CERVIS: Cervical Cancer Early Response Visual Identification System

Kira Palazzo
Lauren Serfas
Juliana Trujillo

Follow this and additional works at: https://scholarcommons.scu.edu/bioe_senior

Part of the Biomedical Engineering and Bioengineering Commons
SANTA CLARA UNIVERSITY

Department of Bioengineering

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

Kira Palazzo, Lauren Serfas, Juliana Trujillo

ENTITLED

CERVIS: CERVICAL CANCER EARLY RESPONSE VISUAL IDENTIFICATION SYSTEM

BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

BACHELOR OF SCIENCE

IN

BIOENGINEERING

Thesis Advisor

6/9/20

Department Chair

6/9/20
CERVIS: CERVICAL CANCER EARLY RESPONSE VISUAL IDENTIFICATION SYSTEM

By

Kira Palazzo, Lauren Serfas, Juliana Trujillo

Senior Design Project Report

Submitted to
the Department of Bioengineering

of

SANTA CLARA UNIVERSITY

in Partial Fulfillment of the Requirements
for the degree of
Bachelor of Science in Bioengineering

Santa Clara, California

2019-2020
Acknowledgments

We would like to acknowledge the following people for their support:

- Dr. Prashanth Asuri, Santa Clara University Bioengineering
- Dr. Michele Parker, Santa Clara University Public Health Science
- Dr. Craig Stephens, Santa Clara University Biology and Public Health Science
- Dr. Emily Park, Santa Clara University Bioengineering
- Dr. Elizabeth Dahlhoff, Santa Clara University Biology
- Mr. Hiram Lozano, Anaerobe Systems
- Lauren Cherrey, Public Health Science
- Will Nelson, Public Health Science

We would also like to thank Santa Clara University School of Engineering and the Xilinx Grant for funding.
Table of Contents

Table of Contents 3
List of Figures 6
List of Tables 7

ABSTRACT 8

CHAPTER 1: INTRODUCTION 9
  1.1 Background 9
  1.2 Significance of Project 10
  1.3 Proposed Goals 10

CHAPTER 2: PROJECT OVERVIEW 11
  2.1 Target Population 11
    2.1.1 Parameters for Deployment 12
  2.2 Review of the Field: Current Detection Methods 12
    2.2.1 Pap Smear 12
    2.2.2 Visual Inspection with Acetic Acid (VIA) 13
  2.3 Vaginal Microbiome 13
    2.3.1 Fusobacteria 14
  2.4 Quantification Methods 15
    2.4.1 Colony Forming Units (CFU) 15
    2.4.2 Microscopy 16
    2.4.3 qPCR 16
  2.5 Team and Project Management 17
    2.5.1 Budget 18
    2.5.2 Timeline 18

CHAPTER 3: CERVIS PROTOTYPE PARAMETERS 19
  3.1 Incubation Time 19
    3.1.1 Methods 19
    3.1.2 Preliminary Results 20
  3.2 Incubation Temperature 21
    3.2.1 Methods 21
    3.2.2 Preliminary Results 21
  3.3 Aerobic Exposure 22
    3.3.1 Methods 22
    3.3.2 Preliminary Results 24
APPENDIX B: Supplemental Information  56
Section 1: General qPCR Procedure  56
Section 2: Preparing Fusobacteria and Primers for qPCR  57
Section 3: Microscopy  58

REFERENCES  61
List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Incubation Time Parameter Results</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>Temperature Parameter Testing Results (72 and 98 hours)</td>
<td>22</td>
</tr>
<tr>
<td>3</td>
<td>Aerobic Exposure Parameter Testing (98 hours)</td>
<td>24</td>
</tr>
<tr>
<td>4</td>
<td>Amplification Plot for Testing Primers</td>
<td>27</td>
</tr>
<tr>
<td>5</td>
<td>Amplification Results for Testing Primers</td>
<td>28</td>
</tr>
<tr>
<td>6</td>
<td>Standard Curves</td>
<td>30</td>
</tr>
<tr>
<td>7</td>
<td>Bacterial Growth Based on Dilution Factor</td>
<td>32</td>
</tr>
<tr>
<td>A</td>
<td>qPCR Plate Setup for Testing primers</td>
<td>54</td>
</tr>
<tr>
<td>B</td>
<td>qPCR Plate Setup for Generating an Absolute Standard Curve</td>
<td>55</td>
</tr>
<tr>
<td>C</td>
<td>Amplification Plot for Generating an Absolute Standard Curve</td>
<td>55</td>
</tr>
<tr>
<td>D</td>
<td>Microscopy Diagram</td>
<td>59</td>
</tr>
</tbody>
</table>
List of Tables

Table 1: Methods of Bacterial Quantification 17
Table 2: Summary of the Comparison of Modalities 36
Table A: Funding Received 48
Table B: CERVIS Expenditures 48
Table C: 2019-2020 Academic Timeline for CERVIS 49
Table D: Universal Master Mix Experimental Setup qPCR 52
Table E: Fusobacteria Master Mix Experimental Setup qPCR 52
Table F: No Primer Control (NPC) Experimental Setup qPCR 53
Table G: Primer Sequences Used in qPCR Experiments 53
Table H: UMM and FMM Total Volumes (Testing Primers) 53
Table J: UMM and FMM Total Volumes (Generating an Absolute Standard Curve) 54
Table K: Bacterial Counts Microscopy 59
ABSTRACT
The goal of CERVIS is to increase screening for cervical cancer through the development of a low-cost, minimally invasive screening procedure for women in low-resource settings that requires minimal healthcare expertise. There currently exist two primary screening procedures: the Pap smear, primarily used in developed countries, and visual inspection with acetic acid (VIA), primarily used in low and middle-income countries (LMICs). Both of these procedures require a high degree of healthcare training in order to administer and interpret, and are highly invasive, requiring direct interaction with the cervix. Our alternative procedure utilizes a particular bacteria, *Fusobacterium nucleatum*, within the cervicovaginal microbiome that has been associated with cervical cancer pathology. Our screening procedure seeks to identify the need for further diagnostic testing based on a vaginal swab representative of fusobacteria colonization of the vagina. Two modalities, growth media containing colorimetric indicators and a qPCR-based assay, are used to qualitatively and quantitatively measure the presence of this bacteria. This collection method is less invasive and does not require healthcare expertise, therefore allowing for self-administration. Outcomes of testing are measured by determining the feasibility of the prototype regarding sensitivity and parameters of bacterial growth, including time for incubation, the temperature of incubation, and aerobic exposure time. Further testing will include quantifying fusobacteria in a cervical cancer patient sample and establishing exact temperature ranges of incubation and incubation time.
CHAPTER 1: INTRODUCTION

1.1 Background
Globally, cervical cancer ranks fourth in incidence and in cancer-related mortality among women, with the majority of cases occurring in low and middle-income countries (LMICs) (WHO, 2019). These high incidence and mortality rates are due to delayed diagnosis, which means that cervical cancer often goes undetected for several years. The WHO states that 90% of those cervical cancer-related deaths occur in LMICs, many of which could be prevented by early screening and diagnosis (WHO, 2019). Human Papillomavirus (HPV) is the most common risk factor for cervical cancer, and proper implementation of an HPV vaccine helps to prevent this disease (Liu & Richardson, 1987). However, while this vaccine is widely used in developed countries, it is not implemented on a national level in many LMICs, and even if programs exist these are not effective (Nakisige, 2017). Vaccines are in the process of being implemented but would be ideally administered to girls in their pre-teenage years, and as such the effects of such a vaccine would take many years to be able to see results. Furthermore, to decrease the incidence of the disease, it is still necessary to address prevention factors while also pursuing cervical cancer screening, diagnostic and treatment measures.

Additional prevention methods that enable detection of precancerous changes before progression to invasive disease are also being developed to help reduce the prevalence of cervical cancer cases (Finocchiaro-Kessler, 2016). Screening is a very common prevention method, but in LMICs, the screening rates remain very low (Gyawali, 2015; Runge, 2019). This is often because there are shortages in the necessary supplies to perform these screening tests and a lack of trained healthcare personnel to administer these tests (PATH, 2019). Cervical cancer education is an additional preventative measure to help inform women of the risks and proper safety practices surrounding unsafe sex, but many LMICs currently lack this sort of education.
1.2 Significance of Project
Although cervical cancer is the 4th most common cancer in women worldwide, there are currently no low-cost, minimally-invasive screening tests available. Due to the prevalence of cervical cancer, the HPV vaccine is not simply a replacement for screening. Implementing the HPV vaccine is a proactive prevention measure, whereas screening is reactionary to the development of cervical cancer, preventing its progression to later stages. Current cervical cancer screening procedures identify DNA and protein biomarkers from cervical samples but are expensive, difficult to administer, and often invasive. These factors limit test feasibility in low-resource areas, creating a need for new screening technologies.

Team CERVIS aims to increase screening in Uganda by developing a low-cost, minimally-invasive screening test that can be self-administered and requires minimal healthcare expertise. Last year’s test created a prototype for this screening test, and this year our focus is on determining the feasibility of that prototype by determining its sensitivity and optimal implementation parameters.

1.3 Proposed Goals
Team CERVIS, a group of undergraduate students from Santa Clara University departments of Bioengineering and Public Health, aims to build on a promising prototype designed to detect the presence of fusobacteria on a vaginal swab. Fusobacteria is an anaerobic bacteria found in the cervical and vaginal microbiomes that is found in increased concentrations in cervical cancer (Audirae Chalifour et al., 2016). The prototype contains media that will change color due to a reaction with fusobacteria present on a vaginal swab. While last year’s team successfully proved that the media was specific to fusobacteria, it is still unclear whether the prototype is sensitive enough to detect the amount of fusobacteria present in vaginal samples of patients with cervical cancer. Furthermore, this year, team CERVIS explored the feasibility of implementing this prototype, including testing the sensitivity of the media along with determining optimal implementation parameters.
CHAPTER 2: PROJECT OVERVIEW

2.1 Target Population
The development of the prototype requires a targeted population to establish a deployment strategy and to measure the current impacts of cervical cancer and its preventative strategies. Our chosen target population is women in Uganda, where one of the highest incidences of cervical cancer in the world is observed, as well as a low screening rate (Black, 2019). Uganda lacks many primary and secondary preventative methods to help reduce cases of cervical cancer. Uganda is a Sub-Saharan country in Eastern Africa, with a population of nearly 32 million. Each year, about 40.5 in 100,000 Ugandan women die of cervical cancer compared to 6.8, the global average (Black, 2019). Cultural reticence to seek routine invasive pelvic examinations also prevents women from seeking treatment until the disease has progressed to severe, symptomatic stages (WHO Training Guide 2015). Up to half of women diagnosed with late-stage cervical cancer die within 3 years of diagnosis (Wabinga, et al., 2000), and up to 80 percent of those women die within 5 years (Gondos, et al., 2005). Increased, minimally invasive screening could prevent such deaths from occurring. Presently, despite Uganda’s nationally-implemented HPV vaccine program, efforts by the Ministry of Health have been uncoordinated and had limited success (Nakisige et.al., 2017). Furthermore, in 2016, only 4.8% of women have been screened (Campos et.al., 2017). Successful implementation of an HPV vaccine program would greatly decrease the incidence of cervical cancer, but screening and diagnostic technology are still required in order to effectively combat mortality rates from this disease. Finally, our team currently has connections to a non-governmental organization (NGO), Rose Academies, and several clinics there. Therefore, it makes it an ideal location to develop a deeper understanding of our target population’s needs, and could also establish a relationship for future product deployment.
2.1.1 Parameters for Deployment

There are several parameters that need to be considered prior to deployment of the prototype, but a select few were determined to be the most pressing to assess feasibility given the limits of conditions in Uganda. Currently, women in rural areas travel to receive healthcare or have outreach teams visit them to provide healthcare services (Jeronimo et al., 2014). We wanted to establish a baseline incubation time for the color change to evaluate the feasibility of our prototype in the field. Incubation temperature of the fusobacteria is another major factor to properly produce an indicative dark gray color. Many areas may not have access to an incubator to maintain the standard temperature of incubation for fusobacteria, so this parameter is explored throughout our experiments to determine whether or not an incubator is a necessary element for proper prototype functionality. Finally, due to the anaerobic nature of our target bacteria, we have considered aerobic exposure as an important parameter to analyze for feasibility. Determining how long a swab can be exposed to air prior to insertion into the media will help to develop a proper protocol for healthcare clinicians administering this test. Cervical cancer is a malignant and proliferative disease in countries like Uganda, but Team CERVIS aims to reduce its severe incidence and mortality that women suffer.

2.2 Review of the Field: Current Detection Methods

The key to preventing and addressing cervical cancer development is preventative medicine and routine screenings. Current guidelines for women recommend annual cervical cancer screening for HPV-positive women, and every 3 years for all others, but in actuality is irregular and often determined by resource availability (CDC, 2018). Pap smear is most common in developed nations while Visual Inspection with Acetic acid (VIA) is used more often in poorly resourced areas (Gaffikin, 2002).

2.2.1 Pap Smear

Pap smears and colposcopies are the most common cervical cancer screening methods to accurately detect cellular processes of both precancerous and cancerous samples within a swab sample of the cervix (Markovic, 1998). Pap smear analysis requires a high degree of technical
and clinical knowledge to identify and swab the cervix correctly to interpret sample results for appropriate diagnosis. Not only is the equipment to analyze the sample expensive and laboratory access required for proper and effective analysis, but these procedures have a high number of false positives. Moreover, individual patients have indicated that Pap smears and colposcopy are invasive, painful, and costly, requiring regular screening every 2-3 years (Markovic, 1998).

### 2.2.2 Visual Inspection with Acetic Acid (VIA)

Visual Inspection with Acetic Acid (VIA) of the cervix is an inexpensive screening method used primarily in poorly resourced areas (Gaffikin, 2002). This screening test is performed by trained health workers and nurses in women’s health clinics as well as mobile screening camps in low-income areas. The test is invasive and can cause some discomfort similar to the Pap smear method because of the insertion of a self-retaining vaginal speculum. After insertion, acetic acid is applied to the cervix and observed for a reaction between the suspected lesion and the acetic acid indicated by a color change (Gaffikin, 2002). Similar to limitations of other screening procedures, VIA requires a high degree of technical knowledge to properly administer the screening, must be done in a clinical environment, and is highly invasive. If the test is not administered properly, bubbles may form, resulting in false positives (Mandelblatt, 2002).

The limitations of these current technologies highlight the need for a cost-effective medical screening procedure that enables women in low resource settings, without access to advanced medical care, to accurately test for cervical cancer. Furthermore, the results of the screening should be easily understood by individuals with little to no technical medical background.

### 2.3 Vaginal Microbiome

One of the newest avenues of cancer detection research involves microbiota, which are the microbial communities present in certain areas of the body. These include the gastrointestinal tract, oral cavity, and the cervicovaginal regions. This class of organisms was chosen as a target for our prototype because current literature indicates that dysbiosis, a microbial imbalance, has
been linked to many diseased states, from irritable bowel syndrome to many types of cancers, including cervical cancer (Kriss et. al., 2018).

There are many factors that influence the composition of the vaginal microbiome. These can include hygiene, lifestyle factors (i.e. sexual health, contraceptives, etc.), pregnancy, and menopause. The composition can change several times throughout a single individual’s lifetime. The bacteria present in the vaginal microbiome can be classified in one of five ways, referred to as Community State Types (CSTs). Each females’ vagina can be classified as CST I-V, with dominant bacteria in each category. The most common CSTs are III and IV, and an individual can transition from CST III to CST IV during their lifetime, and transitions between any two community state types are common (Gupta, et. al, 2019).

Of particular interest to our team is the prevalence of vaginal dysbiosis in Sub-Saharan Africa (van de Wijgert and Jespers, 2017). A definitive causal link has been established between vaginal dysbiosis and cervical cancer from oncogenic HPV acquisition to cervicovaginal precancerous development, meaning that detection by this manner has the capability of detecting early-stage cervical cancer (Brusselaers, 2019)

2.3.1 Fusobacteria

Fusobacteria strains have been detected in pathogenic dysbiosis of microbiomes such as the colon, oral mucosa, and gut (Han, 2015). Recent work has also correlated its presence to cervical cancer pathology. Vaginal dysbiosis is often observed in correlation with a change in the ratio of healthy to pathological microorganisms, including an increase in the amount of fusobacteria. This dysbiosis, caused by infection or other pathology, can increase a patient’s risk to develop more serious conditions such as cervical cancer (Gupta et al, 2019).

Studies have shown that *Fusobacterium nucleatum* is upregulated in correlation with cervical cancer, finding that it constitutes approximately 17% of the cervical microbiome in this disease state (Audirac-Chalifour et al., 2016). *F. nucleatum* is an obligate anaerobe, meaning that it can only grow in conditions where oxygen is absent. This is the bacteria that we are targeting with our research and experiments because it provides the most conclusive link to cervical cancer in
patients diagnosed with the disease, and is not present in high levels in the healthy microbiome.

2.4 Quantification Methods

Sensitivity testing is required in order to determine the accuracy of a screening procedure. This testing measures how often a test correctly generates a positive result when the targeted condition is present. This is known as the true positive rate (King and Mody, 2010). Producing a true positive result is essential for an accurate screening procedure.

The National Institute of Health (NIH) launched the human microbiome project in 2008 to better understand microbial microenvironments in specific areas of the human body and how they contribute to human health and disease (Gevers, 2012). The vaginal microbiome was explored to gain insight into the baseline state of a healthy vaginal microbiome. Some studies also indicated that in women affected by cervical cancer, the composition of fusobacteria in the vaginal microbiome is 17% (Audirac-Chalifour et al., 2016). The composition of cervical and vaginal flora specimens has been proven to be comparable, therefore the vaginal microbiome can be representative of the cervical microbiome (Smith et al., 2014).

2.4.1 Colony Forming Units (CFU)

When performing a quantitative test, there are several available methods for establishing a baseline. The selection of the proper method depends on the degree of precision desired. One of the easiest and most common quantification methods is counting colony-forming units (CFUs). This method is regarded as the gold standard for determining bacterial cell number (Hazan et al., 2012). Utilizing this method is beneficial because only viable bacterial colonies are counted, excluding any dead bacteria and debris (Hazan et al., 2012). The most significant disadvantage of this method is that clumps of bacteria cells can be miscounted as single colonies. While no specialized equipment is required for counting CFUs, there are several limitations to this method, including an extended incubation time of several days in order to obtain observable colonies.
2.4.2 Microscopy
Compound microscopy is another method commonly utilized in bacterial quantification. Hemocytometers are used in conjunction with the microscope in order to determine absolute cell count. Using this method can be difficult because it requires the ability to differentiate between bacterial cells and debris, as well as being able to clearly identify clumps of bacteria (Hazan et. al., 2012). This method requires a specialized microscope and a hemocytometer plate, but results can be obtained in a shorter amount of time than CFU counting because cells can be counted without incubating first.

2.4.3 qPCR
Quantitative Polymerase Chain Reaction (qPCR) or Real-Time PCR is a method commonly used to quantify the abundance of particular microbial DNA. Analysis via qPCR combines traditional end-point detection PCR with detection by fluorescent expression technologies to record amplification in “real time” (Smith et. al., 2009). This method also provides wide sequence coverage, as it can specifically target particular taxonomic or functional markers from bacterial domain down to specific bacterial strains. The results from this method can be obtained in approximately 1.5 hours, but post-analysis can be difficult and time-consuming, particularly in developing an absolute standard curve. The standard curve development itself requires initial quantification by one of the aforementioned methods for qPCR verification.

The table below summarizes the benefits and limitations of each of the accessible microbial quantification methods.
Table 1. Methods of Bacterial Quantification

<table>
<thead>
<tr>
<th>Quantification Method</th>
<th>Benefits</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony-forming units (CFUs)</td>
<td>● Easy to conduct&lt;br&gt; ● Counts only live cells&lt;br&gt; ● Range of detection unlimited</td>
<td>● Time-consuming&lt;br&gt; ● Susceptible to contamination</td>
</tr>
<tr>
<td>Compound Microscopy</td>
<td>● Range of detection unlimited&lt;br&gt; ● Quick results</td>
<td>● Relies on the ability of viewer to count bacterial colonies, large potential for human error</td>
</tr>
<tr>
<td>qPCR</td>
<td>● Counts bacterial DNA from live, dead or injured cells&lt;br&gt; ● Can specifically target a particular bacterial strain&lt;br&gt; ● Quantification of up to 96 samples at once&lt;br&gt; ● Fairly quick results (hours)</td>
<td>● Tedious experimental procedure may lead to errors&lt;br&gt; ● Post-analysis with standard curves is complicated</td>
</tr>
</tbody>
</table>

2.5 Team and Project Management

The project team is composed of three Bioengineering (BIOE) majors in conjunction with five Santa Clara University (SCU) faculty members, two Public Health science majors from the Engineering World Health (EWH) partnership, and Hiram Lozano from Anaerobe Systems. The faculty advisors are Dr. Prashanth Asuri, Dr. Michele Parker, Dr. Craig Stephens, Dr. Emily Park, and Dr. Elizabeth Dahlhoff. Dr. Prashanth Asuri has served as a Bioengineering advisor. Dr. Michele Parker has served as a Public Health advisor and a mentor to the Engineering World Health sector of the project. Dr. Craig Stephens has served as an expert in microbiology and advised the students on both microbiological techniques and characteristics of bacteria. He also provided the students with lab space and supplies. Dr. Park has served as an advisor for designing qPCR experiments. Dr. Dahlhoff provided lab space, materials, and expertise in
running a qPCR experiment. Lauren Cherrey and William Nelson acted as public health student partners for the project, advising the BIOE team about relevant ethical and cultural issues.

This project originally stemmed from the work of a 2017-18 senior design group and subsequent iteration during the 2018-19 school year. Within the BIOE student team, all experimental design, presentations, intra-team communication, and lab work were completed together. In addition to baseline roles, individual team members had responsibilities of their own. Juliana Trujillo managed supply orders and was the point of contact with the School of Engineering, Lauren Serfas organized meetings and was the primary point of contact with advisors, and Kira Palazzo was the lead point of contact with Anaerobe Systems, managing updates and supply shipments from the company to SCU.

2.5.1 Budget

The funds provided for experimentation and development of our senior design project were requested from the Santa Clara University Undergraduate Programs. The awarded funding is summarized in Table A found in Appendix A. Additionally, a detailed outline of the expenditures of the project totaling $1,431.32 can be found in Table B in Appendix A.

2.5.2 Timeline

Reference Appendix A, Table C for an overview of completed action items for the CERVIS project for the 2019-20 school year.
CHAPTER 3: CERVIS PROTOTYPE PARAMETERS

We chose three initial parameters to test conditions for feasibility: incubation time, incubation temperature, and aerobic exposure prior to insertion into the CERVIS media. We chose these parameters as the most pressing questions to assess feasibility given the limits of conditions in Uganda.

3.1 Incubation Time

In rural areas of Uganda, access to clinical settings may be limited. For this reason, it is important to minimize clinical wait times for the CERVIS screening procedure. Once the screening has been performed, results are not immediately available because of the necessity to culture the CERVIS tubes in an incubator. The purpose of the incubation time parameter test is to determine the minimum amount of time necessary for a true positive to be produced. This would be necessary for the deployment of the procedure in Uganda because it would allow for identifiable wait times, minimizing patient anxiety, and increasing ease of processing for healthcare personnel.

3.1.1 Methods

In preparation to grow fusobacteria, the anaerobic chamber was turned on and allowed to purge for 24 hours to remove oxygen and establish an anaerobic environment.

*Entering the Anaerobic Chamber*

Fusobacteria plates, which had been cultured for 96 hours, were removed and photographed. After the plates had been properly documented, viable colonies were removed from plates using sterile inoculating loops and placed into a 9 mL dilution blank. The dilution blank was then resealed and vortexed at speed 7 for ten seconds. Once this was completed, 1 mL of the solution was withdrawn from the tube and added to another 9 mL dilution tube to create a tenfold dilution. Once this was completed, the dilution tube was sealed and then vortexed on 7 speed for 10 seconds to distribute the bacteria through the solution. Following the vortex, 20 μL was added to a sterile swab of the bacteria-containing solution; 20 μL was the amount determined to be the
maximum volume retained on the chosen swabs. This swab was then inserted into the media and sealed. The CERVIS tube was then placed into the passbox, sealed from the inside, and the team member exited the chamber.

*Outside of the Chamber*

Once removed from the chamber, the CERVIS tube was incubated at 37 °C and documented at 0, 24, 48, and 72 hours.

### 3.1.2 Preliminary Results

According to qualitative analysis, a color change did not occur in the media at all until 48 hours, with a more substantial color change occurring at 72 hours. However, these results do not provide conclusive evidence for the exact point at which a true positive can be observed, so further tests would be necessary to confirm this.

<table>
<thead>
<tr>
<th>Incubation Time</th>
<th>0 hrs</th>
<th>24 hrs</th>
<th>48 hrs</th>
<th>72 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
<td></td>
</tr>
</tbody>
</table>

*Figure 1: Incubation Time Parameter Results*
3.2 Incubation Temperature

In rural villages in Uganda, access to an incubator may be severely limited, so we wanted to explore alternative incubation temperatures that could result in a true positive result without the use of a traditional incubator.

3.2.1 Methods

*Within the Anaerobic Chamber*

See section 3.1.1 for details on placing fusobacteria into CERVIS media tubes.

*Outside of the Anaerobic Chamber*

The four CERVIS tubes designated for temperature parameter testing were placed in various temperatures in order to determine if bacterial growth was possible. One tube was designated to be at room temperature, 25 °C, one was placed in a 37 °C incubator, one in a 30 °C incubator, and one in an outdoor area that would experience temperature fluctuations throughout the day. The tube placed outside was in a shaded area so that direct sunlight would not be a confounding variable. Each tube was documented after 72 and 98 hours, though the outdoor tube was only documented at 72 hours.

3.2.2 Preliminary Results

Without access to an incubator, it is likely that access to this procedure would be severely limited. According to preliminary testing, bacterial growth occurs only at 37 °C.
<table>
<thead>
<tr>
<th>Temperature</th>
<th>Varying</th>
<th>25 °C</th>
<th>37 °C</th>
<th>30 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>72 hours</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>98 hours</td>
<td>Not pictured</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
</tr>
</tbody>
</table>

*Figure 2: Temperature Parameter Testing Results (72 and 98 hours)*

### 3.3 Aerobic Exposure

During sample collection, it is likely that the vaginal swab would be exposed to air for at least several seconds to several minutes following collection by the patient. For this reason, tests were conducted to determine the maximum time for aerobic exposure that would allow the procedure to maintain viability. That is, the test was necessary to determine if exposure to air would still allow for a true positive result of the CERVIS procedure. A true positive can be obtained only if fusobacteria growth occurs, and this test would confirm the degree to which exposure of this anaerobic bacteria to air would inhibit growth.
3.3.1 Methods

Within the Anaerobic Chamber

Once it had been confirmed that the chamber was anaerobic, the team opened the incubator and took pictures of each plate. Fusobacteria that was designated for aerobic exposure testing was cultured. The plates were assessed for viable colony growth and set aside. Approximately 30 viable fusobacteria colonies from the plates were ‘picked’ via a sterile 1 μL inoculating loop and placed into a tube containing 5 mL of dilution solution. The inoculating loop was shaken vigorously to release the colonies into the dilution solution and the tube was sealed with the cap. The tube was then vortexed at speed setting seven for ten seconds to ensure even distribution of fusobacteria throughout the solution.

Next, each swab was prepared to be inserted into the media. To do this, we vortexed the tube containing the bacteria for ten seconds, then removed the cap and removed 20 μL of the bacteria-containing solution. This was pipetted onto a swab, turning the swab as the solution was released to coat it in solution. Following this, the swab was then plunged into the CERVIS media and sealed with the cap.

The twelve swabs were designated for aerobic exposure testing. The procedure of vortexing the bacteria-containing solution and adding it to the swab was repeated for each swab, after which the swab was placed in the tube rack with the tip of the swab facing upwards. Prior to exiting the chamber the tube rack with swabs was placed in the passbox and was sealed from the inside.

Outside of the Anaerobic Chamber

Before opening the passbox, we collected a timer and twelve new CERVIS media tubes, along with a tube rack to stand those in. We labeled duplicate tubes for six aerobic exposure times--15, 30, 45, 60, 120, and 300 seconds--for a total of twelve tubes. Next, while one person was ready to start a timer, the other got ready to open the passbox. When the passbox was opened, the timer was started and watched until it was time to insert the swab into the media. Just before a time point was reached, both team members opened the corresponding CERVIS media tubes, selected
the swabs without touching the tip, carefully pushed the swabs into the media tip down, and resealed the tubes. This process was repeated for each time point listed above. Once all tubes contained swabs, they were placed into the 37 °C incubator and checked at 72 and 98 hours, capturing images each time.

3.3.2 Preliminary Results
After 72 hours, little to no distinct growth had been observed in all of the tubes. After 98 hours, growth was observed in all time points except for 300 seconds, but there were large inconsistencies between duplicates at 30, 60, and 120 seconds (see Figure 3). These inconsistencies could be explained by the variability common in bacterial suspensions such as the one used for parameter testing. There is a possibility that the tubes that showed no growth had not received any or received very little fusobacteria in 20 µL of the bacterial suspension that was pipetted onto the swab.

<table>
<thead>
<tr>
<th>Exposure Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 sec</td>
</tr>
<tr>
<td>30 sec</td>
</tr>
<tr>
<td>45 sec</td>
</tr>
<tr>
<td>60 sec</td>
</tr>
<tr>
<td>120 sec</td>
</tr>
<tr>
<td>300 sec</td>
</tr>
</tbody>
</table>

Figure 3: Aerobic Exposure Parameter Testing (98 hours)
CHAPTER 4: DETERMINING SENSITIVITY OF THE CERVIS MEDIA

The overall goal of team CERVIS this year was to determine the feasibility of last year’s prototype. In order to do that, one of the subgoals was to assess the sensitivity of the CERVIS media to ensure it could detect the amount of fusobacteria expected in the vaginal microbiome of a woman with cervical cancer.

4.1 Objectives

As previously mentioned, 17% of the vaginal microbiome is composed of fusobacteria. Based on our assumptions that a vaginal swab is representative of the vaginal microbiome for the sensitivity testing, we can conclude that approximately 17% of a vaginal swab is composed of fusobacteria for women with cervical cancer. Knowing the approximate amount of fusobacteria that would likely be in the vaginal swab of a woman with cervical cancer allows for a target percentage of how sensitive the CERVIS media needs to be in order for team CERVIS to consider last year’s prototype feasible.

It is necessary to generate a percentage that corresponds to the sensitivity of the CERVIS media to compare to our target percentage of 17%. This percentage can be calculated by dividing the amount of fusobacteria necessary to initiate a color change in the CERVIS media by the total bacteria present in a vaginal swab. It has been found that total bacteria in a microbiome does not change depending on disease states, so the number of total bacteria that is found in the vaginal microbiome of a woman with or without cervical cancer should be approximately equal (Vickery and Ramakrishnan, 2017).

Therefore, to evaluate the sensitivity of the CERVIS media, we had to determine:

1) How much initial fusobacteria is necessary to add to the CERVIS media for a color change to occur (Section 4.5.1)

2) How much total bacteria is present on a vaginal swab (Section 4.5.2)
4.2 General qPCR Procedure

In order to quantify the amounts of fusobacteria and total bacteria present in the sample, we used quantitative Polymerase Chain Reaction, or qPCR (see description of this quantitative method in Section 2.4.3). The following section describes the general procedure for preparing and performing qPCR, which are preliminary steps necessary before running our desired experiments.

The qPCR procedure can be broken down into three primary steps:

1) Preparing dilutions of the bacterial DNA
2) Creating the Universal and Fusobacteria Master Mix and NPC
3) Combining the DNA dilutions and Master Mix on a qPCR plate

Further details can be found in Appendix B, Section 1.

4.2.1 Preparing Fusobacteria and Primers for qPCR

Primers are one of most, if not the most important, critical component of any PCR because they control the specificity and sensitivity of the analysis (Bustin, 2017). We needed to find “universal” primers for eubacteria, all bacteria. The universal primers used in our experiments were taken from a study that had previously sequenced the vaginal microbiome (Mao et al., 2012). We found species-specific primers for *Fusobacterium nucleatum* as it was the species associated with cervical cancer pathology (Castellarin, et.al. 2012). The sequences of these primers are located in Table G in Appendix A.

Preparation of our experiments entailed growing fusobacteria in an anaerobic hood and isolating DNA for the qPCR procedure. In addition, primers had to be prepared from a lyophilized state prior to qPCR testing. The detailed methods used to perform this are described in Appendix B, Section 2.
4.3 Testing Primers
Before we could begin quantifying fusobacteria in the CERVIS media or total bacteria on a vaginal swab, we first had to ensure our qPCR procedure was working appropriately and our reaction components were performing as expected. We began by verifying that our Universal and Fusobacteria primers were working properly. To test the universal primers, we first ran a qPCR experiment with Fusobacteria and *E. coli*. We expected both of these bacteria to produce amplification if they were indeed universal and our procedure was appropriate. We also ran a qPCR experiment using Fusobacteria and *E. coli* with the *Fusobacterium nucleatum* primers. In this situation, we expected the fusobacteria to produce amplification while the *E. coli* would not.

4.3.1 Methods
In testing the primers, we followed General qPCR Procedure as described in Appendix B, Section 1. We performed two ten-fold dilutions of the *E. coli* DNA (147 µg/mL) and fusobacteria DNA (2.58µg/mL) and used the $10^1$ and $10^3$ dilutions when pipetting them onto the qPCR plates. In preparing our Fusobacteria and UMM we calculated for 12 reactions (10 necessary + 2 additional). See Table H and Figure A in Appendix A for the total volumes and qPCR plate setup.

4.3.2 Results

![Amplification Plot](image)

*Figure 4: Amplification Plot for Testing Primers*
The amplification for both the fusobacteria and universal primers is shown in Figure 4. The x-axis is the cycle number of the qPCR machine, and the y-axis is the change in the amount of DNA. The lower the cycle number, the greater the amount of DNA initially present in the sample. In order for there to be considered amplification, the curve must rise above the threshold level, determined by the horizontal green and orange lines. The point where those two lines cross is called the Cycle Threshold (or C\textsubscript{T} value). In the lines within area A, we see that there is clearly amplification for both the universal and \textit{F. nucleatum} primers. However, in area B, they are high C\textsubscript{T} values (signifying very low initial amounts of DNA) so the results are inconclusive as to whether there was sufficient amplification or not.

The following figure (Figure 5) shows amplification in another way that is easier to assess whether the primers were performing as expected.

![Figure 5: Amplification Results for Testing Primers](image)

The box in blue shows the controls for the experiment (water instead of any DNA and NPC instead of the UMM or FMM). There should be no amplification for these eight reaction wells (all purple circles). The red circles signify amplification, therefore we recognize that our controls were not working as expected. However, looking at the amplification plot, those curves fall in Area B, so the data was on the borderline of being considered “amplified” or not. We recognized this inconsistency, but since these were preliminary experiments, we continued to move forward recognizing that this data was likely due to contamination from our inexperience of running these experiments.
Returning to Figure 5, the top row is bacteria with the UMM (with the Universal primers), and the bottom row is with the FMM (with F. nucleatum primers). As mentioned previously, we expected there to be amplification with both the E.coli and F. nucleatum (which we see with the red circles) with the UMM (the top row), but there was only amplification with F. nucleatum with FMM (the bottom row). With the E. coli and FMM, we got results that were inconsistent with our hypothesis, as it showed some amplification with one of E. coli concentrations. Once again, because we saw that the FMM was amplifying F. nucleatum (which we were more focused on), we took note of this inconsistency and continued to proceed forward with the rest of the qPCR experiments.

### 4.4 Generating Absolute Standard Curves

Once we felt comfortable with the qPCR procedure and had ensured that our Universal primers were amplifying different types of bacteria (E. coli and F. nucleatum) and our fusobacteria primers were amplifying F. nucleatum, we then proceeded with the next step of our preliminary sensitivity testing which was generating universal and fusobacteria absolute standard curves.

An absolute standard curve is generated from a **known** amount of DNA, so it can be used as a reference for determining an **unknown** amount of DNA in a sample. This was important so that we could determine the unknown amounts of initial fusobacteria in the CERVIS media and total bacteria that were present in a vaginal swab.

We determined our known amount of fusobacteria present in our initial dilution using microscopy (See Appendix B, Section 3). We then used the amount of fusobacteria DNA from our microscopy results and entered them into the standard curve technology programmed into the qPCR machine. This automatically created our Fusobacteria absolute standard curve and universal absolute standard curve.

### 4.4.1 Methods

After determining through microscopy how much fusobacteria in our initial dilution, we then followed the “General qPCR Procedure” as described in Appendix B, Section 1. We performed
five, ten-fold dilutions of fusobacteria DNA (6.08 μg/ml) and used all five dilutions when pipetting them onto the qPCR plate. In preparing our Fusobacteria and Universal master mix we calculated for 13 reactions (11 necessary + 2 additional). The total volumes and qPCR can be found in Table J and Figure B in Appendix A.

4.4.2 Results

![Figure 6: Standard Curves](image)

The fusobacteria standard curve (orange) and universal standard curve (purple) are shown in Figure 6. The amount of bacteria is shown on the x-axis, and a measure of amplification (C<sub>T</sub> value) is shown on the y-axis (see Results section for Testing primers for a more detailed explanation of C<sub>T</sub> value). In order for an absolute standard curve to be considered precise enough for determining unknown DNA amounts, the r<sup>2</sup> value has to be greater than or equal to 0.999. Therefore, while our fusobacteria standard curve (r<sup>2</sup> = 1.000) would be precise enough, our universal standard curve (r<sup>2</sup> = 0.925) would not. Unfortunately, we were in the middle of repeating this experiment to increase our r<sup>2</sup> value when Santa Clara University closed all labs, so we did not get to generate standard curves that could be used for the rest of the sensitivity testing.
4.5 Sensitivity Testing Experiments

Once appropriate standard curves were created, we could then return to the two primary quantities we needed to determine in order to assess whether the CERVIS media was sensitive enough for women with cervical cancer:

1) How much initial fusobacteria is necessary to add to the CERVIS media for a color change to occur?

2) How much total bacteria is present on a vaginal swab of a healthy woman?

4.5.1 Determining Initial Quantity of Fusobacteria Required for Color Change

In order to determine how much initial fusobacteria was necessary for a color change to occur in the CERVIS media, we first had to assess where a dramatic color change was occurring. Therefore, fusobacteria was grown in an anaerobic chamber for 96 hours. Approximately 50 colonies were then placed into a dilution blank containing 9 mL of dilution solution and vortexed well. 1 mL of this bacteria suspension was then added to a tube containing 9 mL of dilution solution (1:10 dilution). Five 1:100 dilutions were then made by taking 100 μL of the bacteria suspension from the previous dilution and adding it to a new tube containing 9.9 mL of dilution solution. The contents were then vortexed well before creating the next dilution. Furthermore, in total there were the following dilutions: the initial dilution, 1:10, 1:10\(^3\), 1:10\(^5\), 1:10\(^7\), and 1:10\(^9\).

From the dilutions following the initial one, 20 μL of each were pipetted onto clean swabs and each placed into a tube containing the CERVIS media. The caps were then placed on each of the tubes and taken outside of the anaerobic hood and placed in the 37 °C incubator. The color change was then observed every 24 hours, and at 72 hours, it was observed that there was a significant color change in at least some of the CERVIS tubes. As shown in the table below, at 72 hours, we observed that the 1:10\(^1\) and 1:10\(^3\) dilutions had undergone significant color changes.
Due to time constraints, we did not get to complete the rest of this sensitivity procedure as planned, but we still had developed the overall procedure of how we would complete the rest of this experiment.

**Proposed Experiment**

After it had been determined where the color change was occurring within the CERVIS media tube, we would then take the $1:10^1$ and $1:10^3$ fusobacteria dilutions which were initially pipetted onto those swabs outside of the hood, and perform DNA miniprep according to Qiagen DNeasy Miniprep kit (cat. nos. 27104). At that point, we would then perform a qPCR reaction by adding the normal qPCR components (SYBR Green Master Mix, qPCR grade water) with the forward and reverse fusobacteria primers (see Appendix B, Section 1 for qPCR experiment methods).
Therefore, the qPCR machine would amplify the fusobacteria in those dilutions. By plotting the Ct values generated from the qPCR machine onto the fusobacteria standard curve generated earlier, it could then be determined how much initial fusobacteria was present in the CERVIS media where the color change occurred. This would serve as one of the critical numbers that would be needed in order to generate a percentage to compare to 17% (the percentage of fusobacteria present on a vaginal swab of a woman with cervical cancer).

4.5.2 Determining Total Bacteria Present on a Vaginal Swab

The second critical number that needed to be obtained was the total amount of bacteria present in a vaginal swab. To do this, we first needed to collect a vaginal swab.

Obtaining a vaginal swab
Three women from Santa Clara University took gloves and a sterile swab placed in a bag to the restroom. The swab was removed from the bag using sterilized forceps and the swab was inserted 1-2 inches into the vagina. After rotating the swab three times inside the vagina, it was quickly inserted into a dilution blank and the lid was closed. The dilution blank was further taken to the lab for DNA miniprep.

DNA Miniprep with Tissue Sample
The samples were vortexed for 30 seconds and then prepared according to the protocol for “cultured cells” in the Qiagen DNeasy Miniprep kit (cat. nos. 27104). The DNA was stored in the 20 °C freezer until ready for use in quantitative PCR (qPCR).

Proposed qPCR experiment with vaginal swab
While we did have the opportunity to obtain vaginal DNA, we did not get the opportunity to perform a qPCR experiment with this DNA to determine how much total bacteria was present in a vaginal swab. However, below is the overall procedure for how we would have done so:
After the vaginal DNA was obtained, it would be added to SYBR Green Master Mix, and the forward and reverse universal primers. This would amplify all of the DNA found in the vaginal DNA. After a normal qPCR test was run (see Appendix B, Section 1 for qPCR procedure), the Ct values given to us from the amplification plot from the qPCR machine would be plotted on the universal standard curve. This would further tell us how many total bacteria is on a vaginal swab, which is the second critical number.

4.5.3 Conclusion of Sensitivity Testing
After determining both the amount of initial fusobacteria necessary for a significant color change to occur in the CERVIS media and the total amount of bacteria in a vaginal swab, a percentage could then be generated (dividing the fusobacteria by the total bacteria). This number could then be compared to 17% of fusobacteria that we would expect to find in the vaginal swab of a woman with cervical cancer. If the percentage was approximately 17%, we could conclude that the CERVIS media is sensitive enough and therefore, last year’s prototype has feasible sensitivity.

Unfortunately, because the amounts of fusobacteria and total bacteria were not able to be determined, we could not generate a percentage. However, we believe that this procedure would lead us to draw a conclusion of whether the CERVIS media has feasible sensitivity.
CHAPTER 5: IMPLEMENTATION MODALITIES

5.1 qPCR as Modality
The original purpose of qPCR experiments was to test the feasibility of the CERVIS media sensitivity. However, we realized that the qPCR procedure could also be used as a clinical modality to screen women for cervical cancer if a qPCR machine was accessible. Using the patient’s vaginal swab, the amount of fusobacteria, and the total amount of bacteria could be determined and plotted on the standard curves to assess the patient for cervical cancer pathology. This would allow us to generate a percentage, which we could compare to the 17% to assess whether the woman likely had cervical cancer or not. Therefore, qPCR in itself could be considered another minimally-invasive implementation modality along with the CERVIS media.

5.2 Comparison of Modalities
We identified two implementation modalities: the CERVIS Media and qPCR experiments. While both are potentially applicable in a clinical setting, there are several key differences between the two. The main points are summarized in a table (Table 3) after the following explanations.

First, the CERVIS media is colorimetric and therefore qualitative, while qPCR is a purely quantitative test. Since the qPCR modality requires specialized equipment, it would be better suited for communities with pre-existing access to the technology, while the CERVIS media is ideal for communities without access. There is also a substantial difference between the wait time for the results of each modality. The CERVIS media relies on the growth of bacteria and thus requires three to four days for incubation, while the qPCR will determine results much more rapidly in only a few hours. Both modalities are potentially low enough cost for LMICs if the qPCR technology is already available.

While the CERVIS media may be more easily implemented in LMICs, preliminary parameter results suggest the requirement of incubation technology and extended incubation times. These characteristics may pose significant barriers to clinical implementation in Uganda. A limitation of the qPCR modality is the requirement of specialized machinery and its accessories, which
could contribute to an initial high cost. The qPCR cost decreases as more tests are run, which could vary individual testing costs.

Additionally, both modalities rely on increased concentrations of fusobacteria in the vaginal microbiome. Fusobacterium colonization of the vagina has also been associated with preterm birth and a few other uterine infections, so women with these conditions may not be able to utilize either procedure. Additionally, there remain some cultural limitations of deployment, such as lack of education surrounding sexual health and the necessity of a community-wide effort to empower women to care for their own health. Fortunately, we had two public health partners on our team that worked this year to bridge this gap of education and women’s health empowerment through the creation of educational brochures. Despite these constraints, we believe that CERVIS has promising future directions.

Table 2: Summary of the Comparison of Modalities

<table>
<thead>
<tr>
<th>CERVIS Media</th>
<th>Both</th>
<th>qPCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>● Qualitative test</td>
<td>● Could be low enough cost for LMICs</td>
<td>● Quantitative test</td>
</tr>
<tr>
<td>● Ideal for communities without access to qPCR technology</td>
<td></td>
<td>● Requires highly specific lab technology</td>
</tr>
<tr>
<td>● Results in days</td>
<td></td>
<td>● Results in hours</td>
</tr>
</tbody>
</table>
CHAPTER 6: SPECIFICITY TESTING
In order for the CERVIS media to be considered functional, it needs to be specific to cervical cancer. This specificity testing is important because the test can be utilized for detecting a particular disease, rather than generally detecting a bacteria. A goal for CERVIS this year was to determine an additional bacterial biomarker that is associated with cervical cancer so that the procedure is more specific. Unfortunately, an extensive literature review has not yet revealed a promising candidate to increase specificity, so in this section, we will speculate on what criteria would make an optimal candidate.

This year, CERVIS focused experiments on the sensitivity of the media to detect fusobacteria. Sensitivity indicates the true positive of a test, or the ability to correctly identify individuals with the disease. Another indicator of how accurate a test relies on specificity, the ability to correctly identify individuals without the disease. Highly sensitive and specific tests are the most accurate, so both must be considered.

6.1 Selection Criteria for Bacteria
One method of increasing specificity would be to identify a second bacteria to be analyzed alongside fusobacteria. Currently, there exists no conclusive data on other microbiota implicated in cervical cancer, but we have developed criteria that this such bacteria would fulfill. In order to be considered for further analysis, the bacteria must be present or capable of being detected in cancerous vaginal microbiomes. This is in order to enable the vaginal swab to be effective, i.e. able to pick up this bacteria when the test is administered. The second criterion is that it be absent or in significantly low quantities in a non-cancerous vaginal microbiome. This is to enhance the sensitivity of both qPCR and CERVIS media screening procedures, ensuring that only cancerous vaginal states are detected. Preliminary parameter testing suggests that incubation at the typical 37°C for over 72 hours is a requirement for fusobacteria, thus any bacteria that grow at that temperature or below and for that time or shorter is acceptable. Furthermore, preliminary tests for aerobic tests indicate the possibility of up to 120 seconds of aerobic
exposure before no growth is seen in a swab, so the bacteria selected would also need to be able to withstand aerobic exposure for a reasonable amount of time.

In qPCR, specificity can be increased by focusing on exclusivity and inclusivity. Exclusivity refers to distinguishing the target strain from similar but genetically distinct non-target strains through highly specific primers for implementation. As mentioned in Chapter 4, primers for the exact species, *Fusobacterium nucleatum*, were used to narrow the scope of detected bacteria. The second method for increasing PCR specificity is inclusivity, or the range of the qPCR used to detect a wide range of targets with defined relations such as taxonomic, immunological, and genetic compositions (Kralik and Ricchi, 2017). Probe-based qPCR assays may also confer an additional level of specificity, because of its ability to multiplex multiple targets, meaning that two targets are amplified at once. If a secondary or alternative bacteria were to be identified and associated with cervical cancer, probe-based qPCR can be used to amplify multiple targets in a single reaction (Thermofisher, 2020).

An additional qPCR application would be to screen for HPV strains 16 and 18 directly due to their strong correlation to cervical cancer. HPV may be present years prior to cervical cancer development, while fusobacteria is more strongly correlated with later stages of cervical cancer. Targeting both in qPCR could be a more accurate indicator of significant and later stages of cancer development.
CHAPTER 7: CONCLUSION & FUTURE DIRECTIONS

7.1 CERVIS Media Parameters
For the three parameters—time, temperature, and aerobic exposure—we have only preliminary data, due to our interrupted time in the lab. However, we were able to determine suggested parameters for our procedure based on our results. Based on these results we found certain limitations and future directions. One limitation common to all of our parameter tests was limited lab time. Time allowing, we would have repeated all parameter experiments and would have run all of our tests in triplicate.

7.2 Preliminary Results for Incubation Time
We concluded that the optimal time range for incubation at 37 °C is between 72 and 98 hours. While standard incubation times for fusobacteria range from 24-48 hours, when grown in the CERVIS media, a significant darkening of the media was only observed within the indicated time range.

7.2.1 Limitations
One of the major limitations of our procedure itself is the extended period of incubation required to produce a true positive result. Results are not immediately available, which poses an ethical dilemma because it could potentially cause heightened anxiety while patients wait for results of the test. This may also require a waiting period for the woman to receive treatment if needed. This is a shorter duration than a Pap smear, which can take up to 3 weeks to see results, but a longer wait time than VIA, which can give results almost immediately. Same-day results are ideal for LMICs, as many women may not have access to a clinic in rural areas and may need to travel to a city to receive treatment.

In order to continue to improve the procedure, it would be necessary to explore avenues to decrease fusobacteria growth time. If possible, there would need to be adjustments to the media in order to promote the growth of bacteria.
7.3 Preliminary Results for Incubation Temperature
Based on preliminary data testing for incubation at room temperature, 25 °C, standard incubation temperature (body temperature), 37 °C, and the temperature of an available incubator, 30 °C, we determined that 37 °C is the preferable incubation temperature. However, we acknowledge that there would need to be additional trials at temperatures between 30 °C and 37 °C as well as extended incubation times at lower temperatures such as room temperature to confirm this result. We also would have conducted testing on the effect of varying temperatures on fusobacteria growth.

7.3.1 Limitations
A major limitation of the observed incubation temperature preliminary findings is that if there is limited access to an incubator at 37 °C, then the procedure may not be able to produce a true positive result. Average temperatures in Uganda are often lower than 37 °C, which would make incubation at room temperature unlikely according to our current findings.

7.4 Preliminary Results for Aerobic Exposure
Based on preliminary data, we concluded that fusobacteria-containing swabs can be exposed to air for at least two minutes and still produce a true positive result. This is a reasonable collection time for a vaginal swab, which indicates that this test could be viable once ready for deployment. Further exploration into the time period between 120 and 300 seconds is required to establish a true threshold for when fusobacteria is no longer viable.

7.4.1 Limitations
Another limitation of our experimental design was inconsistency in results between duplicate tests. Due to the variability of the bacterial suspension, there was no way to tell how much bacteria was contained in the 20 μL of solution that was pipetted onto the swab. In order for consistent results to be obtained, the tests must be repeated to ensure that each swab has fusobacteria on it when placed into the media.
7.5 Sensitivity Results
Time constraints prohibited us from determining whether last year’s prototype has feasible sensitivity to screen for cervical cancer. However, we were able to develop an absolute standard curve and an experimental procedure that would allow us to determine the amount of fusobacteria needed in the CERVIS media for a color change to occur. This amount would have enabled us to generate a percentage that we could compare to 17%, the amount of expected fusobacteria present in a vaginal swab of women with cervical cancer. With such results, we could then conclude whether the CERVIS media was sensitive enough to screen for cervical cancer.

7.6 Future Directions
Our shortened time in the lab left us with preliminary results of the CERVIS media parameters, but there are other elements to consider to further define field testing parameters. These include the effects of varying temperature, humidity, and other related environmental factors. While we largely focused on sensitivity, determining true negatives by increasing specificity may also play a part in creating a more accurate prototype and qPCR procedure. This may come in the form of additional precancerous or cancerous biomarkers, but there are many more to explore. In order to standardize the color change occurring in the CERVIS media, we believe a color gradient chart would be beneficial to accompany the media for deployment. Finally, an important future step in our project would be to find a clinical or industry partner to test both modalities with vaginal swab samples of women with cervical cancer. Establishing this partnership is essential to assess the functionality of each procedure.

This year, team CERVIS designed experiments to confirm the feasibility of screening for cervical cancer using fusobacteria in vaginal samples for LMICs. This procedure is intended to be sensitive, low-cost, and minimally invasive which we believe could help increase screening in Uganda. We established two modalities—the colorimetric CERVIS media and qPCR—and both of these could have future implications. The potential low cost of qPCR creates a market for low-income or uninsured women in developed countries such as the United States. Furthermore,
fusobacteria is associated with other types of cancers such as oropharyngeal & colon cancers and has the potential for screening. These implications indicated that screening for this particular bacteria has potential beyond the scope of our project.
CHAPTER 8: ENGINEERING STANDARDS

8.1 Realistic Constraints
Both the qPCR and colorimetric CERVIS media assay procedures are reliant upon the detection of *Fusobacterium nucleatum* bacteria in the vagina, the strain linked to cervical cancer (Audirac-Chalifour et al., 2016). However, that particular species has also been linked to preterm birth and intrauterine infection (Diguilio, 2012). Additionally, the presence of sialidase, a biochemical marker, promotes growth of fusobacterium nucleatum (Agarwal et al., 2018). Furthermore, women who test positive for bacterial vaginosis are more likely to be vaginally colonized by *Fusobacterium nucleatum*, which may be a cause of the preterm birth (Han et al., 2009). Due to these findings, it may be inferred that the upregulation of fusobacteria may occur in women with bacterial vaginosis or those who have just given birth preterm. Similarly, high levels of sialidase in addition to fusobacteria, there may be causing upregulation. These disease states are not indicative of cervical cancer, and therefore we must suggest that women with these conditions be excluded from screening by our methods.

One of the significant constraints in the deployment of either procedure in Uganda and other LMICs is the cultural limitation. The lack of education surrounding sexual health, in particular cervical cancer screening as well as the necessity of a community-wide effort to empower women to take care of their own health. An additional cultural constraint is the reticence to seek pelvic examinations, so even in countries with screening in place, women may not seek treatment until they present with advanced disease (Sankaranarayanan et al., 1998; Juárez-Figueroa et al., 1998; Safaeian et al., 2007).

8.2 Ethics

*Ethical Justification for Project*

The primary ethical justification for our project centers on the principle of justice. The principle of justice states that all humans have inherent dignity in and of themselves, and as such have fundamental human rights. These rights include access to good healthcare that will allow them to maintain their health to the highest possible degree. Article 25 of the Universal Declaration of
Human Rights written and distributed by the United Nations states that each person “has the right to a standard of living adequate for the health and well-being of himself and his family, including….medical care and necessary social services” (United Nations, 1948). Therefore, anyone who does not have an equal opportunity to access medical care must be prioritized. Additionally, a collaboration between nations with more resources and those with less could lead to better inter-country relationships, establishing a global community.

Our project focuses specifically on women’s health in Sub-Saharan Africa, namely Uganda. Women’s health is a globally significant issue but is especially relevant in this region because of the shortages in equipment and personnel. Our main focus, cervical cancer, is very deadly, especially in areas with limited access to preventative, screening, and diagnostic measures. Without access to these measures, the incidence of cervical cancer and the mortality rate of women in this region of the world will remain high.

Developing a procedure that is minimally invasive, low cost, and can be self-administered has the potential to benefit the common good by increasing screening rates in Uganda. Limiting the number of highly invasive procedures such as VIA or the Pap smear will help to minimize unnecessary procedures and costs respectively.

What Does Our Product Teach Us About the Character of an Engineer?

According to the Biomedical Engineering Society (BMES) code of ethics, biomedical engineers have certain ethical responsibilities that they must adhere to when working in the field. These responsibilities extend to us as undergraduate researchers, especially because our procedure is intended to have clinical applications. These ethical responsibilities can be categorized in three ways: healthcare obligations, professional obligations, and research obligations.

The healthcare obligations that help to elucidate the ethical focus of our project involve “consider[ing] the larger consequences of [our] work in regard to cost, availability, and delivery of healthcare” (BMES Code of Ethics). With the target population of our procedure residing in
Uganda, it is important to create a procedure that can be readily available, low cost, and easy to distribute. Working with the Santa Clara University Frugal Innovation Hub and the connections that we met during the Collaborate 4 Africa event, our team was able to better understand the needs of the women living in Uganda. The aspects of our prototype that focus on addressing the obligations include the low cost, easy accessibility (capability to be self-administered), and small, self-contained design that would allow for ease of distribution.

The professional obligations associated with our project include using our “knowledge, skills, and abilities to enhance the safety, health, and welfare of the public” (BMES Code of Ethics). Using the knowledge we have gleaned as undergraduates and with the help of faculty and industry advisors, we have sought to create a procedure that is safe and acknowledges the needs of the women in rural Uganda. By conducting experiments to enhance our understanding of cervical cancer and the prototype created, we have worked towards enhancing the health of the public.

Finally, the research obligations associated with our project included complying with university standards and documenting our research carefully. In complying with university safety guidelines, we completed biology/microbiology general safety training and lab-specific training, to ensure that our team doesn’t put our own safety or the safety of others in jeopardy. Additionally, we carefully documented all of our work to ensure that future iterations of the project are possible and any work that we performed may be easily repeatable.

**Ethical Challenges Regarding Safety & Risk**

The main ethical challenge regarding the safety of our procedure was the use of anaerobic bacteria in our experiments. Utilizing this type of pathological bacteria meant that we needed to make sure that no contamination occurred and that none came into contact with skin, eyes, etc. To combat this, we followed the safety guidelines dictated by the university as well as national standards, which included keeping the bacteria inside the lab.
8.3 Engineering Standards

Besides ethics, our design incorporates economic, social, and health and safety engineering standards.

Economic

Due to the focus of our project primarily being determining feasibility and assessing preliminary results of the CERVIS media, we understand that the exact cost is not within the scope of our project. Because our target population of women resides in Uganda, we would want the cost of our procedure to be comparable to VIA, which would mean between about $5-20 (Quentin et. al, 2011). Our second modality, the qPCR procedure, relies on a SYBR Green-based assay. We chose this for its economical feasibility while doing a few preliminary tests on single targets. However, if this procedure were to be scaled up, probe-based qPCR assays would be the better option to ensure specificity in addition to sensitivity (Thermofisher, 2020).

Social and Cultural Impact

The primary goal of our project design is to have a significant social and cultural impact. Because of Uganda’s extremely low rate of cervical cancer screening, it follows that cervical cancer is the leading cause of cancer-related deaths in Ugandan women. One reason for low screening is a lack of education surrounding sexual health and the necessity of a community-wide effort to empower women to take care of their own health. Our two public health partners have been working to bridge this gap of education and women’s health empowerment through the design of an educational brochure.

Some factors that inhibit women’s engagement in cervical cancer screening have been identified as fear of the screening procedure, fear of the outcome, residing in a remote or rural area, limited resources/health infrastructure, and limited access to screening care (Black, 2019). These point to immediate ethical concerns of interpersonal communication of women’s health conditions and strategies for treatment as well as considerations for prototype function; therefore, we intend for this prototype to be deployed in a healthcare clinic.
Health and Safety

The most important components of our project are health and safety. Our goal is to improve the health of women by enabling them to screen for cervical cancer, the fourth most common cancer in women around the world. However, the majority of the deaths that occur due to cervical cancer are in LMICs. Many of these deaths could be prevented by screening and early diagnosis. The main objective of the CERVIS procedure is to provide a way to increase screening through the development of a sensitive, low-cost, minimally invasive screening procedure that requires minimal healthcare expertise to administer.
APPENDIX A: Tables & Figures

Table A: Funding Received

<table>
<thead>
<tr>
<th>Source of Funding</th>
<th>Amount Received</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCU School of Engineering</td>
<td>$1500</td>
</tr>
<tr>
<td>Xilinx Grant</td>
<td>$500</td>
</tr>
<tr>
<td><strong>Total Received</strong></td>
<td><strong>$2000</strong></td>
</tr>
</tbody>
</table>

Table B: CERVIS Expenditures

<table>
<thead>
<tr>
<th>Product Category</th>
<th>Product Name</th>
<th>Product Number</th>
<th>Unit Cost</th>
<th>Total Cost</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lab Consumables</td>
<td>Puritan 3306-U Standard PurFlock Ultra Flocked Tip Applicators with Polystyrene Shaft</td>
<td>3306-U</td>
<td>$13.65</td>
<td>$19.00</td>
</tr>
<tr>
<td></td>
<td>Anaerobe Indicator Test for microbiology</td>
<td>59886-1PAK-F</td>
<td>$76.12</td>
<td>$82.97</td>
</tr>
<tr>
<td></td>
<td>Nitrogen Gas Tank</td>
<td>--</td>
<td>$193.00</td>
<td>$460.00</td>
</tr>
<tr>
<td></td>
<td>0.1-10 microliter pipette tips</td>
<td>69504</td>
<td>$91.01</td>
<td>$103.68</td>
</tr>
<tr>
<td>Cell Culture Consumables</td>
<td>Water, Sterile. WFI Quality., Poly Bottle, 500 mL</td>
<td>4.86505.0500</td>
<td>$17.91</td>
<td>$39.23</td>
</tr>
<tr>
<td></td>
<td>iTaq™ Universal SYBR® Green Supermix, 200 x 20 µL rxns, 2 mL (2 x 1 mL)</td>
<td>1725120</td>
<td>$139.00</td>
<td>$173.31</td>
</tr>
<tr>
<td>Tissue kits</td>
<td>DNeasy Blood &amp; Tissue Kit (50)</td>
<td>69504</td>
<td>$171.00</td>
<td>$231.23</td>
</tr>
<tr>
<td>Fusobacteria-related items</td>
<td>Fusobacterium nucleatum subsp. nucleatum Knorr</td>
<td>25586</td>
<td>$61.20</td>
<td>$292.65</td>
</tr>
<tr>
<td>---------------------------</td>
<td>------------------------------------------------</td>
<td>-------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td>Fuso and universal primers</td>
<td>19084632</td>
<td>-</td>
<td>$29.25</td>
<td></td>
</tr>
</tbody>
</table>

**Total Expenditures:**

| - | - | $1,431.32 |

---

**Table C: 2019-2020 Academic Timeline for CERVIS**

<table>
<thead>
<tr>
<th>Week 1</th>
<th>Fall Quarter</th>
<th>Winter Quarter</th>
<th>Spring Quarter</th>
</tr>
</thead>
<tbody>
<tr>
<td>● Partnership formed between BIOE students and EWH students</td>
<td>● Additional materials and primers ordered</td>
<td>● Brainstorming session for new direction of CERVIS after all future lab work is canceled</td>
<td></td>
</tr>
<tr>
<td>● Lab trained to Dr. Whittal’s lab (for qPCR access)</td>
<td>● Wrote overview for sensitivity protocol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>● Wrote overview for sensitivity protocol</td>
<td>● ▲</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Week 2</th>
<th>Fall Quarter</th>
<th>Winter Quarter</th>
<th>Spring Quarter</th>
</tr>
</thead>
<tbody>
<tr>
<td>● First meeting with engineering team members and advisor</td>
<td>● Plate Vaginal bacteria</td>
<td>● Additional Literature Review on Vaginal microbiome</td>
<td></td>
</tr>
<tr>
<td>● Performed CFU experiment with vaginal samples</td>
<td>● Met with Dr. Park and agreed to move forward with qPCR</td>
<td>● Discuss future direction with project advisors</td>
<td></td>
</tr>
<tr>
<td>● Met with Dr. Park and agreed to move forward with qPCR</td>
<td>●</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Week 3</th>
<th>Fall Quarter</th>
<th>Winter Quarter</th>
<th>Spring Quarter</th>
</tr>
</thead>
<tbody>
<tr>
<td>● Define goals and parameters for CERVIS</td>
<td>● Order materials for qPCR</td>
<td>● Meet with advisors to discuss “the story” of our final Senior Design Presentation</td>
<td></td>
</tr>
<tr>
<td>● Literature research on existing diagnostic tests</td>
<td>● Meeting with Dr. Dahlhoff to be trained on running a qPCR experiment in the lab</td>
<td></td>
<td></td>
</tr>
<tr>
<td>● Initial meeting with Dr. Stephens</td>
<td>● Meeting with Dr. Park on how to create a Standard Curve on the</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Week 4 | qPCR machine  
- Develop overview of experimental procedure for sensitivity testing with qPCR |
|---|---|
| **Initial meeting with Dr. Parker, Will, and Lauren**  
- Brainstorming Session on constraints and key questions for CERVIS |
| **Trained by Dr. Ruscetti in the Anaerobic hood** |
| **Presentation to Dr. Parker on first half of Senior Design Presentation** |

| Week 5 | Took vaginal samples and purified them using the DNeasy kit  
- Determined concentration of vaginal samples using Qubit  
- Met with Dr. Park and Dr. Dahlhoff to discuss qPCR experimental design and setup  
- Ran qPCR on E. coli with Universal primer |
|---|---|
| **Meet with Clarie Hultquist from last year’s CERVIS team to discuss recommendations on our future direction**  
- Literature review on biomarkers in the blood, urine, and menstrual blood  
- Senior Design Funding Proposal submitted to School of Engineering |
| **Updated presentation to Dr. Parker** |

| Week 6 | Plated *Fusobacteria*  
- Ran qPCR experiment with Universal and Fusobacteria primers on vaginal bacteria  
- Determine color change of CERVIS media after adding in *Fusobacteria*  
- CFU experiment with *Fusobacteria* |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Research other possible bacteria to increase specificity of CERVIS media</strong></td>
<td></td>
</tr>
</tbody>
</table>
| **Presentation to Dr. Parker and Dr. Asuri**  
- Submit RD of chapters 3-5 of thesis |
<table>
<thead>
<tr>
<th>Week 7</th>
<th>• Research other possible bacteria to increase specificity of CERVIS media</th>
<th>• qPCR experiments with <em>E. coli</em> and <em>Fusobacteria</em> and Universal and Fusobacteria primers</th>
<th>• Run through presentation with technical and non-technical individuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 8</td>
<td>• Safety trained in Dr. Stephens lab</td>
<td>• Plate additional <em>Fusobacteria</em> in anaerobic hood</td>
<td>• Submit recording of Senior Design Presentation</td>
</tr>
<tr>
<td></td>
<td>• qPCR experiments with <em>E. coli</em> and <em>Fusobacteria</em> and Universal and Fusobacteria primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 9</td>
<td>• Presentation for Collaborate 4 Africa</td>
<td>• Replate <em>Fusobacteria</em></td>
<td>• Senior Design Presentation</td>
</tr>
<tr>
<td></td>
<td>• Met with Hiram at Anaerobic Systems</td>
<td>• CFU experiment with <em>Fusobacteria</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Meeting with Dr. Stephens on using a high-powered microscope for counting bacteria</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• qPCR experiments with <em>E. coli, Fusobacteria</em> and vaginal bacteria using Universal and Fusobacteria primers</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Parameter testing with CERVIS media (temperature, aerobic conditions)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Hemocytometer counting</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Create Universal and Fusobacteria standard curves with fusobacteria</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• IEEE Conference Presentation</td>
<td></td>
</tr>
</tbody>
</table>

### Week 10

- Order materials from Anaerobe Systems
- Practice streaking plates with *E. coli*
- Anaerobicity testing with *E. coli* in an anaerobic pouch, anaerobic jar, and aerobic conditions
- Create Universal and Fusobacteria standard curves with fusobacteria
- Classes moved to online platform; labs closed
- Final Thesis Submission

---

Table D: Universal Master Mix Experimental Setup qPCR

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume/reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYBR Green Master Mix</td>
<td>5 μL</td>
</tr>
<tr>
<td>Universal Forward Primer</td>
<td>0.2 μL</td>
</tr>
<tr>
<td>Universal Reverse Primer</td>
<td>0.2 μL</td>
</tr>
<tr>
<td>PCR grade water</td>
<td>2.6 μL</td>
</tr>
</tbody>
</table>

Table E: Fusobacteria Master Mix Experimental Setup qPCR

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume/reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYBR Green Master Mix</td>
<td>5 μL</td>
</tr>
<tr>
<td>Fusobacteria Forward Primer</td>
<td>0.2 μL</td>
</tr>
<tr>
<td>Fusobacteria Reverse Primer</td>
<td>0.2 μL</td>
</tr>
<tr>
<td>PCR grade water</td>
<td>2.6 μL</td>
</tr>
</tbody>
</table>
Table F: No Primer Control (NPC) Experimental Setup qPCR

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume/reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYBR Green Master Mix</td>
<td>5 μL</td>
</tr>
<tr>
<td>PCR grade water</td>
<td>3 μL</td>
</tr>
</tbody>
</table>

Table G: Primer Sequences Used in qPCR Experiments

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Fusobacterium</em></td>
<td></td>
</tr>
<tr>
<td><em>nucleatum</em></td>
<td>F: 5’- CAACCATTACTTTAACTCTACCATGTCA-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-GTGTACTTTACAGAAGGAGATTATGTAATTAATTC-3’</td>
</tr>
<tr>
<td>Universal Primer</td>
<td>F: 5’ CCTACGGGNGGCGCWGAG 3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’ GACTACHVGGGTATCTA ATCC 3’</td>
</tr>
</tbody>
</table>

Table H: UMM and FMM Total Volumes (Testing Primers)

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume/10 μL</th>
<th>Number of reactions</th>
<th>Total Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYBR Green Master Mix</td>
<td>5 μL</td>
<td>12</td>
<td>60 μL</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>0.2 μL</td>
<td>12</td>
<td>2.4 μL</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>0.2 μL</td>
<td>12</td>
<td>2.4 μL</td>
</tr>
<tr>
<td>PCR grade water</td>
<td>2.6 μL</td>
<td>12</td>
<td>31.2 μL</td>
</tr>
</tbody>
</table>
Figure A: qPCR Plate Setup for Testing Primers

Table J: UMM and FMM Total Volumes (Absolute Standard Curve)

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume/10 μL</th>
<th>Number of reactions</th>
<th>Total Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYBR Green Master Mix</td>
<td>5 µL</td>
<td>13</td>
<td>65 µL</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>0.2 µL</td>
<td>13</td>
<td>2.6 µL</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>0.2 µL</td>
<td>13</td>
<td>2.6 µL</td>
</tr>
<tr>
<td>PCR grade water</td>
<td>2.6 µL</td>
<td>13</td>
<td>33.8 µL</td>
</tr>
</tbody>
</table>
Figure B: qPCR Plate Setup for Generating an Absolute Standard Curve

Figure C: Amplification Plot for Generating an Absolute Standard Curve
APPENDIX B: Supplemental Information

Section 1: General qPCR Procedure

1. Preparing dilutions of the bacterial DNA
The bacterial DNA (either fusobacteria or E. coli) was removed from the -20 °C freezer, placed on ice, and given time to defrost. Serial dilutions of the DNA were created in nuclease-free tubes. These tubes were labeled according to their dilution, which was dependent upon the particular experiment and type of bacterial DNA. PCR grade water was the diluent and the total amount of volume for each dilution was 20 μL. Each dilution was mixed by agitation of the tube (“finger flicking”) and centrifuged for five seconds.

2. Creating the Universal and Fusobacteria Master Mix and NPC
The forward and reverse universal and fusobacteria primers along with the SYBR Green Master Mix were removed from the -20 °C freezer, placed on ice, and given time to defrost. Depending on the number of reactions that were being performed for the given experiment, a different amount of the primers, PCR grade water, and SYBR Green Master Mix were combined to form the Universal Master Mix (UMM), Fusobacteria Master Mix (FMM), and No Primer Control (NPC). See Table D-F in Appendix A for the amount of each component added per 10 μL reaction.

The given experiment determined how many reactions were being performed (i.e., how many wells were going to be used on the qPCR plate). Once the total volume for each of the reagents was determined (obtained by multiplying volume/reaction with the total number of reactions), all components for the UMM, FMM, or NPC were combined separately in a labeled 1.5 mL centrifuge tube. The components were then agitated (“finger flicked”) and centrifuged for three seconds.

3. Combining the DNA dilutions and Master Mix on a qPCR plate
To set up the qPCR plate, a 0.1 mL 96-well plate was placed on a qPCR specific metal ice block. The UMM, FMM, NPC, and bacterial DNA dilutions were all placed on ice. Eight μL of either
NPC (for the control), UMM, or FMM was pipetted into each reaction well according to the particular experiment. Bacterial DNA or water for control (2 μL) was then added to each reaction well. Layouts of these experiments can be found in Figures A & B in Appendix A.

Microplate well caps were then sealed onto all the filled wells of the qPCR plate. Once sealed, the qPCR plate was then centrifuged in a salad spinner and spun for 1 minute. The plate was examined for bubbles and centrifuged again until no bubbles were observed in the plate. The plate was loaded into the qPCR machine and began running by pressing “Run Program.” The plate ran for about 1.5 hours and was subjected to further analysis once completed.

**Section 2: Preparing Fusobacteria and Primers for qPCR**

*Preparations for Growing Fusobacteria*

Experiments with fusobacteria were conducted in the anaerobic hood. Before plating, a white to pink anaerobic indicator was opened inside of the chamber to establish that the environment was indeed anaerobic. Using a 1 mL needle syringe, dilution media was withdrawn and added to the fusobacteria pellet to rehydrate the lyophilized fusobacteria cells. The fusobacteria was mixed with dilution media by pipetting up and down. Once mixed, the entire suspension was withdrawn and transferred back to the original dilution tube containing 5 to 6 mL of dilution media.

*Growing Fusobacteria*

Fusobacteria suspension in quantities of 10 μL and 1 μL were pipetted onto blood agar plates and spread using plastic inoculating loops. These dilutions were each plated three times for a total of six plates. The plates were then placed in a 33 °C incubator inside of the anaerobic chamber agar-side up and incubated for 72 hours. After that time, 30 small colonies of fusobacteria were obtained using an inoculating loop and placed in a new tube containing 9 mL of dilution solution. Two milliliters of this dilution solution was used to create dilutions for determining color change with the CERVIS media (see Section 3.1-3.3), and the rest was taken outside of the anaerobic hood.
DNA Miniprep
The fusobacteria suspension was then placed in a microcentrifuge tube 1 mL at a time and centrifuged at 7500 rpm for 1 minute. Following centrifugation, the supernatant was poured off into a waste beaker and an additional 1 mL of the suspension was added. This was repeated for the remainder of the fusobacteria mixture and the pelleted cells were stored in the 20 °C freezer until DNA miniprep could be completed. DNA miniprep was performed according to the Qiagen DNeasy Miniprep kit (cat. nos. 27104).

Preparing Primers
To prepare for the quantitative PCR, universal and *Fusobacterium nucleatum* specific-primers were first hydrated using the following protocol: primers in given tubes were spun down using microcentrifuge tubes, then molecular grade water was added at an amount relative to the primer amount in order to create a 100 µM primer stock. This solution was then allowed to sit at room temperature for 10 minutes. From the stock solution, a working 10 µM stock was created by diluting the original solution 1:10 in a sterile microcentrifuge tube.

Section 3: Microscopy

I. Introduction
There is a need for an alternative quantification method that creates a “known” quantity for generating an absolute standard curve. Our team chose microscopy (hemocytometry) as the best secondary quantification method for our qPCR experiments.

II. Methods
One hundred microliters of the initial fusobacteria dilution were pipetted onto a hemocytometer slide and placed under a Keyence microscope. From the nine 1mm x 1mm boxes on the hemocytometer slide, three 1mm x 1mm boxes were randomly chosen as a representative sample. In each of those three boxes, four 0.25mm x 0.25 mm boxes were randomly chosen among the sixteen boxes, and pictures were taken of the bacteria. The bacteria were counted and recorded. The bacteria counts for each of four 0.25mm x 0.25mm boxes were added together and multiplied by four, as the four boxes represented only one quarter of the total boxes in each 1mm
x 1mm grid. The sum of all of the three 1mm x 1mm was then calculated and multiplied by three, as these three boxes represented \( \frac{1}{3} \) of all the total boxes on the hemocytometer slide. Because the height of the hemocytometer slide is exactly 0.1mm, the total volume of the hemocytometer is \((0.3\text{mm} \times 0.3\text{mm} \times 0.1\text{mm})\) 0.9 mm\(^3\) (or 0.0009 mL). With the total number of bacteria and the total volume of liquid that bacteria were in, the concentration of the bacteria can be determined. The counts and sums of each of the boxes can be shown in the table below.

### III. Results

**Figure D: Microscopy Diagram**

![Microscopy Diagram](image)

**Table K: Bacterial Counts Microscopy**

<table>
<thead>
<tr>
<th>0.25mm x 0.25 mm Boxes</th>
<th>1mm x 1mm Boxes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Box A</td>
</tr>
<tr>
<td><strong>1</strong></td>
<td>288</td>
</tr>
<tr>
<td><strong>2</strong></td>
<td>225</td>
</tr>
<tr>
<td><strong>3</strong></td>
<td>228</td>
</tr>
<tr>
<td><strong>4</strong></td>
<td>272</td>
</tr>
</tbody>
</table>

| Sum of 4 boxes          | 1013  | 961   | 1013  |

| Total estimate of bacteria in each 1mm x 1mm box | 1013 x 4 = 5252 | 961 x 4 = 3844 | 1013 x 4 = 5252 |
Sum of boxes A, B, C: $5252 + 3844 + 5252 = 14,348$ bacteria

Estimate of entire grid: $14,348 \times 3 = 43,044$ bacteria

Concentration of bacteria: $43,044$ bacteria/$0.0009$ mL $= 47,826,667$ bacteria/mL
REFERENCES


https://doi.org/10.1016/j.micpath.2019.103696


https://doi.org/10.2147/MB.S77507


https://sti.bmj.com/content/74/6/448.short


acetic acid (VIA) and cryotherapy in Ghana: the importance of scale. *Tropical medicine & international health, 16*(3):379–389.  
https://doi.org/10.1111/j.1365-3156.2010.02722.x

https://doi.org/10.1016/j.gore.2019.05.008

https://doi.org/10.1097/01.olq.0000243623.67673.22

https://doi.org/10.1016/S0035-9203(02)90317-2


Smith, W.L. et. al. (2014) Cervical and Vaginal Flora Specimens Are Highly Concordant with Respect to Bacterial Vaginosis-Associated Organisms and Commensal Lactobacillus Species in Women of Reproductive Age. *Journal of Clinical Microbiology, 52* (8) 3078-3081; DOI: 10.1128/JCM.00795-14

“The HPV Vaccine: Access and Use in the U.S.” *The Henry J. Kaiser Family Foundation*, 19 June 2019,  


