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MilkGuard: Low-Cost, Polymer-based Sensor for the Detection of Escherichia coli in Donated Human Breast Milk

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MilkGuard: Low-Cost, Polymer-based Sensor for the Detection of *Escherichia coli* in Donated Human Breast Milk

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ABSTRACT

Breast milk, the gold standard for infant nutrition, could prevent up to 13% of child deaths worldwide. However, many mothers are unable to breastfeed due to health conditions and other factors. Because of this, a network of more than 500+ human milk banks, which collect and distribute donated breast milk to infants, have emerged worldwide. However, operational costs to ensure the safety of this milk remain time-intensive and costly.

There are no existing diagnostics for rapid and on-site detection of bacterial contaminants in donated milk. Currently, many milk banks send samples to outside laboratories for bacterial culturing tests, which take 24-48 hours to receive results. In contrast, MilkGuard is an on-site detection method which ensures results in hours rather than days. To determine whether or not *E.coli* is present in donated milk, a drop of milk is deposited onto the sensor. If the milk is contaminated, the sensor will turn a blue color due to an enzyme-substrate reaction of the bacteria.

The goal of the project is to create a cost and rapid alternative to traditional bacterial culturing testing to screen for *E. coli* bacteria in donated human breast milk. This will allow users to ensure that milk samples are sterile enough to provide to young infants, while also providing breast milk banks an alternative that will allow them to screen more samples in a shorter amount of time.

Keywords: breast milk, breast milk banking, breastmilk sensor, polymer-based sensor, microfluidics, multiplexed sensor, paper chromatography, *E. coli* detection

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CHAPTER 1: INTRODUCTION

1.1 Background Motivation and Big Picture Rationale

On a global scale, breast milk is considered the gold standard for infant nutrition. Containing protective antibodies that optimize an infant's chances of survival, breast milk is the ideal substance to provide the necessary nourishment in the first six months of life.[1] In addition to offering essential nutrients, breastfeeding provides significant long-term benefits that can contribute to positive health outcomes. It can lower the risk for infectious diseases like diarrhea-related diseases and respiratory infections, as well as lowering the likelihood of developing chronic conditions such as asthma and allergies in early childhood.[2] Additionally, the nutritive benefits of breast milk can be seen to enhance cognitive development, as demonstrated when comparing intelligence quotients between children who were breastfed for seven months as opposed to those who were only breastfed for one month.[3] Lastly, breast milk provides invaluable immune system benefits, which reportedly could prevent up to 13% of child deaths worldwide.[4]

Although breastfeeding has been proven to be beneficial to infants and mothers alike, many women are unable to breastfeed. One of the major reasons that can prohibit a mother from breastfeeding her child is her own health status, as women who are infected with HIV/AIDS or other chronic, blood-borne conditions are recommended not to breastfeed.[5] Additionally, some mothers do not breastfeed their infants because child may have a metabolic disorder whereby they cannot digest breast milk or they are unable to latch on to the breast.[6] Lastly, some mothers do not breastfeed their children due to cultural factors. For instance, some mothers may work in places that do not allow them to pump milk during the day, while others may not have adequate support at home or not have anyone in their lives who has breastfed in the past.[7] All of these

[1] J. Wagner, C. Hanson, and A. Berry, "Donor Human Milk for Premature Infants: A Review of Current Evidence," *ICAN: Infant, Child, & Adolescent Nutrition*, vol. 5, no. 2, pp. 71-77, 2013.
[2] "Breastfeeding and the Use of Human Milk," *American Academy of Pediatrics*, vol. 129, no. 3, Mar. 2012.
[3] M.S. Kramer, "Breastfeeding and Child Cognitive Development," Archives of General Psychiatry, vol. 65, no. 5, pp. 578-584, 2008.
[4] G. Jones, R.W. Steketee, R.E. Black, Z.A. Bhutta, S.S. Morris, , & Bellagio Child Survival Study Group, "How many child deaths can we prevent this year?" *The Lancet*, vol. 362, pp.65-71, 2003.
[5] "Acceptable medical reasons for use of breast milk substitutes," World Health Organization, Geneva, Switzerland, 2009.
[6] J.H. Kim and S. Unger, "Human milk banking," *Paediatrics Child Health*, vol. 15, no. 9, pp. 595-598, Nov. 2010.
[7] US Department of Health and Human Services. "The Surgeon General's call to action to support breastfeeding." 2011.

factors can discourage mothers from breastfeeding their children, putting these infants at an unfortunate disadvantage.

1.2 Breast Milk Banking Improves Access to Breast Milk for Infants in Need

In an attempt to increase access to human breast milk that is free from contamination there has been a global emergence of human breast milk banks to help infants in need. Table 1 compares the number of milk banks, pasteurization method and quality control procedures of human breast milk banks around the world.

Table 1. Comparison of Human Breast Milk Banks Globally

Country/Region	Number of Milk Banks	Pasteurization Method	Quality Control Procedures
North America	26	Holder Method	Bacterial Culturing
Europe	214	Varies	Varies
Brazil	210	Holder Method	Dornic Acidity, Bacterial Culturing, Crematocrit Testing
South Africa	>12	Flash Pasteurization	Bacterial Culturing
India	14	Holder Method	Bacterial Culturing

Over the past several decades, North America has seen a growth in traditional human breast milk banking, as indicated by the 26 human breast milk banks that are currently a part of the Human Milk Banking Association of North America. In comparison with North America, there are 214 human breast milk banks in Europe that are a part of the European Milk Bank Association. France has the highest number of human breast milk banks with 36, whereas

countries like Russia and Portugal have 1 human breast milk bank each.[8] However, as indicated in **Table 1**, operating procedures vary amongst the EMBA members.

In considering countries in the developing world, Brazil has the most extensive and long standing human breast milk banking system in the world. They have 210 breast milk banks nationwide to support 3 million annual births. This extensive network has been a key factor in decreasing the infant mortality rate by 73% from 1985 to 2013.[9] In contrast to Brazil, South Africa only has 12 breast milk banks. These breast milk banks face challenges of resource-poor settings where they struggle with inadequate staffing and a lack the necessary funds for equipment maintenance.[10] Conversely, India is seeing substantial growth in the milk banking industry. Although are only 14 human breast milk banks nationwide, which is not enough to support over 25 million infants born annually, the Indian Academy of Pediatrics' has made huge strides in creating and updating a comprehensive set of guidelines for milk banks in India.[11]

1.2.1 Breast Milk Banking Process Overview

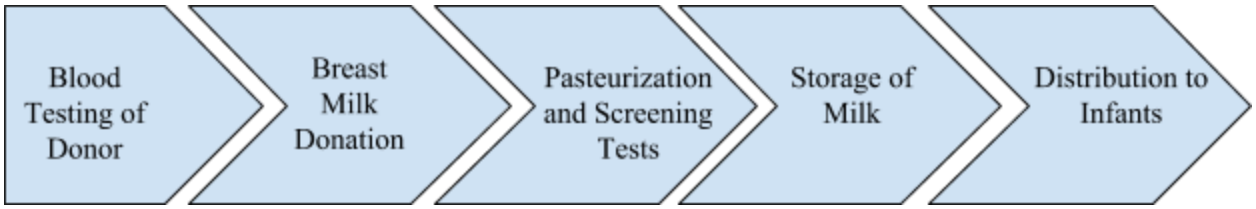

Figure 1. *Schematic of operational flow for breast milk banking.*

To verify the safety of donated human breast milk for consumption, human breast milk banks have implemented controls to ensure there are no contaminants or dangerous substances in the milk. The primary opportunities for contamination include the initial lactation and pumping by the mother, initial storage, transportation to the milk bank, reception at the milk bank, and operational operational processes within the bank itself.

[8] "EMBA," European Milk Bank Association, 2016.
[9] F. Pires. (2014 Dec 2). Learning from Brazil's success with milk banks. *University of Michigan News.*
[10] I. Coutsoudis, M. Adhikari, N. Nair, and A. Coutsoudis, "Feasibility and safety of setting up a donor breastmilk bank in a neonatal prem unit in a resource limited setting: An observational, longitudinal cohort study," *BMC Public Health*, vol. 11, no. 356, 2011.
[11] K. Bharadva et al., "Human milk banking guidelines," *Indian Pediatrics*, vol. 51, no. 6, pp. 469-474, 2014.

As such, a stringent quality control process must be implemented to ensure consumers are not exposed to harmful contaminants.

Through a review of current best practices of safety controls employed by human breast milk banks, the process outlined in **Figure 1** was found to be the general breast milk banking procedure to verify and ensure the safety of donated human breast milk. The general milk banking process includes donor testing, breast milk donation, pasteurization & screening tests, storage of the milk, and distribution to infants.[12]

The process begins with the human breast milk bank testing the donor's blood before she donates to ensure that the milk will not be able to transmit diseases such as hepatitis B/C, syphilis or HIV/AIDS. Further the donor's blood is tested to ensure that it does not contain traces of illegal drugs, certain medications, tobacco products, herbal supplements, or environmental contaminants as the presence of these substances in breast milk can negatively impact a child's health.[13] Once a donor has been successfully screened, she can donate human breast milk that may be collected at home or at the milk bank.

Once the human breast milk has been donated, human milk banks will pasteurize the collected milk to destroy any harmful bacteria present in it. Following pasteurization the donated human breast milk is cultured, often times by a microbiological lab that is external to the milk bank itself.

After the donated human breast milk has undergone pasteurization and culturing, it should be stored at four degrees Celsius for up to 48 hours and frozen at negative 20 degrees Celsius for three to six months.[14] Finally, the donated human breast milk is distributed to mothers and infants in need.

1.2.2 Current Methods for Pasteurization at Milk Banks

The goal of pasteurization is to limit the risk of harmful bacteria present in donated human breast milk. Pasteurization methods use high heat levels to inactivate

[12] K. Bharadva et al.
[13] K. Bharadva et al.
[14] D. Silvestre, M.C. Löpez, L. March, A. Plaza, and C. Martinez-Costa. "Bactericidal activity of human milk: stability during storage," *British Journal of Biomedical Science*, vol. 63, no. 2, pp. 59-62, 2006.

bacteria, viruses, and other potential pathogens.[15] Although industrial-grade pasteurizations are used in the dairy industry to ensure safety of dairy milk, many human breast milk banks are unable to afford this type of technology because they can cost up to $60,000.[16] Instead, Holder pasteurization is a common method where donated human breast milk is heated up to 62.5 degrees Celsius for 30 minutes in a shaker water bath.[17] Another method called flash pasteurization involves heating the donated human breast milk up to 72ºC for 16 seconds.[18] These methods are widely practiced around the world at human breast milk banks. Depending on the human breast milk bank, pooling of the donated human breast milk from multiple donors may occur to maximize nutritional content and uniformity.[19] While pooling may overall enhance the quality of the donated human breast milk, contamination makes it difficult to determine the source.[20]

Unfortunately, there is no guarantee pasteurization will destroy all of the harmful bacteria present in the donated human breast milk. In addition, pasteurization may negatively affect beneficial properties of the donated human breast milk. It generally does not alter nutritional carbohydrate, fat, and salt composition, but the high level of heat may reduce or destroy beneficial immune factors like IgA, IgG, IgM, and lactoferrin.[21] However, without an alternative method, human breast milk banks continue to pasteurize the donated breast milk to to ensure safety for infants.

1.2.3 Cost and Resource Efficiency of Culturing Method for Screening Breast Milk

Bacterial culturing may be done before and/or after to verify the success of pasteurization in the human breast milk banking process. It is practiced pre-pasteurization and post-pasteurization to determine the microbial content in donated human breast milk. Pasteurization methods often do not maintain a high constant temperature resulting in

[15] "Strengthening Human Milk Banking: A Global Implementation Framework," PATH, 2013.
[16] S. Landers, and K. Updegrove, "Bacteriological screening of donor human milk before and after Holder pasteurization," *Breastfeeding Medicine*, vol. 5, no. 3, pp. 117-121, 2010.
[17] K. Bharadva et al.
[18] W. E. Corpelejin, et al.
[19] "Strengthening Human Milk Banking: A Global Implementation Framework," PATH, 2013.
[20] "Strengthening Human Milk Banking: A Global Implementation Framework," PATH, 2013.
[21] W. E. Corpelejin, et al.

remaining bacterial contamination.[22] Consequently, human breast milk banks send out small samples or batches of donated human breast milk to a microbiological lab to determine if any bacteria grows within a 24 hour period. Depending on the guidelines of the human breast milk bank, if bacterial colony forming units (CFUs) are found above a certain limit, the milk is likely discarded to ensure microbiological quality.[23]

Even though donated human breast milk could be heavily contaminated, bacterial culturing is not standardized amongst human breast milk banks around the world. It is a costly and lengthy process, so human breast milk banks must balance quality assurances along with their own resources and financial limitations. Using a sterile laboratory and hiring a highly trained laboratory technician quickly raise the costs for bacterial culturing. Additionally, microbiological testing results are not instantaneous.

Measuring the full financial impact of human breast milk banks around the world is a challenge. However, the processing and operating costs of donated human breast milk appear to be negligible compared to the cost of treating vulnerable infants with immune system diseases and complications.[24] Bacterial culturing is important in monitoring the success or failure of pasteurization methods to determine contamination and prevent future problems for infants. Therefore, human breast milk banks may be a more cost effective solution for infants and mothers in need.

1.3 Problem Statement

Current methods of culturing bacteria when testing samples is expensive and time-consuming for milk banks in both developed and developing communities. To reduce these factors, milk banks are in need of a rapid, affordable, accurate, and sensitive sensor that is able to detect and quantify the presence and concentration of *E. coli*.

[22] K. Bharadva et al.
[23] K. Bharadva et al.
[24] J.H. Kim, and S. Unger, "Human Milk Banking," *Paediatrics & Child Health,* vol. 15, no. 9, pp. 595–598, 2010.

1.4 Literature Review: Existing Diagnostics Technology for Human Breast Milk

The following table compares diagnostic technologies for breast milk and their varying purposes, detection methods, prices, applications, and times required to obtain results. The Happy Vitals test kit and the Milk Screen strips are commercially available, while the Biosensors for Quality Assurance of Dairy Products, Food Biosensors, and Electrochemical DNA-based biosensor, and Poly Methyl methacrylate (PMMA) “Lab-on-a-chip” for Human Breast Milk Defatting are still in research stage.

Table 2. Diagnostic products and technologies for milk on the market and under development.

Product	Component Detected	Detection Method	Price	Application Setting	Time
Happy Vitals[25]	Macronutrient levels, heavy metals, vitamins, minerals	Lab testing by microbiologists	$169.95 - $695.95	Commercially available; lab screening of samples sent from mothers at home	3-5 days
Milk Screen by Upstring Strips[26]	Alcohol, Docosahexaenoic acid	Colorimetric test strip	$14.99 for eight strips	Commercially available; testing at home	2 minutes
NeoGen Tests for Dairy Products[27]	β-Lactoglobulin (BLG), casein, total milk (casein and whey proteins), allergens,	Screening microwell tests, test strips, lateral flow strips, microwell enzyme-linked immunosorbent assay (ELISA) tests	Not advertised	Commercially available; used throughout production process	30 minutes
Soleris System and Vials[28]	*E. coli* O157:H7	Ready-to-use vials with colorimetric indicator, incubator, and system software	Not advertised	Commercially available; used throughout production process	4-24 hours
Biosensors for Quality Assurance of Dairy Products[29]	Nutrients and pesticides	Temperature, light, bacteria	Variable, >$1,000	Laboratory research, primarily cow milk	Variable
Food Biosensors[30]	General quality, carcinagen aflatoxin M1	Optical biosensing of chemiluminescence and fluorescence detection	Variable, >$1,000	Laboratory research, primarily cow milk	Variable
Electrochemical DNA-based biosensor [31]	Bacillus cereus	DNA-based Au-nanoparticle modified pencil graphite electrode (PGE)	Low	Laboratory Research	Variable
Poly Methyl methacrylate (PMMA) "Lab-on-a-chip"[32]	Energy content as measured by fat, protein and lactose	Cross-flow microfiltration structure	Low	Laboratory research	Few minutes-two hours

[25] "Tracking Better Health for Mom and Baby." Happy Vitals. N.p., n.d. Web. 20 November 2017.
[26] "Milkscreen." Milkscreen Home Test for Alcohol in Breast Milk. N.p., n.d. Web. 20 November 2017.
[27] "Total Milk/Casin/BLG." NeoGen. Happy Vitals. N.p., n.d. Web. 20 November 2017.
[28] "*E. coli* Vial." NeoGen. Happy Vitals. N.p., n.d. Web. 20 November 2017.
[29] Pividori, Maria Isabel, Salvador Alegret. "Biosensors in Quality Assurance of Dairy Products." Portable Biosensing of Food Toxicants and Environmental Pollutants. Eds. Dimitrios P. Nikolelis, et al. CRC Press, 2013. 411-442.
[30] Ahmed, M. U., Zourob, M., & Tamiya, E. Food biosensors. Cambridge: Royal Society of Chemistry, 2017. 521 pp.
[31] Izadi, Z., Sheikh-Zeinoddin, M., Ensafi, A. A., & Soleimanian-Zad, S. (2016). Fabrication of an electrochemical DNA-based biosensor for Bacillus cereus detection in milk and infant formula. *Biosensors & Bioelectronics*, *80*582-589. doi:10.1016/j.bios.2016.02.032
[32] Lai, M., Lai, C. T., Keating, A., Dell, J., & Liu, Y. Cross-flow microfiltration for lab-on-chip defatting of human breast milk. *Biomedical Applications of Micro- and Nanoengineering IV and Complex Systems* 2008. Doi: 10.1117/12.810584.

1.5 Literature Review: Existing Low-Cost, Microfluidic Sensors for Bacterial Detection

1. **Paper sensors for bacterial detection in dairy milk and orange juice[33]**

   Paper sensors exist in a simple kit that would allow for untrained personnel to carry out sensitive, multiplexed detection *E. coli* in food samples. These paper based sensors utilize sol-gel-derived silica inks placed by an ink-jet printing technique to produce colorimetric results, which can be judged by the human eye or a combination of digital camera and image analysis software.

2. **Integrated capture, concentration, polymerase chain reaction, and capillary electrophoretic analysis of pathogens on a chip[34]**

   This sensor is comprised of micropumps and valves on a PMMA chip that utilizes PCR and amplicon separation for selective targeting of pathogens. As this sensor maintains advantages such as analysis speed and compact size, it also boasts detection sensitivity and analysis volume that are capable of identifying a dangerous pathogen with contemporary significance at clinically relevant sensitivities (O157 *E. coli* at 0.2 CFU/μL).

3. **Optical enzyme-linked immunosorbent assay on a strip for detection of Salmonella typhimurium[35]**

   This rapid and sensitive enzyme-linked immunosorbent assay (ELISA) on an immunochromatographic strip utilizes colorimetric and chemiluminescent techniques to detect *Salmonella typhimurium*. It can quantitatively measure the pathogen in the range of 9.2×10^3 to 9.2×10^6 CFU/mL within 20 min (colorimetric method) and 16 min (chemiluminescent method).

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[33] Hossain, S. M., Ozimok, C., Sicard, C., Aguirre, S. D., Ali, M. M., Li, Y., & Brennan, J. D. Multiplexed paper test strip for quantitative bacterial detection. *Analytical and Bioanalytical Chemistry* 2012, *403*(6), 1567-1576. doi:10.1007/s00216-012-5975-x

[34] Beyor, Nathaniel et al. "Integrated Capture, Concentration, PCR, and Capillary Electrophoretic Analysis of Pathogens on a Chip." *Analytical chemistry* 81.9 (2009): 3523–3528. *PMC*. Web. 5 Dec. 2017.

[35] Park, Sojung, Yong Tae Kim. "Optical Enzyme-linked Immunosorbent Assay on a Strip for Detection of Salmonella typhimurium." *BioChip Journal* 4.2 (2010): 110-16. Web.

4. **Microfluidic Assay for Continuous Bacteria Detection Using Antimicrobial Peptides and Isotachophoresis**[36]

   This assay utilizes an electrophoretic focusing technique called isotachophoresis to establish a channel of heavily concentrated antimicrobial peptides (AMPs). The contaminated fluid travels down this channel and the bacteria is separated and from the AMPs where its fluorescence signal to provide a detectable and quantifiable measurement.

1.6 Field Review: Capturing Customer Desired Outcomes

1.6.1 Mothers' Milk Bank of San Jose

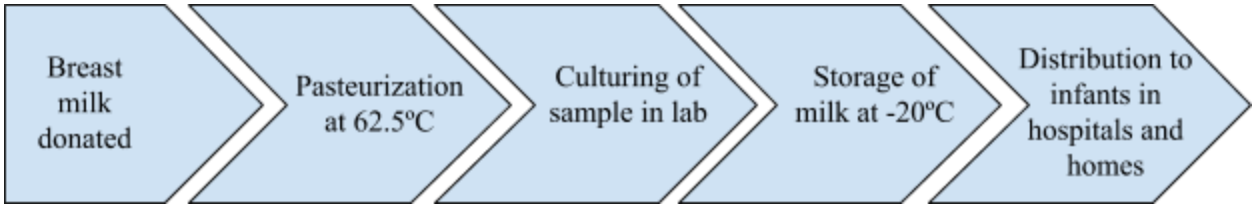


Figure 2. *Schematic of operational flow at Mother's Milk Bank in San Jose, California.*

A visit to Mother's Milk Bank (MMB) in San Jose, California provided insight to the industry and explained the bank's processes.[37]

To begin, mothers who have passed the blood test screening pump their excess milk into glass or plastic containers, package the containers in a cooler, and ship the cooler to MMB. Upon reception of the shipments, MMB slowly thaws the milk breast milk to keep the milks live tissue intact. Donations from about three donors are pooled and stored in glass jars. The jars are then pasteurized at 62.5ºC for 30 minutes. After pasteurization, a sample from each batch of pooled milk is cultured in a lab and a microbiologist screens for colony-forming units. A significant portion of the donated milk is thrown away due to contamination, but loss at MMB San Jose is about half the rate at other milk banks in the United States. Clean milk is stored in -20ºC freezers

[36]Schwartz, O., & Bercovici, M. (2014). Microfluidic Assay for Continuous Bacteria Detection Using Antimicrobial Peptides and Isotachophoresis. *American Chemical Society Publications,86*. Retrieved December 5, 2017.
[37] Sakamoto, Pauline. "Mother's Milk Bank Tour and Informational Interview." 20 Oct. 2018.

before being donated to infants in hospitals and homes, where it can be refrigerated for 24-48 hours.

The most common contaminant in donated human breast milk *after* pasteurization is *Bacillus cereus.* MMB attributed this contamination to improper cleaning of the mother's pumps when they are expressing, but the proliferation of *Bacillus cereus* is accelerated during pasteurization.

The demand for donated human breast milk from MMB surpasses the supply, which is attributed to the promotion of human breast milk over formula by the private, for-profit milk bank Prolacta. The existence of online milk banking—despite being illegal in the state of California—evidences inadequate supply of donated breast milk and mothers' willingness to obtain it. Additionally, a lack of regulation prevents milk from reaching infants high-risk who need it most. Rather, infants drinking milk obtained via online avenues are 6 months old on average. In addition to feeding infants, human breast milk is the only known treatment for necrotizing enterocolitis, a gangrene in the gut.

The existing standards place strict limits on the permissible contamination; federal organization recommend that milk banks "kill all pathogens" in stored milk, creating a zero tolerance for colony forming units. Elimination of these pathogens through pasteurization, however, results in a loss of about 20% of the naturally occurring immune factor IgA.

The criteria for a sensor to detect *E. coli* were outlined as follows:

- Objective results that eliminate the risk of human error in interpreting results, preferable standard device that reads and displays quantifiable results
- Limit of detection 10^4 CFU/mL
- Prove that milk does not need to undergo pasteurization

1.6.2 PATH[38]

PATH is an international non-governmental organization (NGO) that leads global

[38] PATH Informational Interview [Telephone interview]. (2017, December 4).

health innovation by accelerating innovation across five different platforms: vaccines, drugs, diagnostics, devices, and system and service innovations.[39] Dr. Kiersten Israel-Ballard and Kimberly Amundson provided insight to PATH's mission, projects, and resources they apply across the globe.

As a body working to improve health equity and innovating medical technology, PATH provides expertise and tools to niche markets in order to break down barriers and save lives. One of such markets include the Maternal Health sector.

As stated previously, there are no standards for storage and treatment of donated human breast milk in human breast milk banks across the globe. While different governance bodies have individual standards, human milk banks across the globe have various practices to meet such standards. In an effort make improvement, PATH has released national guidelines for human breast milk banks in India to "ensure timely initiation of breastfeeding and promote breastfeeding practices."[40] By collecting feedback from across the globe, PATH has created their Global Implementation Framework to share information on best practices surrounding this sector.

Like Mother's Milk Bank of San Jose, PATH described challenges surrounding the human breast milk banking industry. They highlighted that many banks outsource testing in order to determine if contamination is present within samples. This outsourcing is both expensive and time-consuming, so professionals in underdeveloped settings often do not have access to such resources and are in need of a simple, rapid, and point-of-care solutions.

Lastly, PATH shed light on the implications that the sensor could potentially have with breastfeeding mothers who may eventually see this product go to market. According to feedback they collected, a common theme with mothers is that they never feel like they are enough. Mothers feel like they could produce more milk with greater nutritional value in order to provide the best care possible for their children. As a team working to develop

[39] PATH: Driving Transformative Innovation to Save Lives. (n.d.). Retrieved December 5, 2017, from https://www.path.org/index.php

[40] Gurnani, V., Khera, A., Deb, S., Prabhakar, P. K., Singh, A., Kaujia, R., . . . Bhart, V. (n.d.). *National Guidelines on Lactation Management Centres in Public Health Facilities*. Government of India: Child Health Division: Ministry of Health and Family Welfare.

a resource that could be perceived as a new tool to "judge" a mother's milk, our team must be cognizant of the ramifications that can result and its effects on mothers across the globe.

1.6.3 Sion Hospital, Mumbai, India[41]

In June of 2017, one member of the 2016-17 senior design team, Callie Weber, and one member of the EWH team, Karen Mac, toured the birthing unit, collection sites, milk banking lab, and microbiology departments led by the PATH team at the Sion Hospital in Mumbai, India. Here the team gained valuable insight for the future direction of MilkGuard.

They learned that the sensor must guarantee results with 99% assurance in order to be used in the milk bank setting. It must eliminate false positives, having the ability to differentiate between the detection of *E. coli* and other pathogens. It must also eliminate false negatives due to alternative cell lines of *E. coli* present in the sample.

The sensor must be able to detect other potential contaminants in breast milk in order to be a viable alternative to bacterial culturing. Currently, Sion Hospital is mainly concerned with the contaminants: *Staphylococcus aureus, Asilobacterium, Klebsiella, Enterobacterium, Enterococcus*, and *Bacillus*, and gram-negative bacteria.

The strip's colorimetric results should have a strict limit indicating the permissible level of contaminant versus an unusable one. A quantification method integrating a mobile phone application could potentially be employed, but the use of this platform should take into consideration the willingness and aptitude of the hospital employees to adopt its use. However, should it be used, then the solution should be developed for Android given the predominance of Android users over iPhone users in India. The hospital is currently making the transition from paper-based records to an electronic medical record system, indicating the under resourced context in which the strip could be used.

[41] Weber, Callie, Mac, Karen. "MilkGuard: Mumbai Field Research Travel Report." June 2017.

The Sion Hospital Breast Milk Bank currently performs bacterial culturing before and after pasteurization, but still pasteurizes all of their milk due to limited timing and resources. A sensor with a limit of detection of 10^3 CFU/mL could stand in as a substitute to culturing pre-pasteurization. This would allow for contaminated milk to be thrown out without unnecessarily pasteurizing it. Additionally, the sensor would allow for milk to be safely pooled into batches, which is the preferred method of most milk banks (including Mother's Milk Bank of San Jose) because it standardizes the nutritional content of the donor milk for all recipients.

1.6.4 Mothers' Milk Bank of Aurora

Mother's Milk Bank of Aurora maintains all of the same HMBANA standards and regulations as Mother's Milk of San Jose. Rather than screening their milk on-site, this bank outsources the bacterial culturing tests to a laboratory, which can take up to three days for transportation, testing, and results. Given the bank's holistic approach to maternal health, they brought to attention the psychological impact which a product that qualifies the viability of a mother's breast milk could have on a mother who is already in a compromised or sensitive state.

1.6.5 Northwest Mothers Milk Bank of Portland

Northwest Mothers Milk Bank adheres to the standards set by HMBANA. This bank also outsources their bacterial culturing tests to a laboratory, taking up to three days for transportation, testing, and results. The bacterial tests are unspecific. Although this bank also follows a "zero tolerance" policy for bacterial contamination, they retest any results that come back between 10 and 50 CFU/mL. Sometimes the milk resulting in 10 to 20 CFU/mL contamination can successfully pass a retest and be cleared for distribution. The milk between 20 and 50 CFU/mL usually fails a retest. This bank only performs post-pasteurization tests. They cannot justify paying for pre-pasteurization tests due to the high percentage of milk that passes the tests after pasteurization. However, they did recognize that a fairly cheap method for pre-pasteurization screening, such as

our device, would be useful in eliminating the pasteurization process for milk that is highly contaminated. This milk would instead be thrown away initially, instead of undergoing the pasteurization process, waiting for test results, then throwing it away.

1.7 Project Goal

The overall goal of the project is to develop a low-cost, polymer-based sensor that detects *Escherichia coli* bacteria in donated human breast milk. The sensor aims to enable easy, efficient detection, eliminating the need for pasteurization of uncontaminated donated human breast milk in breast milk banks.

1.8 Project Objectives

The sensor's development is focused on time and cost factors that are associated with the pasteurization and culturing of cells within donated human breast milk at established breast banks. Based upon customer interviews with Mother's Milk Bank of San Jose, PATH, and other milk banks associated with the Human Milk Banking Association of North America, criteria include, but are not limited to:

- Sensing contamination of at least 10^3 CFU/mL
- Obtain objective, precise, and quantifiable results
- Mitigation of false positives and false negatives

Our overall goal is to create a simple, affordable, and easily manufacturable sensor that can consistently test for a variety of pathogens found within donated human breast milk in both underdeveloped and developed industry settings. To do so, we plan on taking the following steps:

- Replicate detection method of ß-galactosidase in breast milk
- Ensure detection method of ß-galactosidase is compatible in breast milk
- Determine sensor sensitivity
- Improve uniformity of biological reagent deposition on sensor
- Accelerate cell-lysing steps

1.9 Solution Ideation

1.9.1 Existing Sensor Mechanism Overview

The proposed method of using the paper based sensor consists of the following steps:

1. *E. coli* cells in human breast milk sample will be lysed prior to use of paper based sensor.
2. The bottom part of the sensor will be dipped into the milk sample.
3. The enzymes from the *E. coli* cells will hydrolyze the substrate on the sensor, causing a blue color to form in the presence of contamination greater than 10^3 CFU/mL.
4. Calorimetric results will be read by user to determine safety for consumption.

1.9.2 Opportunities for Improvement Upon MilkGuard Sensor

1. **Lower limit of detection to 10^3 CFU/mL**

   Since trace amounts of bacteria are capable of compromising an infant's growing immune system, the sensor must be able to detect low concentrations to ensure infant health safety.

2. **Develop a multiplexed sensor to test for additional contaminants**

   The MilkGuard sensor could be adapted to test for additional pathogens that are of primary concern in the storage of breast milk. The sensor could have the ability to test multiple pathogens simultaneously.

3. **Improve dispensing techniques**

   Create an easy method for users to deposit milk on the strip.

4. **Improve manufacturability**

   Implement the use of gel-printing to rapidly create more uniform test strips.

5. **Optimize packaging**

   Design a package that will provide satisfactory storage, enabling the sensor to have a long shelf life and still be easy to open.

6. **Develop mobile image capture for quantification of bacterial contamination**

A mobile phone application that could provide real time quantification of contamination would greatly benefit the end user. As android phones are becoming more popular, a mobile platform would allow greater access to powerful data analysis.

1.9.3 Anticipated Modifications to Mechanism

1. **ß-galactosidase v. ß-glucuronidase.** The current prototype senses the presence of *E. coli* via the presence of ß-glucuronidase, however, sensor's specifications could be improved by changing the detection method to sense ß-galactosidase. The detection of ß-galactosidase is a known and effective standard method of detection for many *E. coli* strains.[42] In collaboration with a microbiologist, Dr. Craig Stephens, PhD, changing the enzyme detected could improve the sensor's functionality as lactose, present in both dairy and human breast milk, catalyzes the production of ß-galactosidase in strains with an intact LacZ gene.[43] This alteration has potential to improve the functionality of the sensor as it is used in a breast milk buffer.
2. **Uniform Printing.** The reagents could be deposited onto the paper surface via ink-jet printing. This structured-layers approach prints a capture layer (polyvinyl amine), a lower sol–gel-derived silica layer, the reagent, and a top silica layer, with reagents entrapped in corresponding zones.[44] To optimize the printing performance (ability to jet the inks) as well as the enzyme activity, the different printing solutions could be modified by the addition of glycerol, to control viscosity, and Triton X-100, to control surface tension.[45] The piezoelectric Epson Stylus C88 Inkjet Printer will be used to

[42] Rivas, L., Mellor, G. E., Gobius, K., & Fegan, N. (2015). *Detection and typing strategies for pathogenic Escherichia coli*. New York: Springer.
[43] MilkGuard Informational Interview [Personal interview]. (2017, November 7).
[44] Li, Jia, Fabrice Rossignal, Joanne Macdonald. Inkjet Printing for biosensor fabrication: combining chemistry and technology for advanced manufacturing. Royal Society of Chemistry 2015 15, 2538-2558. DOI: 10.1039/C5LC00235D
[45] S. M. Zakir Hossain, Roger E. Luckham, Anne Marie Smith, Julie M. Lebert, Lauren M. Davies, Robert H. Pelton, Carlos D. M. Filipe, and John D. Brennan. Development of a Bioactive Paper Sensor for Detection of Neurotoxins Using Piezoelectric Inkjet Printing of Sol−Gel-Derived Bioinks. Analytical Chemistry 2009 81 (13), 5474-5483 DOI: 10.1021/ac900660p

deposit the layers and manufacture sensors on Whatman Grade 1 Qualitative Filter Paper, improving the sensor's deposition uniformity and economy of scale.

3. **Immunomagnetic Separation.** IMS would allow the sensor to selectively detect a target pathogen. It allows a target organism to be separated from a food matrix and background microflora, and concentrated into a smaller sample volume, which would make it easier for a paper strip to detect a concentrated *E. coli* sample.[46]

The sensor's efficiency will be improved by streamlining time-consuming, pre-treatment steps onto the sensor. Its applicability will be enhanced by lowering the sensor's limit of detection, and its reliability will be guaranteed through the mitigation of false negatives and false positives. Finally, its viability as a consumer product will be enabled through manufacturable, scalable preparation.

1.10 Key Deliverables

The key deliverables of the project are the following:

1. Produce consistent prototype sensor.
2. Verify the viability of ß-galactosidase detection in human breast milk.
3. Generate standard concentration curve corresponding to colorimetric results for ß-galactosidase detection.

The expected results are the following:

1. Prepare the sensor for production using IMS for cell-lysing and an inkjet printer for uniform deposition of gel on paper sensor.
2. Continue partnerships with key nonprofits to ensure sensor can be field tested with real impact.

[46] Tu S-I, Reed S, Gehring A, He Y, Paoli G. Capture of Escherichia coli O157:H7 Using Immunomagnetic Beads of Different Size and Antibody Conjugating Chemistry. Sensors (Basel, Switzerland). 2009;9(2):717-730. doi:10.3390/s90200717.

1.11 Team and Project Management

The team has been communicable, organized, and proactive with the goals and progress of the project each week, quarter, and over the course of the year. Effective communication and record keeping prevent misunderstandings and allow for discussion in the case of disagreements. Each teammate's dedication enables discovery to be made, and the team's understanding of how each teammate can contribute given their strengths, schedule, and demands at a given point in time allows for dynamic collaboration and delegation.

The team agreed upon a Team Charter at the beginning of the project. The Charter outlined objectives, responsibilities, consequences, milestones, and expectations for the duration of the project. The agreement ensured that all team members were on the same page regarding what was expected of them and prevented conflict from arising.

Through collaboration Engineering World Health and the School of Engineering, two faculty advisors on the project contributed their expertise. Dr. Parker from the Public Health Department and Dr. Kim from the Bioengineering Department served as these advisors. The bioengineers met with Dr. Kim weekly to discuss experiment results, propose goals for the following week, and talk through concepts. They also met with Dr. Parker periodically to discuss the future of the project and potential opportunities for advancing the project. The EWH students attended a class with Dr. Parker each week as well as a weekly meeting with the bioengineering students to share ideas.

Weekly meetings were a platform to share findings in the lab, explain conceptual design components, brainstorm ideas, hypothesize outcomes, and outline action items. The action items allowed team members to assign tasks, report progress, and hold one another accountable for deadlines.

Table 3: Team Member Roles and Responsibility

Team Member	Role
Nick Kikuchi Bioengineering	● Correspondent with Dr. Mobed-Miremadi ● Lead in gel entrapment procedures ● Maintains experiment log
Maggie May Bioengineering	● Correspondent with Mother's Milk Bank and Dr. Barber ● Thesis organization and documentation ● Runs assays alongside Nick and Matt
Matt Zweber Bioengineering	● Correspondent with Dr. Stephens ● Orders materials and manages inventory ● Organizes results of collected data
Sara Kelly Public Health	● Assists in background research ● Prepares content for conference applications
Karen Mac Public Health	● Mumbai Trip Researcher ● Prepares content for conference applications
Maya Tromburg Public Health	● Assists in background research ● Prepares content for conference applications
Dr. Unyoung Kim Bioengineering (Advisor)	● Medical device advisor provides insight into experimental design ● Guides team in experiment design and data organization
Dr. Michele Parker Public Health (Advisor)	● Advisor provides feedback regarding design application ● Edits conference applications and advises team on future work
Dr. Maryam Mobed-Miremadi Bioengineering (Advisor)	● Biomolecular advisor provides insight into experimental design ● Guides team in experiment design and data organization

1.12 Budget

Our budget mainly consisted of lab supplies. We anticipated buying most of the items

that we did end up purchasing. These items were reasonably priced within our budget. The only surprise was purchasing $800 worth of breast milk, which happened over a slight miscommunication. Fortunately, we were able to pay for the breast milk and continue to purchase other necessary materials.

1.13 Timeline

The progress made on the development of the sensor was limited by the nine month time period allotted for work. The need research and establishing familiarity with previous laboratory protocols took place from September-December 2017. A majority of the laboratory work took place from January-June 2018.

CHAPTER 2: SOLUTION OVERVIEW AND DESIGN RATIONALE

2.1 Overview of Sensor Design and Application

The sensor relies on the indirect detection of *E. coli* through the binding of the enzyme ß-galactosidase with the substrate X-gal. ß-galactosidase is expressed by *E. coli,* and as a reporter gene expressed in the lacZ operon, its presence therefore indicates the presence of *E. coli.* Upon X-gal hydrolysis of ß-galactosidase, galactose and 5-bromo-4-chloro-3-hydroxyindole is yielded. The 5-bromo-4-chloro-3-hydroxyindole then spontaneously dimerizes and is oxidized into 5,5'-dibromo-4,4'-dichloro-indigo, an intensely blue, insoluble product. In order to obtain this blue product, the milk sample must be prepared with *E. coli* and lysed. The sensor must entrap X-gal within a platform upon which the milk sample can be deposited so that the reaction can take place for colorimetric analysis.

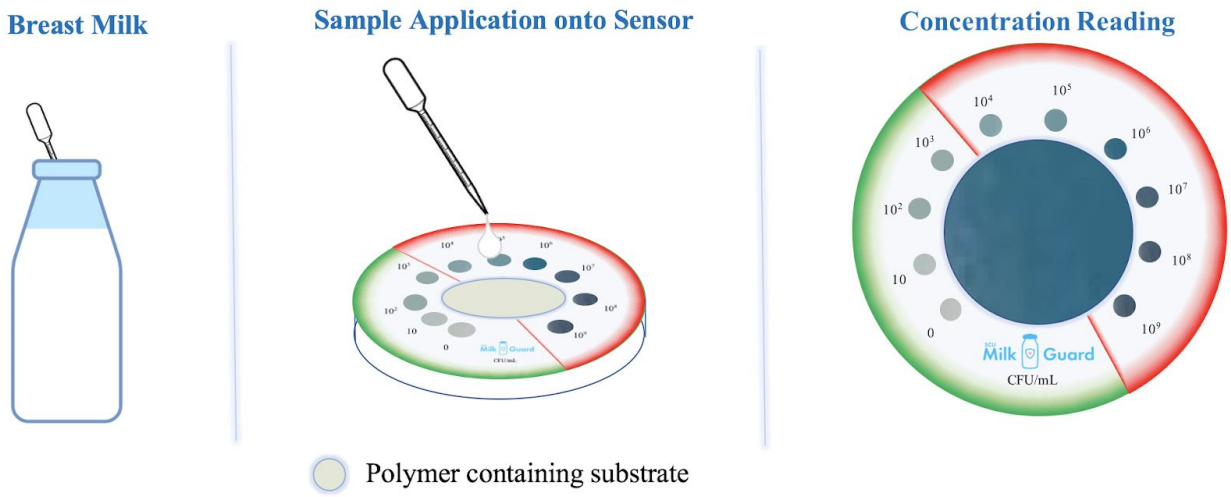


Figure 3. Sensor Schematic and Application. *The ß-galactosidase, expressed by E. coli, resides within the milk sample. A drop from the milk sample is deposited onto the central sensor, which contains the substrate X-gal. The color change produced within the sensor can then be compared to the key, which indicates the concentration of bacteria contamination corresponding to the observed color change.*

2.2 Key Constraints: Customer Needs and System-Level Requirements

The sensor is designed to meet the needs of breast milk banks around the world, particularly those in developing countries. The World Health Organization outlines the ASSURED criteria for point-of-care devices: affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free and deliverable to end-users. Thus, criteria were adopted from these recommendations for the design of the sensor:

Affordable: The device must cost less than $1.00 to ensure accessibility for low-resource settings.

Sensitive: The device must have a low limit of detection (preferably 10^3 CFU/mL).

Specific: The device must consistently detect certain bacteria. Ideally, it must guarantee that it is not detecting interference from a non-targeted bacteria.

Minimal pretreatment of sample: The device must be user friendly and not require additional equipment.

Quantified, Objective Results: The sensor must clearly indicate the presence or absence of bacteria.

Rapid: The device must provide results in under 24 hours.

Frugal: The device must be easy to dispose and portable.

2.3 Detailed Design Description

2.3.1 Indirect Biomolecular Detection Method

ß-galactosidase is induced in *E. coli* cells by the lactose in human breast milk. Based off of this induction, X-gal is allowed to react with ß-gal. The color produced from X-gal hydrolysing ß-gal represents the indirect result of the concentration of *E. coli* cells in a sample.

2.3.2 Lysing Step

The lysing step prepares the cells such that the enzymes β-glucuronidase and β-galactosidase can access the substrate X-glucuronidase and X-galactosidase, respectively. Some methods permeabilize the cell membrane rather than break it down entirely.

2.3.3 Gel Entrapment

Gel entrapment is a key component to our sensors construction that allows for substrates to retain their bioactivity and functionality because they are able to sustain their native-state conformations.[47] Our team used this method to entrap our substrate (X-gal) to retain its activity and replicate our assay on the polymer-based platform.

2.3.3.1 Alginate Gel. Alginate is a naturally-occurring, water-soluble polymer that is composed of (1,4)-linked β-D-mannuronic and (1,3)-α-L-guluronic acid residues. As an organic copolymer, it is a cheap and thoroughly researched biomaterial that has multiple applications in microencapsulation of numerous substrates.[48] Alginate was chosen as a

[47] D.-M Liu, I-W. Chen. "Encapsulation of Protein Molecules in Transparent Porous Silica Matrices via an Aqueous Colloidal Sol-Gel Process." *Department of Materials Science and Engineering, University of Pennsylvania.* 47 (1999). 4535-4544. Web.

[48] Cheryl Simpliciano, Larissa Clark, Behrokh Asi, Nathan Chu, Maria Mercado, Steven Diaz, Michel Goedert, Maryam Mobed-Miremadi. "Cross-Linked Alginate Film Pore Size Determination Using Atomic Force Microscopy and Validation Using Diffusivity Determinations." *Journal of Surface Engineered Materials and Advanced Technology.* 3 (2013): 1-12.

method of entrapment and immobilization of biomolecules for a polymer-based platform. Due to its inertness, alginate gels allow biomolecules to retain their bioactivity without affecting the physical or chemical structures.[49]

2.3.3.2 Rationale for Alginate Gel Compared to Other Entrapment Gels. Alginate gel was chosen as an entrapment technique over other methods including bioaffinity attachment, covalent bonding techniques, and silica sol-gel immobilization due to ease of fabrication, cost, and uniformity of its mesh and pore size. While the previous team used silica-sol gels as their entrapment mechanism, research proved that alginate acted as a more effective option.

Bioaffinity attachment relies on cellulose binding domains (CBD) that tags and fuses the desired protein to the paper-based platform.[50] Covalent chemical bonding immobilization relies on the presence of biomolecules and functional groups to create rapid immobilization on the paper platform through covalent bonds.[51] Silica sol-gels can be created from a number of precursors including $Si(OEt)_4$ (TEOS), $Si(OMe)_4$ (TMOS), or sodium silicate. No matter the method of production, a sol-gel is created that is able to entrap biomolecules without interfering with that substrate's bioactivity.[52] Though all methods are effective for immobilization of biomolecules on paper-based platforms, alginate is superior due to its ease of manufacturability, cost, and uniformity.

2.3.3.3 Comparison of Gel Entrapment Methods. Previously, the sensor's design utilized silica sol-gels for the entrapment of biomolecules on a paper-based format. Our team explored the use of alginate gels due to its ease of manufacturability, cost, and uniformity.

Table 4. Comparison of key characteristics between silica sol-gel and alginate.

[49] Interview with Maryam Mobed-Miremadi.
[50] Fanzhi Kong, Yim Fun Hu. "Biomolecule Immobilization Techniques for Bioactive Paper Fabrication." *Analytical and Bioanalytical Chemistry.* 403 (2012): 7-13. Web.
[51] Björn O. Roos, Antonio C. Borin, Laura Gagliardi. "Reaching the Maximum Multiplicity of a Covalent Chemical Bond." *Angewandte Chemie.* 119 (2007): 1491-1494. Web.
[52] Buckley, A.M., Greenblatt M.J. *Chem. Ed.* 1994, 71 (7), 599. Web.

Characteristic	Alginate	Silica Sol-Gel
Gel Fabrication	Simple	Medium
Cost	Low	Low
Uniformity Control	Automatic Mesh Uniformity	Controllable Pore Size Uniformity
Sensor Fabrication Complexity	Low	Medium

In comparison between the use of alginate and silica sol-gels, four factors were considered: gel fabrication, cost, uniformity control, and sensor fabrication.

Manufacturability. Both gels are easily fabricated. They both only require simple steps in order to create the gel solution (refer to **Materials and Methods)**. However, silica sol-gels require the use of a vacuum filtration system and syringe filters. Silica sol-gels also require the addition of glycerol in order to adjust viscosity and surface tension that affected the gelation of the sol-gel. Alginate simply requires a cross-linking solution and drying times in order to create the gel-like structure.

Cost. Silica sol-gel is very cheap, however alginate is significantly cheaper, therefore alginate proves to be a more economically viable option.

Uniformity Control. The gels' pore size are the key component that allow for the correct function of the gels on the sensor. The pores allow for the desired enzyme to travel through the pore and react with the biomolecules (substrates) that retain bioactivity while entrapped in the gel. Depending on deposition methods and production method, the pore size is variable. Originally, the silica sol-gels were pipetted directly into a mold to create the gel layers on the sensor. However, doing so relies heavily on human production

which often results in human errors and non-uniform deposition of the layer. This creates variable pore sizes. To fix such issue, we attempted use of printers to improve uniformity. **Table 5** compares two printing methods.

Table 5. Comparison of Hp Volumetric Dispenser vs. Inkjet Printing Methods

Printer	**Advantages**	**Disadvantages**
Hp Volumetric Dispenser	● Allows for small volume deposition ● Uniform layers ● Rapid deposition	● Requires the addition of surfactant to reduce surface tension. ● Limits glycerol addition to 0% - 20% ● Limits the amount of sodium silicate present in the gel
Inkjet Printer	● Uniform layers ● Rapid deposition	● Requires compatible viscosity with ink cartridge ● High potential to clog ink cartridge

Though both methods of printing simplify manufacturability of such layers, they also pose additional complications. To meet the requirements of each printers, changes to the viscosity and surface tension of the gels must be variable. The biggest problem is balancing the composition of the gel with the parameters of the printers. The use of alginate allows for a simplified fabrication process of gels. Instead of needing to use a printer or chemical reactions to control and create uniform pore sizes, alginate automatically creates a uniform mesh. No matter the method of fabrication of such gel (refer to **Materials and Methods**), the addition of cross-linking solution forces individual alginate strands to arrange in a uniform mesh with non-variable pore sizes.

Sensor Fabrication. When utilizing silica sol-gel layers, the sensor requires the presence of three separate regions (refer to **Sensor Design**). The first two layers are present to encapsulate ferric chloride and X-gus. The third layer acts as a hydrophobic

barrier to stop the blue color indication from progressing further down the paper. The adaptation of alginate on the sensor simplifies its manufacturability even more. Ferric chloride acts as a trivalent cross-linker for alginate and according to our research, does not necessarily catalyze the reaction between X-gal and ß-galactosidase. So, we can eliminate this region as it is not beneficial to the sensor's functionality as it was previously when our method of indication relied upon the reaction between X-gus and ß-glucuronidase. In addition, the sensor's alginate layer is created by using 3% alginate in order to create a pore size of approximately 3 nm. The alginate is mixed directly with the X-gal, so the cross-linked strands contain the X-gal within its structure, which eliminates the need to "entrap" the substrate between two distinct layers. Due to this fabrication method, we are able to deposit the sample directly onto the gel and, due to its predetermined pore size, enable the substrate to diffuse out of the gel to meet with the ß-galactosidase. The determined volume of sample mixed with the gel eliminates the need for the MSQ layer present in the initial sensor design.

2.3.4 Sensor Design

2.3.4.1 Revised Design. After determining that a lateral flow method was not suitable for our sensor as it would allow for the possibility of contaminant entrapment within the paper pores, our design switched to a drop-based format. Here, a user would simply need to deposit a specific volume directly onto the sensor and allow the reaction to take place over the necessary period of time.

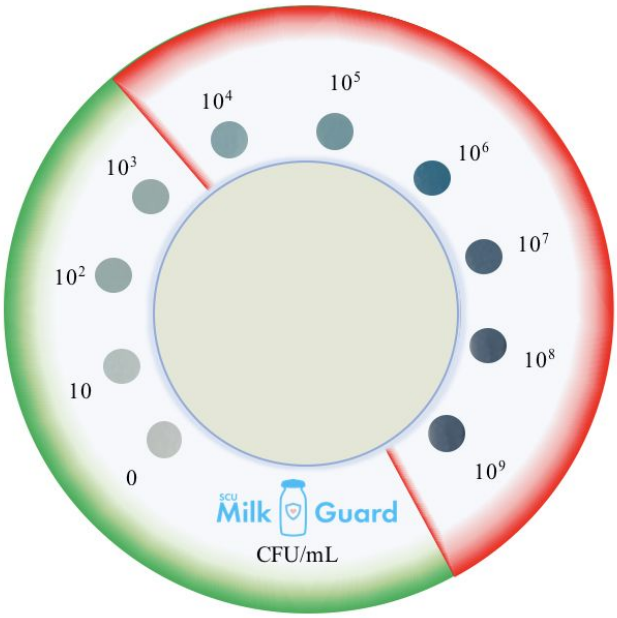


Figure 4. MilkGuard Revised Design. The sensor has a circular shape with an attached legend or key in the outer concentric circle to to allow for direct sample comparison. The alginate-based sample is located in the inner concentric circle where the reaction and blue color change will take place.

2.4 Expected Results

The sensor will produce a blue color change in the presence of *E. coli,* and a colorimetric spectrum corresponding to *E. coli* concentrations will be developed.

2.5 A Backup Plan

In the case that the alginate capsules and slabs do not prove to be valid methods for X-gal entrapment and desired colorimetric indicators, the silica sol-gel method can be further explored and optimized. The HP Volumetric dispenser can be used to print the PVAm and XG layers, while the EPSON inkjet printer can be used to print the silica sol-gel layers.

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

Pasteurized human breast milk was purchased from Mother's Milk Bank (San Jose, CA) to test the efficacy of the detection method in solution and in alginate. SCU-104 *Escherichia coli* cells used to contaminate the human breast milk samples were given to us by Dr. Craig Stephens of the Santa Clara University Biology Department. The cells were grown in LB broth from Sigma Aldrich, Inc. (St. Louis, MI). The cells were lysed with Thermo Fisher's B-PER Direct Bacterial Protein Extraction Kit, Lysozyme, and DNAse (Waltham, MA) or permeabilized with chloroform and sodium dodecyl sulfate (SDS) given to us by the Bioengineering Department of Santa Clara University (Santa Clara, CA). X-gal (5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside) from Thermo Fisher was used as the substrate in the enzyme-substrate reaction and dissolved in N,N-Dimethylformamide from Sigma Aldrich, Inc. The Bioengineering Department of Santa Clara University provided us with materials to make alginate capsules: alginate powder, 1.5% CaCl solution, 0.9% NaCl solution, 1 mL syringe, 18G 1 ½ inch precision needle, and a cell strainer.

3.2 Methods

3.2.1 Growth of *E. coli* Cells

A swab of SCU-104 *E. coli* cells was placed into 2 mL of LB broth in a 10 mL tube. In early experiments, IPTG was mixed into the LB/cell mixture to induce the lacZ gene in the *E. coli* cells. However, IPTG was replaced with human breast milk for the majority of experiments. Once mixed, the 10 mL tube was placed into an incubator at 37˚C and shaken at 225 rpm for approximately 10 hours. This time period allows the cells to surpass the log phase into their fully matured phase. Once incubated, the cells were removed and measured in a spectrophotometer. Absorbance measurements were taken at OD 600 to determine the concentration of *E. coli* in the sample in CFU/mL.

3.2.2 Dilution Preparation

Dilutions are prepared to create a set of contaminated samples of donated human breast milk with known concentrations. Initially, dilutions were prepared concentrations ranging from 10^4 - 10^8 CFU/mL in order to ensure detection of higher concentrations of ß-galactosidase. Once our protocol had been validated at these higher concentrations and we obtained consistent detection, we expanded this range to create dilutions ranging from 10^2 - 10^8 CFU/mL in an attempt to increase the sensor sensitivity.

3.2.3 Lysing Step

The *E. coli* cells are lysed in order to allow the ß-galactosidase protein to escape the cell membrane. To prepare each concentration of the serial dilution for completion of the Miller or B-PER protocols, the solutions are first centrifuged at 3000 g for 5 minutes. The supernatant is then extracted from each tube via pipet, and 400 µL of 50mM NaH_2PO_4 are added to each tube to resuspend the cell. Either B-PER solution or chloroform and SDS (sodium dodecyl sulfate) are added to each tube to lyse or permeabilize the cells. The cell solutions are then shaken at 225 rpm for 15 minutes. Once the cell membrane is lysed or permeabilized, the ß-galactosidase is able to react freely with the X-gal to produce a blue color.

3.2.2.1 B-PER Protocol. To lyse the cell membrane, 100 mL of B-PER solution is added to each concentration of *E. coli* solution in the serial dilution. The solution is made of ~0.8% lysozyme, ~0.8% DNAase, and 98.4% B-PER by volume. The solutions are then incubated in a shaker for 15 minutes before adding X-gal.

3.2.3 Preparation and Construction of Alginate Gel Sensor

Using alginate as an entrapment mechanism allows for the simplification of the sensor design. With several comparably simple preparation methods, substrates can be easily entrapped within this gel and retain their bioactivity. This allows for our sensor to

contain one, all-inclusive layer that not only contains a bioactive substrate, but also contains the blue color change within a defined boundary.

3.2.3.1 Alginate Mixing Preparation. Our group utilized a 3% alginate mixture because we wanted to obtain the structure and viscosity of 2% alginate mixture when cross-linking. Because the alginate is autoclaved to eliminate possibilities of contamination, the extreme heat causes the 3% mixture to shrink to the structure and size of a 2% mixture. To prepare the 3% alginate gel, 3 g of Alginic acid sodium salt from brown algae was mixed with 100 mL of 0.9% NaCl. This mixture was spun for 24 hours at 650 rpm using a magnetic stir bar and corresponding spinner. The mixture was checked periodically during the 24 hours, and in the case of clumping, large pieces were broken apart to increase uniformity. After the alginate solution is prepared, it was then autoclaved to eliminate chances for contamination. Once completed, 19 mL of alginate was mixed with 1 mL of X-gal stock solution (created by mixing 20 mg in 1 mL of dimethylformamide) for 4 hours. This ratio of alginate to X-gal was chosen to previously obtain a 1:1 ratio of alginate to X-gal to produce uniform results within the alginate gels. [53] After optimization, this 1:1 ratio was determined to be non-ideal for our protocol. Therefore, the concentration of X-gal in the alginate was increased.

3.2.4.2 Alginate Capsule Preparation. Alginate capsules were prepared by using 1 mL of alginate mixed with X-gal. To create the slabs, a 1 mL syringe and an 18 gauge, 1.5 in. long needle were used. Using constant and steady pressure application, small and uniform capsules were deposited directly into the 1.5% $CaCl_2$ cross-linking solution. This method of capsules preparation was used to allow for higher optical observations as our team optimized our sensor's functionality. After cross-linking for 30 minutes, the capsules were removed from the solution and filtered using a cell strainer. The capsules were washed three times with 0.9% NaCl to stop all cross-linking activity.[54]

[53] Mobed-Miremadi, Maryam. "Alginate Mixing Preparation ." 2 Feb. 2018.
[54] Cheryl Simpliciano, Larissa Clark, Behrokh Asi, Nathan Chu, Maria Mercado, Steven Diaz, Michel Goedert, Maryam Mobed-Miremadi. "Cross-Linked Alginate Film Pore Size Determination Using Atomic Force Microscopy

3.2.5 Monitoring Biomolecular Activity on the Sensor

Biomolecular activity on the sensor is monitored every hour by one of three senior design team members. The intensity of the blue color is observed by the human eye, and results are recorded by subjective words describing characteristics such as color, turbidity, or separation of layers.

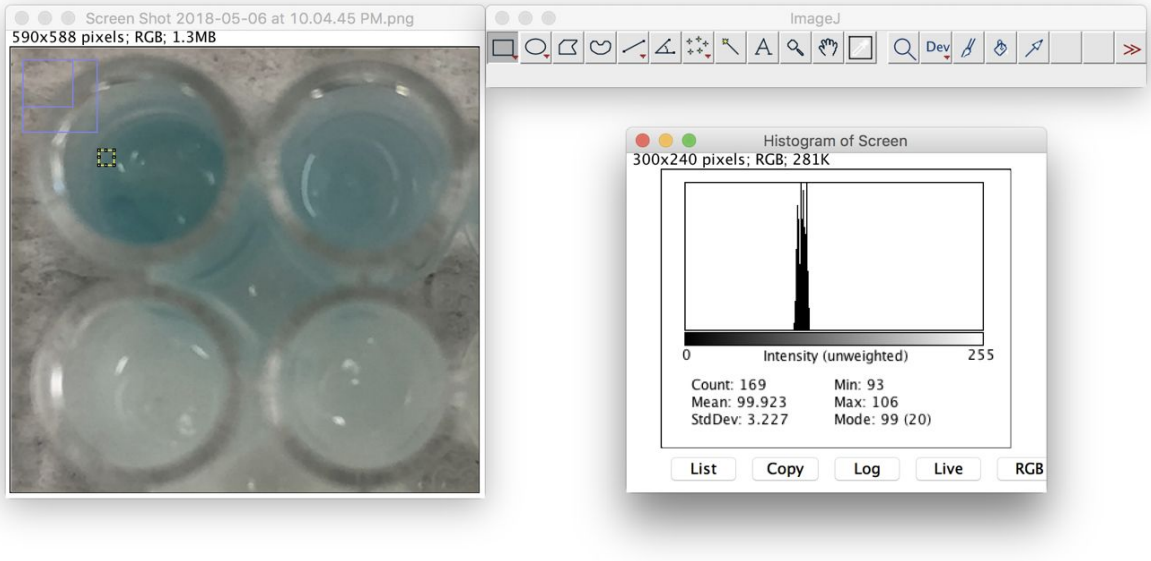
During optimization and confirmation experiments (See 4.22 and 4.23), the intensity of the blue color was observed by the human eye referencing the following scale derived by assigning arbitrary values to the range of blue intensities produced in the experiment. Blue intensity was observed at hourly or bi-hourly time intervals before the optimum time for observation and data collection was determined.

Figure 4. Original used to assign arbitrary values to blue color on the sensor. *This scale was originally used to attempt to quantify blue color change results. This allowed for the normalization of results before our group was able to create an accurate and precise scale according to the actual obtained color change.*

and Validation Using Diffusivity Determinations." *Journal of Surface Engineered Materials and Advanced Technology.* 3 (2013): 1-12.

3.2.6 Measurement of Biomolecular Activity on the Sensor

After 8 hours, when the optimum blue color change was observed, a picture of sensor was taken using an iPhone camera. The image was uploaded to ImageJ, an open source image processing program developed at the National Institutes of Health. As seen in ***Figure 5,*** mean intensity values of each well in the images were extracted and imported into Excel. The intensity values were normalized to the control wells, and standard curves were generated.



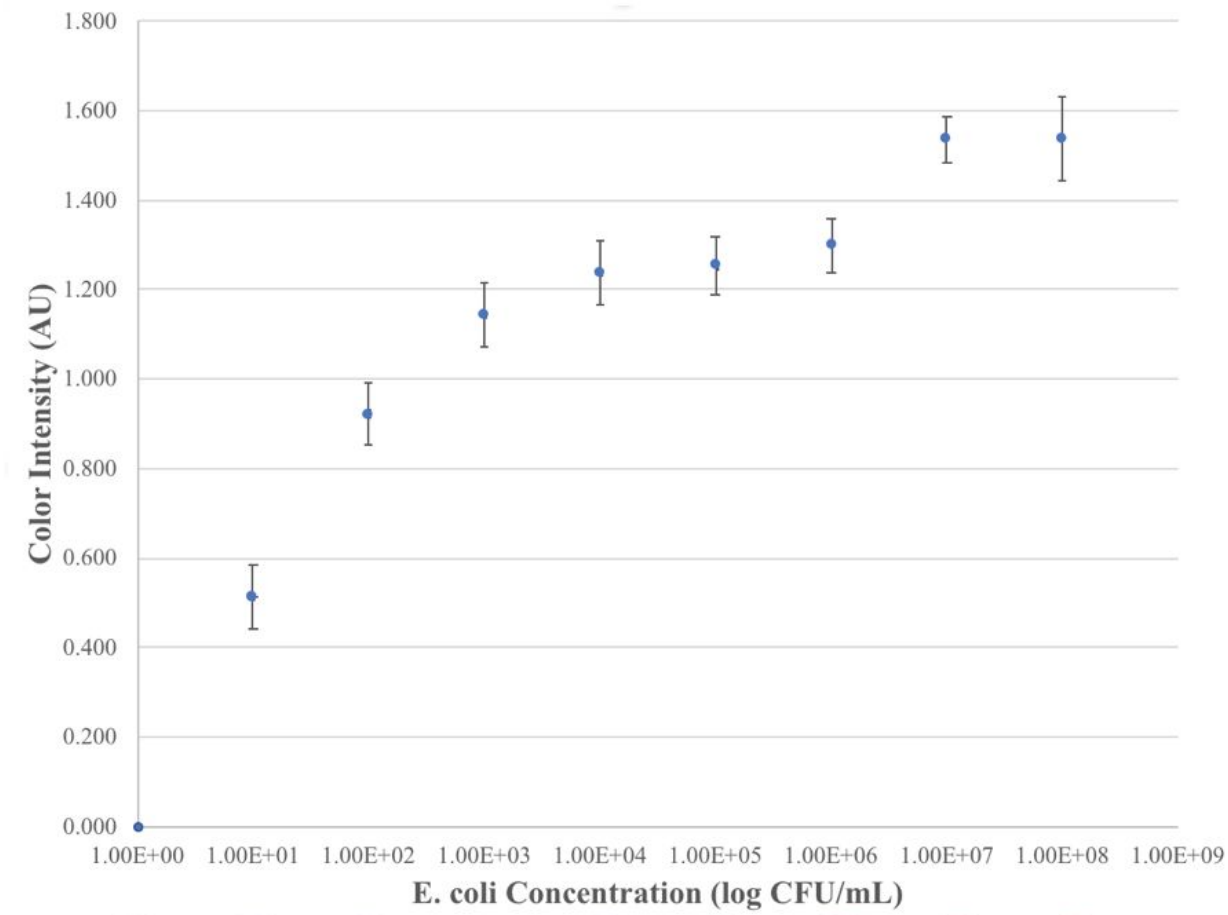


Figure 5. ImageJ tool, analysis methods, and results. *Each well-plate was sampled using the ImageJ tool to allow for the standard curve generation of the mean intensity values.*

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Determination of Optimal *E. coli* Strain and Enzyme Combination

4.1.1 Experiment

ß-glucuronidase and ß-galactosidase were compared as optimal enzymes to detect in BL21 *E. coli* in order to produce the most intense blue color. The *E. coli* was grown in 2 mL of LB broth and 200 µL of IPTG, a molecular mimic of allolactose, in order to induce the ß-galactosidase. Two serial dilutions were prepared using phosphate buffer to obtain concentrations of *E. coli* ranging from 3.16×10^8 to 10^4 CFU/mL. Both dilutions were lysed using the B-PER protocol. One dilution used the substrate X-gus to react with ß-glucuronidase, and the other dilution used the substrate X-gal to react with ß-galactosidase. Lower limits of detection were determined by observing any trace of blue color in each centrifuge by eye.

Another experiment was performed to determine if a natural strain of *E. coli* would best fit the model of our desired final product. The cells were grown with 200 µL of pasteurized human breast milk (HBM), replacing IPTG, in order to induce production of ß-galactosidase. Three serial dilutions were prepared using HBM as the buffer. The naturally occuring strain "SCU-104" was inoculated into one dilution ranging from 3.16×10^8 to 10^3 CFU/mL. BL21 *E. coli* was inoculated into two dilutions ranging from 2.26×10^8 to 10^3 CFU/mL. All dilutions were lysed using the B-PER protocol. The SCU-104 dilution used X-gal, while the BL21 dilutions used X-gal for one and X-gus for the other. Binary existence of blue color was determined by eye in each centrifuge tube.

4.1.2 Results

In the experiment comparing ß-glucuronidase to ß-galactosidase, the X-gal/ß-galactosidase detection method proved to be more sensitive at a 1.00×10^7 CFU/mL lower limit of detection after 10 hours, whereas the X-gus/ß-glucuronidase yielded a lower limit of 3.16×10^8 CFU/mL in the same period of time.

In the experiment comparing the two *E. coli* strains, SCU-104 and BL21, no color was observed in the BL21 dilution using X-gus after 10 hours. For BL21 using X-gal, a 1.00 x 10^6 CFU/mL lower limit of detection was observed after 10 hours. For SCU-104 using X-gal, a 3.16 x 10^6 CFU/mL lower limit of detection was observed after 10 hours.

4.1.3 Discussion

ß-galactosidase was determined to be a more sensitive method of detection than ß-glucuronidase. We decided to continue our experiments using ß-galactosidase and X-gal for the enzyme-substrate reaction. Although the limit of detection for BL21 was slightly lower than that of SCU-104, we decided to use the SCU-104 strain going forward, as the naturally occuring strain better represents *E. coli* that our device would encounter in the real world. The blue intensity for each concentration of *E. coli* was also more intense in the SCU-104 strain.

4.2 Viability of Alginate Gel Entrapment

4.2.1 Experiment

Alginate was prepared in a ratio of 1 mg X-gal:1 mL alginate. 1 mL of alginate was dispensed from a syringe onto a glass slide. The alginate was cross linked with 1.5% CaCl for 1 hour. Cross linking was stopped with 0.9% NaCl. The alginate was cut into 9 approximately equal sizes; each piece was placed in a 12 well plate to await dispensing of lysate. A serial dilution of SCU-104 *E. coli* was prepared in HBM with ranges of 3.16 x 10^8 to 10^4 CFU/mL. The dilution was lysed with the B-PER protocol. Lysate was dispensed in 20 µL volumes onto each piece of alginate in the 12 well plate. The time between the end of cross linking and the dispensing of lysate was 10 minutes. Color was observed by eye in hour increments throughout the day.

4.2.2 Results

The first noticeable color change appeared in the top concentration, 3.16 x 10^8 CFU/mL, at 4 hours. At 48 hours, color was observed down to 10^5 CFU/mL.

4.2.3 Discussion

This experiment simply determined that we can observe color change in alginate. From here we decided to further explore the possibility of alginate as a medium for our enzyme-substrate reaction.

4.3 Effect of Substrate Concentration in Alginate Entrapment

4.3.1 Experiment

A serial dilution was created to obtain concentrations ranging from 3.16×10^8 to 10^4 CFU/mL. Two separate dilutions were created identical and were tested on alginate slabs with different ratios of substrate concentrations entrapped within its matrix. One dilution was lysed with the B-PER Protein Extraction Reagent Kit and an identical dilution was lysed with the standard Miller Permeabilization. Alginate slabs were created in two different ratios: 1:1 X-gal substrate/1 mL of alginate and 2:1 X-gal substrate/1 mL of alginate. The prepared slabs were each cut into two rows of eight segments. All thirty-two slabs were placed within a 48-well plate and received assay samples that underwent both lysis procedures.

4.3.2 Results

The Miller Permeabilization protocol consistently produced faster results compared to the B-PER protocol. For both lysis methods, the 2:1 substrate/alginate mixture originally produced a darker color change, however, over time, the difference between the 1:1 and 2:1 substrate/alginate mixture experienced color change became negligible.

4.3.3 Discussion

Because both ratios of substrate to alginate produced similar color change after the same amount of time, for simplicity, future experiments were conducted using a 1:1 substrate/alginate mixture. Our team deemed that there was no significant difference when using such ratios.

4.4 Evaluation of Uniformity of Color Diffusion in Alginate

4.4.1 Experiment

Samples of milk were inoculated with 3.16 x 10^8 CFU/mL, lysed with two different lysis protocols, and deposited on alginate (1 mg X-gal/1mL alginate) slabs to test for uniformity of the color diffusion throughout the gel upon and after reaction. The prepared slabs were cut into two rows of eight segments. The top eight segments were placed in individual wells in a 48-well plate and received assay samples that underwent Miller permeabilization; the bottom six segments were also placed in individual wells in a 48-well plate, but received assay samples that underwent B-PER lysis.

4.4.2 Results

More intense and uniform color change was observed on slabs which had been covered in assay that underwent Miller permeabilization than those that had been covered in assay that had been lysed using B-PER. Overall, the segments varied in uniformity and diffusion of blue color, and much of the blue color collected on the edges or corners of the slab segments.

4.4.3 Discussion

Because uneven and irregular distribution of blue color change remained an issue, the capsule method was explored because it offers more desirable uniformity.

4.5 Verification of Alginate Contamination

4.5.1 Experiment

A prepared batch of alginate containing X-gal was stored in a sealed beaker for seven days. A second batch of alginate was prepared without X-gal, autoclaved for sterilization, and stored in a sealed beaker for seven days.

4.5.2 Results

Blue color change was observed in the unsterilized beaker after one week of storage. The autoclaved alginate/X-gal mixture remained unchanged after one week of storage; no color change was observed.

4.5.3 Discussion

The blue color change indicated seemingly spontaneous production of the blue precipitate 5,5'-dibromo-4,4'-dichloro-indigo. Because *E. coli* or ß-galactosidase had not been deposited onto or into the prepared alginate, it was unclear what contaminate had been hydrolyzed by the X-gal within the prepared alginate and then dimerized and oxidized into the blue product. In order to rule out the possibility of spontaneous color change without the addition of a ß-galactosidase-expressing contaminate, a sample batch of alginate was autoclaved, mixed with X-gal, stored for observation. The stability of this mixture over time indicates contamination of the first alginate by some sort of contaminate. Sterilization of the alginate mixture prior to the addition of X-gal prevents foreign contamination and supports the shelf life of an alginate-based solution.

4.6 Alginate Capsule Method and Effect of Induction

4.6.1 Experiment

An experiment was created to verify that the colorimetric reaction is replicable on an alginate capsule. 3 mm alginate capsules at a 1:1 substrate/alginate ratio were created using the described manufacturing method in **Section 3.2.4.2**. A single concentration at 3.16×10^8 CFU/mL and was placed onto the prepared alginate capsules to simply test for the replication ability. The capsules were allowed to react for 24 hours prior to observation.

4.6.2 Results

Color change was obtained in the sample after 24 hours. No color change was observed in the negative control.

4.6.3 Discussion

Our team determined that our assay is replicable on alginate capsules.

4.7 Validation of Alginate Capsule Method

4.7.1 Experiment

Previously, alginate capsules were created by mixing the anhydrous form of the substrate X-gal with alginate to create a 1:1 substrate/alginate mixture. To validate the alginate capsule method and produce more uniform color change through the capsule's surface, our team experimented by mixing 20 mg of X-gal in 1 mL of Dimethylformamide (DMF) to create a stock solution. 19 mL of alginate and 1 mL of X-gal stock solution were mixed overnight and 3 mm capsules were created using the described manufacturing method in **Section 3.2.4.3**. A serial dilution was created ranging from 10^8 to 10^2 CFU/mL and was deposited directly onto the capsules.

4.7.2 Results

Hourly observations were recorded. Significant color change was observed in the top two concentrations: 10^8 CFU/mL and 10^7 CFU/mL.

4.7.3 Discussion

The replicability of our entire assay on various concentrations of *E. coli* contaminated milk was clear, however our assay clearly had flaws. In the time period that we recorded observations, we saw minimal color change. In previous experiments, our team witnessed qualitative results in lower concentrations, and in this experiment, we did not observe the same robustness of our assay. While we were able to conclude that our assay is replicable on the dilution ladder, it also displayed flaws in our assay design.

4.8 Optimization of Induction for Cell Growth

4.8.1 Experiment

In effort to optimize the production of our enzyme ß-galactosidase, our team optimized our cell growth process to find the ideal milk induction volume. Twelve different tubes of cells were incubated with varying milk volumes: 0, 20, 40, 60, 80, and 100 µL. Six dilutions were created in water and six dilutions were created in milk to compare our results from a relevant experiment environment to an ideal experiment. Serial dilutions were created by creating samples with various concentration of *E. coli* cells ranging from 10^8 to 10^2 CFU/mL. All cells were lysed using the B-PER lysis protocol. 100 µL of each sample was deposited into a 96-well plate and observed hourly for 8 hours.

4.8.2 Results

An intense blue color change was observed in top two concentrations in all dilutions. Observations were recorded hourly for an 8 hour period and were observed again after 24 hours.

4.8.3 Discussion

While an intense blue color change was observed in all dilutions in the top two concentrations at 10^8 CFU/mL and 10^7 CFU/mL, the dilution with a milk induction volume of 100 µL produced the most intense blue color. Though we optimized the milk induction volume, we saw flaws with the results we obtained. In previous experiments, we witnessed color change in concentrations much lower than the threshold obtained from this experiment, detecting around 10^4 CFU/mL in comparison to the observed 10^7 CFU/mL. In the time period that we recorded observations, we saw minimal color change. In previous experiments, our team witnessed qualitative results in lower concentrations, and in this experiment, we did not observe the same robustness of our assay. While we were able to conclude that the milk induction was optimized further

towards the ideal condition, our experiment also displayed significant flaws in our assay design.

4.9 Optimization of Sample Volume for Detection Intensity

4.9.1 Experiment

To determine the ideal volume of milk sample necessary to achieve optimum blue color change, the sample volume of contaminated milk added to the prepared alginate capsules was varied. Serial dilutions were created by creating samples with various concentration of *E. coli* cells ranging from 10^8 to 10^2 CFU/mL. All cells were lysed using the B-PER lysis protocol. 20, 40, 60, 80, and 100 µL of inoculated milk dilutions were deposited onto the capsules in a 96 well plate and observed after 8 hours.

4.9.2 Results

The blue color change increased in intensity as the volume of milk increased; the most intense blue color was produced in the wells containing 100 µL.

4.9.3 Discussion

Although this result reflects the understanding that a more intense blue corresponds to a greater volume of contaminant, it also indicates that the minimum volume of sample required to produce a reaction observable by the human eye. The most intense blue color change with 100 µL informs the protocol for the end user. 100 µL of sample enables the alginate capsule to be fully emerged and thus take advantage of all of the substrate XG that diffuses out of the capsule—hypothetically radially—and reacts with the ß-galactosidase in the milk.

4.10 Optimization Runs (4-level, 5-variable)

4.10.1 Experiment

In order to determine which variables had the greatest impact on the reaction overall, an L_{16} Taguchi orthogonal array experiment was conducted with a

"larger-the-better" optimization goal.[55] This factorial design entails a four-level, five-variable array of different combinations of the variables. The formulae needed to determine the optimized level of parameters based on the sensitivity (S_m), signal to noise ratios (S/N), difference (D), and rank (R) using larger-the-better-optimization are given in Equations 1–4.

$$D = \frac{\sum_{i,j} [S/N(dB)]}{L} \qquad (1)$$

where i corresponds to the level, j corresponds to the factor, and L is the total number of levels. Rank, *R* is given by:

$$R = Maximum\ (n_i) - Minimum\ (n_i) \qquad (2)$$

Signal-to-noise ratio (dB) for the jth experiment, $(S/N)_j$ is given by:

$$(S/N)_j\ (dB) = -10 \log_{10} \left[\frac{1}{n} \sum_{m=1}^{n} \frac{1}{Y_{mj}^2} \right] \qquad (3)$$

where *n* is the total number of replicates per experiment, and Y_{mj} is the measured viability for the *m*th trial of the *j*th experiment. For the purpose of our timeline, only one experiment was completed using the Taguchi method.

Sensitivity (dB) for the jth experiment $(S_m)_j$ is given by:

$$(S_m)_j\ (dB) = 10 \log_{10} \left[\frac{1}{n} \sum_{m=1}^{n} Y_{mj}^2 \right] \qquad (4)$$

The variables that were analyzed include the volume of milk added to the cell growth tubes for induction, the volume of X-gal substrate added to individual capsules, the lysing incubation time, the temperature at which the reaction took place, and the concentration of *E. coli* cells in the milk sample. The volumes of milk added for induction included 0, 50, 100, and 150 µL. The lysing incubation times included 10, 15, 20, and 25 minutes. The temperatures

[55] Jared Hara, Jordan Tottori, Megan Anders, Smritee Dadhwal, Prashanth Asuri & Maryam Mobed-Miremadi (2016) Trehalose effectiveness as a cryoprotectant in 2D and 3D cell cultures of human embryonic kidney cells. Artificial Cells, Nanomedicine, and Biotechnology, 45:3, 609-616, DOI: 10.3109/21691401.2016.1167698.

included 23, 37, 40, and 45ºC. The concentration of *E. coli* cells included 3.16 x 10^5, 10^6, 10^7, and 3.16 x 10^7 CFU/mL. 16 combinations of these five variables were prepared for deposition on alginate capsules and observed hourly for 10 hours.

4.10.2 Results

Two combinations of variables that produced the most intense blue color in the shortest amount of time were identified: Combinations G and M. "G" consisted of 50 µL milk for induction, 20 minutes of lysing incubation time, 100 µL X-gal, 37ºC reaction temperature, and 3.16 x 10^7 CFU *E. coli*/mL milk sample. "M" consisted of 150 µL milk for induction, 10 minutes of lysing incubation time, 100 µL X-gal, 40ºC reaction temperature, and 3.16 x 10^7 CFU *E. coli*/mL milk sample. Although another combination produced a comparatively dark blue color, it was not identified as a desirable combination because it took longer for the color to develop and it required a 45ºC reaction temperature, which would require the end user to spend more energy and money to maintain a warmer temperature that is less common in conventional laboratory settings. *S/N* charts can be seen in **Appendix VII**.

4.10.3 Discussion

This Taguchi experiment allowed for the optimization of possible combinations for the given five variables. To reduce the number of experiments to a practical level and obtain the most information, only five variables were tested. From the 16 possible combinations, two favorable ones were observed and selected for confirmation runs (**4.11 Confirmation Runs**).

Additionally, the most important variables could be identified using the Signal-to-Noise ratios of each of the variables. The variables that have the greatest impact on the reaction are volume of milk for induction, *E. coli* concentration, and temperature at which the reaction takes place, whereas the lysing incubation time and substrate X-gal concentration have less of an impact on the outcome. We can infer this by looking at the absolute value of the S/N ratio for each variable as well as the difference between each variable's highest and lowest S/N ratio value. A higher absolute value determines a

variable to be more significant. A greater difference in S/N ratios among different conditions for one variable also determines that variable to be more significant in producing blue color at a lower limit of detection in a shorter amount of time.

4.11 Confirmation Runs

4.11.1 Experiment

Experiment 4.10 was repeated with the two best performing combinations G and M. Three replicates of each of the combinations were prepared. Instead of limiting the *E. coli* concentrations to the four original ones chosen in the previous experiment, full dilutions ranging from 10-10^8 CFU/mL as well as a control with no contamination were prepared. These dilutions were prepared for deposition on alginate capsules and observed hourly for 10 hours.

4.11.2 Results

The blue color change was more intense and over the same amount of time in combination M than in G. No blue color change was observed on the control alginate capsule.

4.11.3 Discussion

This experiment confirmed that the procedure was replicable and clarified that the second combination M (150 µL milk for induction, 10 minutes of lysing incubation time, 100 µL X-gal, 40ºC reaction temperature) produced the most ideal results. This guided the current protocol for the use of the sensor. The shorter incubation time reduces overall time for detection and the 40ºC reaction temperature is feasible in milk bank and laboratory settings that already have an incubator or alternative heat source in their possession.

4.12 Verification of Optimization on Alginate Capsules

4.12.1 Experiment

Experiment 4.11 was repeated with only combination M on alginate capsules. B-PER protocol had been employed in experiment **4.11 Confirmation Runs** for its shorter time and overall ease, but given the superiority of the Miller permeabilization in experiments **4.3 Effect of Substrate Concentration in Alginate Entrapment** and **4.4 Evaluation of Uniformity of Color Diffusion in Alginate** on alginate slabs. Thus, the two lysing procedures were also compared on alginate capsules using the identified optimal combination of factors.Full dilutions ranging from 10-10^8 CFU/mL as well as a control with no contamination were prepared. These dilutions were prepared for deposition on alginate capsules and observed hourly for 10 hours. Images of the wells were taken eight hours after the completion of the the protocol and image analysis was performed using ImageJ.

4.12.2 Results

More intense blue color changed was achieved in eight hours using the B-PER protocol as opposed to the Miller protocol. A spectrum key was generated corresponding the blue color to the *E. coli* concentration.

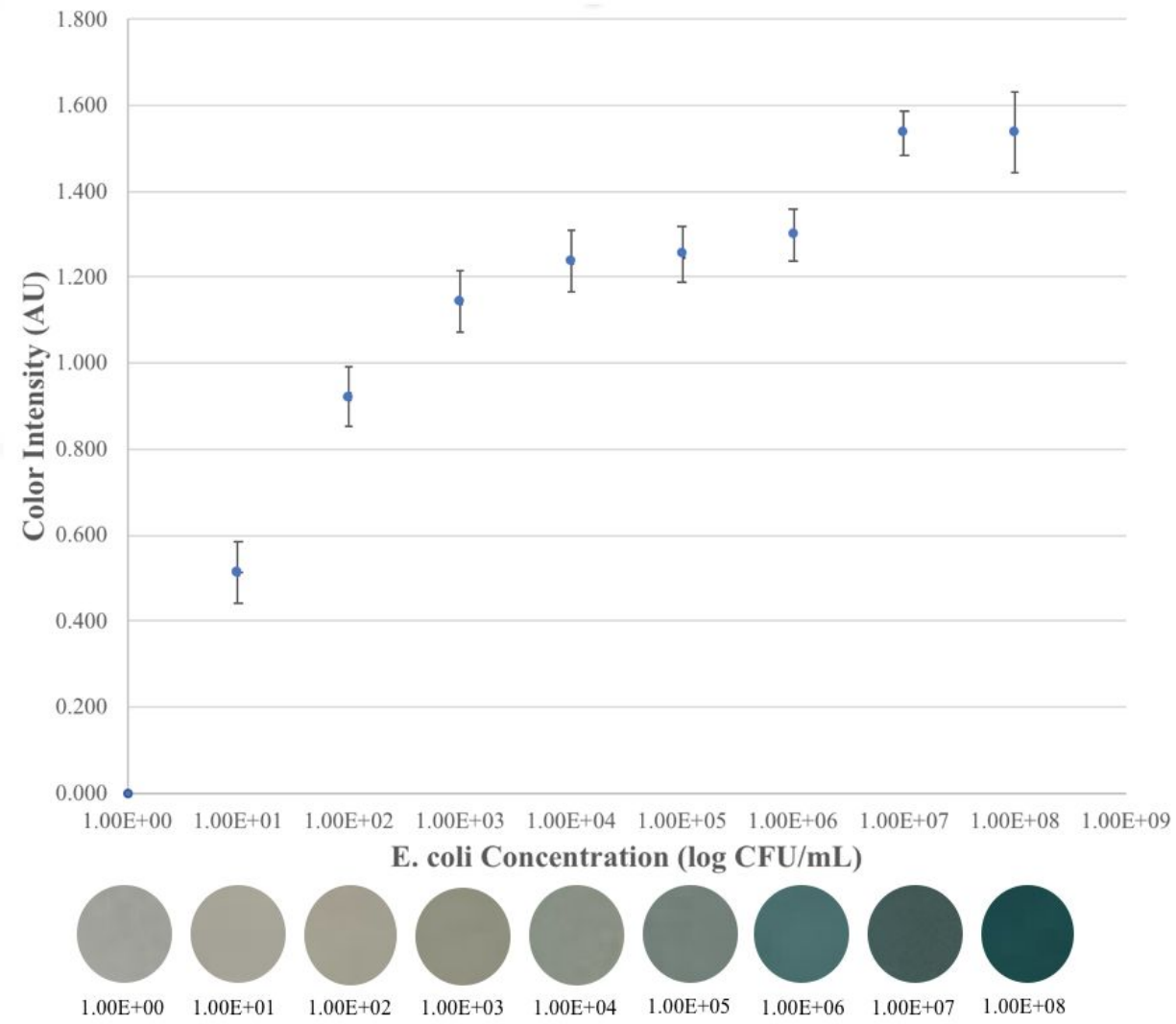


Figure 6. The Quantified Results of Current Prototype Replicates. *Using our quantified, replicable data, we were able to generate a color spectrum that more accurately represents the color change at various concentrations of bacterial contamination.*

4.12.3 Discussion

As the concentration of the *E. coli* in the milk increased, so the did the color intensity of the captured image of the sensor well. The alginate capsule sensors produce the expected result. The negligible standard deviation on the 1 CFU/mL wells indicates that the sensor is highly sensitive. The significant separation between standard deviations on the lower *E. coli* concentrations (10-10^3 CFU/mL) further supports the sensor's sensitivity. The overlapping standard deviations for the middle *E. coli* concentrations, as reflected by the similar blue shades of color that are difficult to differentiate via the

human eye, indicate the variability introduced by the image capture method. Ambient lighting and light refraction through the plastic of adjacent wells in the well plate prevent more distinct blue color from being captured.

CHAPTER 5: COSTING ANALYSIS

Overall, the project fell within the budget and is comparable to current industry costs for bacterial culturing and screening in Mumbai, India according to research conducted at Sion Hospital.

Cost estimates for production using the small-scale materials were derived and are outlined in the table below. The complete product includes the gel capsule prototype, which consists of the X-gal and alginate, in conjunction with the two lysing protocols.

Table 6. The cost estimates for a single use of a complete sensor product.

	Cost Per Sensor Using Miller Protocol ($)	Cost Per Sensor Using B-PER Protocol ($)
XG	0.0003	0.0003
Alginate	0.0047	0.0047
Lysing Protocol	0.0410665	0.31
	0.046	0.32

Although the Miller protocol makes the complete product dramatically cheaper, both the Miller protocol and the B-PER protocol fall under the target cost of $1.00 per sensor. While the Miller protocol would make pricing not only competitive but also cheaper than the current practice of bacterial culturing, it is not particularly user-friendly and frugal. A lab technician may need special training for the careful handling of hazardous chloroform, and it requires a hood. The price of a product utilizing B-PER for lysis is competitive with the cost of current detection methods.

CHAPTER 6: ENGINEERING STANDARDS AND REALISTIC CONSTRAINTS

6.1 Ethical Justification: Lack of Research

Breast milk is known throughout the medical community as the best source of nutrition for infants with countless benefits compared to alternatives such as formula. Though a key source of factors that can aid in development and growth, like the previous team, our team encountered an astounding lack of research surrounding breast milk diagnostics and related banking systems. To further the dedication that last years' team made to narrowing the knowledge gap, our team also devoted ourselves to enhance the scientific knowledge surrounding breast milk.

6.2 Importance of Ethics for MilkGuard

Our team dedicated ourselves to creating a technology that was both scientifically and ethically sound to produce the most accurate, safe, and applicable device to the unmet medical need. The project surrounds an industry that caters directly to those affected by the greatest risk--infants. Below are ethical considerations regarding team organization, product design, and cultural and social ethics:

Table 7. Team Organization to Uphold Project Ethics

Ethical Consideration	**Established Practice**
Team Member Ethics	**Established Weekly Meetings** Weekly meetings were established to ensure that all team members were kept accountable and up to speed with the project. Each meeting was documented with an agenda and thorough minutes taken on a rotating schedule. **Lab Etiquette** Team members worked to establish lab times that were

	consistent for a majority of or all team members. This allowed for thorough collaboration and effective, equal use of lab time.
Team Member Contributions	**Collaborative Environment** All team members were encouraged to share opinions, ideas, and creativity with the team. While team members may have differing opinions, the group was dedicated to staying open-minded and considering all possible perspectives of the team. **Delegation of Tasks** Team members divided certain tasks and became Subject Matter Experts (SME) in specific areas of the project. Whether taking charge of communication with a specific partnership or leading a step during lab time, each member was tasked to staying up to date with the specific subject area.
Ethical Partnership Building and Management	**Contact with Advisors and Collaborators** The team met with highly involved advisors at least once a week to maintain positive trajectory towards team goals. Collaborators and partnerships were contacted periodically to maintain relationship and provide necessary updates. **Establishment of New Contacts** The team traveled to the Bay Area Global Health Innovation Conference on May 4 - 5. Here, the team interacted with organizations and professionals in the global health sector to establish greater connections and collaborations within the field. **Partnership with Engineering World Health (EWH)**

	The team worked closely with the Engineering World Health students to maintain a positive and efficient relationship. Clear goals and tasks were assigned to team members to ensure that both parties were equally as invested in the project's impact.

The National Society of Professional Engineers' Code of Ethics for Engineers[56] and the World Health Organization's (WHO) Guide for Open Point-of-Care Diagnostic Platforms for Low to Middle Income Countries[57] were consulted to ensure that our design met criteria applicable in our target setting.

Table 8. Product Design Ethics

Ethical Consideration	**Established Practice**
Result Reporting and Experiment Reproducibility	**Lab Notebooks and Related Electronic Documents** Each member was required to record results physically in lab notebooks and results and experiment design were transferred into an electronic log of experimentation. Results and experiments were overseen by Dr. Craig Stephens, Dr. Ashley Kim, and Dr. Maryam Mobed-Miremadi as acting advisors, professionals, and collaborators on the project. **Experiment Reproducibility** Experiments were conducted in sets of three replications to confirm results and experimental design. Results were quantified using ImageJ and standard deviations were calculated to analyze result deviations from the norm.

[56] Nspe.org. (2018). *Code of Ethics | National Society of Professional Engineers*. [online] Available at: https://www.nspe.org/resources/ethics/code-ethics [Accessed 14 Jun. 2018].
[57]Who.int. (2018). [online] Available at: http://www.who.int/phi/Open_DX_Platform_WHO_Call_for_Interest.pdf [Accessed 14 Jun. 2018].

Breast Milk Handling	**Donor Confidentiality** Breast milk samples were obtained through the Human Milk Banking Association of North America (HMBANA) through a research application. Raw and pasteurized milk was requested without donor information such as demographics and key identifiers. Sample labels were kept confidential to allow for blind experimentation free of biases. **Mother's Milk Bank of San Jose Partnership** The team visited Mother's Milk Bank of San Jose to build and maintain a relationship with the director. Per request, the team obtained sensitive information and agreed to keep it confidential as we used it to guide the sensitivity of our product. **Sample Handling** Breast milk treated as a hazardous material in lab and followed all applicable waste handling protocols.

Table 9. Cultural and Social Ethics

Ethical Consideration	**Established Practice**
End User	**World Health Organization/Engineering World Health** In an effort to meet parameters established by the World Health Organization and Engineering World Health, costs were kept low, user-friendliness was improved, and sensor robustness was improved. Doing so will allow it to be more economic and will open up distribution to allow for greater access in low-resource, vulnerable areas.
Global Health Awareness	**Partnerships** The team worked directly with PATH. This organization works

	to establish milk banks in low resource settings, so they acted as guidance to ensure that our sensor is functional in the industry. **Field Research** Two team members traveled to Sion Hospital in Mumbai, India. By traveling to a low-resource setting where breast milk banks are prevalent, greater design constraints and criteria were developed to further the applicability of the project.
Maternity	**Breastfeeding Culture** The goal of the project is to promote breastfeeding and breast milk donation so that all infants may have access to such nutritional content. **Maternal Integrity** In effort to promote breastfeeding, our team considered the ethical ramifications of informing mothers of their milk's nutritional content and potential contamination. Our goal is not to dissuade mothers from breastfeeding or breast milk donation, so we collaborated with PATH to maintain the ethical integrity of the project's result documentation and communication.

6.2.1 Potential Risk Modes and Analysis

To address potential failure risks that may result from our product, a Failure Modes Effects and Analysis was conducted to identify key factors that may result in the failure in the device and the related severity of such failure. The following were identified as the greatest sources of failure risk:

- Milk Volume Added
- Cross-Contamination
- Device Reuse

- Lack of Access to Higher Temperatures

While not all of these failure risks were completely addressed by the team, next steps were explored in order to address such risks.

6.2.1.1 Potential Failure Modes and Analysis: Milk Volume Added. While this variable is key to the experimental design *in vivo*, it is not as applicable within a clinical lab setting. In industry, samples are composed entirely of breast milk and related factors, and small milk volume additions are not applicable in a greater testing facility. This factor is key to experimental design but is not applicable in an industry setting.

6.2.1.2 Potential Failure Modes and Analysis: Cross-Contamination.

Cross-contamination is a crucial failure mode of our device. Currently, the specificity of our bacterial contamination is unknown. Because our device relies on an enzyme-substrate reaction specifically for *E. coli* bacteria with an intact LacZ operon and our team specifically inoculated our milk samples with known concentrations of bacteria, we know that our device is applicable with such related bacteria. However, our device is currently not applicable to other species of bacteria that do not rely on such enzyme-substrate reaction.

Our team confirmed that substrate entrapped within a cross-linked alginate matrix allowed for molecules to retain their bioactivity to allow for the enzyme-substrate reaction to take place. Since this method was verified, we anticipate that this mechanism is applicable on a greater scale to other related contaminants. For future work, individual alginate capsules can be manufactured with specific enzymes entrapped in them. These capsules will then act like chambers on the sensor to be able to detect multiple areas of contamination, thus, allowing for a more specificity and reduction of false results due to cross-contamination.

6.2.1.3 Potential Failure Modes and Analysis: Device Reuse. Our device functions as a single-use product. Originally having an opaque coloration, the device changes to a

permanent, stark blue. This color change is irreversible, so it does not allow for the reuse of the MilkGuard product. Because of this stark color change, we anticipate that users will know that our device are single-use products.

6.2.1.4 Potential Failure Modes and Analysis: Lack of Access to Higher Temperatures. Our device produces optimal results at 8 hours given that the reaction is allowed to take place at 40°C. Our team is aware that not all laboratories have access to electricity and power to allow for certain equipment to function indefinitely. Therefore, our team explored other potential biomaterials that will reduce the need for such higher temperatures. We are aware that there are biomaterials that retain a greater potential energy, which reduces the need for consistent access to these higher temperatures.

6.3 Engineering Standards for Consideration

According to the National Society of Professional Engineers' Code of Ethics for Engineers, professional engineers must take into consideration the impact of our design in the areas of ethics, science, technology and society, civic engagement, economic, health and safety, manufacturability, usability, sustainability, and the environmental impact. Such considerations must be considered to ensure that the technology not only benefits the consumer, but also prevents negative impact on the surrounding community. The following delineates MilkGuard's potential impact in a selection of these areas.

6.3.1 Ethical Considerations

When meeting with breast milk banking experts and maternal and infant health experts, they highlighted the importance of supporting and praising mothers for their breast milk. They clearly explained that the results of the testing and diagnostic device should not make a mother feel inadequate or deficient in either her breast milk expression or the nutritional quality of her milk. There is an obvious ethical obligation that our team has to not demean or devalue mothers whose milk is not as nutritious as anothers. MilkGuard is not a device that should cause harm or worry for mothers. Instead, it is

designed as a tool to improve testing capabilities of breast milk in order to detect for bacterial contamination. While the origin of contamination may come from a variety of sources, the most important factor is the device will ensure that the breast milk delivered to at risk infants. As the project continues, it is crucial that ethical obligations are upheld so that mother and infant are encouraged to consistently donate safe breast milk.

6.3.2 Social Impact

Though breast milk banking is prevalent in some countries, like India and Brazil, it is extremely uncommon within the United States. MilkGuard's research will fill a knowledge gap surrounding this industry, and can increase awareness and accessibility of these breast milk banks worldwide. With greater and more developed research and technology within this sector of maternal health, woman can be empowered to further support breastfeeding and breast milk donation.

6.3.3 Health and Safety

MilkGuard is an extremely simple and easy-to-use diagnostic device that will be used primarily by lab technicians with sufficient training in lab settings. Currently, it is *not* meant to be used by any mothers or related consumers. It is not meant to be ingested, nor is it toxic to human touch. The biomaterials used are bioinert, so it will not cause any reaction when in contact with human tissue. In addition, all biomolecules used are used in small trace amounts when constructing the device, and these do not pose an immediate or severe health and safety risks to the end-user.

6.3.4 Regulatory Requirements[58]

According to the Food and Drug Administration, non-invasive diagnostics can be considered Class I Devices. Class I devices can be defined as simple devices that pose low potential for harm on the end-user. In an effort to better understand the regulatory

[58]Fda.gov. (2018). *Class I / II Exemptions*. [online] Available at: https://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/Overview/ClassifyYourDevice/ucm051549.htm [Accessed 14 Jun. 2018].

space, our team consulted John Petrovich, CEO of the Alfred Mann Foundation, at the Bay Area Global Health Innovation Challenge. His feedback reinforced our assumption that MilkGuard would be considered a Class I device if our team decided to pursue FDA approval. Though there are FDA and ISO standards that must be met for medical devices, because our product is considered a Class I device and is completely non-invasive, it does not need to go through certain forms of testing, like biocompatibility or toxicity testing.

6.3.5 Economic Considerations

MilkGuard is designed to be deployed in low to middle income countries, therefore it was designed to be as low-cost as possible. While originally developed on a paper-based platform to reduce costs, our team transitioned to a polymer-based platform because an inexpensive, organic biomaterial was selected and exceptionally reduced the complexity of the sensor. The following is a cost-breakdown of the unit price of a single sensor:

Table 10. Cost-breakdown of a Single Sensor

Items	**Cost Per Sensor ($)**
Alginate	0.0003
XG	0.0047
Lysozyme	0.08
DNAse	0.1456
B-PER	0.086
Total	**0.32**

6.3.6 Manufacturability

In a previous design, the sensor was constructed with three bands, each with several layers deposited. Additionally, it utilized a lateral flow method in order to allow

the desired enzyme to travel through the porous filter paper. Since transitioning from the silica sol-gel entrapment mechanism to an alginate capsule entrapment method, the manufacturability of this device increased exponentially. Once mixed with the X-gal substrate, the alginate capsules can be manufactured uniformly utilizing a syringe pump which allows for the process to be extremely high-throughput. Such process will allow for the rapid and uniform production of the desired alginate capsules which can then be easily deposited directly onto the device's platform before use.

6.3.7 Usability

The design currently requires that trained lab technicians conduct the testing. To be a true point-of-care device in a low to middle income setting according the World Health Organization, the device must be extremely user-friendly. Though the device does require that the milk samples be pre-treated prior to deposition on the device, the sensor is extremely simple and can be easily used even in low-resource settings where professionals may not experience the same training modules as their counterparts in high income settings.

6.3.8 Environmental Impact

MilkGuard is a single-use sensor that is easily disposable without complex or extensive hazardous waste protocols. Because this design iteration transitioned to a alginate-based platform, the environmental impact of our device has decreased. Alginate is an organic biopolymer that is a derivative of algae, so it is easily disposed of. In addition, little amounts of reagents are used within the sensor, so as a whole, it does not have extensive effects on the surrounding environment.

CHAPTER 7: SUMMARY AND CONCLUSION

7.1 Project Summary

MilkGuard began as a project to streamline the testing required within the breast milk banking system. It was centered around advocating for greater maternal health technologies and increased awareness of the importance of breast milk across the globe. When the project was first passed down to our team, it had a limit of detection of 10^5 CFU/mL and utilized a more complex design. Since then, our team made numerous iterations to the design to meet goals originally created at the beginning of the project's duration. By utilizing a different enzyme-substrate reaction for the detection of ß-galactosidase to measure the concentration of *E. coli* bacteria, we anticipate that the sensor's mechanism can now be applied to other bacterial contaminants. In addition, our team identified and optimized the use of an alginate biomaterial, which allowed for a drastic simplification in the sensors design and manufacturability. Lastly, our team optimized the conditions at which we conducted our experiments to find the conditions that results in the greatest signal. The combination of a different detection method, new biomaterial, and optimized conditions allowed for the sensitivity of our sensor to increase to 10 CFU/mL.

Additionally, to further the project's goal of increasing awareness about the importance of breastfeeding and breast milk donation, our team worked to capture new audiences with our project's impact. In May, our team participated and presented in the Bay Area Global Health Innovation Challenge where we networked with established global health organizations and professionals. In addition, our team maintained relationships with organizations like PATH and EWH to ensure that our message impacted those currently in related industries, and we established new contacts with other related organizations, like D-REV, a non-profit organization focused on distributing world-class affordable technologies to those in need, to help further the development and deployment of MilkGuard.

7.2 Future Work

7.2.1 Shelf Life Assessment

The shelf life of the X-gal substrate lyophilized in the alginate gel needs to be assessed in order to figure out how long the substrate will remain effective for use in a reaction with ß-galactosidase.

7.2.2 Image Capturing

Currently, iPhone cameras are used in conjunction with ImageJ to analyze the blue color intensity in the alginate gel and solution. A standardized, high quality method of image capturing and analysis needs to be implemented in order to ensure the confidence of our device's lowest limit of detection. Further methods could include the use of high quality cameras, lights, and microscopes for efficient image capture.

7.2.3 Diffusion Characterization

We would like to mathematically characterize the diffusion of X-gal out of alginate when in contact with a solution containing ß-galactosidase. This research would allow us to determine aspects that significantly affect the diffusion rate so that we can optimize the sensor's design to work around such constraints. This characterization will help improve the sensor's efficiency and effectiveness.

7.2.4 Detecting Multiple Contaminants

Ideally, our device would be able to detect all of the common contaminants in breast milk banks. The most common contaminant is *Bacillus cereus*. Our device would need to be able to detect *Bacillus cereus* at a high sensitivity in order to be used in breast milk banks. *Staphylococcus* is another top concern at milk banks in which we hope to be able to detect.

7.2.5 Field Testing

We would like to connect with multiple banks in varying geographical areas and resource settings to understand exactly how our device would fit into their respective milk banking systems.

7.3 Lessons Learned

The project provided an educational experience through which we developed the ability to design, plan, and execute experiments. In the face of ambiguity or contradicting results from multiple experimented, we learned to identify and troubleshoot different variables to adjust and test in subsequent experiments. This process strengthened our perseverance and persistence.

Our challenges largely stemmed from our overall lack of experience in a research laboratory setting. Because we came from pre-medicine and medical device tracks, we were less familiar with the bioengineering labs and the handling and storage of materials in the labs. A significant portion of the fall quarter was spent developing skills and familiarizing ourselves with the protocol established by the previous year's team. In doing so, we faced difficulty printing the multiple layers of the sensor using the inkjet printer and the volumetric dispenser. The printer was not designed to print more viscous fluids like the polyvinylamine, and the volumetric dispenser was considerably time-consuming.

This challenge hindered efficient manufacturability, ultimately influencing us to explore alternative methods of entrapment and sensor construction. Later, we ran into a problem with the performance of our assay, which we attribute to the somewhat unpredictable and adaptable nature of cell behavior. The same blue intensity was not achieved in low concentrations of *E. coli* dilutions using the same cell line that we had been using. This led us to eliminate the possibility of contamination, re-plate the cells, and conduct the optimization experiment, which produced particularly valuable feedback.

Our latest challenge involved our method of image capture. We opted to start with an iPhone camera for sake of ease, time, and availability as each of our three team members own one. However, iPhone cameras are programmed to automatically adjust exposure settings, thus preventing consistency across data collection at different time periods by different team members

using different iPhones. Additionally, the sensors were located in a plastic well plate in which adjacent wells contained different dilutions of contaminant. Ambient lighting throughout the plate as well as light refraction from sensors in adjacent wells interfered with the intensity values captured by the camera.

Finally, we shared a lot of the same strengths as a team. But, we struggled to reconcile some of our differences. Sometimes, we opted to forego preparatory critical thinking and trust that running an experiment would yield informative results. Other times, an analytical thought or perspective postponed an experiment in effort to critically evaluate the purpose and design of an anticipated experiment. Eventually, we struck a balance between this critical thinking and acceptance with ambiguity before moving forward with our experiments.

We also connected with professionals across a variety of departments (i.e. Public Health, Biology, Physics, and Bioengineering). We effectively leveraged their expertise and synthesized their input throughout our experimental protocol and innovation design. When confronted with little or slow progress, we did not limit ourselves to narrow or obvious ideas. In fact, our exploration of alternative approaches led to our new entrapment and production method altogether. These strengths were all brought about in our hard work throughout the year. Ultimately, this discipline enabled us to passionately communicate our work and vision. And, it enabled the dramatic simplification of the sensor's design as well as expanded manufacturing feasibility and scalability.

Simultaneously, we recognize that we need to further reduce the limit of detection (LOD), improve the color change intensity, extend shelf life and storage viability, expand the sensor to detect additional contaminants, enhance specificity, and further develop the image capture method.

Overall, we encourage future teams to remain open to new approaches, diversify a team's specialties and strengths, dedicate significant time and patience to learning proper and efficient lab skills and protocol, and work with the Engineering World Health team to quantify the social impact of a market-ready product. Finally, an effective team will embrace frequent, clear, and concise communication throughout the design process—and have fun doing it!

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APPENDICES

APPENDIX I: COMPLETE MATERIALS LIST AND BUDGET

The original budget was $1,855.05 dollars for materials. The Santa Clara School of Engineering grant and Engineering World Health grants provided us with $1,500 and $1,000 respectively.

Table 11. Complete Budget and Materials List.

Item	**Use**	**Source**	**Price ($)**
Human breast milk	Sample	Mother's Milk Bank (San Jose, CA)	800
BL21 Escherichia coli cells	Detection pathogen	Dr. Jonathan Zhang, Santa Clara University (Santa Clara, CA)	0
LB Broth	Bacterial growth medium	Sigma Aldrich (St. Louis, MI), purchased by previous team	0
B-PER Direct Bacterial Protein Extraction Kit	*E. coli cell lysis*	Thermo Fisher Scientific (Waltham, MA)	221
Whatman qualitative filter paper, Grade 1	Detection platform	Sigma Aldrich (St. Louis, MI), purchased by previous team	0
Polyvinylamine (PVAm) (Lupamin 9095)	Polymer activates paper; provides adhesive support for sol gel layers	BASF (Mississauga, Canada)	0
X-gus (5-Bromo-4-chloro-3-indolyl-β-D-glucuronide sodium salt)	Substrate for E. coli enzyme β- glucuronidase (GUS)	Sigma Aldrich (St. Louis, MI), purchased by previous team	0
X-gal (5-Bromo-4-Chloro-3-Indolyl	Substrate for E. coli enzyme ß-galactosidase	Thermo Fisher Scientific (Waltham, MA)	190

β-D-Galactopyranoside)			
Sodium silicate solution (SS)	Sol-Gel precursor	Thomas Scientific (Swedesboro, NJ)	47.75
Tetraethyl orthosilicate (TEOS)	Used with anhydrous glycerol to synthesize Diglyceryl Silane (DNS) (Sol-Gel precursor)	Sigma Aldrich (St. Louis, MI), purchased by previous team	0
Dowex 50WX8 hydrogen form	Cation exchange resin replaces Na+ with H+ in SS sol preparation	Sigma Aldrich (St. Louis, MI)	88
Anhydrous glycerol	Used with TEOS to synthesize DNS; 30% added to solutions to adjust viscosity/surface tension for ink-jet printing	Sigma Aldrich (St. Louis, MI), purchased by previous team	0
Iron chloride	Oxidizing reagent	Sigma Aldrich (St. Louis, MI), purchased by previous team	0
Methytrimethoxysilane (MTMS)	Component of the hydrophobic barrier: Methylsilsequioxane (MSQ)	Sigma Aldrich (St. Louis, MI)	36.3
Ethanol (EtOH)	MSQ synthesis	Bioengineering Department, Santa Clara University (Santa Clara, CA)	0
Hydrochloric Acid (HCl)	MSQ synthesis	Bioengineering Department, Santa Clara University (Santa Clara, CA)	0
Buchner Funnel	SS-gel synthesis	Chemistry Department, Santa Clara University (Santa Clara, CA)	0

Syringe Filter, 0.45-µm	SS-gel synthesis	EMD Millipore (Hayward, CA), purchased by previous team	0
Syringe, 5 ml	SS-gel synthesis	Bioengineering Department, Santa Clara University	0
E. coli Antibody (20- ER13)	Conjugated to magnetic beads for E. coli preconcentration	Fitzgerald (Acton, MA), purchased by previous team	0
Hydrazide-Modified Magnetic Beads	Conjugated to E. coli antibodies for E. coli preconcentration	Bioclone (San Diego, CA)	250
Phosphate buffer saline (PBS), NaH2PO4	Storage buffer for MB-Ab coupling	Bioengineering Department, Santa Clara University (Santa Clara, CA)	0
Epson Stylus C88 Inkjet Printer	Improve manufacturability of layering molecules	Best Buy (Richfield, Minnesota), purchased by previous team	0
Ink cartridges	Improve manufacturability of layering molecules	Amazon (Seattle, WA), purchased by previous team	0
Chloroform	Used in Miller protocol for permeablization (replaces lysing)	Bioengineering Department, Santa Clara University (Santa Clara, CA)	0
0.1% Sodium dodecyl sulfate (SDS)	Used in Miller protocol for permeablization (replaces lysing)	Bioengineering Department, Santa Clara University (Santa Clara, CA)	0
Lysozyme	Used in lysing process in tangent with B-PER	Thermo Fisher Scientific (Waltham, MA)	50
DNAse	Used in lysing process in tangent with B-PER	Thermo Fisher Scientific (Waltham, MA)	91

ß-Galactosidase from Escherichia coli	Positive control for X-gal - ß-gal reaction	Sigma Aldrich (St. Louis, MI)	81
Alginate powder	Used with 0.9% NaCl solution to make alginate hydrogel	Bioengineering Department, Santa Clara University (Santa Clara, CA)	0
1.5% CaCl solution	Used to crosslink alginate hydrogel	Bioengineering Department, Santa Clara University (Santa Clara, CA)	0
0.9% NaCl solution	Used with alginate powder to make alginate hydrogel. Also used to stop crosslinking of alginate.	Bioengineering Department, Santa Clara University (Santa Clara, CA)	0
1 mL syringe	Used to make alginate capsules	Bioengineering Department, Santa Clara University (Santa Clara, CA)	0
18G 1 ½ inch precision needle	Used with with 1 mL syringe to make alginate capsules	Bioengineering Department, Santa Clara University (Santa Clara, CA)	0
Cell strainer	Used to filter alginate capsules out of CaCl solution	Bioengineering Department, Santa Clara University (Santa Clara, CA)	0
		Total:	1855.05

APPENDIX II: GANTT CHART AND DESIGN PROCESS TIMELINE

Task	Sept. 2017	Oct. 2017	Nov. 2017	Dec. 2017	Jan. 2018	Feb. 2018	Mar. 2018	Apr. 2018	May 2018	June 2018
Identify Problem Statement										
Familiarize with 2016-17 Team's Experimental Protocols and Instrumentation										
Take Inventory and Order Materials										
Field Research at Milk Banks										
Understand Customer Needs and Establish Criteria										
Review of Literature and Exploration of Alternative Solutions										
State Project Goal and Objectives										
Submit Proposal for Undergraduate Programs Funding										
Replicate Previous Team's Assay in Water Buffer										
Conceptual Design Iteration										
Write Thesis Introduction										
Conduct Assay in Donated HBM										
Simulate Sensor in Well Assay										
Optimize Lysing Procedures										
Print Sensor Layers Using HP Volumetric Dispenser										

Test Feasibility of Alginate Entrapment										
Apply to Bay Area Global Innovation Challenge										
Run Experiments to Optimize Detection										
Optimize Enzyme-Substrate Reaction in Alginate Slab and Capsules										
Prepare for BAGHIC and Senior Design										
Run Final Experiments with Improved Image Capture										
Train New Team in Lab										

Figure 7. Gantt chart timeline and project deadline goals. *This Gantt chart helped our team map out our project goals for the year and established strict deadlines for us to meet. These deadlines allowed our team to act efficiently and timely with all submissions.*

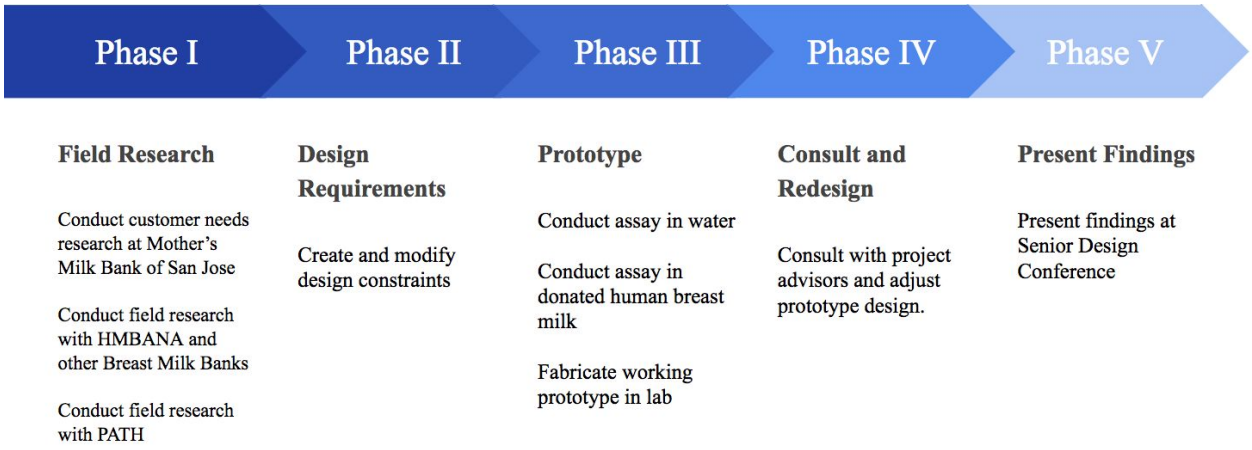


Figure 8. Design process timeline. *MilkGuard's design process timeline helped us establish realistic goals in lab milestones and solutions to ensure we met deadlines and aspirations created in the Gantt chart (**Figure 6**).*

APPENDIX III: ß-GALACTOSIDASE AND X-GAL REACTION

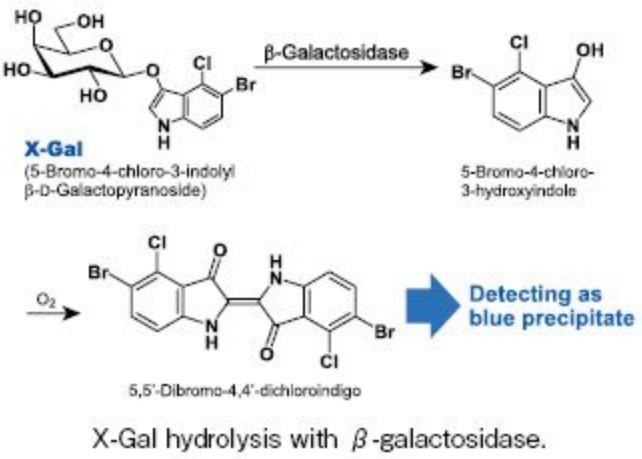


Figure 9. X-gal/ß-galactosidase reaction. *When reacting with ß-gal, the X-gal will cleave and hydrolyze. The hydrolysis will allow for the formation of a blue precipitate that our sensor utilizes to indicate the concentration of bacterial contamination.*

APPENDIX IV: PREVIOUS ENTRAPMENT MECHANISMS AND FABRICATION

Silica Sol-Gel

Silica Sol-Gels can entrap and immobilize biomolecules on paper platforms. Silica's lattice structure stabilizes biomolecules without affecting their physical properties or chemical structure. These gels can also be loaded into inkjet printers and easily and rapidly deposited onto paper without altering its chemical structure.[59]

Preparation and Construction of Silica-Sol Gel Sensor

To prepare the silica sol-gel, 10 mL of ddH_20 were mixed with 2.9 g of sodium silicate solution to obtain a pH of 13. 5 g of Dowex cation exchange resin were added to the mixture to replace Na^+ with H^+. The mixture was stirred for 30 seconds to reach a final pH of 4. The gel was

[59] S. M. Zakir Hossain, Roger E. Luckham, Anne Marie Smith, Julie M. Lebert, Lauren M. Davies, Robert H. Pelton, Carlos D. M. Filipe, and John D. Brennan. Development of a Bioactive Paper Sensor for Detection of Neurotoxins Using Piezoelectric Inkjet Printing of Sol−Gel-Derived Bioinks. Analytical Chemistry 2009 81 (13), 5474-5483. DOI: 10.1021/ac900660p

vacuum-filtered through a Buchner funnel. That filtrate was filtered the filtrate through a 0.45-um membrane syringe filter.[60] To prepare the X-gal stock solution, 20 mg of X-gal was dissolved in 1 mL dimethylformamide. The polyvinylamine (PVAm) was used in its pure form.

To prepare the sensor, each of the layers were sequentially pipetted onto the paper within a well plate such that the entire surface of the well was coated in the fluid. The layers were deposited in the following order: 20 µl PVAm, 20 µl silica sol-gel, 5 µl X-gal, 20 µl silica sol-gel. The completed construction was set out to dry for at least 20 minutes before application of the lysate.

Alginate Slab Preparation

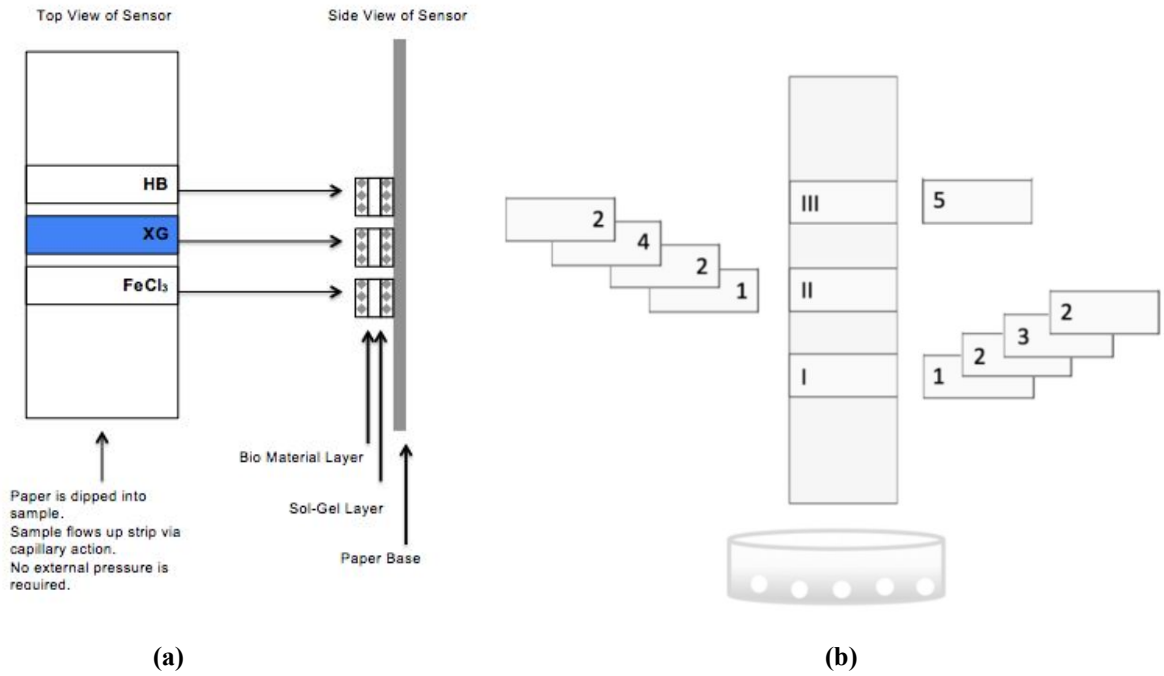
Alginate slabs were prepared by depositing 1.5 mL of alginate mixed with X-gal onto a polylysine coated slide and maneuvered in order to create a slab-like geometric shape. Through several experiments, our team concluded that a wet-slab method was ideal for our application. Following this method, once the initial slab was created, it was immediately cross-linked in 1.5% $CaCl_2$ for approximately 1 hour. This divalent cross-linker was chosen to create a uniform pore size of approximately 3 nm, which is ideal for our application based upon the size of our enzyme (ß-Galactosidase) and substrate (X-gal). Once cross-linking was completed, the slab was were washed three times with 0.9% NaCl to stop all cross-linking activity and allowed to dry for 20 min. Prior to segmentation into the appropriate size for the sensor.[61]

[60] S. M. Zakir Hossain, Roger E. Luckham, Anne Marie Smith, Julie M. Lebert, Lauren M. Davies, Robert H. Pelton, Carlos D. M. Filipe, and John D. Brennan. Development of a Bioactive Paper Sensor for Detection of Neurotoxins Using Piezoelectric Inkjet Printing of Sol−Gel-Derived Bioinks. Analytical Chemistry 2009 81 (13), 5474-5483 DOI: 10.1021/ac900660p

[61] Y. Nakashima, K. Tsusu, K. Minami, Y. Nakanishi. "Development of a cell culture surface conversion technique using alginate thin film for evaluating effect upon cellular differentiation." *Review of Scientific Instruments.* 85 (2014). Web.

APPENDIX V: MILKGUARD INITIAL DESIGN

The initial design of the sensor contains three separate regions each with multiple layers that can be seen in ***Figure 10***.



(a) **(b)**

Figure 10. Initial sensor design and region breakdown. *The schematic of the layers that entrap the biomolecule and makeup the sensor.*[62] *The original sensor design is composed of three bands with various layers in each.* ***(a)*** *displays the general concept of original sensor design, and* ***(b)*** *breaks down each band into the number of layers deposited within it.*

[62] Morrison, Nina; O'Connor, Samantha; and Weber, Callie, "MilkGuard: Low Cost Paper Sensor for the Detection of Escherichia coli in Donated Human Breast Milk" (2017). Bioengineering Senior Theses. 64.

Table 12. Breakdown of each band deposited on the sensor and its corresponding layers.

Region	**Layers**
Region I	Layer 1: 0.5% poly(vinyl amine) Layer 2: Silica sol-gel Layer 3: $FeCl_3$ Layer 4: Silica sol-gel
Region II	Layer 1: 0.5% poly(vinyl amine) Layer 2: Silica sol-gel Layer 3: X-Gus Layer 4: Silica sol-gel
Region III	MSQ

0.5% poly(vinyl amine) (PVAM). The initial design utilized a thin coating of 0.5% of PVAM at the base of Region I and Region II. This thin layer was used in order to create a barrier between the silica sol-gel and the paper platform. Such a layer was there in order to prevent the silica sol-gel from leaching into the paper platform to preserve the integrity of the sensor itself.

Silica Sol-gel. As discussed in previous sections, the silica sol-gel layer was used to entrap biomolecules and preserving such bioactivity.

Ferric Chloride ($FeCl_3$). Ferric chloride acted as an oxidizing agent in order to catalyze the reaction to produce a blue color change at a faster rate.

X-Gus. Previously, a nonpathogenic strain of *E.coli* (BL.21) was chosen as the cell line in the experimental procedure with the idea that once the sensor was optimized, its functionality could be applied to other contaminants. BL.21 have a specific enzyme

ß-glucuronidase (GUS) that upon its reaction with the substrate 5-bromo-4-chloro-ß-D-glucuronide sodium salt (X-Gus), produces a blue color indication.[63]

Methylsilsequioxane (MSQ). MSQ was used as a hydrophobic barrier in order to stop the lateral flow of the sample. This concentrated the blue color change in Region II to ensure for accurate readings that could be correlated to quantitative results.

APPENDIX VI: MILLER PROTOCOL

Instead of "lysing" the cell membranes, the Miller protocol permeabilizes the *E. coli* cells by widening the pores of the membrane. The widening of the pores allow for ß-galactosidase to exit the cell and furthermore mix with X-gal. The cells are permeabilized by adding 100 µl chloroform and 50 µl 0.1% sodium dodecyl sulfate (SDS).[64]

APPENDIX VII: SIGNAL TO NOISE RATIOS

Our group compared signal to noise ratios of the five variables we selected as the most significant in the experimentation. Such values can be seen in ***Figure 11*** and were used to establish which of the five variables created the best signal.

[63] Morrison, Nina; O'Connor, Samantha; and Weber, Callie, "MilkGuard: Low Cost Paper Sensor for the Detection of Escherichia coli in Donated Human Breast Milk" (2017). Bioengineering Senior Theses. 13.

[64] "A Better Miller Assay." *OpenWetWare.* Aug 26, 2014 (LIU).

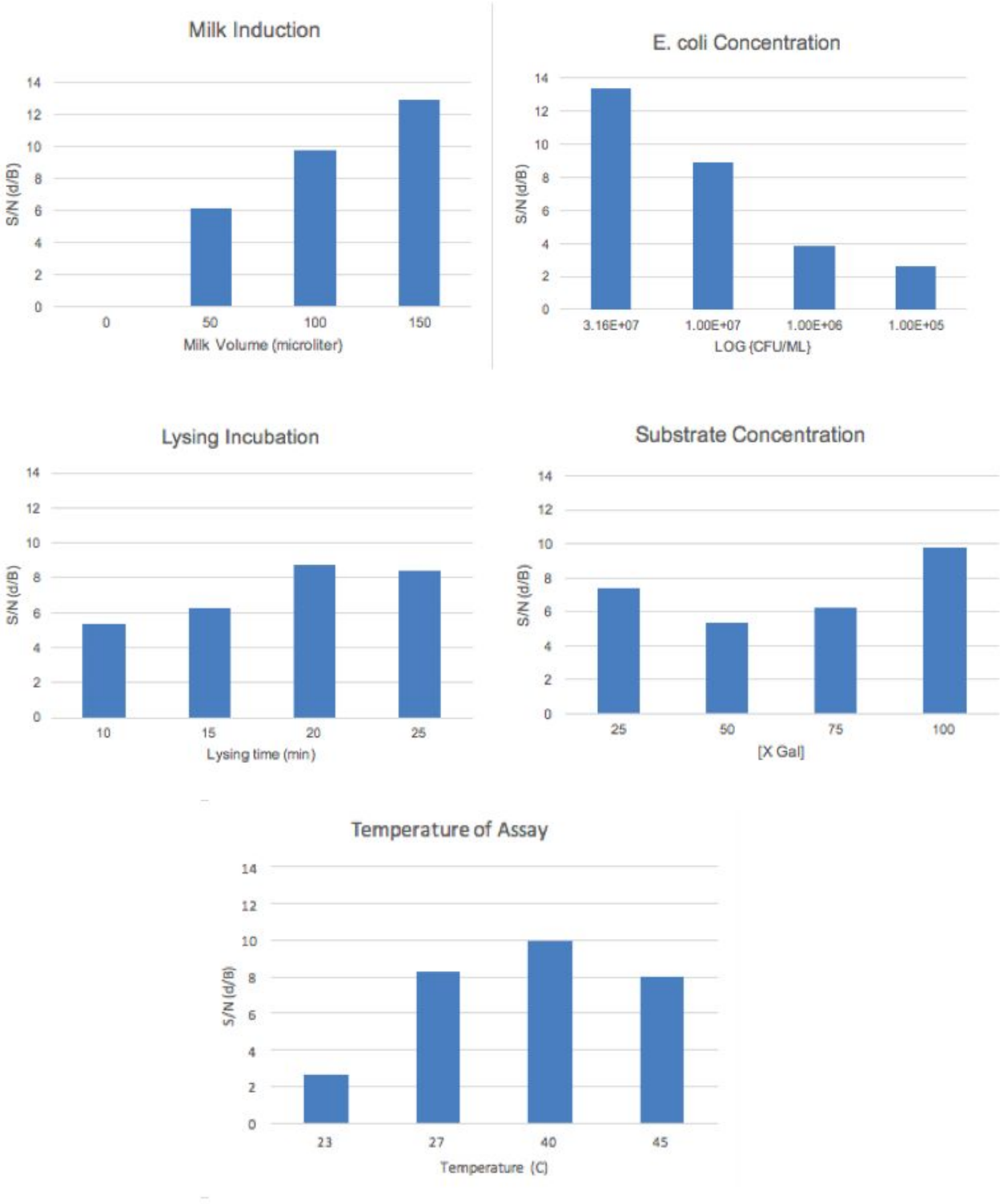


Figure 11. Signal to noise ratios. *The signal to noise ratios for the following variables were analyzed to determine which produced the most significant signal in experimentation.*