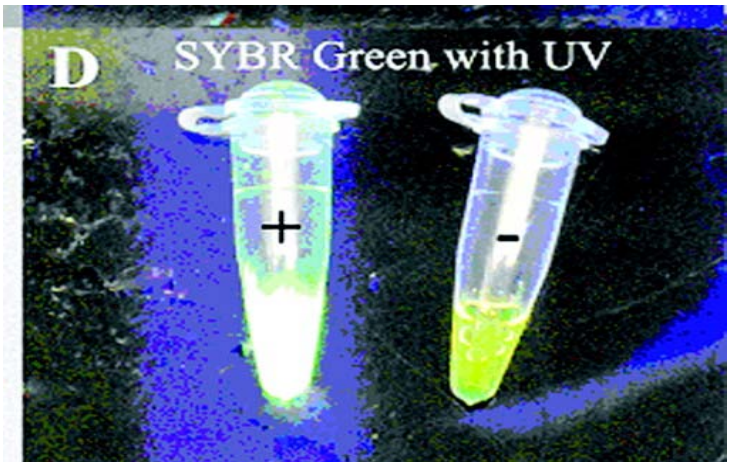


***Figure 3*: Distinction between infected (left) and non-infected (right) Lamp Products under UV transillumination. Taken From Reference 1.**

Since LAMP simply requires heating of an already pre-concocted solution it can be used to implement a simple point of care diagnostic box. The amplification takes place in real time and therefore a diagnosis can be made within an hour. This is the purpose of this project – to implement LAMP in a portable, affordable, user friendly instrument for pathogen detection. Shown in *Figure 4* is a picture of a UV transilluminator.

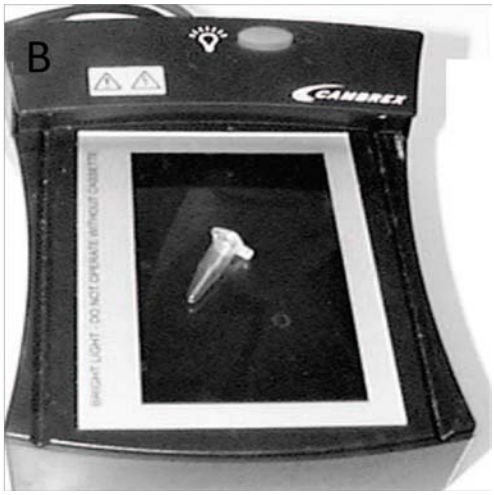


***Figure 4: A UV transilluminator is shown with a test tube on top. The UV transilluminator is not portable. Taken from reference 2.***

## 2.5 FLUORESCENCE

When light interacts with matter, it can either transmit through the matter or it can be absorbed by the matter either entirely or in part. Whether the light transmits through or is absorbed by the substance is dependent on the photonic properties of the substance. In cases where the light is absorbed, energy is fed to the molecules of the substance and they become "excited". This absorption of energy must occur in discrete units or quanta. The quanta – energy relationship is given by equation 2.1 below:[12]

$$E = \frac{hc}{\lambda} \quad (2.1)$$

In the above formula, "E" is the light energy absorbed by the molecule measured in joules. The term "h" is Planck's constant equal to $6.63 \times 10^{-34}$ joule -seconds, the term "c" is the speed of light equal to $3 \times 10^{8}$ meters per second and the term "λ" is the wavelength of the light.[12] Every molecule exhibits allowable energy levels for electrons to occupy. When light is absorbed by a molecule, the photons absorbed by the molecule cause a transition of the electrons to higher energy states. This absorption of radiation is unique for each molecule as the electron is elevated to an upper excited energy level. These transitions often occur from the lowest energy level of the molecule or the ground state to higher states. While the electron is in the excited state, some energy greater than the lowest vibrational level of the molecule is rapidly dissipated. Consider this " energy A". In addition, as electrons are excited to higher levels of energy, vacancies in the lower states begin to occur. The electron eventually dissipates all of its remaining excited energy and returns to the lower states while emitting its energy as a packet of light. This phenomenon is known as "fluorescence". [12] In light of the fact that some energy was lost in the brief period before the electron returned to the lower states (energy A), the

emitted energy is less than the absorbed and is hence always of longer wavelength than the excitation wavelength that was absorbed. This can be observed by analyzing equation 2.1 where a larger wavelength is indicative of less energy. [12]

For our application, the key molecule responsible for photon absorption and re-emission is SYBR green dye. Since this dye fluoresces at roughly 520 nm, it is imperative to use an excitation light of wavelength smaller than that to ensure fluorescence occurs.

## 3.0 INITIAL TESTING

Initially, amplified LAMP products were used for testing. That is, samples that had already been subjected to the LAMP process by the biologists at Children's Hospital were tested. This was still a preliminary phase. Thus, heating was not an initial consideration. The dye chosen for this project was SYBR green dye. After the LAMP, reaction, this dye would be mixed with the positive and negative LAMP samples. This dye has some distinctly different excitation and emission spectrums. This attribute is vital in that it allows for a photo-detection scheme to be devised such that only the fluorescence of the sample is absorbed by the detector and not the excitation light. Shown in *Figure 5* is the excitation and emission spectrum of the SYBR green dye.

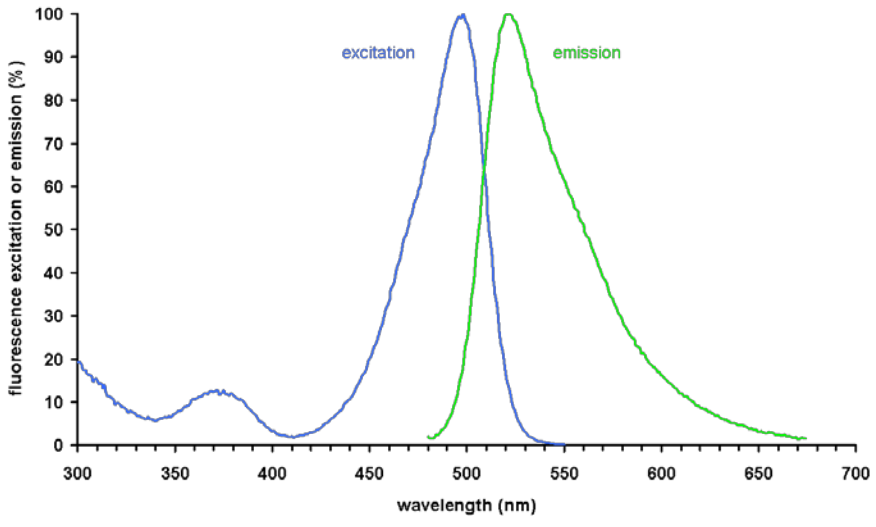


***Figure 5: Excitation and emission spectrum of SYBR green dye. Taken from Reference 13***

As noted from the *Figure 5*, SYBR green dye has a strong absorption in the blue range at approximately 490 nanometers. However, there is significant emission in this range as well. Thus, for blue excitation it becomes difficult for a precise detection to be made and to ensure that only the fluorescence is detected and not the excitation. However from *Figure 5*, it is evident that a local maximum occurs in the UV spectrum at approximately 370 nanometers. This spectrum is distant enough from the emission spectrum of the SYBR green dye such that a precise detection can be made. That is, a wavelength-specific detector can be utilized that only accepts the green fluorescence spectrum of the dye/pathogen combination at 520 nm, while rejecting the UV excitation light at 370 nm. This ensures the user that the sample is infected when a positive detection signal is obtained.

In accordance with keeping the project cost low, a series of UV LEDs were bought. The first such UV LED was the NSPU510CS made by Nichia. This is a lamp –type LED which is also breadboard compatible thereby facilitating quick prototyping. *Figure 6* shows the light spectrum of the UV LED.

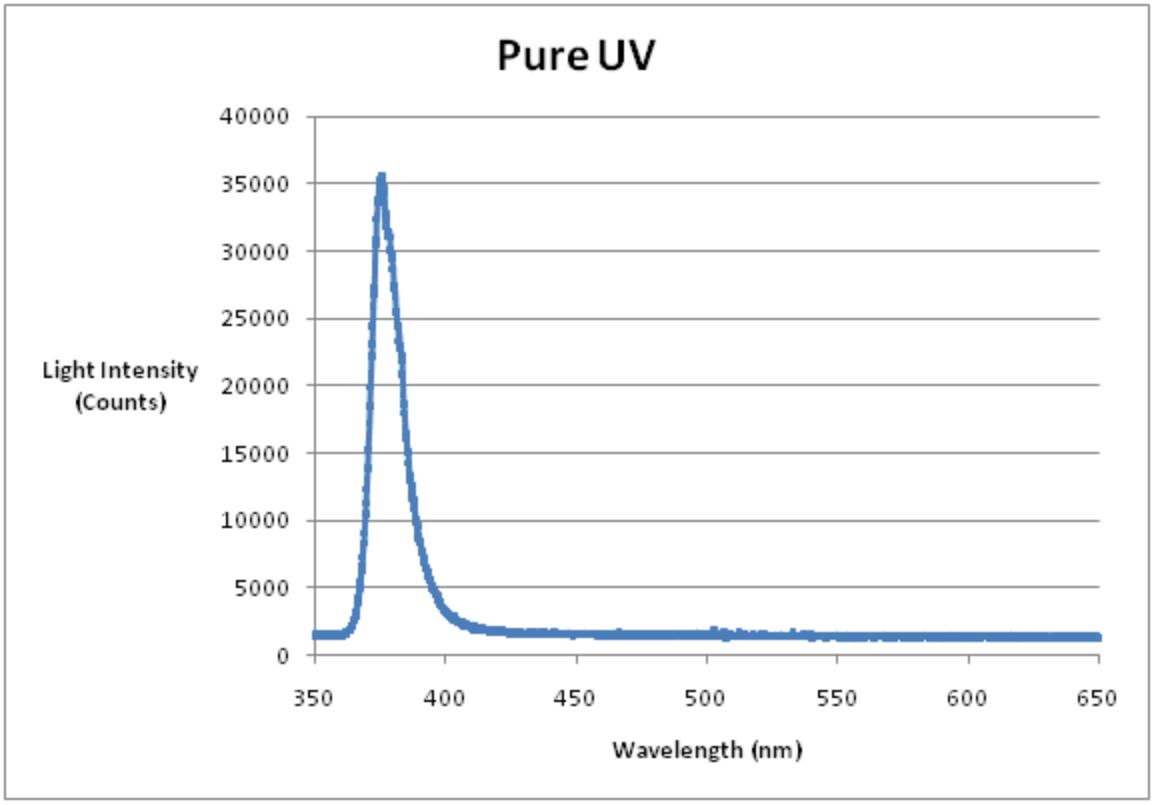


***Figure 6: Emission spectrum of UV LED***

This data was obtained in Dr. Di Gao's laboratory using a spectrophotometer from Ocean Optics (model # Ocean Optics 3000). *Figure 7* depicts a flow-chart of the equipment used in the spectrophotometry lab. The spectrophotometer output shows the light intensity of any light source as function of its wavelength. The data can be converted to a Microsoft Excel file and analyzed. The tall spike of *Figure 6* occurs at a wavelength of 375 nanometers. The light intensity is 40, 000 "counts". The graph displays the strength of a single UV LED and also that no other significant wavelength components (other than the UV, 370nm) exist in the UV light.

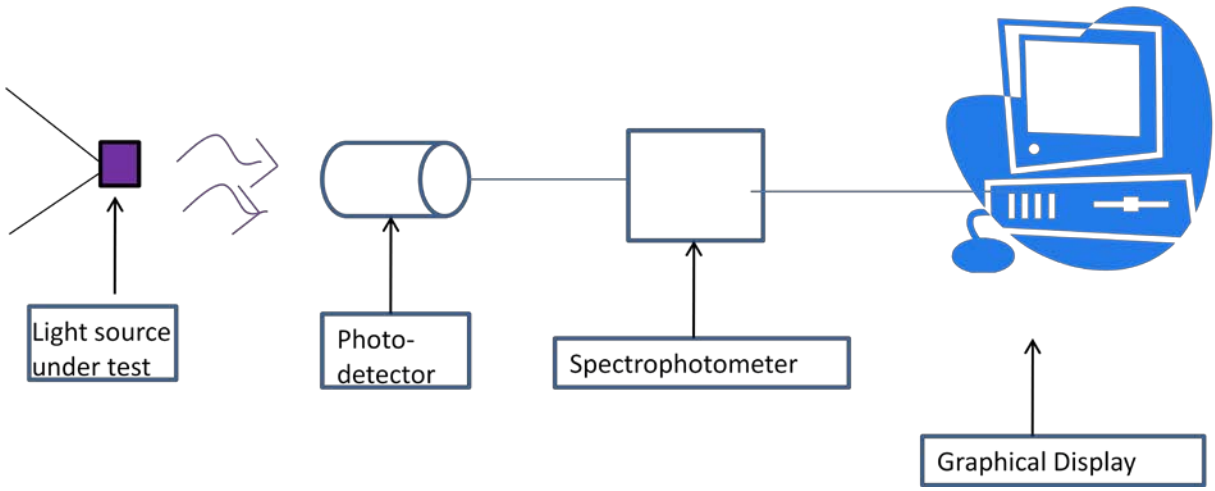


***Figure 7: Equipment setup in spectrophotometry lab.***

In order to confirm that this wavelength of excitation would work with the SYBR green dye, LAMP samples were prepared for direct exposure to UV light from the Nichia LEDs. Shown in *Figure 8* is a test tube array under the Nichia UV LED excitation. The first (left most) tube contained a positive sample of ecoli while all other tubes were negative LAMP samples. A distinct green fluorescence is seen in the first tube demonstrating the expected effect.

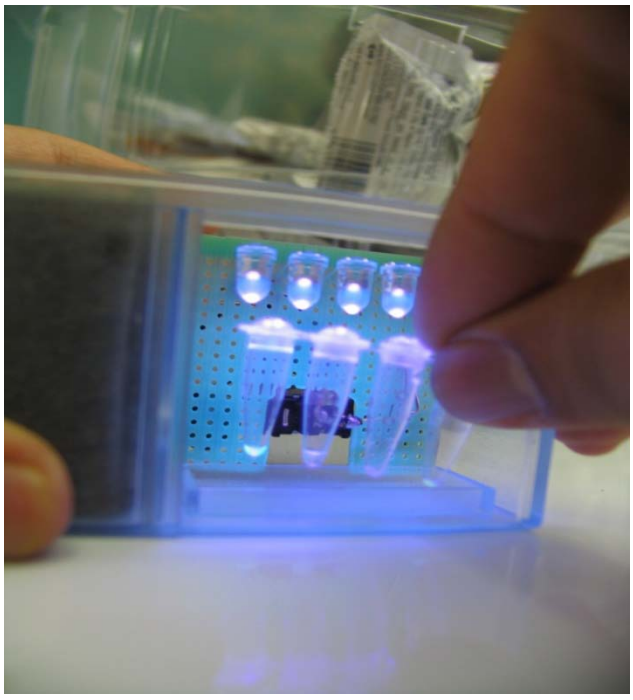

***Figure 8: Using UV LED to obtain fluorescence.***

The next task was to focus on the development of detection circuitry. Through research, it was found that the Cadmium Sulfide (CdS) photoresistor would be ideal for this application. Not only is the CdS photoresistor inexpensive, but it has an appropriate specification for this project. Shown in *Figure 9* is the wavelength / light intensity specification for the CdS photoresistor.

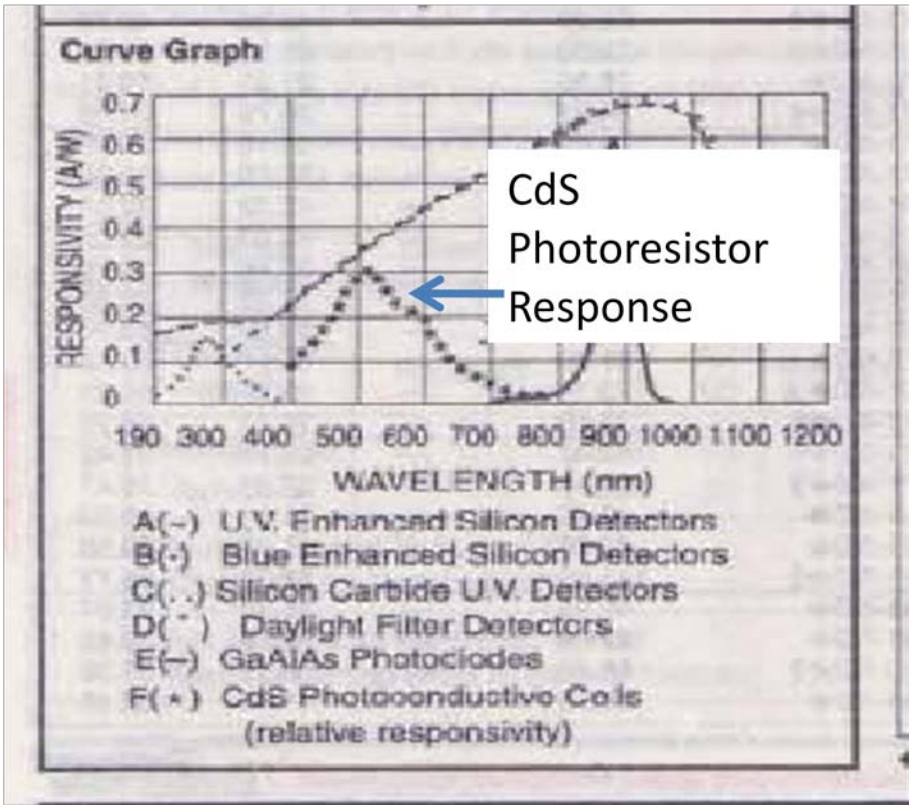


***Figure 9: Light intensity versus wavelength specification for the CdS photoconductive cells. The graph for this device is indicated by the stars, which begin response at approximately 400nm, peak at approximately 520 nm at which point response decreases. Taken from reference 14.***

*Figure 9* shows that the CdS photoresistor has a relatively high response between 400 and 600 nanometers. The photoresistor is supposedly non-responsive outside this regime. Therefore, the CdS photoresistor would presumably be ideal for this application, where UV excitation is used to obtain green fluorescence. Using the CdS photoresistor would ideally only sense the green fluorescence and not the UV light. This would mitigate possibilities of obtaining a false positive diagnosis. The CdS photoresistors were tested in conjunction with the UV LED. It was

determined through experimentation that the CdS photorsesistors responded to UV light even before a pathogen sample was placed above the UV light. This was unexpected as noted from *Figure 9* the CdS photoresistors seemingly have no response in the UV regime (~370nm). If the photoresistors would conduct with UV light, false positives could be obtained in the field where physicians would use this equipment. This was a parameter to be avoided. The reason that the CdS photoresistors conducted on UV light can likely be attributed to one of two possible theories. The first theory is that the UV diode contained a green component, strong enough to make the photoresistor conduct. This however was very unlikely given that the wavelength spectrum of the UV diode had already been characterized; see *Figure 6*. No significant green component existed in the UV spectrum. The second theory was that the photoresistor was not operating to specification. One of these theories could be validated by introducing optical filters into the system. Before doing that, an idea of the precise wavelength of the emitted green fluorescence was needed. This allows for the appropriate filter to be selected. Thus, a positive amplified pathogen sample was taken to the spectrophotmetry lab and illuminated with UV light. The entire optical response was once again analyzed by the Ocean Optics spectrophotometer previously described. Shown in *Figure 10* is the result obtained by illuminating a positive LAMP sample with the UV light. *Figure 10* is used to locate the wavelength of the fluorescence.

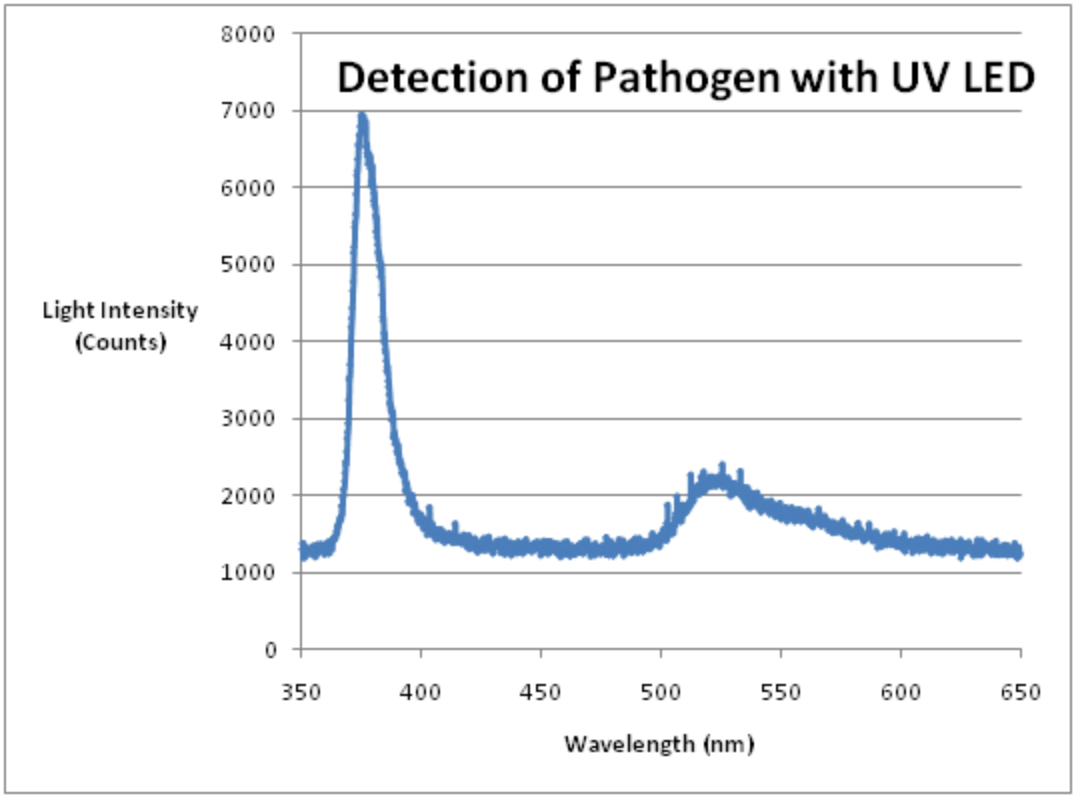


***Figure 10: Detection of pathogen in spectrophotometry lab.***

*Figure 10* shows the fluorescence occurring between the wavelengths of 500 and 570 nanometers. The maximum intensity occurs at 525 nanometers. Therefore several filters with appropriate specifications to admit these wavelengths were bought. It is also apparent from *Figure 10* that the intensity of the UV light is far greater than that of the fluorescence. This further supports the hypothesis that optical filters were in need. The objective in using filters would be to reject as much UV light as possible while admitting as much of the fluoresence wavelength as possible. *Figure 11* depicts the test setup in the spectrophotometry lab demonstrating how the data in *Figure 10* was found.

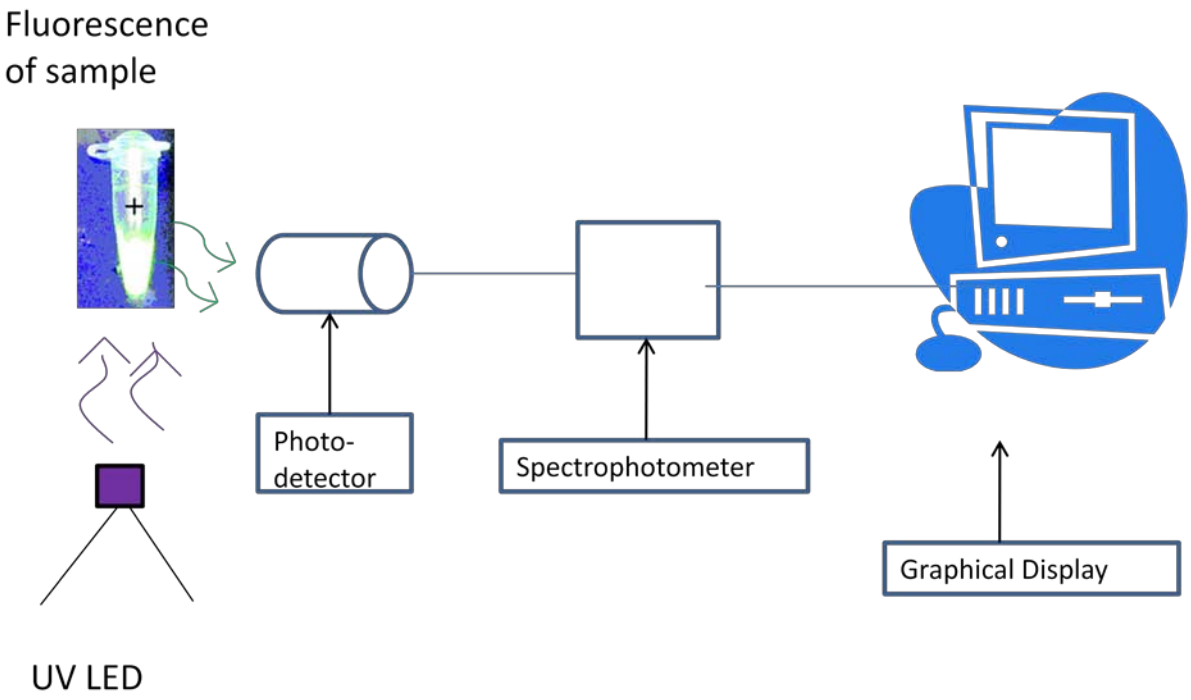


***Figure 11: Test setup in spectrophotometry lab for characterizing the bio-sample fluorescence wavelength and intensity***

# 4.0 FILTER ANALYSIS

The filter that was tested was the NT43-173 made by Edmund Optics. The NT43-173 is a bandpass filter with peak transmittance at 532 nm. It has a full-width half maximum rating of plus or minus 5 nm . The filter was taken to the spectrophotometry laboratory to test its wavelength absorption and rejection characteristics with amplified positive LAMP samples. The filter was placed behind the UV light and fluorescence but in front of the detector as seen in *Figure 12*.

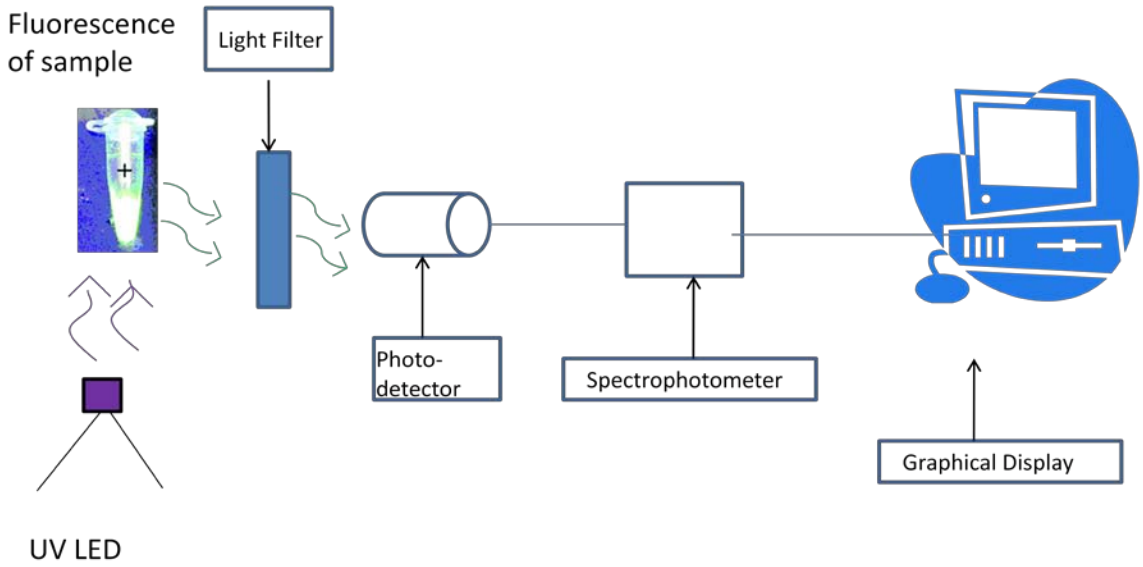


***Figure 12: Test setup utilized to detect fluorescence with addition of light filters to system. This setup is used for all the plots produced in sections 4.1 and 4.2.***

Shown in *Figure 13* is the fluorescence spectrum obtained using UV light and a positive LAMP sample in addition to the bandpass filter.

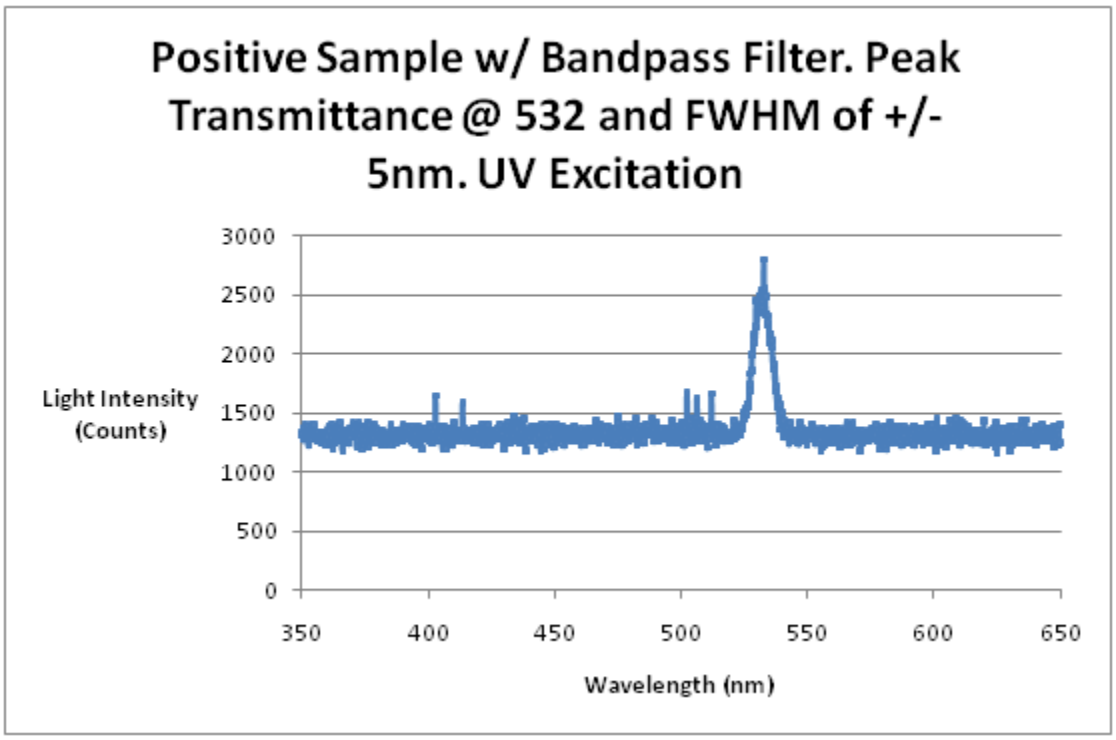


***Figure 13: Bandpass filter analysis, 532 nm***

*Figure 13* shows a peak transmittance at a wavelength of 533 nm. This is consistent, within uncertainty with the filter specification of having a peak transmittance at 532 nm. The peak light intensity is 2748 counts. Additionally, the filter seems to completely reject the UV light which is a necessity. Therefore, UV excitation would be used with the bandpass filter for all subsequent LAMP testing

# 5.0 DETECTION ANALYSIS OF AMPLIFIED PRODUCTS

With the optimal LED/filter combination chosen, it was then necessary to design a UV LED excitation circuit and an analog detection circuit for amplified products. The excitation circuit consisted of a power supply in series with the UV diode and a 1200 ohm resistor. The detection circuitry consisted of a power supply in series with the following: the photoresistor, a 100 ohm resistor in series with a multimeter displaying current. The bandpass filter of peak transmittance at 532 nm was placed between these two circuits. *Figure 14* shows a picture of this preliminary testing circuitry

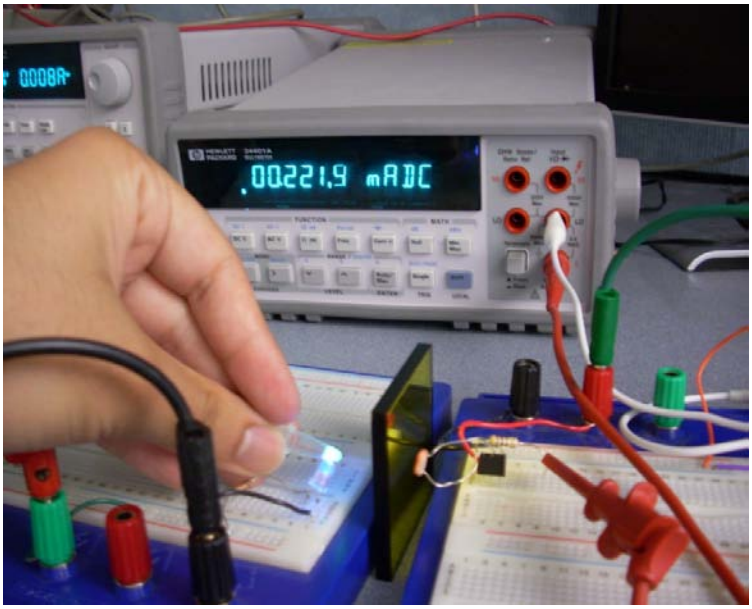


***Figure 14: Preliminary excitation and detection circuitry: UV diode, sample, bandpass filter, photoresistor and multimeter.***

Experiments were performed in order to design a circuit that would conduct for a positive LAMP sample and would not conduct for a negative LAMP sample. It was determined that additional circuitry would be required than what is depicted in *Figure 14* in order to produce such a circuit. This was due to the slight difference in fluorescence intensity of the positive LAMP sample versus the negative LAMP sample. This slight disparity in fluorescence led to a minimal difference in power outputted by the photodetector during positive and negative transitions. This made it difficult to design a circuit that would conduct for positive samples exclusively unless additional circuit components were added.

One possible solution to this problem is the use of an op-amp cascaded into a comparator. The op amp would amplify the signal coming from the photodetector. The output of the op amp would then feed the non-inverting terminal of a comparator. A reference voltage would appear at the inverting terminal of the comparator. This reference voltage would be intelligently designed such that the comparator would send out a "voltage high" when an infected sample was tested and a "voltage low" would be sent out when an uninfected sample was tested.

This circuit implementation is shown in *Figure 15*.

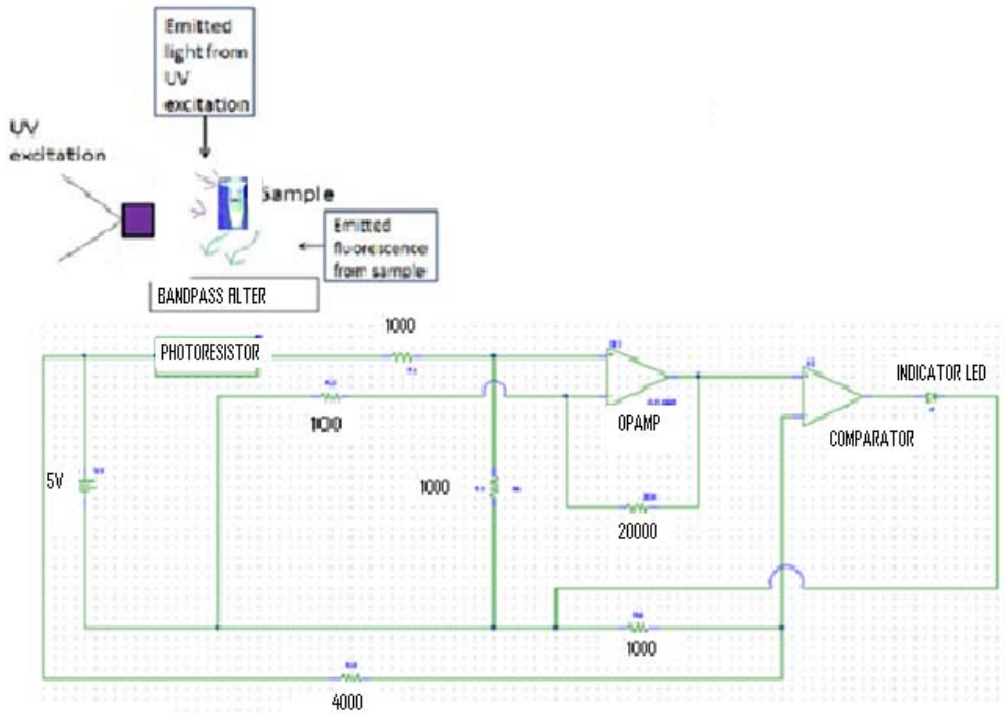


***Figure 15: Amplified detection circuit. The integrated chip towards the left is an op amp. The integrated chip towards the right is a comparator.***

The output of the photoresistor is sent to the positive terminal of the operational amplifier via a voltage division between two 1 kilo-ohm resistors. The inverting terminal is grounded through a resistor with a value of 100Ω. A feedback loop is also connected from the output of the amplifier to the inverting terminal via a resistance of 20 kilo-ohm. Thus, the amplifier is in the non-inverting configuration with a gain calculated below:

$$\frac{Vo}{V^+} = 1 + \frac{R2}{R1} = 1 + \frac{20 \times 10^3}{100} = 201.$$

The output of the operational amplifier is then routed into the non-inverting terminal of the comparator. The reference voltage seen at the negative terminal of the comparator was chosen to be 1.00 volts. This is because the expected voltage for a positive test sample at the

output of the op-amp was at least 1.00 volts. For the negative sample, the highest voltage expected at the output of the op-amp was 0.950 volts. The reference voltage is achieved through a voltage division of a 5 volt regulator (driven by a 9 volt battery) in series with a 4 kilo-ohm resistor and a 1 kilo-ohm resistor – the latter of which is connected to ground. Therefore, the comparator would only send out a "voltage high" when an infected sample was tested. A red indicator LED is placed at the output of the comparator. Thus, the red light would come on only when an infected sample was tested.

On 100 trials, this circuit worked 78 times indicating that a false positive was obtained 22% of the time. This was not preferable for the final design. However, this did establish a proof of concept so that real-time LAMP testing could be performed. Recall that the objective is to design a box that implements LAMP in real time which means to heat/amplify as well as optically excite and detect the sample simultaneously. Until this point, all samples were already subjected to the LAMP process. That is, the samples had already been heated and amplified. Since the observations from this chapter showed that data for the positive sample is above that of the negative sample for a majority of the time, a real time LAMP test could be performed in order to reproduce the characteristic LAMP graph shown in *Figure 2*. Recall that reproducing this graph using low cost instrumentation was a primary objective for this project. A real time LAMP test implies that positive and negative samples will be heated to 65 degrees Celsius while being excited with UV light. The resulting fluorescence during the heating and UV excitation will be detected from the photoresistor and amplified by the opamp. A multimeter will measure the analog signal from the op amp output of *Figure 15*. The resulting data will be plotted over time. There is no need for a comparator or indicator LED in this circuit as the goal would be to reproduce *Figure 2*. Chapter Six discusses the implementation of real time LAMP tests.

# 6.0 REAL TIME LAMP ANALYSIS

## 6.1 USING A STANDARD HOTPLATE FOR THE HEATER AND SUBSEQUENT REAL TIME LAMP DATA

A rectangular aluminum block was machined to accommodate the bio sample test tubes and for heating. The top of the metal block is shown in *Figure 16*.

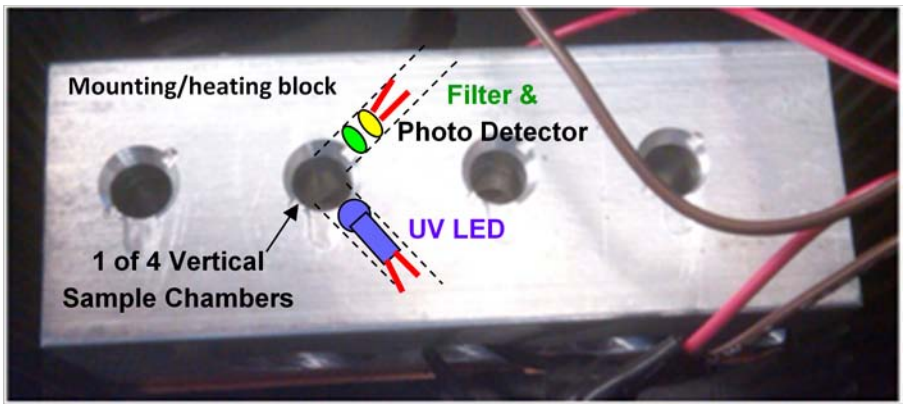


***Figure 16: Top View of aluminum block***

Channels transverse through each of the sample slot axes and were machined in the block to provide optical channels and mounting support for the optical excitation and detection components. The LED, the filter and the photoresistor are lodged inside these optical channels of the metal block of *Figure 16*. The output of the photodetector is connected to the battery and the op amp as shown in the schematic on *Figure 15*. This is depicted in a picture on *Figure 17*. A comparator is not used.

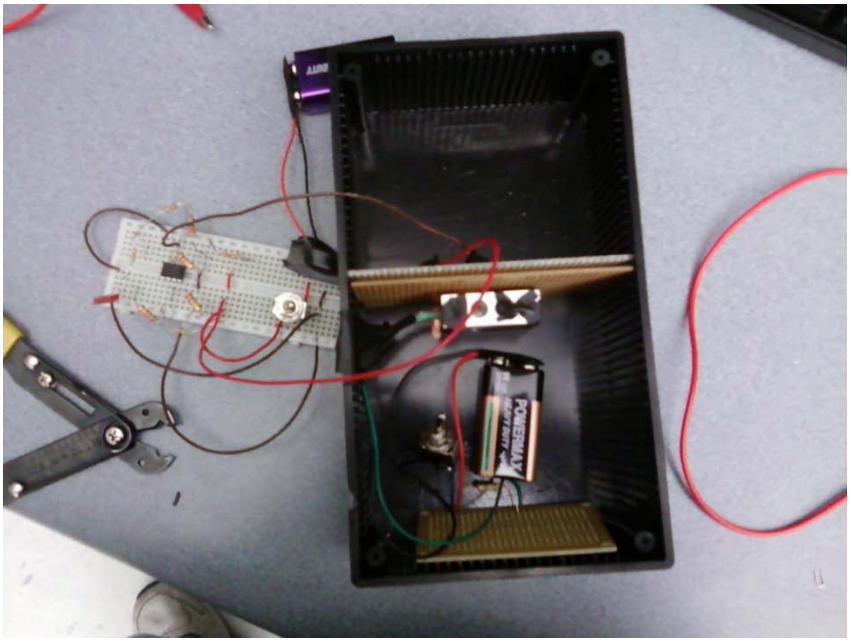

***Figure 17: Connection of the output of the photoresistor to the op-amp.***

The bottom of the aluminum block was attached to a circular copper plate which sits on top of a heat source – initially a hot plate. Eventually, a custom made heater for this application will be implemented. This copper plate is shown in *Figure 18*.

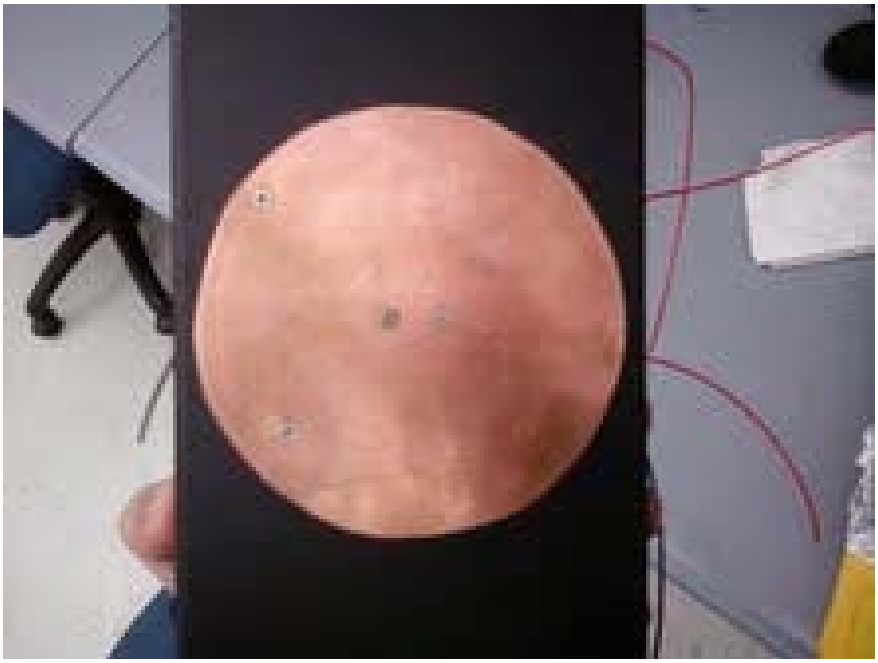

***Figure 18: Copper plate attached to bottom of aluminum block***

The aluminum block and attached copper plate sit inside a plastic project box of approximate dimensions (inches): 7.5*4.5*2.5. The box then sits on top of a hot plate as depicted in *Figure 19*.

***Figure 19: Aluminum block / copper plate sitting on hot plate. The opamp circuit is not visible in this picture***

The following describes the initial test procedure and results of the prototype LAMP system. The hot plate was heated to 65 degrees Celsius. A positive sample was placed inside the slot containing the optical excitation and detection components. The UV light was turned on and measurements were taken every 30 seconds at the output of the op amp using a multimeter. The test was run for an hour. Next, the positive sample was replaced with a negative sample and the test performed again. The outcome of this test is depicted in *Figure 20*.

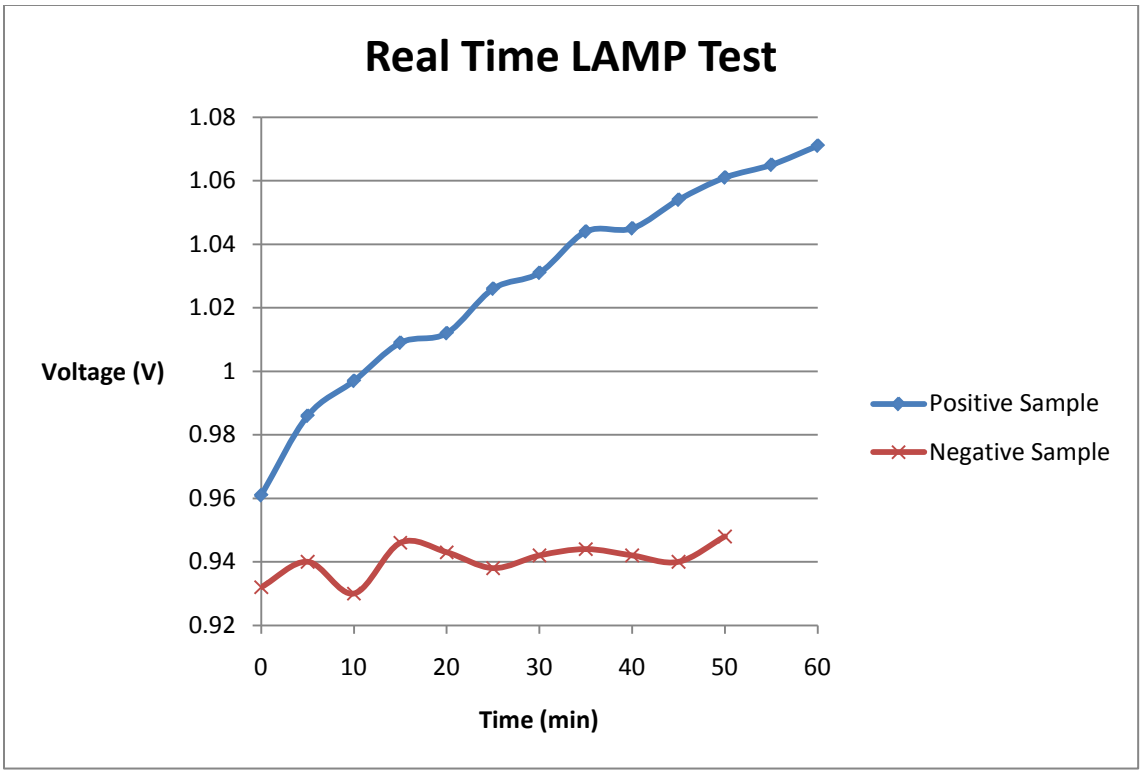


***Figure 20: Real Time LAMP Test***

As expected, an exponential increase for the positive sample is achieved while a relatively flat line is achieved for the negative sample. This is pathogen detection with LAMP. Next, it was necessary to perform the positive and negative tests at the same time. This required that all the channels in the metal block accommodate the optical excitation and detection components. Shown in *Figure 21*, *Figure 22* and *Figure 23* are the results of this test for different pathogens. Each test shows a positive sample and a negative sample heated and detected at the same time. Also, it was preferable to normalize the data so that both the positive and negative samples started at the same point, (0,0).

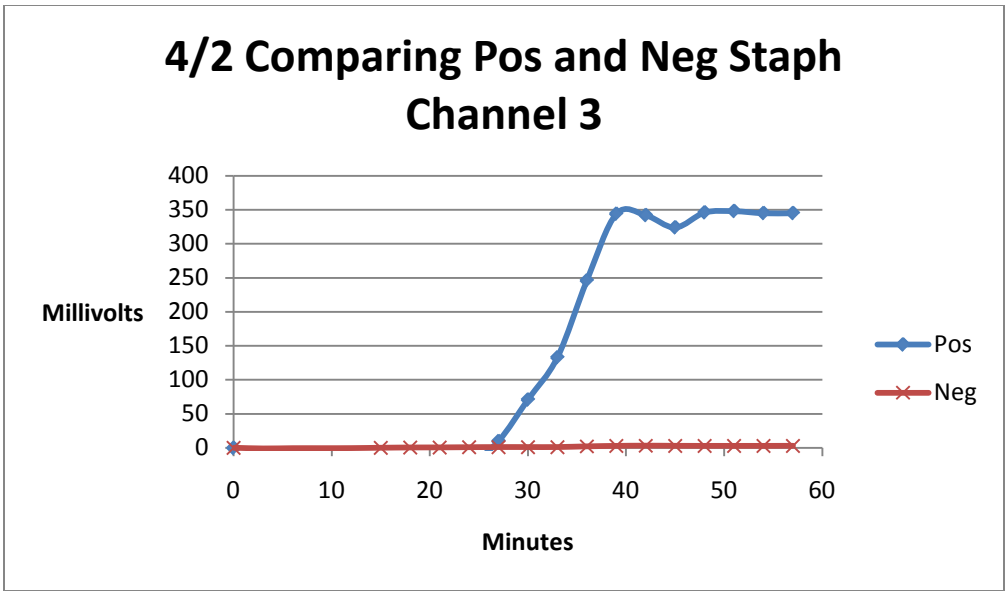


*Figure 21: Detection of Staphylococcus using real-time LAMP*

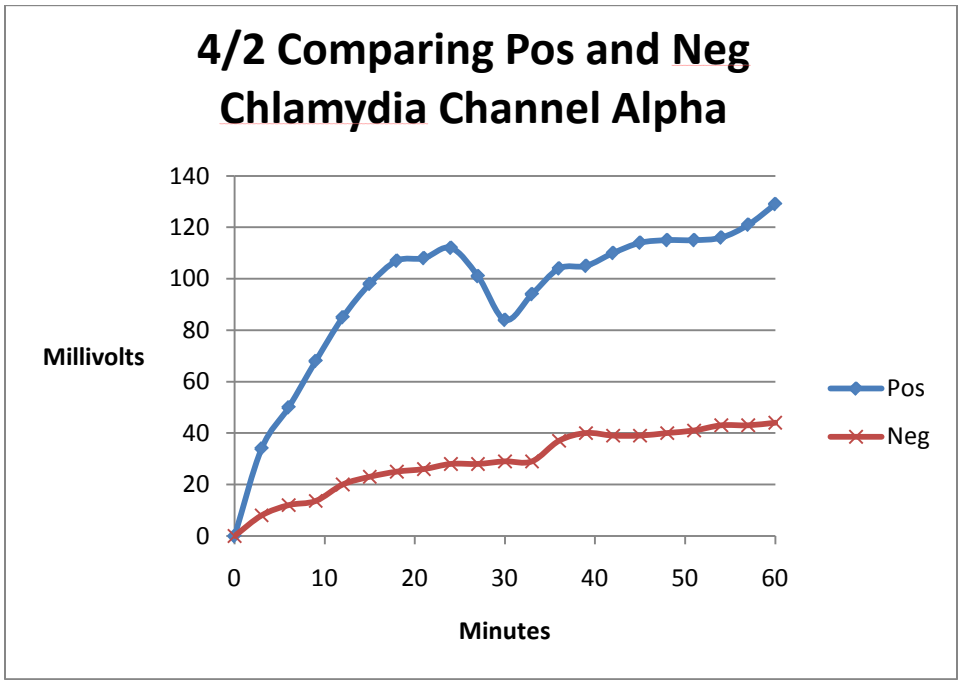


*Figure 22: Detection of Chlamydia using real-time LAMP*

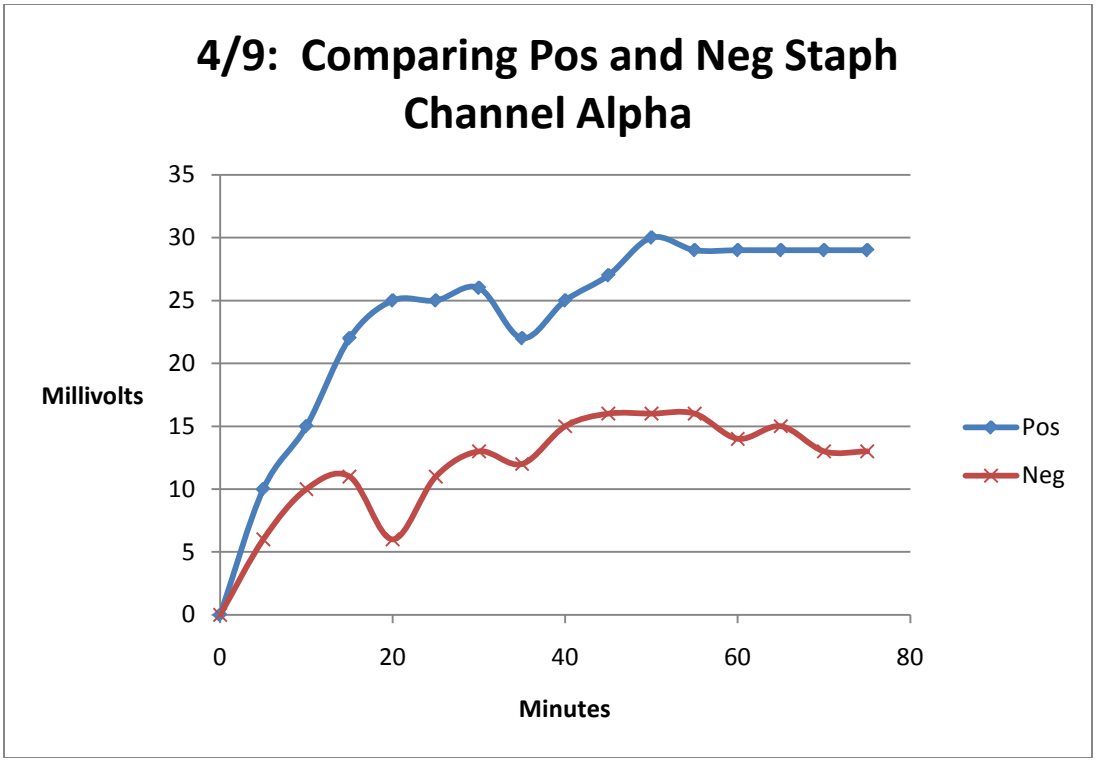


***Figure 23: Detection of Staphylococcus using real-time LAMP***

These results satisfy part of the objective for this project. That is to design a low cost instrument capable of producing data similar to the known characteristic trend of LAMP shown in *Figure 2*. However, it was still necessary to implement a custom made, affordable and portable heater.

## 6.2 HEATING CONSIDERATIONS AND SUBSEQUENT REAL TIME LAMP DATA

Since the final device is to be portable, a standard hot plate cannot be used as the heater. Currently, the use of a USB powered coffee mug warmer is being investigated for implementation of the heater. A picture of the heater is shown in *Figure 24*.

***Figure 24: USB coffee warmer. The USB lead would be cut off to be powered by external power sources.***

This device runs off standard USB specifications which is 5 volts at 500 milliamps. At this specification, the heater obtains a temperature of 50 degrees Celsius which is not sufficiently high for the LAMP reaction to take place. Thus, a preliminary test was run to see if the heater could attain 65 degrees Celsius when the device was driven up to 6 volts. This drew a current of 570 milliamps. Shown in *Figure 25* is a graph depicting temperature of the coffee warmer over time when driven with 6 volts.

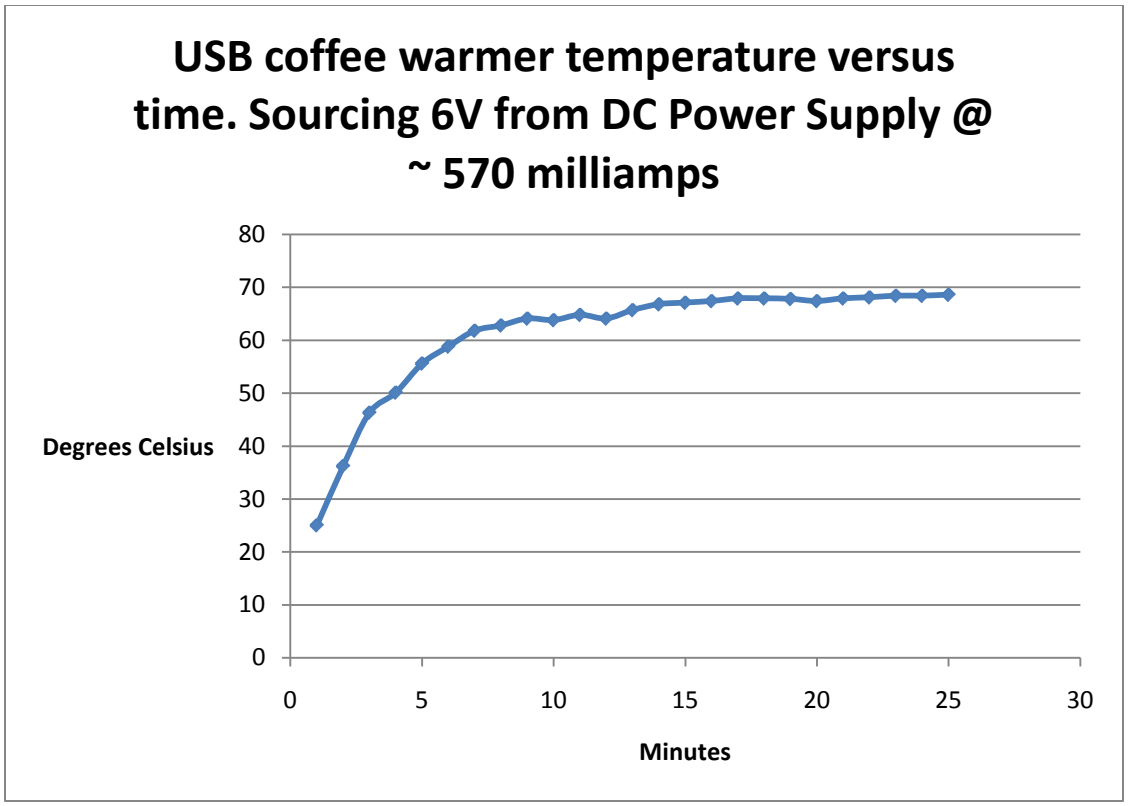


***Figure 25: Driving USB coffee mug warmer with 6 volts at 570 milliamps.***

As seen from *Figure 25*, the device can get up to approximately 65 degrees Celsius in less than 25 minutes.

Next, the aluminum block was placed on top of this heater to see if the temperature of the block could reach 65 degrees C. The large thermal mass of the aluminum block prevented this from happening. Therefore, it became necessary to redesign a new aluminum block. For this new design, individual aluminum pillars that can accommodate the test tube and the optics were machined. The aluminum pillars are then placed in a "delrin" casing which insulates the heat thereby preventing the electronics from overheating and subsequently degrading. Again, optical channels were built so that the UV LED can excite the dye inside the test tube and the photodetector can sense the resulting fluorescence. The excitation, detection and heater circuits

(discussed later) were all soldered onto boards and placed inside the box as depicted in *Figure 26*.

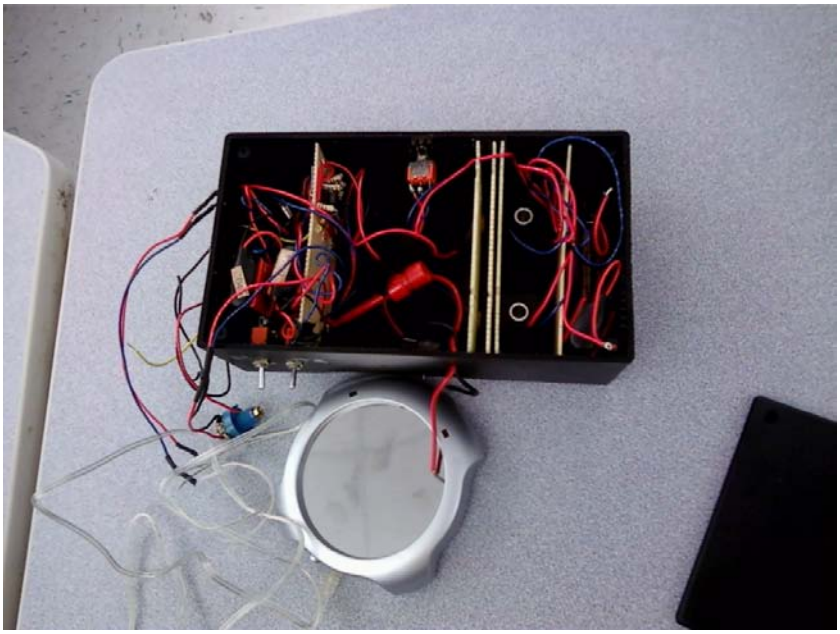

***Figure 26: Inside of new design. All circuitry and components mounted inside the box. Aluminum pillars inside delrin casings on right hand side.***

The bottoms of the individual aluminum pillars have metal knobs that fit through holes that are machined through the box. This allows the box to sit on top of the USB heater and for the samples inside the pillars to heat. Screws are utilized to balance the entire box as it sits on a table. This is shown in *Figure 27*.

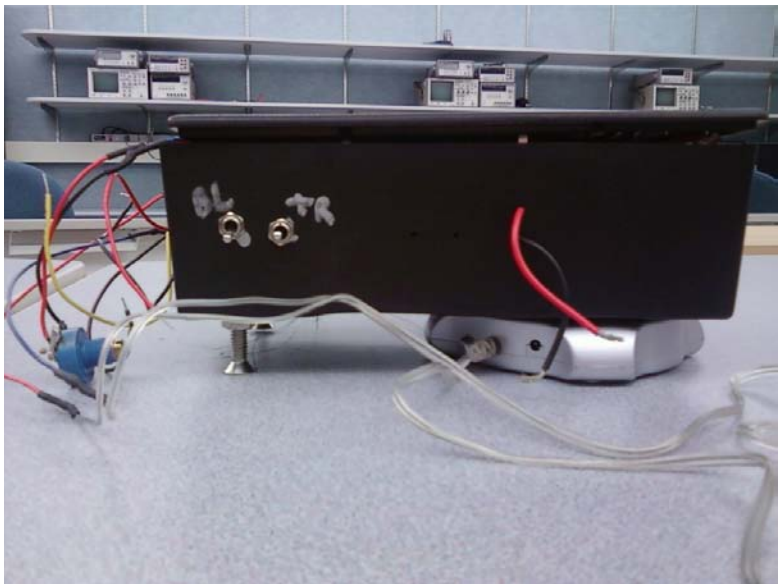

***Figure 27: LAMP box sitting on top of USB heater. Screws used for balancing on left hand side.***

Additionally a new "heater circuit" was implemented. This circuit consisted of two AC to DC transformers in series to produce 21 volts DC. This voltage is routed into a 12 volt regulator. A resistive load is connected to the 12 volt regulator. This load consists of five 39 ohm resistors in parallel with a 50 ohm potentiometer. By adjusting the potentiometer, the power dissipated by the circuit is adjusted. This in turn affects the amount of heat delivered to the aluminum pillars. Thus, the pot entiometer i n effect act s as a t emperature cont rol. The r esistive loa d is t hen connected to the USB heater. *Figure 28* shows this heater circuit.

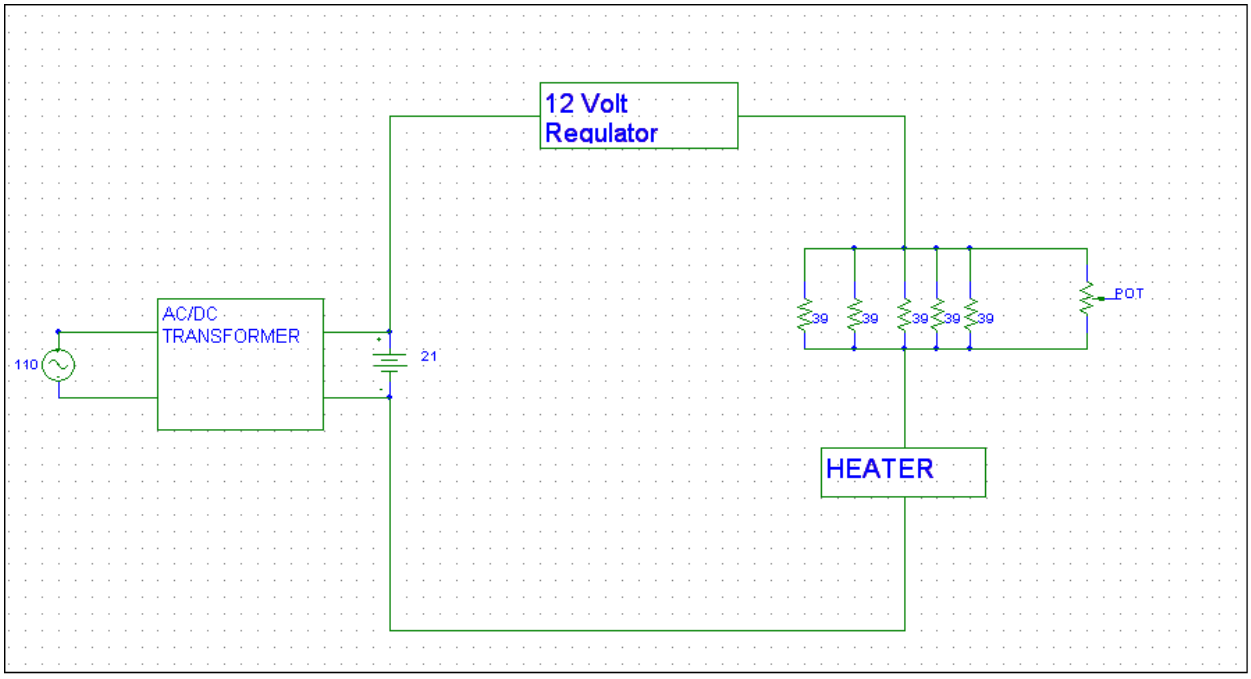


***Figure 28: Heater Circuit***

The reason for the parallel resistive load network is because the heater circuit draws a significant amount of current capable of burning out any one resistor. The parallel combination prevents this from happening. When the potentiometer is set to 50 ohms, the equivalent resistance of the load is approximately 7 ohms. In this configuration, the resistive load has approximately 4.65 volts across it. Using KVL, it is apparent that the heater drops 7.35 volts. The current through the entire circuit is 660 milliamps. Thus the heater dissipates 4.85 watts. This is the configuration that allows the heater to heat the aluminum pillars to reach 65 degrees C. The temperature of the aluminum pillars as they sit on top of the USB heater is shown in *Figure 29* as a function of time. The "heater circuit" of *Figure 28* was utilized to achieve this data.

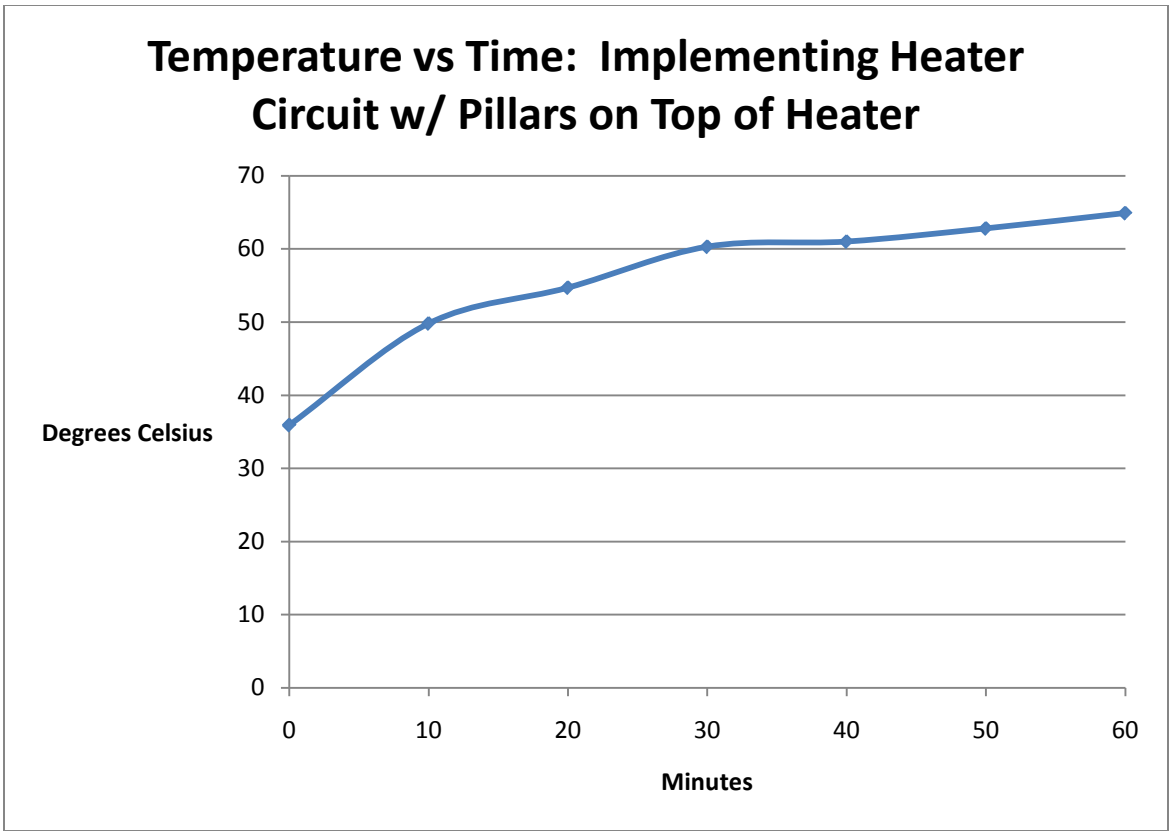


***Figure 29: Temperature of aluminum pillars as they are heated by USB heater in series with resistive load network***

As seen from *Figure 29* it takes nearly an hour for the pillars to reach the required temperature. This was taken into consideration for any subsequent LAMP tests. Shown in *Figure 30*, *Figure 31*, and *Figure 32* are the results from three successful LAMP tests detecting Ecoli and Staph using the diagnostic device in conjunction with the USB heater and USB heater circuit.

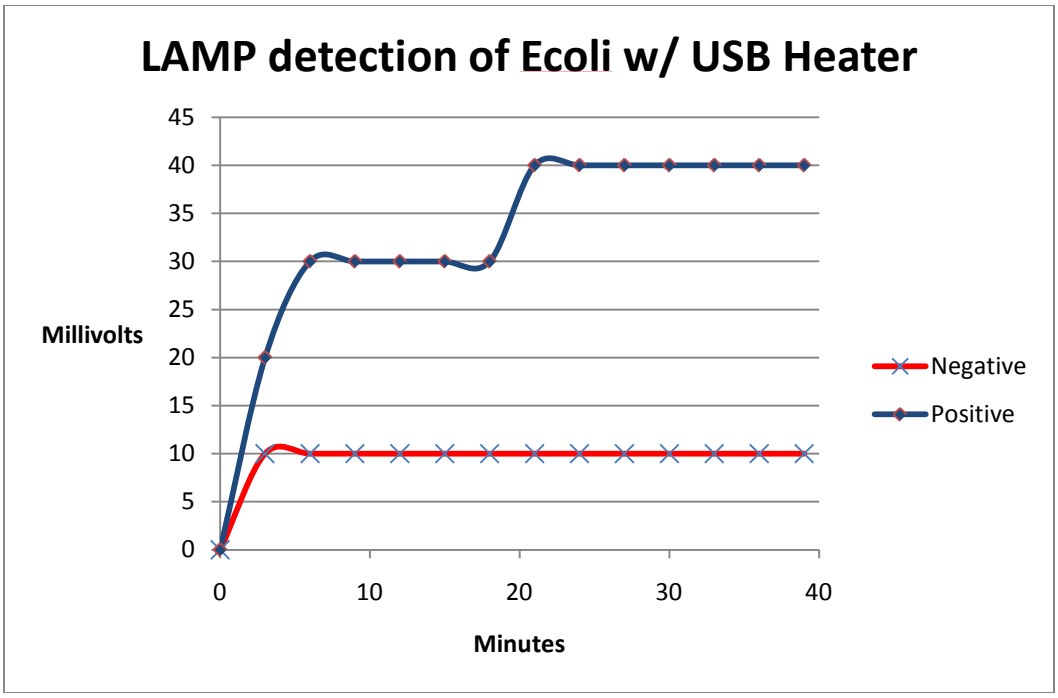


*Figure 30: Detection of Echoli using LAMP box and USB heater*

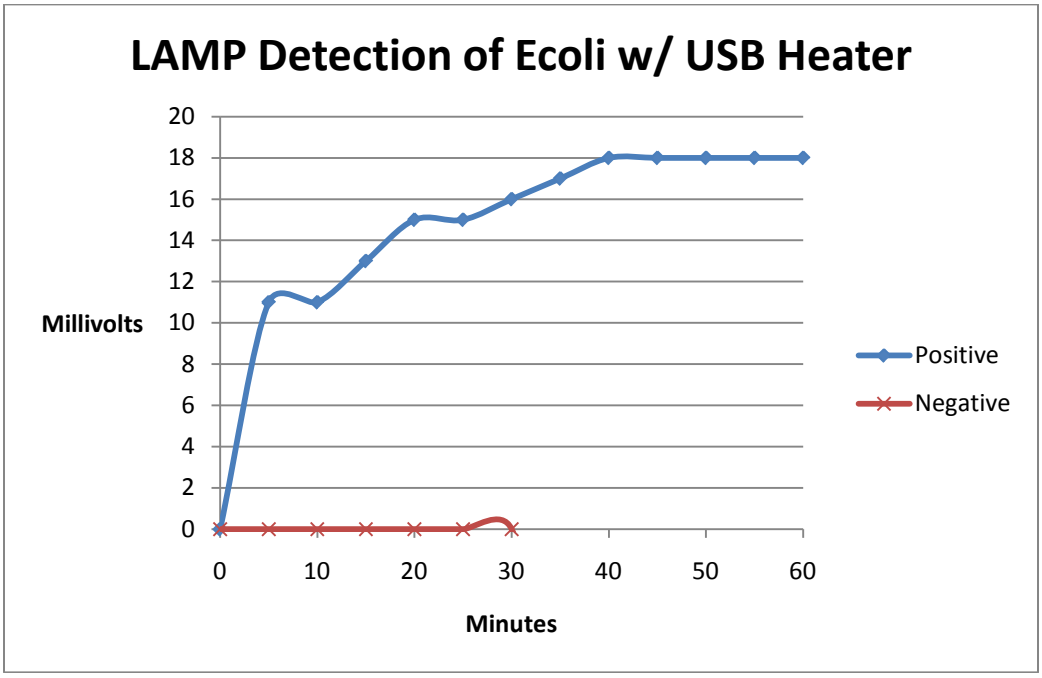


*Figure 31: Second Detection of Echoli using LAMP box and USB heater*

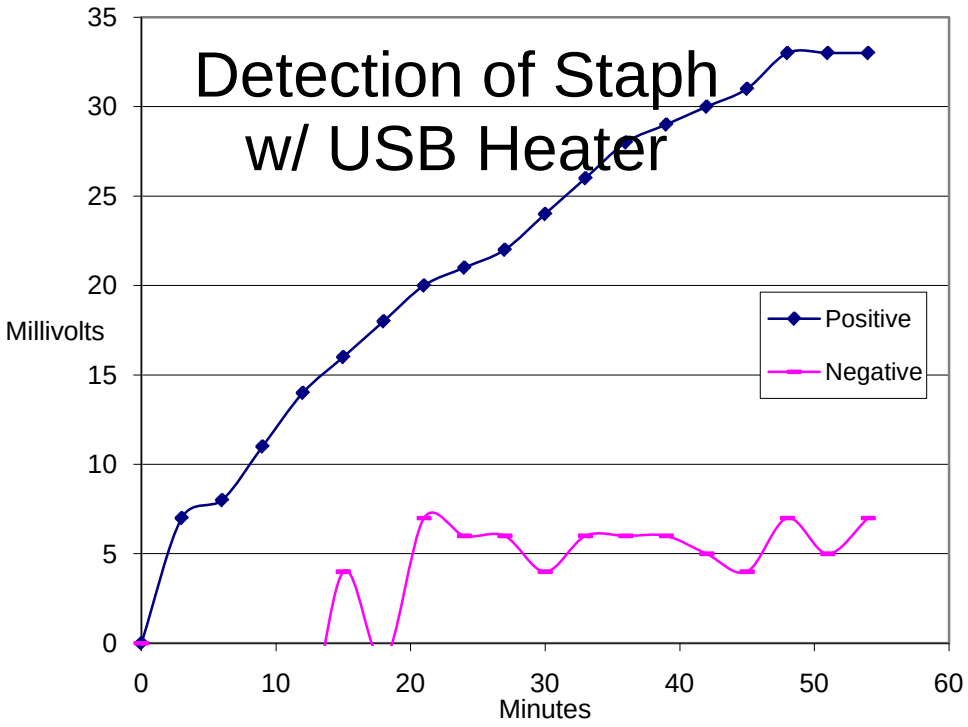


***Figure 32: Detection of Staphylococcus using LAMP box and USB heater***

During some of the tests, the output voltage for the uninfected sample would drop below its original value because the signal was oscillating like electrical noise – as expected. Thus, on a normalized scale as depicted, the uninfected sample would show negative amplification over time which is meaningless. Therefore, the "negative" values of the uninfected sample are not shown. These results completely satisfy the objective of this project. To confirm the validity of these results, the standard and reliable methodology of electrophoresis was used. The very same amplified products that gave the results from *Figure 32* were taken to Children's Hospital. There, the biologists working with Dr. Vats subjected the products to both UV transillumination and electrophoresis. These results are depicted in *Figure 33* and *Figure 34* respectively.

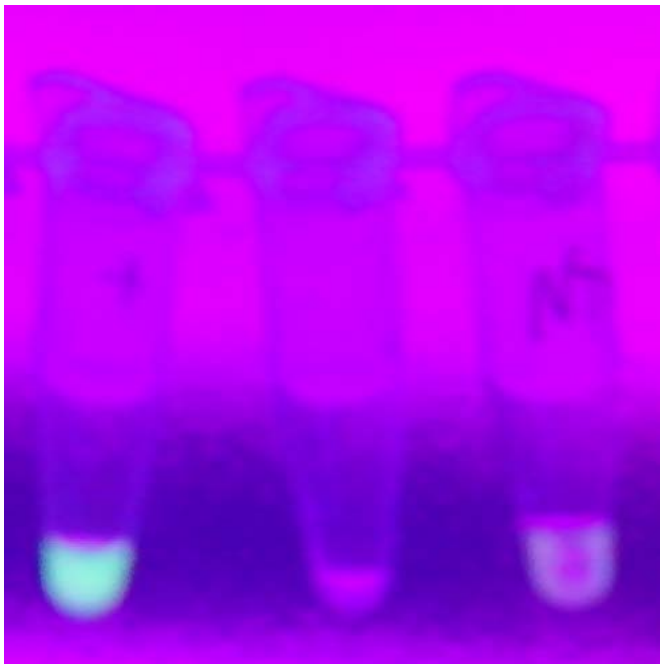

***Figure 33: Using UV transillumination for detection of same solution from Figure 32. This is done to verify the results from Figure 32. The left sample is positive, the right sample is negative. The middle tube is empty.***


***Figure 34: Using electrophoresis for detection of same solution from Figure 32.The left hand lane shows DNA extraction indicating an infection. The right hand side shows no DNA extraction. This image is taken when the products have been electrophoresed and are excited with UV light.***

These results prove the validity of the LAMP device implemented here.

# 7.0 CONCLUSION

## 7.1 FINAL DISCUSSION

Contagious infections are a problem all over the world. An older technique called Polymerase Chain Reaction is employed to amplify potentially infected DNA and detect infections via electrophoresis. Unfortunately, this process is expensive, time consuming and cumbersome. In some areas of the world, there is a dire demand for affordable quality diagnostic tools which PCR is not.

Recently, a novel technique which is inexpensive, fast and can be performed in a straight-forward manner called Loop-mediated isothermal Amplification has proven to alleviate the drawbacks of PCR. At UPMC, Dr. Abhay Vats and his technical group continue to investigate techniques of implementing LAMP for a routine diagnostic tool.

Reported here was a new device using simple low cost components which implements LAMP using the heating and optical excitation and detection of pathogens. The total component cost of the test instrument was approximately $100. This includes the excitation and detection circuitry, the filter, the heater, and the project box. The labor cost for the production of one metal pillar was expensive but would not be as such for mass production. Thus the hardware may potentially be manufactured quite inexpensively. Additionally, the electronic designs lend

themselves to DC power either by using batteries or low cost AC-DC conversion through a simple, common plug in connector.

The output signal of the box depicts a diagnosis telling whether the sample is "positive" or "negative". To accomplish this, an understanding of how LAMP is better utilized for diagnostic use than PCR was obtained. The specification for the SYBR green dye was studied and appropriate excitation LEDs were bought and utilized in obtaining fluorescence. Detection components were tested in order to characterize the fluorescence. It was determined that in addition to optical filters, a circuit consisting of an operational amplifier would be needed to differentiate between positive and negative samples. This circuit is used to take voltage readings over time and to plot the obtained data. The use of a USB coffee warmer in series with a heater circuit is utilized as the necessary heater of LAMP. A proof of concept for displaying this data on palm tops will be demonstrated in the next section.

The problem addressed here is the need for affordable accurate diagnostic tools in developing areas. The promise of LAMP allows for the findings here to be built on and to serve as a basis for further designs. Without the incentive of incorporating various concepts to devise a scheme meant to help others, this preliminary basis could not have been achieved.

## 7.2 FUTURE CONSIDERATIONS: LOW-COST PORTABLE MEDICAL INSTRUMENTATION

All the data in Chapter Six was taken from a multimeter and manually recorded. Recently, Dr. Alex Jones of the Electrical and Computer Engineering department at the University of Pittsburgh joined the project to explore transmitting the data from the diagnostic box to a portable digital device such as a smart phone. A synopsis of a proposal written by Drs. Jones and Stanchina and Raghav Khanna is given below to provide a window on possible extensions to the device described earlier:

To create a system that displays the data graphically, we will exploit both novel techniques that use inexpensive, off-the-shelf components to create detection circuits and the computing power of palm-top computers including smartphones, iPods, etc. The detection circuitry in this case would store raw data from the test setup. The 802.11 wireless standard will be used (often referred to as WI-FI) to communicate between the test instrument and a palm-top computer. WI-FI would be an appropriate option for developing countries because no additional infrastructure is required to use the technology. Additionally, using a palm-top computer as opposed to an on-board display would be beneficial because only one device would be needed to communicate with several instruments.

A pre-prototype system showing proof of concept is shown in *Figure 35*. The "custom detection setup" is the diagnostic box in which the samples are placed. The output is then relayed to the "Sample Storage & Network Control". The network allows the data to be transmitted through the "WI-FI Communications" to a remote palm-top computer.

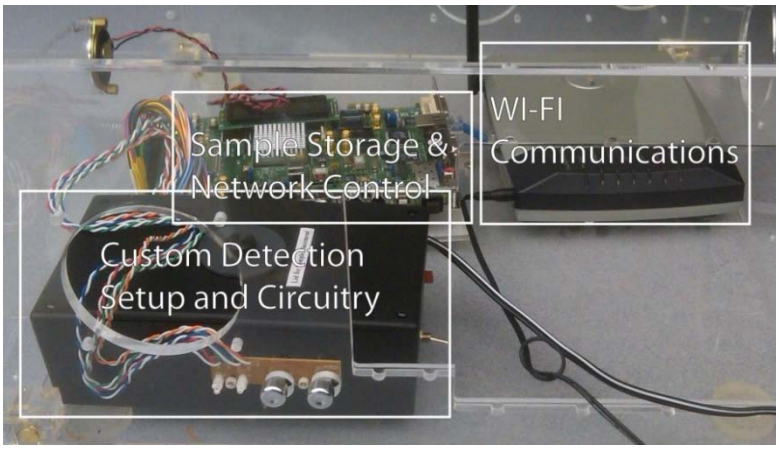


***Figure 35: Pre-protoype LAMP instrument***

A palm-top computer can then acquire the data after the LAMP process and testing have been completed. The device from *Figure 35* can be used to compare the output obtained for a positive sample and a negative sample. The data would be sent to a palm-top computer as shown in *Figure 36* and *Figure 37*. *Figure 36* depicts the data in a tabular format and *Figure 37* depicts the characteristic curve of the data.

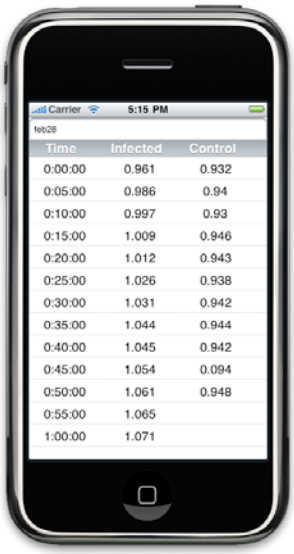


***Figure 36: Palm-top computer display of actual experimental data.***

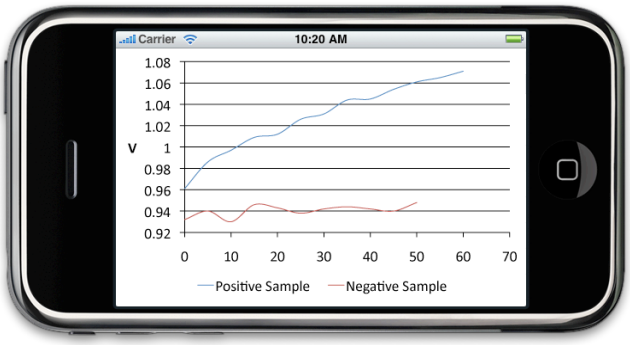


***Figure 37: Palm-top computer display of actual experimental data.***

In accordance with the low cost objective of this project, all these components are relatively inexpensive. The excitation and detection circuitry components and the heater together cost $100 and the WI-FI communications set up costs $100. The total cost for the entire device would not exceed $300. The palmtop computers can be used repeatedly for many different test setups. Thus, a proof of concept is demonstrated providing a foundation for continuation of optimizing this diagnostic instrument for implementation in developing countries.

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