SAND2016-12346R

2016 HS-STEM Summer Internship Program

Portable Diagnostics and Rapid Germination

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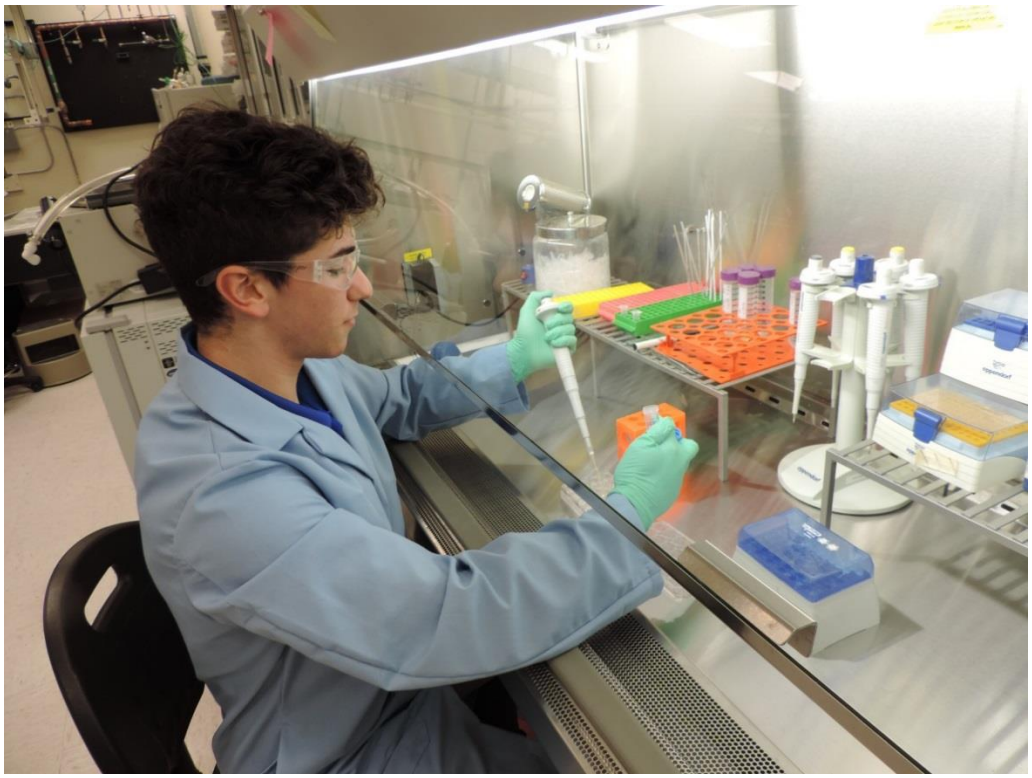
Hosting Site: Sandia National Laboratories – New Mexico
Mentor(s): Dr. Jason C. Harper

Abstract. In the Bioenergy and Defense Department of Sandia National Laboratories, characterization of the BaDx (*Bacillus anthracis* diagnostic cartridge) was performed and rapid germination chemistry was investigated. BaDx was tested with complex sample matrixes inoculated with *Bacillus anthracis*, and the trials proved that BaDx will detect *Bacillus anthracis* in a variety of the medium, such as dirt, serum, blood, milk, and horse fluids. The dimensions of the device were altered to accommodate an *E. coli* or *Listeria* lateral flow immunoassay, and using a laser printer, BaDx devices were manufactured to identify *E. coli* and *Listeria*. Initial testing with *E. coli* versions of BaDx indicate that the device will be viable as a portable diagnostic cartridge. The device would be more effective with faster bacteria germination; hence studies were performed the use of rapid germination chemistry. Trials with calcium dipicolinic acid displayed increased cell germination, as shown by control studies using a microplate reader. Upon lyophilization the rapid germination chemistry failed to change growth patterns, indicating that the calcium dipicolinic acid was not solubilized under the conditions tested. Although incompatible with the portable diagnostic device, the experiments proved that the rapid germination chemistry was effective in increasing cell germination.



1. Internship Project

Unbeknownst to many, Albuquerque, New Mexico, is a hot bed of scientific innovation and discovery. Nestled in Kirkland Airforce Base lies the Sandia National Laboratories, a sprawling facility in which numerous matters of national security are researched. From research in nuclear weapons design to improved methods of disposing hazardous waste, the workforce of Sandia National Laboratories tirelessly works to find new and better ways to combat our nation's problems. At Sandia, in the Bioenergy and Defense Department, Dr. Jason C. Harper focuses on a range of topics. I had the honor of conducting research under Dr. Harper and my investigations centered on low-cost bioanalytical platforms as well as accelerated bacterial germination.

Anthrax is a deadly disease caused by the spore-forming bacteria *Bacillus anthracis*, prevalent in low resource areas in the Middle East, western Asia, and northern Africa. Designated a Tier I pathogen by the Center of Disease Control, *Bacillus anthracis* is included in the set of pathogens that "present the greatest risk of deliberate misuse with significant potential for mass casualties or devastating effect to the economy, critical infrastructure, or public confidence" [1]. In 2014, Dr. Harper is a member of a team that invented BaDx, a *Bacillus anthracis* diagnostics cartridge. Named to the R&D Top 100 Inventions of 2014, BaDx is a portable, pocket-sized anthrax detector that is robust, inexpensive, easy to operate, and self-decontaminates. Furthermore, the device requires no power, external equipment, nor refrigeration, and its sensitivity rivals that of a lab culture. BaDx is the culmination of chemical engineering – it takes several key, separate components and combines them in a new and useful manner. As shown and described in Figure 1, each module serves a unique purpose vital to the success of the product.

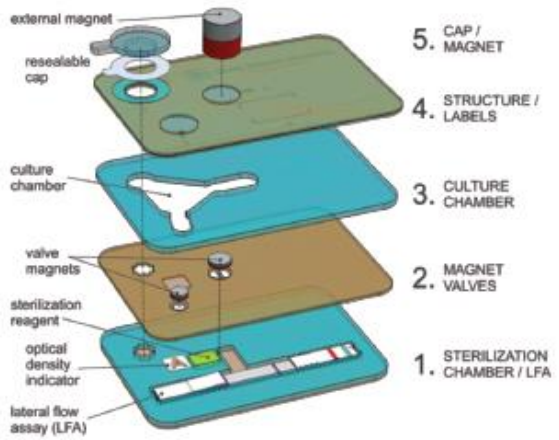


Figure 1. The resealable cap ensures that the sample remains contained. The external magnet paired with the valve magnets (which have since been replaced with steel discs) eliminates the need for power to operate and enables a cell culture, lateral flow assay, and sterilization agent to exist on one device. The BaDx contains a culture chamber with specific growth media, and the "device amplifies the *B. anthracis* so it can detect as few as 100 spores instead of the typical 1-10 million required for detection" [2]. The sterilization reagent safeguards against use of the sample for bioterrorism. The optical density indicator guarantees adequate bacteria growth. The lateral flow assay determines if the pathogen is present in the sample.

Dr. Harper and his intern Andrew Hunt were conducting studies with the BaDx for a paper about the device when I joined the team. Prior to performing hands-on lab work, I completed several general safety courses, as well as additional courses because I would be working with Bio-Safety Level 2 organisms. After completing all the coursework, I began my lab experience by learning how to operate a laser printer. I started by cutting out the seven plastic layers using CorelDraw, a vector graphics editor, and applied adhesive to the proper side of each layer. Then I assembled module 1, 2, and 3. This included adding the lateral flow immunoassay, decontamination powder, steel metal disks, a small magnet, and combining certain layers in a prescribed order. Steel metal disks replaced the magnet valves because the magnet valves were prone to shift out of place while in storage, due to magnetic forces being applied if the devices were stored close to each other or a ferromagnetic material. Each stage required the use of a hydraulic press for joining the plastic layers together. Within a few hours, after pressing the modules together, I had fabricated several fully-functioning BaDx devices.

After manufacturing the device, the next step in the investigative process was testing the BaDx for durability, specificity, and accuracy. The durability tests consisted of running the device after it was dropped from a variety of heights or held at a wide range of temperatures. Specificity testing, which entailed determining the minimum number of *Bacillus anthracis* (Stern strain) spores for positive detection, had been completed prior to my arrival. The tests I conducted focused on the accuracy of BaDx when run with complex matrices. The complex matrixes consisted of horse nasal samples, horse mouth samples, horse stable dirt, fluctuating concentrations of bovine serum, whole milk, or blood. To test the complex sample matrices in the device, a 1 mL sample of the given matrix was obtained and placed in an epitube. If the sample was to be positive, an aliquot of *Bacillus anthracis* would be added to the epitube to obtain a final spore count of 10,000 spores when 350 uL of the sample was loaded into the device. After vortexing the epitube for 10 seconds, a small pipette was used to load the device (fill the culture chamber to the upper black line, 350 uL total volume). The cap was then placed over the culture loading entrance, and the device subsequently placed in an incubator set at 37ºC for 24 hrs. I loaded the BaDxs in the biological safety cabinet.

The next day the device was removed from the incubator and the detection assay was performed. This consisted of using an external magnet to move the disk/valve out from over the lateral flow assay port. On occasion the steel disk would resist movement, but upon slight shaking and repeated drags with the

external magnet the pathway from the cell culture chamber to the lateral flow assay chamber would be cleared. Once liquid began to flow into the assay, a timer was started. At twenty and sixty minutes the results were recorded and pictures taken. For each device there were six possible results: Positive, Negative, False Positive, False Negative, No Control, or Leaked. Almost all the devices resulted in a positive or negative, but on occasion the other results were obtained. Regardless, the results, notes, and pictures were added to a PowerPoint file consisting of every device trial, and to a data spreadsheet. To ensure adequate trials for the variation of the specificity testing, I built more BaDxs. During my internship I ran horse fluid samples and various blood concentration samples. The sample results and observations are included in Dr. Harper's to be published extensive paper on the BaDx deivce of which I will be a co-author. Each test determined whether or not the product would be accurate upon distribution.

Aside from all the aforementioned benefits of the BaDx – low cost, powerless, ease of use, accuracy – the Badx is also revolutionary due to its versatility. The Badx can be readily modified to detect other pathogens. If a pathogen can be identified by a lateral flow assay and can be cultured, it is likely a version of the BaDx can be manufactured to recognize the pathogen. Before arriving in New Mexico, Dr. Harper discussed with me his plans for his invention and I was immediately enthralled. Dr. Harper envisions the BaDx as a go to diagnostic device for food-borne bacteria like *E. coli* and *Salmonella* and bacteria of

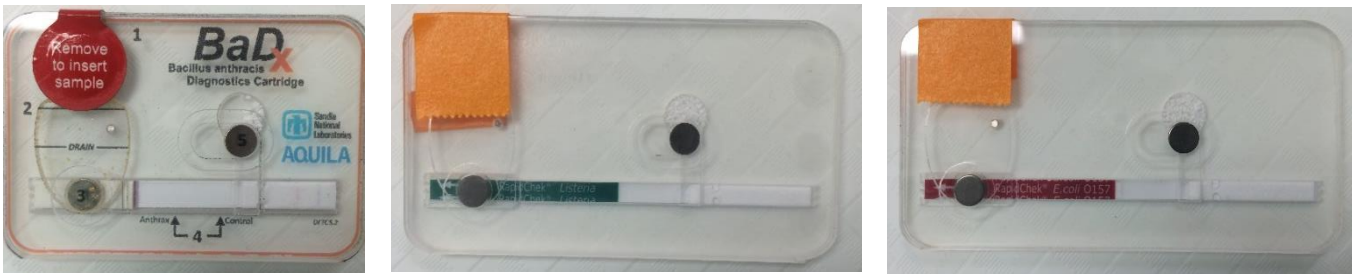


Figure 2. The original BaDx (left), a BaDx modified to identify *Listeria* (middle), and a BaDx modified to identify *Escherichia coli* (right). The orange tape is used to cover the entrance to the sample chamber rather than a cap to save resources during experimentation.

medical interest such as *Staph* and *Strep*. This customizable platform can thus change the monitoring of bacteria within hospitals, health clinics, and homes. Ultimately, instead of traveling to a doctors' office for a simple diagnostic test a person would be able to visit a local pharmacy or supermarket and purchase a BaDx for a very affordable price, and self-test. If the result comes back positive, a person will be able to get the medication needed, faster and more conveniently. During my internship, I have built and run tests on BaDxs modified to detect *Escherichia coli* and *Listeria*. As displayed in Figure 2, the dimensions of the BaDx had to be altered to accommodate the different sized lateral flow immunoassays.

E. coli and *Listeria*, foodborne pathogens, are a constant danger to human health, even in developed countries. As shown by the Chipotle *E. coli* outbreak and the frozen vegetable *Listeria* upsurge, precautionary measures must not be overlooked. A simple test with our device can ensure the safety of countless lives. As soon as the *E. coli* and *Listeria* samples arrived, they were cultured and tested in their respective devices. The *E. coli* version tested positive on the first try, but the *Listeria* version did not. The *Listeria* lateral flow assay was subsequently administered outside of the device and still resulted in negative result, indicating that an incorrect strain of *Listeria* was used. This discovery shifted my focus to the *E. coli* device while the correct *Listeria* strain was ordered. My first research goal was to determine the limit of detection, which was identifying the minimum number of bacteria necessary for positive detection. Andrew calculated the colony forming units per milliliter for the *E. coli* stock solutions, but as glycerol was added to the stock to preserve the cells I needed to determine the new concentration of the cells. In order to do so, I obtained an aliquot of the E. coli stock, stained the cells with fluorescent dye (SYTO 9 green-fluorescent nucleic acid stain and a red-fluorescent nucleic stain, propidium iodide, of the LIVE/DEAD BacLight kit), and used a fluorescent microscope to observe the cells on a hemocytometer. I proceeded to calculate the number of spores in the stock, and then produced aliquots and determined the necessary dilutions to test the *E. coli* device's ability to identify decreasing amounts of the pathogens. The first dilutions tested false negative, which came as a surprise. This caused me to test the E. coli strips, with cultures from stock and from an agar plate, and only the culture from the agar plate came up positive. The results were commensurate with the false negative devices, and I ran another trial to verify my findings. With one culture with cells from the glycerol stock, one from a washed glycerol stock, and one from an agar plate, I was able to determine that the stock alone

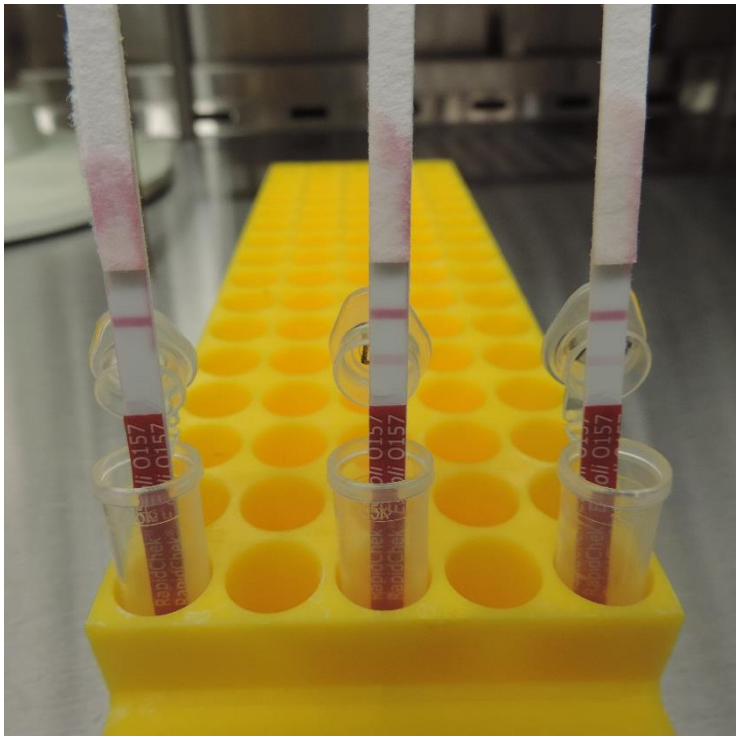


Figure 3. The left culture inoculated with spores from the glycerol stock solution – negative result; middle inoculated with spores from an agar plate – positive result; right culture inoculated with spores from the glycerol stock solution that had been washed (rinsed with media) – positive result

could not be used to inoculate the media for a successful detection (Figure 3). A washing step had to be added to remove the glycerol, which was interfering with the lateral flow assay. I proceeded to load an *E. coli* device with a myriad of cells that had been washed twice, but the device displayed a negative result. To directly compare the device result versus the epitube culture result, I am currently running a split test in which the same sample of growth media and cells is split in half, with half of the solution allocated into the device and the other half into the test tube. After a 24 hr culture, I will place a test strip into the epitube culture and run the *E. coli* device. This test will allow me to determine if it is the device design that prevents the washed cells from being identified. Due to the lack of detection of cells from the glycerol stock by the device, I was unable to determine the limit of detection. My results and discoveries will be included in a paper on the *E. coli* device, as well as prove the viability of an *E. coli* device (given the positive results from the agar plate cell trials). The plated cell results predict future successful products, and I look forward to seeing the *E. coli* versions of the BaDx mass produced for governmental and commercial use.

The BaDx, similar to most diagnostic tools involving cell cultures, is severely limited by the time it takes the bacteria to grow. What if the bacteria germination process could be accelerated? This was the goal of my parallel research endeavour. After reading and studying several papers that stated calcium dipicolinic acid (CaDPA) can increase bacteria germination, I began conducting experiments to ensure that the rapid germination chemistry would increase the bacteria growth of *Bacillus anthracis*. If this was the case, then CaDPA solution in the BaDx device would produce accurate results faster and more efficiently. My first experiments involved running cell cultures of *Bacillus anthracis* with varying concentrations of CaDPA in the media solution, and monitoring the bacteria growth hourly. After calculating the gram amounts of calcium nitrate and dipicolinic acid needed for each molarity, the solution had to be titrated to maintain a pH viable for bacteria growth – a pH of 6.5-7. According to literature, the CaDPA molarity of 30 mM to 40 mM is ideal for inducing cell germination, but that was near the solubility limit of CaDPA. To delay the precipitation process, I utilized a buffer. Once the rapid germination solution had the correct concentration of CaDPA, which functions best as a 1:1 chelate, and the solution held a pH close to 7, the solution was mixed with growth media

specific to *anthracis*, called PLET. For each concentration increment I designated three samples and one control. Throughout all the trials, the control was of the same concentration as the samples, with no bacteria spores added. Additionally, the three samples for each concentration for all experiments were inoculated with one thousand *anthracis* spores. After preparing the well plates with CaDPA solution, media, and spores, the plate was taken to Synergy microplate reader (Figure 4). The microplate reader measured optical density at a wavelength of 600 nm every hour over 24 hrs at 37ºC. The higher the absorbance, the more bacteria present in the sample.

In my first experiment, I prepared the CaDPA and PLET solutions separately, so when I mixed the two the concentration of CaDPA was cut in half. Given that the 60 mM and 90 mM CaDPA had already experienced significant precipitate, I ran the experiment with the 30 mM solution, which when diluted produced concentrations of 0 mM, 5 mM, 10 mM, and 15 mM. The first experiment, although run at lower concentrations than the desired 30-40mM, the results illustrate the benefit of the CaDPA (see Figure 5(a)). Panels (a)-(d) of Figure 5 are plotted as the average absorption of the three replicates of the specified concentration, subtracted by the control sample measurement to take into account the absorption caused by the media and CaDPA solution.

Figure 4. Using Gen 5 software, I programmed the plate reader to run a 24-hour culture, at 37ºC, shaking the plate before every read, and taking readings every hour (starting with zero hours). After the 24-hour cycle, I downloaded the readings, organized the readings by concentration and calculated the average, the average zeroed, the average minus the control, and the average minus the control zeroed. The control would account for the optical density of the solution and media itself, as well as the optical density of any precipitation that may occur.

My next mission was to test the rapid cell germination chemistry at higher concentrations. By adding the PLET directly to the CaDPA solution I was able to double the concentration. In an effort to use less resources, I designed the next trial in a 96 well plate. Unfortunately, the 96-well plate experienced a significant amount of bubbles and condensation in the sample wells due to shaking steps in the low volumes. This appears to have affected the reads, as shown in Figure 5(b), in which the 96-well plate readings do not have the steady growth curves displayed by the first experiment. In an effort to re-obtain the smooth growth curves of the first plate read, I ran a subsequent experiment in the 24-well plate with a maximum CaDPA concentration of 40 mM (Figure 5(c)). The graph shows that the 40 mM concentration produces the most cell germination, but the raw data indicates that the 40 mM growth curve may have confounding from the precipitation formed in the wells. As increased precipitation would hinder lyophilization and re-solubilization, so the subsequent research was performed

with a rapid germination concentration of 30 mM, and one more plate read was run to verify the effectiveness of a 30 mM concentration (Figure 5(d)). 30 mM concentration resulted in a final density 40% higher and reached grow saturation at 12 hrs compared with 18 hrs for no CaDPA.

After obtaining sufficient data proving that the calcium dipicolinic acid increases *anthracis* growth, I had to ensure that rapid germination chemistry would work within the Badx device. I tested this by running a BaDx device as I had previously, but I added the PLET to a 30 mM CaDPA solution, inoculated the combination with about thirty-five *Bacillus anthracis* spores, and then loaded the culture chamber of the device. The 24-hour culture resulted in substantial visible bacteria growth, and the device displayed a faint positive after twenty and sixty minutes. From this result I could extrapolate that the CaDPA was improving the accuracy of the device, as a positive after twenty minutes with only thirty-five spores was difficult to achieve prior to the introduction of the rapid germination chemistry. Unfortunately, the control BaDx device leaked, so I could not do a comparison with the original product. For a more conclusive verdict on the power of the germination chemistry I ran a second trial, with one hundred spores of *anthracis* in the sample and the control. The positive on the control was clearer and darker than on the device with the rapid germination chemistry, indicating that the chemistry may not aid in cell germination within the BaDx. With confirmation that the rapid germination chemistry did not impair the functionality of the device, I continued to the next phase of the experimentation – lyophilizing the calcium dipicolinic acid solution so that it could be packaged with the device. After utilizing a lyophilizer, I ran a plate read with the re-solubilized CaDPA solution to determine the germination effect. As shown by Figure 5(e), the re-solubilized rapid germination chemistry failed to increase germination. The plotted data is the average absorption zeroed to compare the growth curves – the control for the rapid germination samples was abnormally large, which made average absorption subtracted by control data unrepresentative of the bacteria growth. For a definitive read on the lyophilized solution, I ran another plate read with a positive control (in addition to the negative control of no rapid germination chemistry) that included calcium dipicolinic solution that did not undergo lyophilization. I also included a read for samples in which the rapid germination chemistry was lyophilized separately from the specific growth media as the CaDPA solution lyophilized with the growth media may impede solubilization of the CaDPA. Figure 5(f) shows the average of the absorption subtracted by the control, with all runs zeroed to the initial value. It was zeroed because, although the separately lyophilized rapid germination chemistry appeared to improve the bacteria growth, when compared to the initial zero-hour plate read the additional growth was not

significant. Once again, the rapid germination chemistry was shown to increase bacteria germination (positive control), but not when the chemistry underwent a lyophilization cycle. The rapid germination chemistry, lyophilized with the specific growth media (T) or separately (S), failed to increase growth. As the device is designed to be simple to use, with minimal processing steps, it was concluded that the rapid germination chemistry should not be included. Given the CaDPA chemistry loses effectiveness upon lyophilization, most likely do to a lack of solubility, a customer would have to make the calcium dipicolinic acid solution, titrate it, and then add it to device when the device is being loaded. Although an unrealistic option given the nature of the product, the experimentation proved the merit of the rapid germination chemistry.

2. Impact of Internship on My Career

My internship at Sandia National Laboratories marks a critical turning point in my chemical engineering career. My career goals, repertoire of skills, research ability, and determination to thrive in academia have been shaped by my time in New Mexico.

Prior to arriving in Albuquerque, I had gained research experience in Dr. Huinan Liu's Biomaterials and Nanomedicine Lab at the University of California Riverside. I studied the application of hydroxyapatite for orthopedic devices, and my primary obligations were to prepare hydroxyapatite samples, make simulated body fluid, pH various trials, analyze cell culture images, and run immersion tests. An invaluable experience, I was able to step into the research realm and contribute to my first scientific paper. But after my Sandia internship my knowledge has increased tenfold. Completely immersed in research for forty hour weeks, in addition to offsite research, I learned numerous techniques, lab procedures and protocols, and above all else cultivated my scientific mentality.

In manufacturing BaDx and the other devices, I learned how to use CorelDraw and a laser printer. In running and loading BaDx and *E. coli* devices, I learned how to culture cells in epitubes and on agar plates, count cells, calculate number of spores, dilute cells down to a specific count, make spore aliquots, pipette minuscule volumes, and work with multiple samples at once. But, more importantly, I learned to be

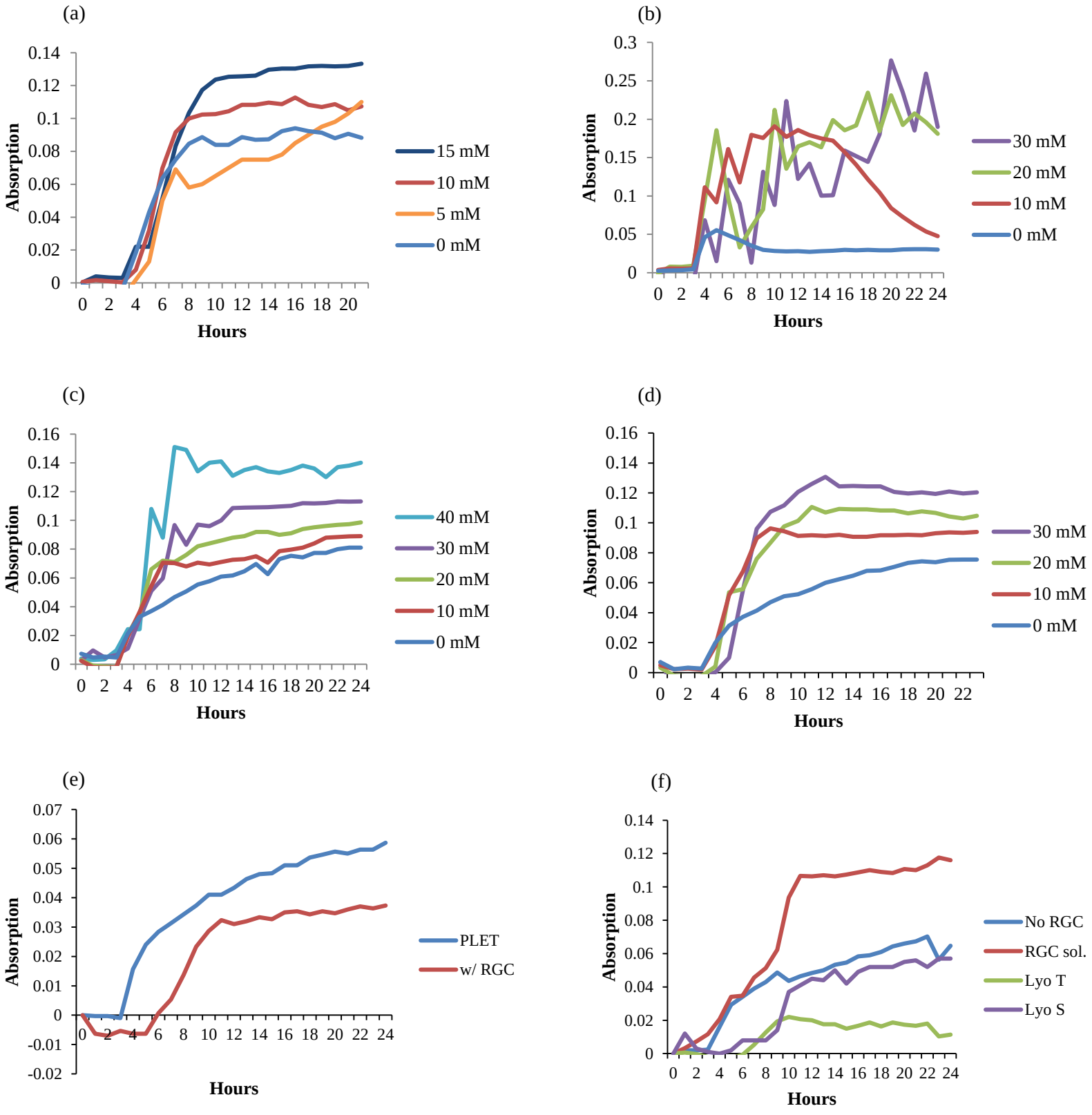


Figure 5. (a) 0-15 mM concentration of the rapid germination solution, 24 well plate (b) 0-30 mM concentration of the rapid germination solution, 96 well plate (c) 0-40 mM concentration of the rapid germination solution, 24 well plate (d) 0-30 mM concentration of the rapid germination solution, 24 well plate (e) 30 mM rapid germination chemistry and specific growth media (PLET) lyophilized together (f) 30 mM rapid germination chemistry in solution (RGC sol.), 30 mM rapid germination chemisty and PLET lyophilized together (Lyo T), 30 mM rapid germination chemistry lyophilized separate from the PLET (Lyo S)

meticulous. All the samples were prepared in a biosafety cabinet, as *Bacillus anthracis* (Sterne strain) is a Biosafety Level 2 organism. I have become proficient at sterile practice, and I keep my lab work organized. Making labels, recording dates, measuring pH's, storing samples in the proper conditions, vortexing samples prior to transferring aliquots, drawing and releasing fluid with the pipette to obtain and dispense a proper sample, constantly spraying hands and sources of contamination with 70% ethanol solution – what may seem to be minute details in the scheme of an entire project – are essential to successful experiments. Furthermore, in conducting the *E. coli* limit of detection tests, I gained skills in the use of a hemocytometer, a fluorescent microscope, a centrifuge, and cell dyes. The rapid germination chemistry investigations broadened my skill set further, as I performed well plate cultures, utilized a microplate reader (Biotek microplate reader) and its corresponding Gen5 software, measured out miniscule masses, incorporated a buffer, administered titrations, operated a pH meter, and executed data analysis. I also gained experience making specific growth media and testing its effectiveness, operating a lyophilizer, using a -80ºC freezer and using an autoclave.

In addition to gaining extensive lab experience, the internship has allowed me to grow as a man of science. I have been constantly solving problems, analyzing results, planning experiments, and executing procedures with precision. My techniques for taking notes, organizing data, managing multiple simultaneous tasks, understanding complex literature, and brainstorming all possible variations, sources of errors, and solutions have improved. I can function in a lab, be it maintaining the lab and the lab equipment (especially cleaning the biosafety cabinet after each use), ordering supplies, collaborating with my superiors and colleagues, or performing research on my own. I developed a deep respect for the world of research – a world I can contribute to and thrive in. Prior to my work at Sandia I was unsure of my post-graduate plans, but now I am committed to obtaining a doctorate in chemical engineering. My goal is to attend a top twenty-five chemical engineering doctorate program, graduate in five years and then ultimately head an engineering department in a company after publishing works and making my own discoveries.

Multiple times each week I met with Dr. Harper, and on each occasion I gained new insight into my experiments and chemical engineering in general. His lessons not only expanded my knowledge and skill set – he taught me how to approach problems and devise solutions. I was also taught by Dr. Harper's two other interns – Andrew Hunt, who will be starting Northwestern's chemical engineering Ph.D. program in the fall, and Robert Johnson, who will be graduating from New Mexico Tech with a Ph.D. in chemical

engineering after the fall semester. In addition to the constant instruction provided by Dr. Harper and his team, I participated in the Bioenergy and Defense Department intern forum. My presentation provided me with crucial experience in public speaking and answering questions in front of distinguished researchers. Making discoveries and performing research are wonderful, but being able to present ideas and sell oneself are just as important.

My summer internship research made significant headway in helping the Department of Homeland Security accomplish its mission and goals. The BaDx device and its alternate versions can be used in pathogen diagnostics abroad and at home, which in turn can prevent bioterrorism and improve national security. The rapid germination chemistry, although ultimately incompatible with the BaDx, has many Homeland Security applications. It can increase the percentage of spores that germinate, thus improving any procedures that rely on high cell culture yield. Furthermore, as endospores are much more difficult to kill than germinated spores, a rapid germination chemistry spray can be applied to accelerate the endospore eradication process. Further research into the versatility of the BaDx design and into rapid germination chemistry can aid in DHS's quest to make our nation safe.

3. Acknowledgments

First and foremost, I would like to thank Dr. Harper. I could not ask for a better mentor. Incredibly knowledgeable, assiduous, and instructive, Dr. Harper will always be a role model for me. I would also like to thank Andrew Hunt, who helped me tremendously during my first six weeks, Robert Johnson for teaching me several cell analysis techniques and sharing his experiences with me, and Jaclyn Murton and Dr. Bryan Carson for allowing me to use their lab equipment and chemicals. Lastly, I would like to thank the Department of Homeland Security for giving me this opportunity – this internship has laid down the first stepping stones on my path to success in the field of chemical engineering. Sandia National Laboratories is a multi-mission laboratory managed and operated by Sandia Corporation, a wholly owned subsidiary of Lockheed Martin Corporation, for the U.S. Department of Energy’s National Nuclear Security Administration under contract DE-AC04-94AL85000.

4. References

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