Chapter 1. Introduction to DNA Microarrays

1.1 Overview of the Howard Hughes Medical Institute (HHMI) Project:

The Howard Hughes Medical Institute (HHMI) award is focused on developing new methods to promote scientific education. At Tufts University, this award is being used to implement genomic testing into undergraduate Organic Chemistry laboratories and the K-12 classroom. By employing DNA microarray technology, a straightforward and cost-effective mitochondrial DNA (mtDNA) detection system will be designed.

The human mitochondrial genome is comprised of approximately 16,500 nucleotides and is inherited maternally, making it effective in studying ancestral origins [1]. Ancestral origins are tracked by monitoring single nucleotide polymorphisms (SNPs), a variation of one nucleotide, on the mtDNA. mtDNA has a nucleotide base substitution rate that is 5-10 times higher than nuclear DNA [2]. Such a high substitution rate generates many SNPs, which are categorized into specific sets known as haplogroups. By analyzing mtDNA SNPs, the geographic origins of haplogroups have been established [3-6], enabling determination of an individual’s ancestral origins.

The traditional method for identifying an individual’s haplogroup requires sequencing the entire mitochondrial genome to examine the polymorphisms that define certain haplogroups. A more effective technique would be to use a microarray containing DNA probes designed specifically to examine the SNPs necessary for haplogroup classification. The HHMI award is being used to develop a DNA microarray capable of haplogroup classification in the undergraduate laboratory setting.

This grant is a multi-year project in which two different types of DNA microarrays will be created. The first aspect of the project involves the use of commercially available technology to design a DNA microarray composed of thousands of probes, specific to SNPs present in the
mtDNA. The goal is to design an experiment in which the students will extract their own mtDNA and analyze it using the microarray. Examination of the hybridization results will determine an individual’s haplogroup and provide information about the person’s maternal ancestry. The microarray used was purchased from Illumina, Inc. and can be analyzed by instrumentation available in the Walt Laboratory. The protocol for running the custom designed mtDNA microarray will need to be simplified so it can be easily integrated into the Organic Chemistry laboratory for the 2007-2008 school year.

The second aspect of the project, and the focus of this thesis, is the creation of a DNA microarray to detect SNPs in mtDNA without relying on costly technology. The development of such a microarray would allow universities to expose undergraduate students to genomic testing and microarrays. The goal is to implement this type of experiment in the Organic Chemistry laboratory for the 2008-2009 school year. Various methods of attaching DNA probes to a solid surface for hybridization experiments are investigated in this thesis. Once a successful protocol has been determined, it will be developed into a DNA microarray and modified for the transition into K-12 science classes. By creating a DNA microarray for integration into the classroom, students will be exposed to genomic testing and the excitement of scientific discovery.

1.2 Use of DNA Microarrays

A DNA microarray is an ordered arrangement of oligonucleotides attached to a solid support used to analyze nucleic acid samples via hybridization. The term “DNA microarrays” was first used in an assay that examined of the expression of multiple genes in parallel [7, 8]. The development of this technique was originally derived from Southern Blotting, developed by Professor Ed Southern of the University of Oxford in which fragments of DNA were relocated
from an agarose gel to a cellulose nitrate filter where they were hybridized to radioactive RNA probes. Following the hybridization, autoradiography was employed to confirm the presence of the labeled DNA based on the sequence(s) of the RNA probes [9]. From this protocol the field of DNA microarrays has exponentially grown due to the fact that it can be implemented in a large number of scientific fields.

Beginning with Southern’s methodology, the use of DNA microarrays has become a standard tool for molecular biology research and clinical diagnostics [10]. DNA microarrays have been used to detect bacterial pathogens commonly found in water and food such as *Escherichia coli*, *Vibrio cholerae* [11], and *Salmonella* spp. [12]. Due to the severity of illness caused by these bacteria, it is necessary to identify the agent present as soon as possible. Previously reported methods of pathogen identification include culture-based methods [13, 14], immunological assays [15, 16], PCR-based assays [17-19], electrochemical assays [20, 21], and array-based biosensors [22, 23]. Problems with these techniques range from possible lengthy time periods to the lack of sensitivity and specificity needed to correctly identify the pathogen present [12].

Microarray platforms have also been implemented for genotyping highly related bacterial strains of *Listeria monocytogenes* [24], *Escherichia coli* [25], and several *Campylobacter* species [26]. The genomes of over two dozen bacterial pathogens have been sequenced, fostering studies on genomic substitutions, deletions, repetitions, insertions, and other types of variations [27]. By examining these modifications in the genome, the evolution of a species or genus can be better understood revealing evolutionary patterns, causes of antibiotic resistance, and areas of the genome responsible for virulence [27].
Single nucleotide polymorphisms (SNPs) are an extensive area of focus for DNA microarrays [28-30]. These variations can lead to various strains of bacterial pathogens [31] and can be markers of more complex diseases such as cancer [32]. A single base change can have a wide range of effects. A polymorphism within a non-coding region of the gene will have no effect on processes and reactions within the body. No protein is generated from this region of the DNA because the DNA is not transcribed into mRNA. If the change in nucleotide base occurs within the coding sequence of the gene, the protein generated will contain the polymorphism. A single base pair modification can change the identity and the function of the protein and therefore downstream processes and reactions.

Polymorphisms create various strains within a species and the highly parallel nature of DNA microarrays lends itself to SNP analysis and strain differentiation. By performing hybridization reactions with a DNA microarray composed of various probes containing SNPs, a yes/no response for every probe is obtained for each strain tested [25]. It is important to note that SNPs are not only implemented in bacterial genotyping experiments but have also become frequently used to genotype the coding region of human mitochondrial DNA (mtDNA), which is inherited from the mother [33]. mtDNA has been a focus in population genetic studies due to its high rate of polymorphisms and because it does not undergo recombination [33]. The absence of recombination among maternal and paternal mtDNA facilitates the tracing of an individual’s genetic line [3]. This is done by examining very closely related individuals and monitoring polymorphisms due to mutations and the high substitution rate in mtDNA [3]. Databases have been compiled, grouping together specific SNPs within the mtDNA that indicate specific geographic dispersal routes and identify ancestral origins [34].
The field of forensic chemistry has implemented DNA microarrays for SNP genotyping. Analysis of SNPs within the entire genome, by investigating short sequences of DNA that include a single base-pair change, can be used to create a unique profile of SNPs for an individual [35]. The use of SNPs in forensics is common because they occur frequently, with estimates of 1 for every 1,000 base pairs and over three million present in the genome [36]. An array-based format not only can test thousands of SNPs at once but is highly successful when handling degraded or minute amounts of DNA such as in crime scene investigations [37]. The field of forensics often must solve problems requiring parental identification. Individual identification using microarray platforms designed to genotype SNPs provide a viable method of such testing [38].

The use of DNA microarrays as an efficient assay to sequence a genome through multiple hybridization experiments in parallel has been previously reported [39]. Sequencing the genome, and more specifically mtDNA, is necessary in order to begin identifying mutations that could lead to the onset of diseases such as cancer. Zhou and coworkers[40] reported the use of an array that allows for sequencing of the complete mitochondrial coding region with >99.99% reproducibility. Their assay demonstrated the reliability and speed of DNA microarrays as well as a higher level of sensitivity than other sequencing methods [40]. Experiments like these have been used to begin to identify specific variations in genes that lead to the onset of diseases.

One of the most promising applications of DNA microarrays focuses on identifying genes linked to serious illnesses such as cancer. Cancer is not caused by a single factor; rather, it arises due to mutations within the body. For example, an amplification of the genome, a deletion, an insertion, a mutation, etc, can all directly affect and change the expression of genes that are involved in regulating important processes such as the cell cycle, proliferation, DNA repair, and
apoptosis [41]. Alterations in these processes can eventually cause the onset of genetic
instability, the initiation of tumorigenesis, and even lead to the development of an increasingly
malignant, invasive, and resistant phenotype [41]. DNA microarrays have been used to identify
genes involved in the multi-step development process of cancer [41]. Nacht and coworkers used
microarrays to identify genes consistently expressed during the progression of breast cancer
tumors and were able to compare and contrast the expression of genes in normal cells, primary
breast cancer, and metastatic breast cancer [42]. The integration of microarrays into cancer
research has led to the identification of genes pertinent to other cancers such as ovarian[43],
lung[44], colon[45], prostate[46], intestinal[47], and head and neck cancer [48]. The
identification of these relevant genes can not only be used as diagnostic markers but also can
potentially be employed for the identification of new treatments based on the function of these
specific genes [41]. Even more promising is the use of DNA microarrays to study
chemoresistance by observing changes in gene expression. For instance, by monitoring gene
expression through the use of DNA microarrays, Kudah and coworkers noted that the efficacy of
an anti-tumor drug on some malignant cells decreased, allowing them to reform a tumor on gene
expression in breast cancer cells [49]. Through the use of microarrays, scientists are now able to
study multiple aspects of oncology leading to a better understanding of a complex disease.

The widespread use and important contribution to science that DNA microarrays have
provided and will continue to impart makes it important to expose future scientists to this
research field. The goal is to integrate the next generation of scientists with a cutting-edge
technique while fostering the excitement for scientific discovery.
1.3 Details of Howard Hughes Medical Institute (HHMI) Grant and Objectives

The Howard Hughes Medical Institute Professorship (HHMI), awarded to twenty professors around the country, has one main focus: to make science more interesting and exciting for students through mentoring and teaching [50]. This award is not only a high honor of distinction but also brings a one-million dollar grant to aid in each professor’s quest to ignite passion for scientific discovery in students [50].

Walt’s vision for the use of this award is a multiple year plan to introduce new laboratory experiments that utilize modern methodologies with a subject matter to which students can relate. Along with this goal, the objective is to extensively get involved with K-12 students so that a passion for science may be instilled in future scientists. More specifically, Walt’s research efforts in developing DNA microarrays[51] will be leveraged to implement genomic testing in undergraduate laboratories and eventually the K-12 curriculum. Current techniques are beyond the scope of a typical organic chemistry laboratory but are a significant part of the scientific research effort.

In order to implement genetic testing in the Organic Chemistry laboratory course, there are several constraints that need to be considered. First, an undergraduate laboratory is on average a three hour time block, placing a time restriction on any experiment. This time limitation creates a problem: genomic testing requires a high enough concentration of DNA for implementation in a concentration-dependent assay. Research labs use the polymerase chain reaction (PCR) to solve this problem, amplifying DNA exponentially, but PCR can be time intensive depending on the temperature profile and number of cycles required. Avoiding such techniques would allow a majority of the undergraduate laboratory time block to be spent performing other more interesting parts of the experiment.
A second constraint is cost. Since this new experiment is designed to be integrated into a university curriculum, the assay cost per student needs to be minimized. In the case of PCR, the cost of operation including sterile pipet tips, a supply of reagents such as Taq polymerase and d nucleotide triphosphates (NTPs), along with the one time instrumentation cost add up to a figure that could overwhelm a university’s laboratory budget. A genomics experiment ideally should avoid such expensive technology but still utilize methodologies that will expose the students to current research protocols. Finally, the level of difficulty of the new experiment needs to be considered. The chemical reactions utilized must have mechanisms understandable to a student with general organic chemistry knowledge. With these constraints in mind, our goal is to develop an experiment so that it can be executed successfully in an undergraduate laboratory.

This thesis is the beginning of the HHMI project in which numerous methods of attaching strands of DNA to inexpensive surfaces are investigated. As various protocols are explored, issues of concern include obtaining a high density of DNA probe attachment to the solid support as well as creating an experiment in which the probes undergo specific hybridization reactions when tested with target DNA.
Chapter 2. Materials and Methods

2.1 Materials

Fluorescein isothiocyanate, hydrogen peroxide, hydrochloric acid, silica gel grade 60 70-230 mesh, 3-aminopropyl-functionalized silica gel, 8% glutaraldehyde, p-nitrophenylchloroformate, sodium chloride, 99.0% formamide, 99.5% ethanolamine, anhydrous toluene, ethanol, diethyl ether, cyanogen bromide, sodium bicarbonate, Sephadex G-100, cyanuric chloride, triethylamine, succinic anhydride, potassium carbonate, dimethyl sulfoxide (DMSO), and acetonitrile were purchased from Sigma Aldrich (St. Louis, MO). For all experiments, except the DNA probe dilution, Millipore 18 MΩ water from Millipore Gradient A10 was used (Billerica, MA). Plain Microscope Slides, Tris EDTA (2-amino-2-hydroxymethyl-1,3-propanediolethylenediamine tetraacetic acid) buffer, acetone, sodium hydroxide, acetic acid, chloroform, Accumet Basic pH/mV/°C meter, and 1.5 mL microcentrifuge tubes were purchased from Fisher Scientific (Pittsburgh, PA). 2-amino-4,6-dichlorotriazine was purchased from Toronto Research Chemicals Inc. (Ontario, Canada). Sterile water to dilute oligonucleotides was purchased from Hospira, Inc. (Lake Forest, IL). 3-aminopropyltrimethoxysilane was purchased from United Chemical Technologies, Inc. (Bristol, PA). *UltraPure SilicaBond*® Dichlorotriazine Functionalized Silica Gel was purchased from SiliCycle® (Quebec, Canada). Microgranular cellulose powder and cellulose chromatography paper was purchased from Whatman (Middlesex, England). Phosphate Buffered Saline was purchased from Fluka & Riedel (Switzerland). P(95% GlycidylMethAcrylate/ 5% EDMA) + EDA microspheres (5.99 µm) were obtained from Bangs Laboratories, Inc. (Fishers, IN). Micron centrifugal filter devices, Ultracel YM-3 3000 Da MWCO, were purchased from Millipore
Billerica, MA). stx1 probe: 5’-Amine-CTT-ATg-TAA-TgA-CTgCTg-AAg-ATg-TTg-ATC-3’ (30 bp, Tm= 55.9°C), InvA probe: 5’-Amine-CTg-AAT-TAC-TgA-TTC-Tgg-TAC-TAA-Tgg-TgA -3’ (30 bp, Tm= 56.2°C), stx1 target: 5’-Cy3-gAT-CAA-CAT-CTT-CAg-CAg-TCA-TTA-CAT-AAg-3’ (30 bp, Tm= 55.9°C), InvA target: 5’ Cy3- TCA-CCA-TTA-gTA-CCA-gAA-TCA-gTA-ATT-CAg -3’ (30 bp, 56.2°C) and OmpU target: 5’-Cy3-gAT-TCC-gTC-AgC-gTA-AgC-g-3’ (22 bp, Tm= 56.2°C) were synthesized by Integrated DNA Technologies, Inc (Coralville, IA). Cy3 (excitation: 550 nm, emission: 564 nm) and FITC (excitation: 492 nm, emission: 518 nm) filter cubes were obtained from Chroma Technology Corporation.

2.2 Imaging System

The imaging system used was a custom built upright Olympus BX-61 microscope system coupled to a Sensicam QE CCD camera (Cooke Corporation; Romulus, Michigan). This epi-fluorescence microscope uses a single objective to focus and collect the light. The excitation light source used was a 100 W mercury arc DC lamp. Optical channels were selected by filter cubes consisting of an excitation filter, emission filter, and a dichroic mirror.

2.3 Methods

2.3.1 Preparation of Amine-Modified Silica Gel [52]

Ten grams of dry silica gel was combined with a solution of 50 mL of anhydrous toluene and 12 mL of 3-aminopropyltrimethoxysilane. The mixture was heated to reflux overnight and allowed to cool to room temperature. The solution was filtered using suction filtration while
washing with 5 mL aliquots of anhydrous toluene, ethanol, and diethyl ether. The silica gel was
dried in an oven at 65°C for 8 hours.

2.3.2 Verification of Amine Groups Present on Silica Gel

1 mL of 1X phosphate buffered saline (PBS) was added to 0.15 g of the commercially
available 3-aminopropyl functionalized silica gel, the amine-modified silica gel generated in
2.3.1, and the unmodified silica gel. 50 µL of fluorescein iosthiocyanate (FITC) (1 mg/1 mL in
acetone) was added dropwise to each silica gel sample and vortexed. The mixture was incubated
at 4°C for one hour in the dark. The solid was centrifuged and the supernatant removed. Each
silica gel was washed with 1 mL of 1X PBS and with 1 mL of acetone two times. Samples were
placed on glass microscope slides and examined using the microscope with a FITC filter cube.

Summary of Results: The presence of amine groups was verified on the amine-modified silica gel
(2.3.1) and on the commercially available 3-aminopropyl functionalized silica gel through the
generation of a fluorescent signal using the FITC filter cube. The fluorescent intensity observed
was more intense for the 3-aminopropyl functionalized gel indicating a greater number of amine
groups present. There were no amine groups present on the unmodified silica gel.

2.3.3 Reaction of the 3-aminopropyl Functionalized Silica Gel and Microspheres with
Glutaraldehyde [53]

1 mL of 8% glutaraldehyde was added to 100 µL of amine-modified polymer
microspheres and 0.15 g of 3-aminopropyl functionalized silica gel separately and placed on the
circular rotator for two hours. The solid was washed twice with 1 mL of 1X PBS. 200 µL of 100
µM stx1 probe was added, diluted with sterilized water, and placed on a circular rotator for two hours. The solution was centrifuged and the supernatant removed. 1 mL of 1 M NaCl was added and shaken for three hours. 1 mL of the capping reagent, 50% ethanolamine, was added and shaken for 30 min and then washed twice with 1 mL of 1X PBS. 200 µL of 1 µM stx1 target (complement) and 1 µM OmpU target (non-complement), diluted with 1X PBS, were each added and allowed to hybridize for 30 minutes at 54°C. The solution was centrifuged and the supernatant removed, washed with 1 mL of Tris EDTA (TE) buffer and 1 mL of 1 mL of 1X TE Buffer + 20% formamide. Samples were placed on glass microscope slides and examined using the microscope with a Cy3 filter cube.

**Summary of Results:** When examining the silica gel after only the addition of glutaraldehyde, fluorescence was observed when using the Cy3 filter cube. Therefore, glutaraldehyde is not a suitable reagent when implementing amine-modified silica gel. Successful linker chemistry and hybridization reactions occurred when implementing amine-modified polymer microspheres.

**2.3.4 Reaction of 3-aminopropyl Functionalized Silica Gel and Microspheres with p-nitrophenylchloroformate (NPC) [53]**

Solutions of 1%, 2%, and 4% NPC in chloroform were prepared. 1 mL of each NPC concentration was added to 100 µL of microspheres and 0.15 g of Sigma Aldrich 3-aminopropyl functionalized silica gel separately and placed on the circular rotator for varying amounts of time—2 hr for 1% NPC, 1 hr for 2% NPC, and 30 min for 4% NPC. The solid was washed twice with 1 mL of 1X PBS. 200 µL of 100 µM stx1 probe, diluted with sterilized water, was added and placed on a circular rotator for two hours. The solid was centrifuged and the supernatant
removed. The solid was washed with 1 mL of 1 M NaCl for a variety of time periods. 1 mL of the capping reagent, 50% ethanolamine, was added and shaken for 30 min and then washed twice with 1 mL of 1X PBS. 200 µL of varying concentrations of stx1 target (complement) and OmpU target (non-complement) were added, diluted with either 1X or 5X PBS, and allowed to hybridize for 30 minutes at 54°C. The solid was centrifuged, the supernatant removed, and was washed with 1 mL of 1X TE Buffer and 1 mL of 1X TE Buffer + 20% formamide. Samples were then placed on glass microscope slides and examined using the microscope with a Cy3 filter cube.

To test specificity of the probe in this protocol, four separate hybridization reactions were performed in which 100 µM stx1 and InvA probes were individually hybridized with 1 µM stx1 and InvA target DNA.

**Summary of Results:** All concentrations of NPC provided successful linker chemistry and successful hybridization reactions. A concentration gradient of the stx1 target DNA added was tested and 1 µM solution provided a clear fluorescent signal when compared to the other concentrations. Using 5X PBS to dilute the target DNA solutions yielded a more significant fluorescent signal, when hybridizing with the stx1 target DNA, than when compared to reactions diluted with 1X PBS. Varying the amount of time the solution was washed with 1 M NaCl did not affect hybridization signal intensities.

When testing the specificity, a fluorescent signal was generated when hybridizing the stx1 probe with the stx1 (complement) target DNA as well as when hybridizing the InvA probe with the InvA (complement) target DNA. No fluorescent signal was generated when hybridizing the stx1 probe with the InvA (non-complement) target DNA as well as when hybridizing the InvA
probe with the stx1 (non-complement) target DNA. Since these specificity reactions were not extensively investigated, additional experiments should be performed in attempt to generate a more intense fluorescent signal.

2.3.5 Use of UltraPure SilicaBond® Dichlorotriazine Functionalized Silica Gel

200 μL of 100 μM stx1 probe was added, diluted with sterilized water, and placed on a circular rotator for two hours. The solid was centrifuged and the supernatant removed. 1 mL of 1 M NaCl was added for a variable amount of time. The solid was centrifuged and the supernatant removed. 1 mL of a capping reagent was added and shaken for 30 min. (Two separate capping reagents were used: 50% ethanolamine and a solution of 0.1 M succinic anhydride in 90% DMSO and 10% Sodium Borate Buffer.) The solution was then washed twice with 1 mL of 1X PBS. Varying concentrations of stx1 target (complement) and OmpU target (non-complement), diluted in 5X PBS, were added, and hybridized for 30 minutes at 54°C. The silica gel was centrifuged and the supernatant removed. The remaining pellet was washed with 1 mL of 1X TE Buffer and 1 mL of 1X TE Buffer + 20% formamide. Samples were placed on glass microscope slides and examined using the microscope with a Cy3 filter cube.

To test specificity of the probe in this protocol, four separate hybridization reactions were performed in which 100 μM stx1 and InvA probes were individually hybridized with 1 μM stx1 and InvA target DNA.

Summary of Results: Successful hybridization occurred when using ethanolamine as the capping reagent. Implementation of the succinic anhydride solution did not successfully cap the remaining sites subject to nucleophilic attack, yielding a fluorescent signal when hybridized with
the non-complement target DNA (control) and the complement target DNA. A concentration
gradient of the stx1 target DNA added was tested and 1 μM solution provided a clear fluorescent
signal when compared to the other concentrations tested. Varying the amount of time the
solution was washed with 1 M NaCl did not affect hybridization signal intensities.

When testing the specificity of the probe in this protocol, a fluorescent signal was
generated for all four hybridization reactions indicating that this protocol did not generate an
experiment capable of differentiating between two different strands of target DNA. These
reactions should be further investigated for this protocol was only utilized one time.

2.3.6 Reaction of Cellulose and Sephadex with Cyanogen Bromide [54]

100 mg of cellulose (powder or sheet) or Sephadex was added to 4 mL of water and
magnetically stirred for 30 minutes. 2 M NaOH was added to raise the pH to 11.5 and stirred for
15 minutes. 55 mg of cyanogen bromide was added and stirred for 15 minutes. Suction filtration
was performed while washing with 4°C water and 4 °C 10 mM sodium bicarbonate. 2 M NaOH
was added to maintain the pH at 11.5. The solid was added to a microcentrifuge tube and 200 μL
of 100 μM stx1 probe was added, diluted with sterilized water, and placed on a shaker overnight.
The solid was then washed with 1 mL of 1 M NaCl. 200 μL of varying concentrations of stx1
target (complement) and OmpU target (non-complement), diluted in 5X PBS, were added and
hybridized for 30 minutes at 54°C. The supernatant was removed after centrifugation and then it
was washed with 1 mL of 1X TE Buffer and 1 mL of 1X TE Buffer + 20% formamide. Samples
were placed on glass microscope slides and examined using the microscope with an Cy3 filter
cube. Variations of the amount of cyanogen bromide were added ranging from 55 mg to 165 mg.
An additional modification included a final wash with water.
Summary of Results: The addition of 55 mg and 110 mg of cyanogen bromide cause insufficient amine-modified probe to be attached. When using 165 mg of cyanogen bromide, successful hybridization occurred with the complement target DNA and limited hybridization occurred with the non-complement (control) target DNA. Varying amounts of complement target DNA were hybridized for the same amount of time and demonstrated that 1 \( \mu \text{M} \) solutions provided the most intense fluorescent signal. Stringency was increased with a final water wash.

Reaction of Cellulose and Sephadex with Cyanogen Bromide and a Capping Reagent

The same protocol from 2.3.6 was repeated but 1 mL of 50% ethanolamine was added and placed on the circular rotator for 30 min after washing with 1 mL of 1 M NaCl. The solution was then washed twice with 1 mL of 1X PBS and then target DNA was added.

To test specificity of the probe in this protocol, four separate hybridization reactions were performed in which 100 \( \mu \text{M} \) stx1 and InvA probes were individually hybridized with 1 \( \mu \text{M} \) stx1 and InvA target DNA.

Summary of Results: Hybridization reactions performed on the powdered cellulose and the cellulose sheets yielded a fluorescent signal when hybridized with the stx1 target DNA and no fluorescent signal with OmpU DNA. When implementing Sephadex, hybridization reactions using the complement and non-complement did not yield significantly different fluorescent intensities. Stringency was increased with a water wash, for the hybridization reactions of the stx1 probe with the complement target DNA, a fluorescent signal was produced that was slightly more intense than the hybridization with the non-complement target DNA.
When testing the specificity of the probe in this protocol, a fluorescent signal was generated when hybridizing the stx1 probe with the stx1 (complement) target DNA as well as when hybridizing the InvA probe with the InvA (complement) target DNA. No fluorescent signal was generated when hybridizing the stx1 probe with the InvA (non-complement) target DNA as well as when hybridizing the InvA probe with the stx1 (non-complement) target DNA.

2.3.7 Reaction of Cellulose with Cyanogen Bromide and Triethylamine [55]

200 mg of cellulose (powder or sheet) was washed with 10 mL of 30% (v/v) acetone to water solution. The solid was then suction filtered and cooled to -20°C by placing it in the -20°C refrigerator for 10 minutes. A solution of 330 mg of cyanogen bromide in 5 mL of 30% (v/v) acetone to water solution was added to the cellulose. 190 µL of 1.5 M triethylamine solution was added to the cellulose and stirred. Parafilm was placed over the beaker and cooled to -20°C by placing it in the -20°C refrigerator for 10 minutes. The solution was suction filtered while washing separately with 2 mL aliquots of cold 30% acetone to water + 0.1 M hydrochloric acid solution, cold 30% acetone to water solution, 4°C water, and 4°C 0.1 M carbonate buffer. The solid was added to a microcentrifuge tube, 200 µL of 100 µM stx1 probe was added, diluted with sterilized water, and placed on shaker for overnight. The solid was then washed with 1 mL of 1 M NaCl. 200 µL of 1 µM solutions of stx1 target (complement) and OmpU target (non-complement), diluted in 5X PBS, were added and hybridized for 30 minutes at 54°C. The supernatant was removed after centrifugation and then washed with 1 mL of 1X TE Buffer and 1 mL of 1X TE Buffer + 20% formamide. Samples were placed on glass microscope slides and examined using the microscope with a Cy3 filter cube.
Summary of Results: Successful hybridization occurred when using 1 µM complement target DNA and no fluorescence was visible when hybridizing with the non-complement target DNA.

2.3.8 Reaction of Cellulose with Cyanuric Chloride (A) [56]

300 mg of cellulose was added to 10 mL of 2 M NaOH and stirred magnetically for 30 minutes. The solution was then filtered using suction filtration. The cellulose powder was added to a solution of 0.28 g cyanuric chloride in 12.5 mL acetone and stirred for 15 minutes. 5 mL of water was then added followed by 5 mL of 20% acetic acid. Suction filtration was performed while washing with cold acetone. The solid was placed in a microcentrifuge tube and 200 µL of 100 µM stx1 probe was added, diluted with sterilized water, and placed on shaker overnight. The solid was then washed with 1 mL of 1 M NaCl. 1 µM solutions of stx1 target (complement) and OmpU target (non-complement), diluted in 5X PBS, were added and hybridized for 30 minutes at 54°C. The supernatant was removed after centrifugation and then it was washed with 1 mL of 1X TE Buffer and 1 mL of 1X TE Buffer + 20% formamide. Samples were placed on glass microscope slides and examined using the microscope with a Cy3 filter cube.

Summary of Results: Fluorescence from the hybridization of the stx1 and OmpU target DNAs was visible and therefore modifications need to be made to improve the procedure so the control experiment does not yield a fluorescent signal.

Reaction of Cellulose with Cyanuric Chloride and a Capping Reagent
The same protocol from 2.3.8 was repeated but 1 mL of 50% ethanolamine was added and placed on the circular rotator for 30 min after washing with 1 mL of NaCl. The solution was then washed twice with 1 mL of 1X PBS and then target DNA was added.

To test specificity of the probe in this protocol, four separate hybridization reactions were performed in which 100 µM stx1 and InvA probes were individually hybridized with 1 µM stx1 and InvA target DNA.

**Summary of Results:** It was difficult to differentiate between the fluorescent intensities generated when hybridizing with stx1 and OmpU target DNA. An additional wash with water increased the stringency and generated a clear difference between the two hybridization reactions.

When testing the specificity of the probe, a fluorescent signal was generated when hybridizing the stx1 probe with the stx1 (complement) target DNA as well as when hybridizing the InvA probe with the InvA (complement) target DNA. However, the intensities of these signals were not as intense as the signals produced with using other protocols in this thesis. These experiments should be repeated for they were only performed once due to time restraints. As expected, no fluorescent signal was generated with hybridizing the stx1 probe with the InvA (non-complement) target DNA as well as when hybridizing the InvA probe with the stx1 (non-complement) target DNA.

**2.3.9 Reaction of Cellulose with Cyanuric Chloride (B) [57]**

100 mg of powdered cellulose was treated with 1 M NaOH and stirred for two hours. The solution was suction filtered to remove the liquid and a solution of 55 mg of cyanuric chloride in 2.5 mL of acetone was added to the cellulose and stirred for five minutes. 2.5 mL of water was
added and stirred for five minutes. Following this, 2.5 mL of 10% acetic acid was added to quench the reaction. The solution was suction filtered while washing three times with a 5 mL solution of 1:1 solution of acetone to water, then twice with 5 mL of water, and finally twice with a 5 mL solution of 0.1 KH$_2$PO$_4$. The solid was placed in a microcentrifuge tube and 200 µL of 100 µM stx1 probe was added, diluted with sterilized water, and placed shaker overnight. The solid was then washed with 1 mL of 1 M NaCl. 200 µL of 1 µM solutions of stx1 target (complement) and OmpU target (non-complement), diluted in 5X PBS, were added and hybridized for 30 minutes at 54°C. The supernatant was removed after centrifugation and then it was washed with 1 mL of 1X TE Buffer and 1 mL of 1X TE Buffer + 20% formamide. Samples were placed on glass microscope slides and examined using the microscope with a Cy3 filter cube.

**Summary of Results:** Initial hybridization experiments generated a fluorescent signal when hybridizing with both the stx1 (complement) and OmpU (non-complement) target DNA. This non-specific fluorescent signal requires modification to the protocol. An additional wash with water was performed in an attempt to increase the stringency but there was no difference in fluorescence between the samples hybridized with the complement and non-complement target DNA.

**Reaction of Cellulose with Cyanuric Chloride and a Capping Reagent**

The same protocol from 2.3.9 was repeated but 1 mL of 50% ethanolamine was added and placed on the circular rotator for 30 min after washing with 1 mL of NaCl. The solution was then washed twice with 1 mL of 1X PBS and then target DNA was added.
To test specificity of the probe in this protocol, four separate hybridization reactions were performed in which 100 µM stx1 and InvA probes were individually hybridized with 1 µM stx1 and InvA target DNA.

Summary of Results: The addition of a capping reagent eliminated the non-specific attachment of target DNA to the cellulose. Fluorescence was visible when hybridizing with stx1 (complement) target DNA and no signal was detected when hybridizing with OmpU (non-complement) target DNA, indicating the success of this protocol.

When testing the specificity of the probe in this protocol, a fluorescent signal was generated when hybridizing the stx1 probe with the stx1 (complement) target DNA as well as when hybridizing the InvA probe with the InvA (complement) target DNA. No fluorescent signal was generated when hybridizing the stx1 probe with the InvA (non-complement) target DNA as well as when hybridizing the InvA probe with the stx1 (non-complement) target DNA. Since these specificity reactions were not extensively investigated, additional experiments should be performed in attempt to generate a more intense fluorescent signal.

2.3.10 Reaction of Cellulose with 2-amino-4,6-dichlorotriazine [58]

0.28 g of 2-amino-4,6-dichlorotriazine was added to 12.5 mL acetone and 12.5 mL water at 50°C. To a 5 mL aliquot, still at 50°C, 1 g of cellulose was added. 2 mL of a stock solution made up of 10 mL of 15% (w/v) sodium carbonate and 6 mL of 1 M hydrochloric acid was added to the cellulose mixture at 50°C. The solution was magnetically stirred for five minutes. 1 M hydrochloric acid was until the pH dropped below 7 as determined by a pH meter (no
change in composition). The mixture was then suction filtered while washing with 50% acetone/water and water. The solid was immediately added to 1X PBS. The resulting solution was centrifuged and the supernatant removed. 200 µL of 100 µM stx1 probe was added, diluted with sterilized water, and placed on a shaker overnight. The solid was then washed with 1 mL of 1 M NaCl. 200 µL of 1 µM solutions of stx1 target (complement) and OmpU target (non-complement), diluted in 5X PBS, were added and hybridized for 30 minutes at 54°C. The supernatant was removed after centrifugation and then washed with 1 mL of 1X TE Buffer and 1 mL of 1X TE Buffer + 20% formamide. Samples were placed on glass microscope slides and examined using the microscope with Cy3 filter cube.

**Summary of Results:** Fluorescence from hybridization with the stx1 and OmpU target DNA was visible. Modifications need to be made to eliminate the fluorescent signal present in the control experiment.

**Reaction of Cellulose with 2-amino-4,6-dichlorotriazine and a Capping Reagent**

The same protocol from 2.3.10 was repeated except after washing with 1 mL of 1 M NaCl, the solution was washed with 1 mL of 50% ethanolamine and placed on the circular rotator for 30 min. The solution was then washed twice with 1 mL of 1X PBS and then the target DNA was added.

To test specificity of the probe in this protocol, four separate hybridization reactions were performed in which 100 µM stx1 and InvA probes were individually hybridized with 1 µM stx1 and InvA target DNA.
Summary of Results: It was difficult to differentiate between the initial fluorescent intensities generated when hybridizing with stx1 and OmpU target DNA. An additional wash with water increased the stringency and generated a clear difference between the two hybridization reactions.

When testing the specificity of the probe for this protocol, a fluorescent signal was generated when hybridizing the stx1 probe with the stx1 (complement) target DNA as well as when hybridizing the InvA probe with the InvA (complement) target DNA. No fluorescent signal was generated with hybridizing the stx1 probe with the InvA (non-complement) target DNA as well as when hybridizing the InvA probe with the stx1 (non-complement) target DNA. Stringency was increased with a water wash.

2.3.11 Reaction of Cellulose Activated with Cyanogen Bromide and Polyethylenimine with Cyanuric Chloride-Activated DNA

To activate the stx1 DNA probe, 250 μL of 100 μM stx1 probe was added to 40 μL of cyanuric chloride (90 mg in 16 mL of acetonitrile) and placed on the circular rotator for 2 hours. The DNA-cyanuric chloride mixture was added to the filter portion of a YM-3 (3,000 Da MWCO) centrifugal concentrator unit and centrifuged at 10,000 rpm for 30 min. The filter portion was rinsed three times by adding 70 μL of SBB to the filter portion of the centrifugal concentrator and centrifuged for 30 min. The filtered cellulose was then collected by addition of 100 μL of SBB the top portion of the centrifuge vial and mixed well. The filter portion of the centrifugal concentrator unit was placed upside down in a new microcentrifuge tube and centrifuged for 1 min at 2000 rpm. The filter portion was removed and an additional 100 μL of
SBB was added to the filter area and mixed well. It was inverted into the same microcentrifuge tube and centrifuged for 1 min at 10,000 rpm.

To activate the cellulose, 100 mg of powdered cellulose was added to 4 mL of water and magnetically stirred for 30 minutes, 2 M NaOH was then added to raise the pH to 11.5 and stirred for 15 minutes. 165 mg of cyanogen bromide was added and stirred for 15 minutes. 2 M NaOH was added to maintain the pH at 11.5. Suction filtration was performed while washing with 4°C water and 4°C 10 mM sodium bicarbonate. The cellulose was added to the microcentrifuge tube and 1 mL of 5% polyethylenimine (PEI) in water was added and placed on the circular rotator for 2 hours. This solution was centrifuged and the remaining PEI was removed. The solid was resuspended in 400 µL SBB and combined with the activated DNA solution and placed on the shaker overnight. The solution was washed twice with SBB and 600 µL of 0.1 M succinic anhydride in 90% DMSO and 10% Sodium Borate Buffer was added. The solution was placed on the circular rotator for 1 hour and then washed twice with 1 mL of SBB and once with 1 mL of 1 M NaCl. 200 µL of 1 µM solutions of stx1 target (complement) and OmpU target (non-complement), diluted in 5X PBS, were added and hybridized for 30 minutes at 54°C. The supernatant was removed after centrifugation and then it was washed with 1 mL of 1X TE Buffer and 1 mL of 1X TE Buffer + 20% formamide. Samples were placed on glass microscope slides and examined using the microscope with a Cy3 filter cube.

**Summary of Results:** Initial hybridization experiments created a fluorescent signal after hybridizing with the stx1 (complement) and OmpU (non-complement) target DNA. An additional wash with water was carried out in an attempt to increase the stringency but there was no
difference between the samples hybridized with the complement and non-complement target DNA.
Chapter 3. Results and Discussion for the Attachment of Amine-modified Oligonucleotides to Solid Supports

This chapter describes the results of various chemical reactions used to link oligonucleotides to a solid surface. After successfully coupling the oligonucleotide to the solid surface, hybridization reactions were performed to evaluate the success and specificity of each protocol.

3.1 Uses of Silica Glass as the Solid Support

Various platforms have been developed for the attachment of oligonucleotides to surfaces and the earliest involved attaching single strands of DNA to glass slides [7]. Glass slides are used extensively due to their inherently uniform and non-porous surface [59]. Another advantage of glass slides, and other silica-based materials, is the ability to modify the surface. The glass surface is comprised of silanol groups that are readily available for further chemical reactions [53]. These hydroxyl groups can react with silanizing reagents to provide surface amino- or mercapto-groups that enable the attachment of DNA to build a microarray [60, 61]. This thesis focuses on the formation of primary-amine groups attached to the silica glass surface through the use of 3-aminopropyltrimethoxysilane. Through the use of 3-aminopropyltrimethoxysilane, the silanol groups are modified with primary-amine moieties capable of being further modified.

When chemically modifying the glass surface, there are various requirements to ensure successful attachment of DNA probes to the glass slide. The linker, the molecule that connects the glass slide to the oligonucleotide, must be long enough to eliminate steric interference so that more probes attach to the surface [62]. The attachment must be chemically stable and prevent
nonspecific binding to the glass [62]. Finally, the linker should be hydrophilic to guarantee its solubility in aqueous solutions [62]. 3-aminotripropylmethoxysilane meets these requirements and was the reagent of choice in the work reported in this thesis to generate amine groups on the surface of glass.

While glass microscope slides are often the support of choice, alternative materials also provide available hydroxyl groups. The advantage of microscope slides is that there is a uniform surface available to perform the necessary chemistry. One such substitute material is silica gel, which allows for the chemical reactions to take place in solution, thereby increasing the surface area available. Roy and Kundo detail the use of silica gel as suitable material for the immobilization of enzymes through the addition of amine groups to the silica gel surface [63].

### 3.1.1 Preparation of Amine-modified Silica Gel

The first task of this thesis was to generate primary-amine groups on unmodified silica gel. This was achieved through the use of 3-aminopropyltrimethoxysilane. The presence of primary-amine groups is necessary to perform the chemical reactions investigated in this section.

### 3.1.2 Verification of Amine Groups Present on Silica Gel

To determine the existence of primary-amine groups on silica gel, fluorescein isothiocyanate (FITC) was used. FITC is a fluorescent label that attaches to the silica gel through a reaction at the carbon of its isothiocyanate group with a primary-amine moiety. A solution of FITC was allowed to react with three samples: the commercially available 3-aminopropyl functionalized silica gel, the amine-modified silica gel generated in 2.3.1, and the unmodified silica gel as a control sample. A fluorescent signal was observed for the 3-aminopropyl
functionalized silica gel and the amine-modified silica gel, indicating the presence of primary-
amine groups attached to the surface of the silica gel (Fig 1). The unmodified silica gel yielded
no fluorescent signal, verifying the absence of amine groups (Fig 1). The fluorescent intensity
was greater for the 3-aminopropyl functionalized silica gel indicating a greater number of amine
groups present. Due to the higher concentration of primary-amine moieties, the 3-aminopropyl
functionalized silica gel was used in all of the following experiments.

![Figure 1](image)

**Figure 1.** FITC fluorescent images of (a) unmodified silica gel, (b) 3-aminopropyl functionalized, (c) and amine-
modified silica gel after reaction with FITC. Reaction conditions, exposure time (1 ms), and magnification (5X)
were identical.

3.1.3 Reaction of the 3-aminopropyl Functionalized Silica Gel and Microspheres with

Glutaraldehyde

Several chemical reactions can be utilized to covalently attach oligonucleotides through
the modification of primary-amine groups on the silica gel surface. The use of glutaraldehyde
has been extensively studied in the Walt Laboratory[64] and by Yang and coworkers [53].
Glutaraldehyde presents two aldehyde moieties capable of undergoing a reaction with the
primary-amine groups attached to the solid support and amine-modified oligonucleotides (Fig
2a) [53]. Glutaraldehyde has been previously reported as an effective fixative agent because of
its ability to cross-link proteins to polymers and antibodies or antigens to solid supports [64].
Chemistry involving glutaraldehyde is more complicated than outlined by Yang because when it
is dissolved in aqueous solutions, it equilibrates between a variety of structures which can
undergo a multitude of reactions (Fig 2b and 3) [64].

Figure 2 a) Scheme for covalent attachment of oligonucleotide onto a silica glass surface through use of 3-
aminopropyltrimethoxysilane (APTES) and 8% glutaraldehyde b) Various structures of glutaraldehyde possible
when in solution.

Figure 3: Additional cross-linking side reaction possible when glutaraldehyde is present in an acidic environment.

The 3-aminopropyl functionalized silica gel and microspheres were reacted with
 glutaraldehyde. A background image was taken to compare the fluorescent signal generated from
later hybridization reactions. Fluorescence was observed from the silica gel sample, indicating
that glutaraldehyde was not a suitable reagent in experiments utilizing the Cy3 filter cube (Fig 4).
Glutaraldehyde is known to gradually cross-link and it yielded a fluorescent compound with an
emission spectrum that overlaps the emission of Cy3.
Comparatively, amine-modified microspheres were allowed to react with glutaraldehyde followed by the coupling of an amine-modified oligonucleotide, stx1. Following this step, a capping reagent, ethanolamine, was added to react with any remaining aldehyde functional groups present. The use of a capping reagent is important in this protocol for when the amine-modified oligonucleotide is added to the glutaraldehyde-activated surface, not all the available sites successfully attach an oligonucleotide. Therefore, ethanolamine is added to cap the available sites and to prevent non-specific binding with later reagents (Fig 5). The lack of a fluorescent signal present in the background image of the microspheres indicated that the amount of cross linking was limited. This result could be attributed to the fact that the total number of primary-amine groups attached to the microspheres was lower than the 3-aminopropyl functionalized silica gel. After hybridization with Cy3-labeled target, stx1 (complement) and OmpU (non-complement), a fluorescent signal was measured from the complement target and no signal was observed from the non-complement target (Fig 4). These results agree with previous research done in the Walt Laboratory when reacting microspheres with glutaraldehyde [12].
3.1.4 Reaction of 3-aminopropyl Functionalized Silica Gel and Microspheres with p-nitrophenylchloroformate (NPC)

Yang and coworkers detailed a second reaction to covalently link oligonucleotides to amine-modified silica glass surfaces. The carbonyl group of p-nitrophenylchloroformate (NPC) underwent a nucleophilic attack by the nitrogen of a primary-amine moiety while chloride acts as the stable leaving group [53]. Next, the oligonucleotide attaches at the carbonyl carbon and the alkoxide becomes the leaving group (Fig 6) [53]. This methodology utilizes simple organic
chemistry reactions and is appropriate within the scope of an undergraduate Organic Chemistry course.

**Figure 6:** Scheme for covalent attachment of oligonucleotides onto a silica glass surface through use of 3-aminopropyltrimethoxysilane (APTES) and 1% p-nitrophenylchloroformate (NPC).

Initially, 3-aminopropyl functionalized silica gel was allowed to react with a 1% solution of p-nitrophenylchloroformate (NPC) in chloroform, followed by the attachment of amine-modified stx1 probe. The oligonucleotide-modified silica gel was allowed to react with a capping reagent, ethanolamine, to occupy any other available NPC molecules attached to the silica gel that did not couple with an amine-modified oligonucleotide (Fig 7). The capping reagent is important due to its ability to react and occupy sites subject to nucleophilic attack that could undergo non-specific binding and interfere with the results. Hybridization reactions with stx1 (complement) target DNA and OmpU (non-complement) DNA indicated that NPC is a viable linker reagent to attach oligonucleotides. Various concentrations of the stx1 target DNA were implemented in hybridization reactions to test the sensitivity of the assay. After comparing the
fluorescent signal generated by each concentration of target solution hybridized, the limit of
detection was determined to be 1 µM after a 30 minute hybridization time period (Fig 8).

Figure 7: Scheme for the reaction of the capping reagent, ethanolamine, with the NPC-activated silica gel surface.

![Scheme for the reaction of the capping reagent, ethanolamine, with the NPC-activated silica gel surface.]

Figure 8: Cy3 fluorescent images of 3-aminopropyl functionalized silica gel allowed to react with 1% NPC
followed by the attachment of amine-modified stx1 probe (a) before hybridization, (b) after hybridization of 1 µM
OmpU (non-complement) target DNA, (c) after hybridization of 1 µM solution of stx1 (complement) target DNA,
(d) after hybridization of 100 nM solution of stx1 target DNA, and (e) after hybridization of 10 nM solution of stx1
target DNA. All reactions were run in parallel with identical conditions, 5X magnification, and with a 1 ms exposure
time.

Modifications of the protocol were performed to reduce the time required included
increasing the concentration of NPC while decreasing the reaction time. Successful
hybridizations were observed after reacting 2% NPC for 1 hour and 4% NPC for 30 minutes (Fig
9). By varying concentrations of the stx1 (complement) target DNA, from 10 nM to 1 µM, it was
determined that 1 µM provided the fluorescent signal was clearly visible (Fig 10 & 11). This
reaction generated a homogenous fluorescent signal on the particles when hybridizing with 1 µM stx1 target DNA, indicating the success of this experiment.

Figure 9: Variations in the fluorescence created by allowing 3-aminopropyl functionalized silica gel to react with a range of NPC concentrations followed by coupling stx1 probe and hybridizing with 1 µM stx1 (complement) target DNA: a) 1% NPC allowed to react for 2 hours. b) 2% NPC allowed to react for 1 hour. c) 4% NPC allowed to react for 30 minutes. All reactions were carried out following the same general protocol and all images were taken using 5X magnification with a 1 ms exposure time.

Figure 10: Cy3 fluorescent images of 3-aminopropyl functionalized silica gel allowed to react with 2% NPC followed by the attachment of amine-modified stx1 probe (a) after hybridization of 1 µM solution of stx1 (complement) target DNA, (b) after hybridization of 100 nM solution of stx1 target DNA, and (c) after hybridization of 10 nM solution of stx1 target DNA. All reactions were run in parallel with identical conditions, 5X magnification, and with a 1 ms exposure time.

Figure 11: Cy3 fluorescent images of 3-aminopropyl functionalized silica gel allowed to react with 4% NPC followed by the attachment of amine-modified stx1 probe (a) after hybridization of 1 µM solution of stx1 (complement) target DNA, (b) after hybridization of 100 nM solution of stx1 target DNA, and (c) after hybridization of 10 nM solution of stx1 target DNA. All reactions were run in parallel with identical conditions, 5X magnification, and with a 1 ms exposure time.

It is important to note that by increasing the concentration of PBS used to dilute the target DNA solutions, from 1X PBS to 5X PBS, additional hybridization occurred between
complementary DNA strands. This adjustment generated a more intense fluorescent signal. Also, it was determined that variations in the amount of time the silica gel was washed with 1 mL of 1 M NaCl, after addition of the stx1 probe, did not affect the hybridization and the fluorescent intensity observed.

After using 1% NPC to link the amine-modified microspheres to the amine-modified stx1 probe, successful hybridization and a fluorescent signal was observed after hybridizing the stx1 probe and stx1 (complement) target DNA. As expected, no fluorescence was observed when the stx1 probe was hybridized with the OmpU (non-complement) target DNA (Fig 12).

3.1.5 Use of UltraPure SilicaBond® Dichlorotriazine Functionalized Silica Gel

Various companies have begun developing products to eliminate the time restraints imposed by linker chemistry. For example, SiliCycle® sells UltraPure SilicaBond® Dichlorotriazine Functionalized Silica Gel in which cyanuric chloride (2,4,6-trichloro-1,3,5-triazine) is attached to the silica gel, providing a linker available to attach DNA probes to the glass surface (Fig 13). The use of this product has the potential to decrease the time needed to attach the oligonucleotides to the silica gel. While this reagent is costly, compared to the other solid supports investigated in this thesis, a report by Ditzler and coworkers details the activation of silica gel with cyanuric chloride that could be alternatively used [65].
After allowing the amine-modified oligonucleotide to couple with the cyanuric chloride molecule, a capping reagent must be added to react with any remaining available sites on the cyanuric chloride molecule. This eliminates the attachment of unwanted molecules that could interfere with the fluorescent signal obtained from the hybridization experiments. When investigating the use of the SiliCycle® UltraPure SilicaBond® Dichlorotriazine Functionalized Silica, two different capping reagents were utilized, ethanolamine and succinic anhydride.

The succinic anhydride capping reagent has been previously reported when using dichlorotriazine derivatives, prompting this comparison[12]. The fluorescent intensities observed when hybridizing the stx1 probe with stx1 (complement) target DNA were clearly visible (Fig 17). The control hybridization, with OmpU (non-complement) target DNA, non-specifically hybridized or adhered to the silica gel when using a solution of 0.1 M succinic anhydride in 90% DMSO and 10% sodium borate buffer (SBB) as the capping reagent (Fig 14). The signal generated from hybridization with the non-complement target DNA was due to the fact that succinic anhydride does not cap the reactive sites on the dichlorotriazine molecule as initially perceived. Rather, succinic anhydride is a useful capping reagent when free primary-amine groups are present. Ethanolamine proved to be a more effective capping reagent for it was able to react with the active sites of the triazine molecule that did not successfully couple with the
amine-modified oligonucleotides (Fig 15). By capping these reactive groups, the fluorescence in the control hybridization was reduced because the capping reagent eliminated any non-specific binding of the cy3 target DNA molecules to the solid support (Fig 14). Hybridization reactions were performed varying the concentration of stx1 (complement) target DNA. The 1 µM concentration of stx1 target DNA was found to be the concentration needed to generate a visible fluorescent signal after a 30 minute hybridization time period (Fig 16).

![Figure 14](image)

Figure 14: Cy3 fluorescent images of UltraPure SilicaBond® Dichlorotriazine Functionalized Silica Gel after coupling with the stx1 probe and capping with ethanolamine, (a) prior to hybridization, (b) after hybridization of 1 µM OmpU (non-complement) DNA, and (c) after hybridization of the stx1 probe with 1 µM stx1 (complement) DNA. Cy3 fluorescent image of UltraPure SilicaBond® Dichlorotriazine Functionalized Silica Gel after coupling with the stx1 probe and capping with a solution of 0.1 M succinic anhydride in 90% DMSO and 10% SBB (d) prior to hybridization, (e) after hybridization of 1 µM OmpU (non-complement) DNA, and (f) after hybridization of the stx1 probe with 1 µM stx1 (complement) DNA. All hybridization reactions were done in parallel, with a magnification of 5X and an exposure time of 1 ms.

![Figure 15](image)

Figure 15: Mechanism of the reaction of the capping reagent, ethanolamine, with the remaining active sites on the dichlorotriazine-functionalized silica gel.
Figure 16: Cy3 fluorescent images of *UltraPure SilicaBond*® Dichlorotriazine Functionalized Silica Gel after coupling with the stx1 probe and capping with ethanolamine (a) after hybridization of 1 µM solution of stx1 (complement) target DNA, (b) after hybridization of 100 nM solution of stx1 target DNA, and (c) after hybridization of 10 nM solution of stx1 target DNA. All reactions were run in parallel with identical conditions, 5X magnification, and with a 1 ms exposure time.

3.2 Use of Cellulose and Sephadex as the Solid Support

In this thesis, not only is the use of silica glass investigated but also the implementation of cellulose, a polysaccharide of β-D-glucose in which there are equatorial hydroxyl groups present, was investigated as a solid support. Cellulose is available in a wide variety of forms including fibers, microgranules, microcrystals, beads, gel particles, capsules, and membranes. It is favorable due to low cost, accessibility, hydrophilic characteristics, and most importantly for this discussion, the surface hydroxyl groups available for chemical reactions [55]. Cellulose has been used most commonly to attach amine-modified enzymes through the use of linker molecules [56, 58]. Additional studies have detailed the attachment of amine-modified oligonucleotides to cellulose using the same methodologies as for attaching enzymes [54, 57]. Also, while cellulose has the ability to undergo successful surface chemistry to attach amine-modified oligonucleotides, the use of cellulose sheets is the most attractive possibility for the future. Advantages of this material include the elimination of timely centrifugation steps as well the potential development of a spotting technique to initiate the chemical reactions, increasing the simplicity of the experiment.
Another material that has been chemically modified to attach enzymes is Sephadex, a cross linked dextran [66]. Similar to cellulose, it is a complex branched polysaccharide composed of (1-6)-linked α-D-glucose molecules with branches extending off the main chain [66]. Due to the polysaccharide’s high surface area, hydrophilic character, and hydroxyl groups available for chemical modification, it also could be employed as the solid substrate capable of attaching oligonucleotides [67].

3.2.1 Reaction of Cellulose and Sephadex with Cyanogen Bromide

Cyanogen bromide has been comprehensively studied as the linker reagent for attaching enzymes [54, 55, 67]. Axen and Ernback successfully attached chymotrysin and other proteins to dextran (Sephadex), agarose beads, and cellulose powder [67]. The mechanism of the attachment of amine-modified proteins to cellulose using cyanogen bromide was studied by Bartling and coworkers [68]. The reaction investigated in this thesis entails using this chemistry but substituting the amine moiety of a protein with an amine-modified oligonucleotide (Fig 17) [68]. In this reaction, an inert, stable, and neutral carbamate as well as a reactive cyclic imidocarbonate are formed (Fig 17) [69]. The cyclic imidocarbonate is responsible for the coupling reaction (Fig 17) [67].
Figure 17: Scheme of chemical activation of polysaccharides through the use of cyanogen bromide with subsequent attachment of amine-modified oligonucleotide to immobilize onto a solid support.

In this investigation, varying amounts of cyanogen bromide were used to modify the surfaces of powdered cellulose, cellulose paper, and Sephadex. 55 and 110 mg of cyanogen bromide were allowed to react with cellulose powder, but did not generate a significant fluorescent signal after target hybridization, indicating insufficient stx1 probe was coupled to the solid support (Fig 18). Hybridization of stx1 (complement) target DNA on the cellulose sample, resulted in a sparse fluorescent signal and the hybridization of the OmpU (non-complement) target DNA generated no fluorescence. Addition of 165 mg of cyanogen bromide to the cellulose supports, followed by the coupling of the stx1 probe, resulted in a higher fluorescent signal when hybridized with the stx1 (complement) target DNA (Fig 19-20). Comparatively, there was no visible fluorescence detected when hybridizing with the OmpU (non-complement) target DNA, further verifying the success of cyanogen bromide as the linker molecule (Fig 19-20). Various concentrations of stx1 target DNA were hybridized to the stx1 probe coupled to the solid support and 1 μM target provided a visible fluorescent signal throughout the cellulose sample.
When utilizing the Sephadex to attach the probe through the use of 165 mg of cyanogen bromide, fluorescent signal was generated when hybridizing with 1 µM stx1 and 1 µM OmpU target DNA indicating preliminary failure in this protocol. Stringency was increased with a water wash, but caused most of the fluorescent signal from both the Sephadex samples to dissipate (Fig 21).
Figure 21: Cy3 fluorescent images of Sephadex reacted with 165 mg cyanogen bromide followed by the attachment of amine-modified stx1 probe (a) before hybridization, (b) after hybridization of 1 µM OmpU (non-complement) stx1 target DNA, (c) after hybridization of 1 µM stx1 (complement) stx1 target DNA, (d) after hybridization of 1 µM OmpU target DNA and washing with water, and (e) after hybridization with 1 µM stx1 target DNA and washing with water. Reaction conditions, exposure time (1 ms), and the magnification (5X) were identical.

Reaction of Cellulose or Sephadex with Cyanogen Bromide and a Capping Reagent

These experiments following the same protocol as VI with the addition of the capping reagent, ethanolamine. Ethanolamine was allowed to react with cyanogen bromide-activated sites on the cellulose or Sephadex that did not couple with the amine-modified oligonucleotide in the prior step (Fig 22). Ethanolamine is able to occupy any site, easily subject to nucleophilic attack, to prevent the possible attachment of unwanted molecules to the solid support. When hybridizing 1 µM stx1 (complement) target DNA to the stx1 probe attached to the cellulose powder and the cellulose sheets, fluorescence was detected (Fig 23). Parallel hybridization experiments were carried out by hybridizing with 1 µM OmpU (non-complement) target DNA and produced no detectable fluorescence indicating the success of ethanolamine at preventing non-specific attachment (Fig 23). The fluorescent signals generated by the complement and lack of fluorescence created by hybridizing with the non-complement indicate the overall success of this protocol.

Figure 22: Mechanism of the reaction of the capping reagent, ethanolamine, with the remaining available sites of the cyanogen bromide-activated cellulose.
When identical reaction conditions were applied to the solid support Sephadex, fluorescent signal was detected after hybridizing with both 1 µM stx1 (complement) and OmpU (non-complement) target DNA (Fig 24). Hydrogen bonds may be forming between the hydroxyl groups present on the Sephadex and the DNA, which would allow the non-complement target DNA to non-specifically bind to the solid support. An additional wash with water, a strong hydrogen bond disruptor, eliminated this interference and all fluorescent signal in the hybridization reaction with the non-complementary target DNA (Fig 24).
Figure 24: Cy3 fluorescent images of Sephadex reacted with 165 mg cyanogen bromide followed by the attachment of amine-modified stx1 probe (a) before hybridization, (b) after hybridization of 1 µM OmpU (non-complement) stx1 target DNA, (c) after hybridization of 1 µM stx1 (complement) stx1 target DNA, (d) after hybridization of 1 µM OmpU target DNA and washing with water, and (e) after hybridization with 1 µM stx1 target DNA and washing with water. Reaction conditions, exposure time (1 ms), and the magnification (5X) were identical.

3.2.2 Reaction of Cellulose with Cyanogen Bromide and Triethylamine

Further research utilizing cyanogen bromide has led to the use of a cyano-transfer reagent, triethylamine (TEA), to increase the overall yield of the attachment of amine-modified molecules [55]. Kohn and coworkers detail the use of TEA to generate a highly reactive triethylammonium nitrile complex (1) (Fig 25) [70]. TEA increases the electrophilicity of the cyano-group, allowing it to react with the hydroxyl groups present on the glass surface [70]. Following the creation of the cyanate ester, the protein can be coupled to this activated support (Fig 25). This thesis investigates the use of this reaction as a protocol to couple sufficient probe DNA to the solid support to achieve fluorescence upon successful hybridization.

Figure 25: a) Activation of a glass surface through the use of a cyano-transfer reagent, TEA, to generate a highly electrophilic triethylammonium nitrile complex (1) to create cyanate ester moieties [70]. B) Scheme of using cyanogen bromide as the linker molecule to attach oligonucleotides [55].

After hybridizing the stx1 probe attached to the cellulose sheet with 1 µM stx1 (complement) target DNA, a low fluorescent signal was detected while hybridization on the cellulose powder resulted in a much higher signal (Fig 26). A possible explanation for the
difference can be attributed to inadequate coupling of the probe to the sheet, which may not have been fully submerged in the solution. As expected for both reactions, hybridization of the attached stx1 probe with 1 μM OmpU (non-complement) target DNA did not generate a fluorescent signal (Fig 26 & 27).

Figure 26: Cy3 fluorescent images of cellulose sheets reacted with 165 mg cyanogen bromide and TEA followed by the attachment of amine-modified stx1 probe (a) before hybridization, (b) after hybridization of 1 μM OmpU (non-complement) stx1 target DNA, and (c) after hybridization of 1 μM stx1 (complement) stx1 target DNA. Reaction conditions, exposure time (1 ms), and the magnification (5X) were identical.

Figure 27: Cy3 fluorescent images of powdered cellulose reacted with 165 mg cyanogen bromide and TEA followed by the attachment of amine-modified stx1 probe (a) before hybridization, (b) after hybridization of 1 μM OmpU (non-complement) stx1 target DNA, and (c) after hybridization of 1 μM stx1 (complement) stx1 target DNA. Reaction conditions, exposure time (1 ms), and the magnification (5X) were identical.

3.2.3 Reactions of Cellulose with Cyanuric Chloride and its Derivatives

Activation of cellulose can also be accomplished through the use of cyanuric chloride (2,4,6-Trichloro-1,3,5-Triazine) as a linker molecule [56-58]. Kay and Crook reported a method of cyanuric chloride attachment followed by the coupling of an enzyme to the solid support (Fig 28) [56]. This thesis investigates the attachment of amine-modified oligonucleotides to solid supports through the use of cyanuric chloride and its derivatives.
Figure 28: Overall mechanism of activation of cellulose hydroxyl groups using cyanuric chloride with subsequent attachment of amine-modified oligonucleotides onto the solid support.

3.2.3.1 Reaction of Cellulose with Cyanuric Chloride (A)

Initial experiments using cyanuric chloride as the linker molecule to attach the DNA probe generated a non-specific fluorescent signal (Fig 29). Hybridization of 1 µM OmpU (non-complement) target DNA yielded a comparable fluorescent intensity when compared to hybridization with 1 µM stx1 (complement) target DNA (Fig 29). Modifications of this protocol were attempted to circumvent non-specific attachment of any target DNA.

a)  

b)  

c)

Figure 29: Cy3 fluorescent images of powdered cellulose reacted with cyanuric chloride followed by the attachment of amine-modified stx1 probe (a) before hybridization, (b) after hybridization of 1 µM OmpU (non-complement) stx1 target DNA, and (c) after hybridization of 1 µM stx1 (complement) stx1 target DNA. Reaction conditions, exposure time (1 ms), and the magnification (5X) were identical.

Reaction of Cellulose and Cyanuric Chloride with the Addition of a Capping Reagent.

Additional experiments were performed utilizing the capping reagent ethanolamine to ensure that all reactive sites available on cyanuric chloride underwent a nucleophilic attack with a primary-amine group. In this reaction, there is a maximum of two available sites on the cyanuric chloride molecule, after its attachment to the cellulose, that can undergo a chemical reaction. If one amine-modified oligonucleotide coupled with the cyanuric chloride molecule...
then there is one available site for the capping reagent to bind with (Fig 30). The addition of the capping reagent is necessary to eliminate sites on the activated cellulose that can easily undergo nucleophilic addition thereby preventing unwanted molecules from attaching. Hybridization of 1 µM stx1 (complement) and OmpU (non-complement) target DNA both yielded fluorescent signals but with stx1 being more intense than OmpU (Fig 31). Success of this protocol was achieved with a final water wash to eliminate any possibly hydrogen bonding present between target DNA strands and the hydroxyl groups of cellulose. Following this wash, the expected fluorescent signal remained for the sample hybridized with stx1 (complement) target DNA and no fluorescent signal was present for the control hybridization of the OmpU (non-complement) target DNA (Fig 31).

**Figure 30:** Mechanism of the reaction of the capping reagent, ethanolamine, with the remaining available sites on the cyanuric chloride-activated cellulose.

**Figure 31:** Cy3 fluorescent images of powdered cellulose reacted with cyanuric chloride followed by the attachment of amine-modified stx1 probe and capping with ethanolamine (a) before hybridization, (b) after hybridization of 1 µM OmpU (non-complement) stx1 target DNA, (c) after hybridization of 1 µM stx1 (complement) stx1 target DNA, (d) after hybridization of 1 µM OmpU target DNA and washing with water, and (e) after hybridization with 1 µM stx1 target DNA and washing with water. Reaction conditions, exposure time (1 ms), and the magnification (5X) were identical.
3.2.3.2 Reaction of Cellulose with Cyanuric Chloride (B)

This second protocol, implementing cyanuric chloride as the linker molecule, differs from the initial experiment in the treatment of cellulose prior to the addition of the cyanuric chloride solution and in the concentration of cyanuric chloride added. After hybridizing with 1 µM stx1 (complement) target DNA, the expected intense fluorescence was visualized (Fig 33). Equally as intense, was the fluorescence generated when hybridizing with 1 µM OmpU (non-complement) target DNA (Fig 32). Due to the non-specific fluorescent signal present in this experiment, the protocol was modified.

Figure 32: a) Image of powdered cellulose was allowed to react with cyanuric chloride followed by the coupling of the stx1 probe. b) Image of the fluorescent signal generated after hybridizing the stx1 probe coupled to the powdered cellulose with 1 µM OmpU (non-complement) target DNA. c) Image of the fluorescent signal generated after hybridizing the stx1 probe coupled to the powdered cellulose with 1 µM stx1 (complement) target DNA. Hybridizations were performed in parallel with identical procedures. All images were taken under 5X magnification with a 1 ms exposure time.

Reaction of Cellulose with Cyanuric Chloride (B) and a Capping Reagent

To eliminate the non-specific fluorescence intensity generated in 3.2.3.2, the capping reagent, ethanolamine, was used to react with any available sites on the cyanuric chloride linker molecule. By washing thoroughly with this reagent, the non-specific attachment was eliminated and the fluorescent signal of OmpU disappeared (Fig 33). Additional success of this protocol was shown by hybridizing with stx1 (complement) target DNA to yield a strong fluorescent signal (Fig 33). The use of a capping reagent with this cyanuric chloride protocol generated an experiment capable of undergoing successful and specific hybridization reactions.
Figure 33: a) Image of powdered cellulose was allowed to react with cyanuric chloride followed by the coupling of the stx1 probe and capping with ethanolamine. b) Image of the fluorescent signal generated after hybridizing the stx1 probe coupled to the powdered cellulose with 1 µM OmpU (non-complement) target DNA. c) Image of the fluorescent signal generated after hybridizing the stx1 probe coupled to the powdered cellulose with 1 µM stx1 (complement) target DNA. Hybridizations were performed in parallel with identical procedures. All images were taken under 5X magnification with a 1 ms exposure time.

3.2.3.3 Reaction of Cellulose with 2-amino-4,6-dichlorotriazine

A slight variation from 3.2.3.1 and 3.2.3.2 involved the use of a cyanuric chloride derivative, 2-amino-4,6-dichlorotriazine to attach to the cellulose. After coupling the stx1 probe to the cellulose through this molecule, initial hybridization reactions were performed with 1 µM stx1 (complement) and 1 µM OmpU (non-complement) target DNA. After examining the results of this hybridization under the Cy3 filter cube, the was no difference between the fluorescent intensities of these reactions (Fig 34). Next, 99.0% formamide was added to the cellulose to eliminate non-specific attachment of the non-complement Cy3-labeled target DNA (Fig 34). This wash caused both the complement and non-complement fluorescent signals to be dramatically reduced indicating that the initial fluorescent signal present was not due to successful base-pairing. Some fluorescent signal was randomly dispersed throughout the sample but not enough to clearly differentiate between the two hybridizations performed (Fig 34).


Reaction of Cellulose with 2-amino-4,6-dichlorotriazine and a Capping Reagent

The protocol from 3.2.3.3 was modified by adding a capping reagent in an attempt to circumvent any non-specific attachment of DNA. Hybridization experiments again resulted in non-specific binding of the OmpU (non-complement) target DNA (Fig 35). Following hybridization, a final wash with water to disrupt hydrogen bonding was performed, which reduced the fluorescent signal from the non-complement target DNA while preserving the signal from the complement target DNA (Fig 35). The addition of a capping reagent and washing with water increased the stringency of the reaction creating a platform capable of specific hybridization experiments.
3.2.3.4 Reaction of Cellulose Activated with Cyanogen Bromide and Polyethylenimine with Cyanuric Chloride-Activated DNA

Cyanuric chloride can also be used in attachment chemistry by first undergoing a reaction with the amine-modified DNA. Experiments reported by Ferguson et al. treat the amine-modified DNA with cyanuric chloride in acetonitrile to activate the DNA directly [71]. Amine-modified silica microspheres were modified with polyethylenimine (PEI) (Fig 36) through the use of glutaraldehyde as the linker molecule [71]. Upon attachment, PEI offers additional free primary-amine groups capable of undergoing a reaction with the cyanuric chloride activated DNA [71]. This mechanism allows for an increase in the number DNA probes attached to the solid surface. In this thesis, the use of PEI to react with the cyclic imidocarbonate via the cyanogen bromide reaction is investigated to increase the number of amine groups available for attaching DNA probes (Fig 37).

![Structure of polyethylenimine (PEI)](image)

Figure 36: Structure of polyethylenimine (PEI)
Figure 37: Mechanism of cyanogen bromide activation of cellulose or Sephadex followed by the addition of polyethylenimine (PEI) to create additional primary-amines capable of undergoing a reaction with cyanuric chloride-activated DNA.

In this reaction, the cyanuric chloride-activated oligonucleotide was reacted with the primary-amine groups attached to the cellulose and then the solution was capped with succinic anhydride. Succinic anhydride is capable of reacting with any available primary-amine moiety eliminating its ability to act as a nucleophile in other reactions (Fig 38). Capping reagents are important in the protocols explored in this thesis in order to prevent unwanted side reactions that could interfere with the reactions of interest. Upon hybridizing with the stx1 (complement) target DNA and the OmpU (non-complement) target DNA, a fluorescent signal was generated for both reactions (Fig 39). To eliminate the non-specific fluorescent signal, the solution was washed with water in hopes of disrupting any hydrogen bonding present. A fluorescent signal was present for the samples hybridized with the complement and non-complement target DNA strands. One possible explanation could be that not enough capping reagent was added, leaving available sites
vulnerable to nucleophilic attack by other molecules. Due to the presence of the non-specific fluorescent signal, it can be concluded that this protocol was not successful.

![Reaction scheme of succinic anhydride molecule capping an available primary-amine](image)

**Figure 38:** Reaction scheme of one succinic anhydride molecule capping an available primary-amine

![Cy3 fluorescent images](image)

**Figure 39:** Cy3 fluorescent images of powdered cellulose activated with PEI through the cyanogen bromide reaction followed by the attachment of cyanuric chloride activated amine-modified stx1 probe (a) before hybridization, (b) after hybridization of 1 μM OmpU (non-complement) stx1 target DNA, (c) after hybridization of 1 μM stx1 (complement) stx1 target DNA, (d) after hybridization of 1 μM OmpU target DNA and washing with water, and (e) after hybridization with 1 μM stx1 target DNA and washing with water. Reaction conditions, exposure time (1 ms), and the magnification (5X) were identical.
3.3 Determination of the Specificity of Probes Attached to Various Solid Supports

Experiments were repeated using two DNA probes, stx1 and InvA and two strands of Cy3-labeled complement DNA, stx1 and InvA. A total of four hybridization reactions were performed, reacting each probe with its complement and non-complement target. A Cy3 image was taken after each hybridization to compare the fluorescent intensities generated with the probe’s complement and non-complement strand of DNA.

3.3.1 Reaction of Silica Gel with 1% NPC

After performing the four hybridization reactions and examining the fluorescent intensities generated, it was determined that this protocol was successful. This experiment allowed for attachment of the amine-modified oligonucleotides to the silica surface and demonstrated that when hybridizing the probe with the target DNA, only exact base-pairing occurred. This is shown by the generation of a fluorescent signal when the probe hybridized with its complement and no fluorescent signal when the probe hybridized with its non-complement (Fig 40 & 41). This experiment was only performed one time and should be repeated in the future.

Figure 40: Cy3 fluorescent images of 3-aminopropyl functionalized silica gel allowed to react with 1% NPC followed by the attachment of amine-modified InvA probe and the addition of ethanolamine (a) before hybridization, (b) after hybridization of 1 μM stx1 (non-complement) target DNA, (c) after hybridization of 1 μM solution of InvA (complement) target DNA. All reactions were run in parallel with identical conditions, 5X magnification, and with a 1 ms exposure time.
3.3.2 Use of UltraPure SilicaBond® Dichlorotriazine Functionalized Silica Gel

In an attempt to bypass the time intensive step of modifying the silica gel with cyanuric chloride, a commercially available dichlorotriazine-functionalized silica gel was tested. After addition of the probes, the same four hybridization as in the previous procedure were performed. The fluorescence generated by all four hybridizations indicated that the use of this product resulted in non-specific attachment of the Cy3-labeled target DNA (Fig 42 & 43). These results were surprising due to the fact that a capping reagent was used to eliminate any available sites for attachment on the cyanuric chloride molecules. A possible explanation is that the pores present in the silica gel were large enough that the Cy3-labeled target DNA could enter and remain even after successive washings. These specificity tests should be repeated in the future, for only preliminary studies were performed due to time restraints and access to target DNA.
3.3.3. Reaction of Cellulose with Cyanogen Bromide and Ethanolamine

The reaction of cyanogen bromide with cellulose for enzyme attachment has been extensively studied [55, 58, 68, 69, 72] and therefore this protocol was expected to be equally effective in the attachment of amine-modified oligonucleotides. After these hybridization reactions were performed, the results indicated that the probe, when attached to the cellulose through cyanogen bromide, provided a specific hybridization reaction that only allowed for base-pairing with its complement. This conclusion was drawn after comparing the individual hybridization reactions of the InvA probe with stx1 and InvA target DNA. A fluorescent signal was only generated when hybridizing with the complement target (Fig 44). Similar results were obtained, when hybridizing the stx1 probe with InvA and stx1 target DNA (Fig 45). The
fluorescent signals generated when hybridizing the probe and complement target of the cyanogen bromide-activated cellulose (Fig 44 & 45) were significantly more intense than the NPC-activated silica gel (Fig 40 & 41). Depending on the fluorescence-detecting instrument available in an undergraduate laboratory, this may affect the need for a more intense fluorescent signal and therefore the use of the cyanogen bromide protocol. These reactions were only performed one time and should be repeated in the future to determine the consistency of the results obtained.

Figure 44: Cy3 fluorescent images of powdered cellulose allowed to react with cyanogen bromide followed by the attachment of amine-modified InvA probe and the addition of ethanolamine (a) before hybridization, (b) after hybridization of 1 µM stx1 (non-complement) target DNA, (c) after hybridization of 1 µM solution of InvA (complement) target DNA. All reactions were run in parallel with identical conditions, 5X magnification, and with a 1 ms exposure time.

Figure 45: Cy3 fluorescent images of powdered cellulose allowed to react with cyanogen bromide followed by the attachment of amine-modified stx1 probe and the addition of ethanolamine (a) before hybridization, (b) after hybridization of 1 µM InvA (non-complement) target DNA, (c) after hybridization of 1 µM solution of stx1 (complement) target DNA. All reactions were run in parallel with identical conditions, 5X magnification, and with a 1 ms exposure time.

3.3.4 Reaction of Cellulose and Cyanuric Chloride (A) and Ethanolamine

The use of cyanuric chloride as a linker molecule to attach amine-modified oligonucleotides to cellulose was extensively studied in this thesis. Initial success of the protocol was achieved when the expected fluorescence was generated when hybridizing the stx1 probe,
coupled to the cellulose, with the stx1 complement target DNA as well as the lack of fluorescence generated when hybridizing with the non-complement target DNA, OmpU. In attempts to determine the specificity of the probe, attached to the cellulose via a cyanuric chloride molecule, four different hybridizations were performed using only two probes, stx1 and InvA, with two DNA targets, stx1 and InvA. Upon examination of the results, a fluorescent signal was generated when the probe and its complement were hybridized and no fluorescent signal was generated when the probe was hybridized with its non-complement strand of DNA. The specificity of the probe in this experiment was successfully verified by the fluorescent signal generated when hybridizing the probe and its complement. The fluorescence generated when using this protocol was not as intense as expected when being compared to the initial reactions performed in 3.2.3.1. These reactions were only performed one time and should be repeated in the future.

![Figure 46](image-url)

**Figure 46:** Cy3 fluorescent images of powdered cellulose allowed to react with cyanuric chloride followed by the attachment of amine-modified InvA probe and the addition of ethanolamine (a) before hybridization, (b) after hybridization of 1 µM stx1 (non-complement) target DNA, (c) after hybridization of 1 µM solution of InvA (complement) target DNA. All reactions were run in parallel with identical conditions, 5X magnification, and with a 1 ms exposure time.
Figure 47: Cy3 fluorescent images of powdered cellulose allowed to react with cyanuric chloride followed by the attachment of amine-modified stx1 probe and the addition of ethanolamine (a) before hybridization, (b) after hybridization of 1 µM InvA (non-complement) target DNA, (c) after hybridization of 1 µM solution of stx1 (complement) target DNA. All reactions were run in parallel with identical conditions, 5X magnification, and with a 1 ms exposure time.

3.3.5 Reaction of Cellulose with Cyanuric Chloride (B) and Ethanolamine

This protocol, using cyanuric chloride, only differs from the previous protocol in the concentration of the cyanuric chloride solution and the treatment of the cellulose prior to addition of the cyanuric chloride solution. After performing these four reactions and examining the results, the DNA probe demonstrated a high level of specificity when it hybridized to the stx1 and InvA target DNA (Fig 48 & 49). As shown in the Cy3 images, a fluorescent signal was only generated when hybridizing the probe and its complement (Fig 48 & 49). These reactions were only performed one time and should be repeated in the future.

Figure 48: Cy3 fluorescent images of powdered cellulose allowed to react with cyanuric chloride followed by the attachment of amine-modified InvA probe and the addition of ethanolamine (a) before hybridization, (b) after hybridization of 1 µM stx1 (non-complement) target DNA, (c) after hybridization of 1 µM solution of InvA (complement) target DNA. All reactions were run in parallel with identical conditions, 5X magnification, and with a 1 ms exposure time.
Figure 49: Cy3 fluorescent images of powdered cellulose allowed to react with cyanuric chloride followed by the attachment of amine-modified stx1 probe and the addition of ethanolamine (a) before hybridization, (b) after hybridization of 1 µM InvA (non-complement) target DNA, (c) after hybridization of 1 µM solution of stx1 (complement) target DNA. All reactions were run in parallel with identical conditions, 5X magnification, and with a 1 ms exposure time.

3.3.6 Reaction of Cellulose with 2-amino-4,6-dichlorotriazine and Ethanolamine

A high level of specificity was obtained for the use of 2-amino-4,6-dichlorotriazine to attach amine-modified stx1 and InvA probe to cellulose. Each probe was individually hybridized with the stx1 and InvA complement DNA target and examined under the Cy3 microscope. The hybridization of each probe DNA with its complement generated a clear fluorescent signal (Fig 50 & 51). Comparatively, no fluorescence was observed when hybridizing the probe with its non-complementary target DNA (Fig 50 & 51). Due to the high fluorescence generated, a water wash was added to eliminate any non-specific fluorescent signal that was present due to hydrogen bonding. The images taken only contained fluorescent signal generated by complementary base pairing. The use of 2-amino-4,6-dichlorotriazine generated expected results from these four hybridization reactions, indicating the success and specificity of these probes. These reactions were only performed one time and should be repeated in the future.
Figure 50: Cy3 fluorescent images of powdered cellulose allowed to react with 2-amino-4,6-dichlorotriazine followed by the attachment of amine-modified InvA probe and the addition of ethanolamine (a) before hybridization, (b) after hybridization of 1 µM stx1 (non-complement) target DNA, (c) after hybridization of 1 µM solution of InvA (complement) target DNA, (d) after hybridization of 1 µM stx1 target DNA and washing with water, and (e) after hybridization with 1 µM InvA target DNA and washing with water. All reactions were run in parallel with identical conditions, 5X magnification, and with a 1 ms exposure time.

Figure 51: Cy3 fluorescent images of powdered cellulose allowed to react with 2-amino-4,6-dichlorotriazine followed by the attachment of amine-modified stx1 probe and the addition of ethanolamine (a) before hybridization, (b) after hybridization of 1 µM InvA (non-complement) target DNA, (c) after hybridization of 1 µM solution of stx1 (complement) target DNA. (d) after hybridization of 1 µM InvA target DNA and washing with water, and (e) after hybridization with 1 µM stx1 target DNA and washing with water. All reactions were run in parallel with identical conditions, 5X magnification, and with a 1 ms exposure time.

3.3.7 Summary and Future Work

After performing only initial specificity reactions there is not one protocol that suits the overall goal of this project explicitly. Based on the initial evaluation of probe specificity in the reactions, there are three successful protocols that should be investigated further: the reaction of the amine-modified silica gel with NPC, the reaction of cellulose with cyanogen bromide and ethanolamine, and the reaction of cellulose with 2-amino-4,6-dichlorotriazine and ethanolamine. These experiments should first be repeated using the stx1 and InvA probe with the stx1 and InvA target DNA to determine the reproducibility of the initial results for these four specificity hybridization reactions were only performed one time due to time restraints. After repetition of these experiments to determine the consistency of these specificity reactions, minor alterations should be performed. Such changes can include an increase in the concentration of the linker
molecule, a decrease in time to couple the amine-modified oligonucleotide to the activated support, a decrease in hybridization time, etc. Each one of these parameters can be modified in hopes of decreasing the overall time of the experiment so that it can be performed in an Organic Chemistry laboratory class.

Before eliminating the possibility of the reaction of cyanuric chloride with cellulose, the specificity experiments should be repeated. Due to time restraints, these experiments were only performed one time and repetition would conclusively determine if the cyanuric chloride protocols could be successfully modified for future use. Cyanuric chloride and cellulose are the most inexpensive materials used making this an attractive possibility. If the results of this protocol demonstrate that the probe is highly specific then there exists the potential to use this platform to create a DNA microarray capable of analyzing SNPs within the mitochondrial genome.

After reviewing all the protocols and results of the reactions investigated in this thesis, each successful protocol has positive and negative aspects that need to be considered for future directions. Currently, based on initial specificity reactions, the reaction of cellulose with cyanogen bromide has the most immediate promise for several reasons. First, the cost of unmodified cellulose is at a minimum when compared to other solid supports. A potential drawback is that cyanogen bromide is very poisonous and if present in acidic conditions the toxicity factor increases exponentially. Also, only an overnight time period has been investigated to couple the cyanogen bromide-activated cellulose with the amine-modified oligonucleotide. Additional experiments should be performed to determine if such a long coupling period is necessary. Lastly, if this protocol is perfected with the powdered cellulose it would be beneficial
to perform the same reactions on cellulose paper. Not only is this material easier to use but it would eliminate timely centrifugation steps when performing the necessary washing steps.

If the reaction of 3-aminopropyl functionalized silica gel with NPC is to be pursued further it is important to note that this silica gel is more expensive than unmodified solid supports. Additional concerns include the intensity of the signal generated. If this protocol is to be implemented in an undergraduate laboratory then there must be a fluorescent microscope or fluorescent box present to detect the signal generated. The fluorescent signals, generated using the 3-aminopropyl functionalized silica gel, were not as intense as those produced in other experiments. Therefore, further experiments should be carried out to attempt to increase the fluorescence observed.

The use of the 2-amino-4,6-dichlorotriazine allowed for successful coupling of the amine modified oligonucleotide to cellulose and provided a platform for specific hybridization reactions. This material, however, is a derivative of cyanuric chloride, increasing its cost. Since the chemistry of the reaction of 2-amino-4,6-dichlorotriazine is the same for cyanuric chloride, the perfection of the cyanuric chloride protocols would provide a much cheaper experiment to be implemented in an undergraduate laboratory and should be investigated in the future.

The attachment of probe DNA to various solid supports through the use of organic molecules was investigated in this thesis. The success of the coupling of DNA probe to a solid support and the preliminary specificity of the hybridization of this probe to target DNA was evaluated. Additional modifications can be made to improve the fluorescent intensity generated in each experiment for the eventual creation of a DNA microarray. The next step in this project is to perform additional specificity reactions using two probes from the same bacterium. Through the examination of sequences with limited differences, it can be determined if one of the
protocols could be used to differentiate between SNPs for the analysis of the mitochondrial genome for ancestral origin determination. While one protocol has not been determined to be the most ideal for the HHMI grant, the foundation for the creation of a DNA microarray suitable for an Organic Chemistry laboratory and a K-12 classroom has been established.
Chapter 4: Use of DNA Microarrays for Bacterial Identification:

4.1 Introduction to Illumina Inc.

Optical fiber-based arrays have been extensively utilized in the Walt Laboratory to detect a multitude of analytes including, DNA[71], organic vapors[73], and single enzyme molecules[74]. An optical fiber bundle is composed of thousands of individual waveguide cores, hexagonally packed, which share a common cladding[51, 75]. The cladding glass has a lower refractive index than the core glass, which causes light propagating in the core to be totally internally reflected at the interface with the cladding.[51]. After polishing the faces of the bundle, the cores can be etched using hydrofluoric or hydrochloric acid to create wells of a specific depth[76]. When a slurry of oligonucleotide-functionalized silica microspheres (also referred to as beads) is added to the etched fiber end, the microspheres self-assemble into the wells, creating a DNA microarray [75].

The concept of bead-based DNA microarrays has been commercialized by Illumina Inc. into two platforms: the Sentrix® BeadChip™ and the 96-fiber Sentrix Array Matrix™. BeadChips are 2.5 × 8.25 cm silicon substrates[77] in which wells have been etched by standard lithographic techniques (Fig 52) [78]. 3 µm diameter DNA-modified microspheres are then placed in the wells [78]. BeadChips have 16 arrays each containing approximately 50,000 beads. Each array holds 1,520 bead types in approximately 30× redundancy [79].
Figure 52: Sentrix BeadChips are made by etching microwells and placing beads inside the wells.

4.1.1 Microsphere Encoding

The beads, which randomly assemble into the wells, are encoded to identify the specific location and identity of each bead [75, 79]. One encoding protocol involves trapping or attaching dyes to the microspheres. This encoding method is time consuming and limits the number of different bead types [75]. To encode a greater number of bead types, an alternative decoding method has been developed by Gunderson et al. known as sequential hybridization. This method enables as many as 1,520 different bead types to be successfully encoded [79].

To decode and verify the position of each bead type present on the BeadChip, sequential hybridizations are performed using fluorescently labeled decoding oligonucleotides. For example, if there are eight different bead types, 0-7, present on the array, and two different fluorescent labels available, the array could be decoded with three stages of hybridization reactions according to the following equation (Fig 53):

\[ S = \lceil \log_k N \rceil \]

where \( S \) represents the number of stages required, \( k \) corresponds to the number of different fluorescent labels, and \( N \) represents the number of bead types within the array [79]. This exponential decoding process allows many different bead types to be decoded with relatively few
colors and stages [79]. For example, four fluorescent labels and six hybridization stages can be used to decode 4,096 different bead types ($4^6$) [79].

To decode an array with eight different bead types using two fluorescent dyes, three hybridization reactions are required (Fig 53). The hybridization reactions are performed by pooling complementary strands for each bead type and incubating the array in these pools. First, complementary strands for each bead type are created with both fluorescent dyes (i.e. red and green), for a total of 16 different decoding oligonucleotides (8 red and 8 green). Three pools, corresponding to three decoding stages, are then created from these 16 solutions, as shown in figure 10c. An initial reaction is performed hybridizing the red-labeled complements, specific for half the bead types present, and the green-labeled complements, specific to the other four bead types. This process generates a red and green fluorescent pattern that separates the eight bead types into two groups. All the labeled oligonucleotides are then dehybridized. Subsequent hybridizations divide each group of the $n$ bead types into $n/2$. After three sequential hybridization stages, each bead type has a unique three digit binary barcode [79].
Figure 53: Scheme of the decoding process of an Illumina Inc. Sentrix BeadChip involving eight different bead types. (A) The hybridization steps necessary to identify the location of each bead type on the array. (B) Image of the fluorescent signals generated by each hybridization reaction. (C) Decoding fluorescent results to produce a unique binary three-digit barcode for all eight bead types present [79].

4.1.2. Sample Preparation and Data Analysis Using Illumina Inc. Systems

In this thesis, the Illumina Direct Hybridization Assay™ was used to genotype *Listeria monocytogenes*. This platform involves the hybridization of a particular sample directly to the gene-specific probes present on the BeadChip (Fig 54). Prior to performing the hybridization reactions on the BeadChip, the sample DNA must first be amplified through PCR with primers designed specifically for the loci of interest (listed in Appendix I). One of the primers used for the PCR is labeled for hybridization detection. Biotinylated primers are incorporated into the strand complementary to the BeadChip probes and subsequently stained with streptavidin-bound Cy3. The PCR reaction mixture is combined with a specified volume of Illumina Hyb E1 buffer
and applied to one array on the BeadChip. The sample is then allowed to hybridize and the array is washed with the appropriate reagents.

Figure 54: a) Simplified schematic diagram of an individual silica microsphere with one probe attached. The address portion (red) is used to encode the bead [80]. (b) Picture of Sentrix BeadChip 16 x 1 used in the Direct Hybridization Assay.

To analyze the hybridizations that occur when using a BeadChip array, a scanning laser confocal imaging system, known as the Sherlock scanner, is used[81]. This scanner, also called the BeadArray Reader, has a resolution of 0.8 µm and is highly sensitive with a low limit of detection[29, 81]. The BeadArray Reader scans the BeadChip at two wavelengths, 550 and 630 nm, and uses bead map files, which were created when the array was decoded by Illumina Inc. [29]. For the BeadChips utilized in this thesis, only the Cy3 optical channel (550 nm) was used. For typical gene expression or genotyping studies, BeadStudio software extracts raw data and generates reports based on control beads that are on each array.

4.2 Use of Microarrays to Genotype Listeria Monocytogenes

Illumina Inc. technology has been used extensively to genotype and analyze SNPs [82-86]. While previous reports have detailed successful genotyping of viral pathogens[82], this thesis focuses on the potential of Illumina Inc. Sentrix BeadChips to be used in a direct hybridization assay to genotype the bacterial pathogen Listeria monocytogenes. This bacterium,
the only species from *Listeria* spp. that is pathogenic to humans, is often transferred through contaminated food and can cause a wide range of complications within the human body[87] such as intra-uterine infection, meningitis, and septicaemia[88]. Other pre- and post-natal effects of this bacterium include severe systemic infection in a fetus or newborn [88] and can cause an increase in abortive fetuses in the third trimester of pregnancy [87]. The direct hybridization assay uses beads having probes, 22 or 30 bases in length, specifically designed for *Listeria monocytogenes* (listed in Appendix II).

The Illumina Inc. platform was used to genotype 28 strains of *Listeria monocytogenes* and four strains of non-pathogenic *Listeria* spp. The four strains of non-pathogenic *Listeria* spp. were included to determine if this platform can differentiate between non-pathogenic and pathogenic strains [89]. Oligonucleotide probes were designed focusing on highly polymorphic regions of 20 loci to maximize differentiation of each strain. My goal is to generate a binary yes/no response scheme for each probe, analogous to the array-based binary analysis for *Escherichia coli* reported by Shepard et al.[25]. Due to the severity of *Listeria monocytogenes* as a human pathogen, differentiation is important in food safety and clinical practice[89]. Using microarray technology from Illumina Inc., such differentiation should be possible. Thus, the practical application of this project is to develop a method to rapidly identify pathogens, such as *Listeria monocytogenes*, in hopes of limiting exposure and preventing harm.

4.3 Materials

Sentrix BeadChip and accompanying CD, BeadChip hyb cartridge, Hybex® Microarray Incubation System Heating Base, (SciGene), BeadChip wash rack, glass wash dishes, tweezers, Illumina® BeadArray Reader GX, Illumina® hybridization oven, full-scale plus thermometer,
Illumina® BeadStudio application, BeadChip deep well wash tray, BeadChip deep well wash cover, BeadChip coverseals, Hyb E1 buffer, wash E1 buffer, block E1 buffer were all supplied by Illumina, Inc. (San Diego, CA). Ethanol (200 proof) was obtained from Sigma Aldrich. Streptavidin-Cy3 was purchased from Zymed Laboratories, Inc. (San Francisco, CA). RNase-free water was purchased from Promega (Madison, WI). PCR products were amplified from *Listeria monocytogenes* with primers listed in Appendix I and were provided by the lab of Yechezkel Kashi in the Department of Biotechnology & Food Engineering, Techion- Israel Institute of Technology (Haifa, Israel). Millipore 18 MΩ water was used for the washing steps and obtained from a Millipore Gradient A10 (Billerica, MA).

### 4.4 Methods

A wash solution of E1BC was made by diluting 2.25 mL E1BC buffer in 750 mL water. A solution of 2 mL E1 buffer and 2 µL streptavidin was made and stored in the dark. PCR was performed on all 32 strains of *Listeria monocytogenes* at all 20 loci of interest. 10 µL of every PCR product from one strain were combine and thoroughly vortexed. 13.3 µL of a Hyb E1-water stock solution (147 µL Hyb E1 buffer and 88 µL RNase-free water) was combined with 6.7 µL of the PCR mixture. Reaction volumes were heated to 90°C for 5 minutes and snap cooled by placing on ice. The BeadChip was removed from its packaging and secured in a hyb cartridge. A coverseal was introduced into the slot of the coverseal alignment plate. Using tweezers, the adhesive release liner over the BeadChip was removed and 20 µL assay samples were pipetted onto each of the 16 arrays on the BeadChip. The coverseal alignment plate was secured to the hyb cartridge. The assembly was shaken vigorously, making sure that the air bubbles trapped within the hyb cartridge moved in all directions. The hyb cartridge was placed on the BeadChip
hyb wheel in the Illumina hybridization oven and rotated for one hour at 45°C. The BeadChip was then removed from the oven. The Sentrix coverseal was removed and the BeadChip was inserted into a wash rack and placed in a staining dish containing 250 mL of the wash E1BC solution. The staining dish was agitated on a rocking rack for 5 min. The rack was then transferred to a new staining dish containing 250 mL of 100% ethanol and placed on the rocking rack for 5 min. Following this procedure, the rack was transferred to a staining dish contain 250 mL of fresh E1BC solution and placed on the rocking rack for 5 min. Then 4 mL of Block E1 buffer was pipetted into a Wash Tray and the BeadChip was placed faced up in the tray. The tray was placed on the rocking rack for 5 min. The BeadChip was then positioned face up in a wash tray containing the solution of block E1 buffer and Cy3-strepaavidin and placed on the rocking rack for 10 minutes. The rack was transferred to a staining dish contain 250 mL of fresh E1BC solution and placed on the rocking rack for five min. The BeadChip was removed from the rack and centrifuged at 275 rcf for four min. The chips were then scanned using the BeadArray Reader and raw fluorescence intensities were extracted using the BeadStudio application.

4.5 Results and Discussion for *Listeria Monocytogenes* Genotyping

The Illumina Direct Hybridization Assay™ was used to investigate nucleotide variation within different loci of closely related strains of *Listeria monocytogenes*. Preliminary bacterial strain typing was previously reported using PCR methodology [89]. Probes for each strain were designed based on the DNA sequences generated through PCR by the appropriate primers (listed in Appendix I). All loci investigated contained at least one nucleotide variation in the DNA sequence to differentiate between each strain.
The binary responses from the probe sequences on the array to different strains of bacterial provide a ‘barcode’, which is unique for each strain. Initial hybridization reactions generated such a barcode for all strains investigated (Appendix III). Comparison of these experimental barcodes to the predicted barcodes from the strains tested, revealed that the Direct Hybridization Assay™ did not generate the predicted hybridization responses (Fig 55). While the expected hybridization results were not obtained in the initial experiments, a unique pattern of hybridization intensities for each strain was measured (Fig 56 & 57). Since each strain had its own corresponding barcode, the measured barcodes should still be useful for bacterial typing, even if they differ from the theoretical results. A barcode and a phylogenic tree were created based on the hybridization results from various probes (Appendix III & VII). The phylogenic tree is a visual representation of the differences between the Listeria monocytogenes strains.
**Figure 55:** Fluorescent intensities generated by the hybridization of *Listeria monocytogenes* strain Lm14-1/2a with 54 probes using the Direct Hybridization Assay™. Dashed pink line at 166 a.u. represents the hybridization threshold for the reaction based on the background and three times the standard deviation. Color representation: green indicates expected successful hybridization, red represents unexpected successful hybridization, and blue indicates expected unsuccessful hybridization results when comparing the sequence of strain Lm14-1/2a with the probes on the BeadChip.

**Figure 56:** Comparison of fluorescent intensities obtained from the hybridization of three strains, Lm14-1/2a, Lm10 and Lm19/1-1/2a, of *Listeria monocytogenes* with probes present on the BeadChip.
The Direct Hybridization Assay™ was repeated and the barcodes generated did not correspond with those from the first experiment. This result was determined by comparing the positive hybridizations of both experiments and examining where the two assays were consistent and inconsistent (Appendix VIII). These irreproducible results nullify the barcode and phylogenic tree created using the data from the first experiment and eliminate the Direct Hybridization Assay™ as a possible method to genotype *Listeria monocytogenes*.

Recently, attempts to genotype *Listeria monocytogenes* have been investigated with greater success using the Illumina GoldenGate Assay™, an extension ligation reaction with allele specific oligonucleotide primers, designed for SNP detection [90].
Appendix

Appendix I: Forward and reverse primers for each loci of *Listeria monocytogenes* examined.

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<th>Locus</th>
<th>Accession no</th>
<th>Forward primer 5’ - 3’</th>
<th>Reverse primer 5’ - 3’</th>
<th>Tm (°C)</th>
<th>Length (bp)</th>
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<td>ACGATTAATAATACCAACC</td>
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<td>CTCAAACTATTTGGAAAAA</td>
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Appendix II: Probes specific to *Listeria monocytogenes* present on the Sentrix BeadChip and used in the Direct Hybridization Assay™.

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<th>Length (bp)</th>
<th>Tm (°C)</th>
<th>Probe</th>
<th>Sequence</th>
<th>Location</th>
<th>Length (bp)</th>
<th>Tm (°C)</th>
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Appendix III: Barcode created after hybridizing DNA of 33 strains of *Listeria monocytogenes* with probes containing SNPs. 

| Strain   | Lm15/1-1a | Lm11/1-1b | Lm54-1b | Lm17-4c | Lm11 | Lm8/1-4b | Lm8-1b | WH019/3-3b | WH017/3-3b | WH016-4b | WH016-3a | WH016-3c | WH085/3-3c | WH015-1c | Lm14-11a | L.Seelegi | Limcpoua | L.Kanovit | WH088-3a | Lm10 | Lm19/4-4a | Lm16-11a | L.Wishimeri |
|----------|-----------|-----------|---------|---------|------|----------|--------|-------------|-------------|----------|---------|---------|-------------|-------|---------|----------|--------|----------|---------|-----------|
| attn2-S  | 0         | 0         | 0       | 0       | 0    | 0        | 0      | 0           | 0           | 0        | 0       | 0       | 0           | 0     | 0       | 0        | 0      | 0        | 0       | 0         |
| betL-1-S | 0         | 0         | 0       | 0       | 0    | 1        | 1      | 1           | 1           | 1        | 1       | 1       | 1           | 1     | 1       | 1        | 1      | 1        | 1       | 1         |
| betL-2-S | 0         | 0         | 0       | 0       | 0    | 0        | 0      | 0           | 0           | 0        | 0       | 0       | 0           | 0     | 0       | 0        | 0      | 0        | 0       | 0         |
| cheR-1-S | 0         | 0         | 0       | 0       | 0    | 0        | 0      | 1           | 0           | 0        | 0       | 0       | 0           | 0     | 0       | 0        | 0      | 0        | 0       | 0         |
| cheR-2-S | 1         | 1         | 1       | 1       | 0    | 1        | 1      | 1           | 1           | 1        | 1       | 1       | 1           | 1     | 1       | 1        | 1      | 1        | 1       | 1         |
| clpE-1-S | 0         | 0         | 0       | 0       | 0    | 0        | 0      | 0           | 0           | 0        | 0       | 0       | 0           | 0     | 0       | 0        | 0      | 0        | 0       | 0         |
| clpE-2-S | 1         | 1         | 1       | 1       | 1    | 1        | 1      | 1           | 1           | 0        | 1       | 1       | 1           | 1     | 1       | 1        | 1      | 1        | 1       | 1         |
| clpE-3-S | 1         | 1         | 1       | 1       | 1    | 0        | 1      | 0           | 0           | 1        | 1       | 0       | 0           | 0     | 0       | 0        | 0      | 0        | 0       | 0         |
| fhuB-1-S | 0         | 0         | 0       | 0       | 0    | 0        | 0      | 0           | 0           | 0        | 0       | 0       | 0           | 0     | 0       | 0        | 0      | 0        | 0       | 0         |
| fhuB-2-S | 0         | 0         | 0       | 0       | 0    | 0        | 0      | 0           | 0           | 0        | 0       | 0       | 0           | 0     | 0       | 0        | 0      | 0        | 0       | 0         |
| fhuB-3-S | 1         | 1         | 1       | 1       | 1    | 0        | 1      | 0           | 0           | 1        | 1       | 1       | 1           | 1     | 1       | 1        | 1      | 1        | 1       | 1         |
| gid-1-S  | 0         | 0         | 0       | 0       | 0    | 0        | 0      | 0           | 0           | 1        | 1       | 1       | 0           | 1     | 1       | 1        | 1      | 1        | 1       | 1         |
| gid-2-S  | 0         | 1         | 0       | 1       | 0    | 1        | 1      | 0           | 0           | 1        | 1       | 1       | 1           | 1     | 1       | 1        | 1      | 1        | 1       | 1         |
| gid-3-S  | 1         | 1         | 1       | 1       | 0    | 1        | 1      | 1           | 0           | 0        | 1       | 0       | 0           | 0     | 0       | 0        | 0      | 1        | 1       | 0         |
| gid-4-S  | 0         | 0         | 0       | 0       | 0    | 0        | 0      | 0           | 0           | 1        | 1       | 0       | 0           | 0     | 0       | 0        | 0      | 1        | 1       | 0         |
| lisR-1-S | 0         | 0         | 0       | 0       | 0    | 0        | 0      | 0           | 0           | 0        | 0       | 0       | 0           | 0     | 0       | 0        | 0      | 0        | 0       | 0         |
| lisR-2-S | 0         | 0         | 0       | 0       | 0    | 0        | 0      | 0           | 0           | 0        | 0       | 0       | 0           | 0     | 0       | 0        | 0      | 0        | 0       | 0         |
| lisR-3-S | 0         | 0         | 0       | 0       | 0    | 0        | 0      | 0           | 0           | 0        | 0       | 0       | 0           | 0     | 0       | 0        | 0      | 0        | 0       | 0         |
| lmo0023-1-S | 1   | 1         | 1       | 1       | 0    | 0        | 1      | 0           | 0           | 0        | 0       | 0       | 0           | 0     | 0       | 1        | 1      | 0        | 1       | 0         |
| lmo0023-2-S | 0   | 0         | 0       | 1       | 0    | 0        | 0      | 0           | 0           | 1        | 0       | 0       | 0           | 0     | 0       | 0        | 0      | 0        | 0       | 0         |
| lmo0023-3-S | 0   | 0         | 0       | 0       | 0    | 0        | 0      | 0           | 0           | 0        | 0       | 0       | 0           | 0     | 0       | 0        | 0      | 0        | 0       | 0         |
| lmo0023-4-S | 0   | 0         | 0       | 0       | 1    | 0        | 0      | 0           | 0           | 0        | 0       | 0       | 0           | 0     | 0       | 0        | 0      | 0        | 0       | 0         |
| lmo0042-1-S | 1 | 1         | 0       | 1       | 0    | 0        | 1      | 1           | 1           | 1        | 1       | 1       | 1           | 1     | 1       | 1        | 1      | 1        | 1       | 1         |
| lmo0042-2-S | 1 | 1         | 0       | 1       | 0    | 0        | 0      | 1           | 1           | 1        | 1       | 1       | 1           | 1     | 1       | 1        | 1      | 1        | 1       | 1         |
| lmo0075-1-S | 0 | 0         | 0       | 1       | 0    | 0        | 0      | 0           | 0           | 0        | 0       | 0       | 0           | 0     | 0       | 0        | 0      | 0        | 0       | 0         |
| lmo0075-2-S | 0 | 0         | 0       | 0       | 0    | 0        | 0      | 0           | 0           | 0        | 0       | 0       | 0           | 0     | 0       | 0        | 0      | 0        | 0       | 0         |
| lmo0075-3-S | 0 | 0         | 0       | 0       | 0    | 0        | 0      | 0           | 0           | 0        | 0       | 0       | 0           | 0     | 0       | 0        | 0      | 0        | 0       | 0         |
| lmo0176-1-S | 0 | 0         | 0       | 0       | 0    | 0        | 0      | 0           | 0           | 0        | 0       | 0       | 0           | 0     | 0       | 0        | 0      | 0        | 0       | 0         |
| Lm15/1-1a | 0         | 0         | 0       | 0       | 0    | 0        | 0      | 0           | 0           | 0        | 0       | 0       | 0           | 0     | 0       | 0        | 0      | 0        | 0       | 0         |
| Lm0176-2-S | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 |
| Lm0176-3-S | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Lm0176-4-S | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| Lm0176-5-S | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 |
| Lm0196-1-S | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| Lm0196-2-S | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| Lm0672-1-S | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 |
| Lm0672-2-S | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| Lm0672-3-S | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| Lm0672-4-S | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| Lm0672-5-S | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Lm0672-6-S | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Lm0841-1-S | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| Lm0841-2-S | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 |
| Lm0841-3-S | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Lm0841-4-S | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| Lm1982-1-S | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| Lm1982-2-S | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| Lm2142-1-S | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| Lm2142-2-S | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| Lm2560-1-S | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 |
| Lm2560-2-S | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| Lm2560-3-S | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| Lm2560-4-S | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 |
| motB-1-S | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| motB-2-S | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| motB-3-S | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unkn-1-S | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

*a* Direct Hybridization Assay™ results from 56 sites for 32 strains of *Listeria monocytogenes*. Numbers represent no successful hybridization (0) and successful hybridization (1).
Appendix VII: The phylogenetic tree using the binary hybridization response of the strains of *Listeria monocytogenes* hybridized with specific probes, to differentiate between strains.
Appendix VII: Summary of the binary hybridization results obtained using the Direct Hybridization Assay™ after repeating the hybridization reactions of the strains of *Listeria monocytogenes*.\(^b\)

\(^b\) Color representation: green indicates successful after the first hybridization trial, pink represents successful hybridization after the second hybridization trial, blue indicates successful hybridization results for both trials, and white represents unsuccessful hybridization for both hybridization reactions complete.
References


90. Dr. Kathryn Brogan; Manuscript in Progress.