Georgia State University
Digital Archive @ GSU

Chemistry Theses | Department of Chemistry

4-13-2010

Development of a Novel DNA Microchip for Pathogen Detection

Khin Lay Maw
Georgia State University, khinlmaw@gmail.com

Follow this and additional works at: http://digitalarchive.gsu.edu/chemistry_theses

Recommended Citation
Maw, Khin Lay, "Development of a Novel DNA Microchip for Pathogen Detection" (2010). *Chemistry Theses.* Paper 34.

This Thesis is brought to you for free and open access by the Department of Chemistry at Digital Archive @ GSU. It has been accepted for inclusion in Chemistry Theses by an authorized administrator of Digital Archive @ GSU. For more information, please contact digitalarchive@gsu.edu.

DEVELOPMENT OF A NOVEL DNA MICROCHIP FOR PATHOGEN DETECTION

by

KHIN LAY MAW

Under the direction of Dr. Zhen Huang

ABSTRACT

Although DNA microarray can detect multiple DNA samples simultaneously, current detection techniques involve PCR and other traditional procedures. In this study, a sensitive, specific and rapid detection method, which eliminates PCR and other lengthy processes, for pathogenic DNA is presented. This technology is based on the hybridization of target DNA to the immobilized probe, extension of probe DNAs using the target-DNA as a template and signal generation by streptavidin-horseradish peroxidase and substrate. This method is highly specific and sensitive, allowing single-nucleotide-base mismatches discrimination and the detection at femtomole level. The experiments are designed to achieve short hybridization time. Therefore, satisfactory signal can be detected within minutes, allowing the rapid detection of multiple pathogenic DNA. Most importantly, the *E. coli* genomic DNA can be detected using this technology. In conclusion, this detection method is useful for applications including on-site pathogenic disease detection, crime scene investigation, and pathogen inspection in the environment.

INDEX WORDS: DNA microchip, DNA microarray, DNA chemiluminescent detection, Pathogenic DNA detection, Genomic DNA detection.

DEVELOPMENT OF A NOVEL DNA MICROCHIP FOR PATHOGEN DETECTION

by

KHIN LAY MAW

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

in the College of Arts and Sciences

Georgia State University

2010

Copyright by

Khin Lay Maw

2010

DEVELOPMENT OF A NOVEL DNA MICROCHIP FOR PATHOGEN DETECTION

by

KHIN LAY MAW

Committee Chair: Dr. Zhen Huang

Committee: Dr. Markus Germann

Dr. Gangli Wang

Electronic Version Approved:

Office of Graduate Studies

College of Arts and Sciences

Georgia State University

May 2010

DEDICATION

I would like to dedicate this work to someone I believe in most. Without Him and His blessings, I would not be able to achieve what I have achieved so far in my life. I am thankful for the vision, knowledge, talents, success and the people that He has blessed me with. I would also like to thank my family and love for their kindness, unconditional love and support during my studies and throughout my life. I would especially like to thank my parents for the encouragement and guidance to help me achieve my goal. It would not have been possible without their blessings. Lastly, I would like to thank all other family members, teachers and true friends, who have helped me in some way or the other to be who I am today.

ACKNOWLEDGEMENTS

I would like to acknowledge Dr. Zhen Huang, my thesis advisor and committee chair, for his support, guidance, encouragement and passing on his knowledge to me. In addition, I would like to thank the two thesis committee members, Dr. Markus Germann and Dr. Gangli Wang, for their support, suggestions, and guidance. Also, I would like to thank all the lab members for being very supportive, friendly and helpful. Moreover, I would like to thank all the faculty members from Georgia State University, for their teaching and supervision, which have helped me obtain this degree. Furthermore, I would like to acknowledge the Biotechnology Scholars Program of the Department of Biology and the Graduate Assistantship Program of the Department of Chemistry (Georgia State University), the National Institutes of Health (A1058051) and the Georgia Cancer Coalition Distinguished Cancer Clinicians & Scientist Award for their financial support.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS v

LIST OF FIGURES ix

1 INTRODUCTION 1

1.1 DNA Microarray and Microchip Technology 1

1.2 Basic Principles and Techniques 4

1.2.1 Streptavidin-biotin Technology 4

1.2.2 SuperSignal ELISA Femto Maximum Sensitivity Substrate 5

1.3 Current Methods of Pathogen Detection 6

1.4 Objective of This Study 7

2 MATERIALS AND METHODS 9

2.1 Oligonucleotides 9

2.2 Other Materials 11

2.2.1 Reagents and DNA Samples 11

2.2.2 Buffers 11

2.2.3 Kits 12

2.2.4 Instruments 12

2.3 Oligonucleotides Synthesis, Deprotection and Precipitation 12

2.4 Microchip Activation 13

2.5 Probe Immobilization 14

2.6 Target Hybridization 14

2.6.1 Detection Using the Synthesized *lacZ E. coli* Target DNA 14

2.6.2 Detection of the *E. coli* Genomic Target DNA 15

2.6.3 Specificity in DNA Detection: Mismatched Target DNA Sequences 15

2.6.4 Specificity in DNA Detection: Different DNA Probes Sequences 15

2.6.5 Sensitivity in DNA Detection: 15mer with 24mer-1 16

2.6.6 Combination of Specificity and Sensitivity Detection 16

2.6.7 *E. coli* Genomic Target DNA Doped with Synthesized Target DNA 16

2.7 Klenow Extension, Biotin-labeling, and Chemiluminescent Detection 17

2.8 DNA Hybridization Kinetics 18

2.9 Multiple Pathogenic DNA Detection 19

3 DNA CHEMILUMINESCENCE DETECTION 21

3.1 Overview of DNA Detection 21

3.2 Processes Involved in DNA Detection 23

3.2.1 Microchip Activation 23

3.2.2 Immobilization of DNA Probes 23

3.2.3 Target Hybridization 24

3.2.4 Klenow Extension, Biotin- labeling, TSA Amplification and Detection 24

4 SPECIFICITY IN DNA DETECTION 26

4.1 Introduction 26

4.2 Results 26

4.2.1 Specificity in Detection: Four Different Probes 26

4.2.2 Specificity in Detection: Mismatched Sequences of 24mer-1 27

4.2.3 Specificity in Detection: Multiple Pathogenic DNA Detection 27

4.3 Conclusion 29

5 SENSITIVITY IN DNA DETECTION 30
5.1 Introduction 30
5.2 Results 30
5.2.1 Detection Limit of the Synthesized *E. coli* Target DNA 30
5.2.2 Combination of Specificity and Sensitivity in DNA Detection 31
5.3 Conclusion 31
6 DNA HYBRIDIZATION KINETICS 32
6.1 Introduction 32
6.2 Results 33
6.2.1 DNA Hybridization Kinetics Curve 33
6.3 Conclusion 33
7 DETECTION OF GENOMIC DNA SAMPLES 34
7.1 Introduction 34
7.2 Results and Discussion 35
7.2.1 Detection of the *E. coli* K-12 Strain Genomic DNA 35
7.3 Conclusion 35
8 Doping of Genomic DNA with Synthesized Target DNA 36
8.1 Introduction 36
8.2 Results and Discussion 36
8.2.1 Doping of *E. coli* Genomic DNA with 24mer-1 36
8.3 Conclusion 37
BIBLIOGRAPHY 38

LIST OF FIGURES

Figure 1: Chemical structure of biotin. 5

Figure 2: Microchip activation and probe immobilization. 14

Figure 3: DNA chemiluminescent detection overview. Image A: Immobilization of DNA probes; Image B: Hybridization of the target DNA to the immobilized probes; Image C: Klenow extension using biotin-14-dATP; Image D: Poly-HRP-streptavidin binding to biotin and substrate to produce energy in a form of light; Image E: Virtual image of signal production overview; Image F: Detection using a microscope and CCD camera; Image G: Computer-generated, detectable chemiluminescent signal. 22

Figure 4: Probe immobilization on the activated silicon surface..... 24

Figure 5: The virtual microchip surface during the DNA chemiluminescent detection..... 25

Figure 6: The virtual microchip with immobilized DNA probes (Image A), and the virtual microchip when the chemiluminescent signal is detected (Image B)..... 25

Figure 7: The specificity of 24mer-1 (5 fmol) to 15mer. Image 1: 5XSSC buffer; Image 2: 15mer; Image 3: DNA-RNA-DNA chimeric probe of rRNA *E. coli* (26mer-P), Image 4: Bird flu DNA-RNA-DNA chimeric probe. (Detection conditions: 5 sec exposure, 2×2 binning, 2X lens) 27

Figure 8: The specificity of probe DNA (15mer) to the target DNA (mismatched sequences of 24mer, 50 fmol). Image 1A, 1B, and 1C: 24mer-1; Image 2: 24mer-2; Image 3: 24mer-3; Image 4: 24mer-4; Image 5: 24mer-5; Image 6: 24mer-6; Image 7: 24mer-7; Image 8: 24mer-8. (Detection conditions: 5 sec exposure, 2×2 binning, 2X lens)..... 27

Figure 9: Multiple Pathogen Detection. Four different probes DNA were spotted on the chips (from top down): *Bacillus anthraces* (BA-P), Sample 1 (S1-P), Sample 2 (S2-P), and

15mer. Then, target DNA (5 pmol) is applied to observe specificity. Image A: *Bacillus anthraces* target DNA (BA-T); Image B: Sample 1 target DNA (S1-T); Image C: 24mer-1; Image D: *Bacillus anthraces* target DNA (BA-T) and Sample 2 target DNA (S2-T); Image E: Sample 1 target DNA (S1-T) and 24mer-1; Image F: *Bacillus anthraces* target DNA (BA-T), Sample 1 target DNA (S1-T) and 24mer-1. (Detection conditions: 5 sec exposure, 2×2 binning, 2X lens) 28

Figure 10: Synthesized target DNA detection at different absolute quantities. Image 1: 5 pmol; Image 2: 50 fmol; Image 3: 10 fmol; Image 4: 5 fmol; Image 5: 3 fmol ; Image 6 : 2 fmol ; Image 7 : 1 fmol ; Image 8: 0 fmol. (Detection conditions: 15 sec exposure, 2×2 binning, 2X lens) 31

Figure 11: Combination of specificity and sensitivity experiments. Image A: 5XSSC buffer, Image B: Bird flu DNA-RNA-DNA chimeric probe; Image C: 26mer-P; Image D: 15mer. Absolute quantity of the target DNA (24mer-1) is 2 fmol. (Detection conditions: 5 sec exposure, 2×2 binning, 2X lens) 31

Figure 12: DNA hybridization kinetics of the interaction of the target DNA (Biotin-24mer-1: 5'-biotin-ATGTGGATTGGCGATAAAAAACAA-3') and the probe DNA (15mer: 5'-NH_2-TTGTTTTTTATCGCC-3'). 33

Figure 13: Detection limit of the *E. coli* K-12 strain using chemiluminescent detection method. Image 1: 10 fmol 24mer-1; Image 2: 10 μg *E. coli* genomic DNA; Image 3: 5 μg *E. coli* genomic DNA; Image 4: 3 μg *E. coli* genomic DNA; Image 5: RNase-free water. (Detection conditions: 15 sec exposure, 2×2 binning, 2X lens) 35

Figure 14: Doping of *E. coli* genomic DNA with 24mer-1. Image A: 0.5 μg *E. coli* genomic DNA and 10 fmol 24mer-1; Image B: 10 fmol 24mer-1; Image C: 0.5 μg *E. coli*

genomic DNA and 5 fmol 24mer-1; Image D: 5 fmol 24mer-1; Image E: 0.5 µg *E. coli* genomic DNA and 2 fmol 24mer-1; Image F: 2 fmol 24mer-1; Image G: 0.5 µg *E. coli* genomic DNA; Image H: RNase-free water. (Detection conditions: 5 sec exposure, 2×2 binning, 2X lens) 37

1 INTRODUCTION

1.1 DNA Microarray and Microchip Technology

DNA microarray is a recent technology that allows the detection of multiple DNA samples as well as multiple pathogens from the same sample. [1]Microarray technology has been advanced from radiolabeled, two- and three- dimensional and suspension bead microarrays, to qualitative and quantitative microarrays that produce gene expression and disease diagnostic results. [1, 2] According to Miller et al., microarray technology and its use in detection of pathogenic diseases, has been growing vastly from the year 2000 to 2008. [1]

Microarray is a common and effective technique for the medical field as well as the scientific field because it allows the detection of pathogens, inspection of microorganism strains in the environment [3], diagnosis of diseases, discovery of genes [4]and analysis of the single-nucleotide polymorphism(SNP). Genotyping of SNPs is important in disease studies. [5] Genetic disorders, pathogenic bacteria and infectious diseases can also be detected using DNA microarrays. [6] Recently, there have been many flu outbreaks including swine flu, seasonal flu and avian influenza. [7, 8] This is due to the fact that diseases spread easily from one continent to the other, and it is necessary that detection must be done rapidly[9] and the disease can be detected on-site to slow down the spreading. Furthermore, it is essential to have a detection method that selective, sensitive and cost-effective. [9] DNA microarray is a good method for these reasons.

In a DNA microarray, certain concentration of DNA can be spotted manually or robotically. Generally, in a microarray experiment, RNA is extracted from a biological sample using certain buffers and reagents. This RNA is then copied as a cDNA using the reverse transcriptase, and normally, to multiply the DNA, polymerase chain reaction (PCR) is performed. [9, 10]

To further carry out a microarray experiment, the double-stranded DNA (dsDNA) or synthesized long oligonucleotides with certain concentrations are printed on a microchip. The microchip serves as a solid support and is activated with chemical groups prior to the printing of the probes. The immobilization process of the probe to the chip occurs when the chip coated with certain chemicals react with what is labeled on the 5' or 3' end of the probe DNA. [11, 12] There are many steps involved in these DNA microarray experiments. Because many steps are required before the detection process, detection on-site with the collected samples, takes longer and less-efficient. [1, 9, 13, 14] In this study, a novel method for detection of pathogenic DNA using DNA chemiluminescent microarray is established. This method is not only efficient, rapid and cost-effective, but also, it is simple, specific and sensitive.

Today, there exist many types of DNA microarrays such as printed microarrays and electronic microarrays. Printed microarrays are relatively inexpensive and the glass chips are normally used in printed microarrays due to their stability, inexpensiveness and nonporous support. [1] In some studies, the microchips are three- dimensional microchannel, flow-thru chips and it was observed that the sensitivity is much higher compared to glass chips. [11, 15, 16] However, this study introduces DNA microarray using silicon microchips. Silicon microchips are easy to handle, strong, binds to the probes efficiently, inexpensive, stable at high temperatures, produce low background noise, and high signal intensity. [11] Thus, silicon microchips are useful in the DNA chemiluminescent detection. [10, 17]

Current pathogen detection using microarray is amplified using the polymerase chain reaction (PCR) [1, 9, 13, 14] and also, use either dsDNA or long single-stranded DNA (ssDNA) to immobilize on the chip. [1] This slows down the hybridization kinetics of the probe and the target DNA since the duplex probe DNA needs to be denatured first to bind to the target DNA. In this study, the

probes do not need to be denatured or amplified. They bind efficiently to the silicon chip surface since the probes are modified at the 5' end with an amino group. Also, the probes are short ssDNA and allow straightforward hybridization either with the single-stranded target DNA or the denatured, double-stranded target DNA.

Another advantage of this DNA chemiluminescent microarray technology is, it eliminates the reverse transcription, PCR [1, 9, 13, 14] and transcription altogether. Some methods require extracting the mRNA from cells, and then, transforming the mRNA into cDNA to perform the detection. [18, 19] The single-stranded probe DNA in this study is a short, synthesized oligonucleotide that can bind to the target DNA, which can be directly extracted from the bacterial culture or biological samples. Moreover, because these probes are short, they also show a great selectivity. [1, 3]

In addition, this technology eliminates laser excitation fluorescent or radioactive labeling and gel electrophoresis. It only uses the chemiluminescent signal produced via the substrate catalyzed by horseradish peroxidase (HRP). Moreover, in this method, antibodies are not needed unlike other detection methods. [20] Instead, biotinylated-dNTPs are used to bind to the target DNA via hydrogen bonding and Poly-HRP-streptavidin via non-covalent bonding.

In conclusion, a novel method to detect pathogenic DNA, which eliminates lengthy procedures, and is sensitive and highly specific, will be established. In addition, it is also cost-effective and rapid. Thus, this method will make the disease detection much easier and will be useful for many other applications.

1.2 Basic Principles and Techniques

1.2.1 Streptavidin-biotin Technology

Streptavidin is a protein found in the bacterium *Streptomyces avidinii* and consists of 159 residues. [21] These residues are folded to form four identical subunits known as tetramer. Each subunit is composed of a beta barrel formed by eight beta strands. [22, 23] All four of these subunits have identical biotin-binding sites with the same strong affinity for biotin. [21-23] Recently, many researchers have designed to block the three biotin-binding sites of streptavidin to have a specific binding to one site. This type of streptavidin is known as monovalent streptavidin.[23] Some other modifications can also be done to the tetrameric protein to achieve more stability and durability in certain chemical environments. [12, 23]

Biotin is a vitamin known as vitamin H, and it also acts as a coenzyme or cofactor in many enzymatic and metabolic reactions including the citric cycle. Biotin is also water-soluble. [12, 24, 25] As shown in Figure 1, biotin has a valeric side chain, which is useful in linking with other molecules. Since biotin is a small molecule, for some application, it is convenient to link it with different molecules including proteins and nucleotides. When a biotin is linked with another molecule, the process is called biotinylation. Oligonucleotides are well known to link with biotin. In this study, different biotinylated-dNTPs will be used to show the selectivity in the DNA microchip detection. In other words, all types of biotinylated-dNTPs can be used in the detection of pathogenic DNA. [12, 24, 25]

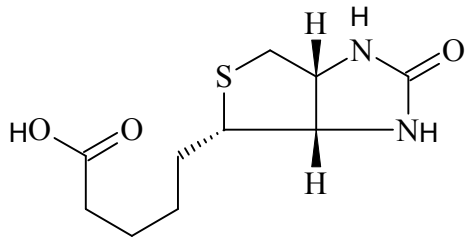

Figure 1: Chemical structure of biotin.

Streptavidin can bind biotin strongly though a non-covalent bond. This strong non-covalent binding between biotin and streptavidin has a dissociation constant of approximately 10^{-15} M. [12, 24-26] In this study, taking advantage of this streptavidin-biotin technology, and availability of biotinylated-dNTPs, the DNA chemiluminescent detection is carried out.

1.2.2 SuperSignal ELISA Femto Maximum Sensitivity Substrate

The SuperSignal ELISA Femto Maximum Sensitivity Substrate is a sensitive substrate that uses the horseradish peroxidase as a catalyst to produce energy in the form of light. This substrate is highly sensitive. In addition, the amount of time it takes for the substrate to reach its maximum sensitivity is only 1 min.

The SuperSignal ELISA Femto Maximum Sensitivity Substrate includes two components. These two components are luminol enhancer solution and stable peroxide solution. These two solutions are used in equal amounts in this study to detect the signal produced in the form of chemiluminescent light. For detection of the chemiluminescent signal, a microscope with a CCD camera is used.

1.3 Current Methods of Pathogen Detection

In recent years, rapid detection of pathogens becomes essential. As the transportation systems are enhanced, the diseases are spread easily through carriers and contacts. Although a disease outbreak can occur in North America, it can spread to other continents in hours. Thus, it is necessary to detect pathogens on-site to prevent the disease from spreading at a high speed. By doing so, the spread of disease can be contained better. In addition to the need for on-site detection, it is essential to rapidly detect the diseases even in clinics and hospitals.

One of the disease detection methods currently available is to perform biochemical tests. Tests, such as the MacConkey agar test that detects the presence of *E. coli,* take a long time due to overnight incubation and so forth. [27, 28] There are many types of *E. coli* strains, [29] and some *E. coli* strains are more dangerous than others. *E. coli* can be present as normal flora; however, *E. coli* is very dangerous when present in the urinary or gastrointestinal tracts and can cause infections including urinary tract infections in humans. Recently, *E. coli* has become the food borne pathogen [30] and many people are ill from eating infected food. Thus, the methods to detect such types of bacteria must be effective and rapid to eliminate unnecessary illnesses.

Another technique that is available for disease detection uses microarray technology and polymerase chain reaction. This method requires extraction of mRNA from the tissue sample, reverse transcription of extracted mRNA into cDNA, and polymerase chain reaction (PCR) to multiply the cDNA obtained. [1, 9, 13, 14, 19] PCR is a useful amplification method to multiply the cDNA copies obtained from the reverse transcription. [1, 9, 13, 14] According to Nam et al., polymerase chain reaction normally requires many steps, takes a long time, and is expensive and difficult to perform. [31] In other words, PCR involves repeated steps of denaturation, hybridization, and elongation of the DNA. [1, 9, 13, 14] These steps take a long time and contain

lengthy procedures. Thus, it is necessary to establish a simple and effective procedure to detect pathogenic DNA. Also, the procedure should have a great sensitivity and specificity.

1.4 Objective of This Study

Recently available techniques either contain complicated procedures or are not sensitive and specific enough in detection. Thus, the objective of this study is to establish a DNA detection method that is simple, rapid, sensitive and specific using the DNA microchip and microarray technology. This detection method will be useful for medical field as well as on-site detection of pathogenic diseases. This study will focus mainly on the detection of synthesized DNA and genomic DNA. In addition, detection sensitivity and specificity will also be studied.

For this study, the probe DNA will be synthesized, printed on the silicon-coated microchip and used as a primer. The complementary target DNA will then bind to this primer via hybridization at a high temperature. This target DNA is longer in sequence, and therefore, biotin-labeled dNTPs can, in turn, bind to the extra nucleotides on the target DNA using the Klenow Fragment of DNA polymerase I. Klenow Fragment is a large fragment with a molecular weight of approximately 68 kDa. [32, 33] This fragment is a result of proteolysis of DNA polymerase I. It mainly performs two activities. These activities include DNA polymerase activity and 3' to 5' exonuclease activity. The 3' to 5' exonuclease activity proofreads the newly synthesized DNA strand and removes mismatched nucleotides. In this experiment, the Klenow Fragment helps extend the probe DNA that is immobilized on the chip using the target DNA as a template. [32, 33] Poly-HRP-streptavidin will then bind to biotin forming a streptavidin-biotin complex, and proceed through the oxidation-reduction reaction when the SuperSignal ELISA Maximum Femto

Sensitivity Substrate is added. Luminol is oxidized and the chemiluminescent signal that is given out is detected using the microscope with a CCD camera.

2 MATERIALS AND METHODS

2.1 Oligonucleotides

Original *lacZ* mRNA [*lacZ*, *E. coli lacZ* mRNA: 724-747 nt]:

5'-AUGUGGAUUGGCGAUAAAAAACAA-3'

Synthesized *lacZ E. coli* target DNA (24mer-1): 5'-ATGTGGATTGGCGATAAAAAACAA-3'

Synthesized *lacZ E. coli* probe DNA (15mer): 5'-NH_2-TTGTTTTTTATCGCC-3'

Original 23S rRNA *E. coli* [rrlA, *E. coli str.* K-12 substr. MG 1655, 23S ribosomal RNA of rrnA operon : 483-508 nt] : 5'-ACCCCGGCGAGGGGAGTGAAAAAGAA-3'

Synthesized 23S rRNA *E. coli* Chimeric Probe (26mer-P): 5'-d(TTCTTTTT)-2'-O-Me(CACTCCCCTC)-d(GCCGGGGT) -NH_2-3'

Bird flu DNA-RNA-DNA Chimeric Probe:

5'-d(TCGTTTTT)-2'-O-Me(GGUAGGUCUGCAAAAUUUU)-d(CAAGAAGATT)-NH_2-3'

Original Sample 1 RNA: 5'-AGAUCGCGCAGAGACUGGAAAGUGU-3'

Synthesized Sample 1 Target DNA (S1-T): 5'-AGATCGCGCAGAGACTGGAAAGTGT-3'

Synthesized Sample 1 Probe DNA (S1-P): 5'-NH_2- ACACTTTCCAGTCTC-3

Original Sample 2 RNA: 5'-AAUCUUCUUGAAAAUUUGCAGACCUACCAAAAACGA-3'

Synthesized Sample 2 Target DNA (S2-T):

5'-AATCTTCTTGAAAATTTGCAGACCTACCAAAAACGA-3'

Synthesized Sample 2 Probe DNA (S2-P): 5'-NH_2- TCGTTTTTGGTAGGTCT-3'

Original *Bacillus anthraces* RNA [BA, *B. anthraces* lethal factor mRNA: 855-891 nt]:

5'-AUCUUUAGAAGCAUUAUCUGAAGAUAAGAAAAAAA-3'

Bacillus anthraces Target DNA (BA-T):

5'-ATCTTTAGAAGCATTATCTGAAGATAAGAAAAAAA-3'

Bacillus anthraces Probe DNA (BA-P): 5'-NH_2- TTTTTTTCTTATCTT-3'

Mismatched 24mer sequences of *lacZ E. coli* target DNA for Specificity Study:

24mer-1: 5'-ATGTGGATTGGCGATAAAAAACAA-3'

24mer-2: 5'-ATGTGGATTCGCGATAAAAAACAA-3'

24mer-3: 5'-ATGTGGATTGACGATAAAAAACAA-3'

24mer-4: 5'-ATGTGGATTGGGGATAAAAAACAA-3'

24mer-5: 5'-ATGTGGATTGGCGACAAAAAACAA-3'

24mer-6: 5'-ATGTGGATTGGCGATAAATAACAA-3'

24mer-7: 5'-ATGTGGATTGGGGACAAAAAACAA-3'

24mer-8: 5'-ATGTGGATTGGGGATAAATAACAA-3'

DNA for hybridization kinetics study (Biotin-24mer-1):

5'-biotin-ATGTGGATTGGCGATAAAAAACAA-3'

2.2 Other Materials

2.2.1 Reagents and DNA Samples

- *Escherichia coli* K-12 strain genomic DNA: 10 µg/ µL
- DNA Polymerase I Klenow: 5000 u mL^{-1} (New England Biolabs, Ipswich, MA)
- Biotinylated- dNTPs: 0.4 mM (Invitrogen, Eugene, OR and Perkin Elmer, Waltham, MA)
- Poly-HRP Streptavidin: 1.0 mg/ mL (Thermo Fisher Scientific, Rockford, IL)
- 3- aminopropyltrimethoxysilane (Aldrich, St. Louis, MO)
- 1,4-phenylene diisothiocyanate: 10 mM (PDITC, Fluka, Buchs, Switzerland)

2.2.2 Buffers

- 5x concentrated sodium chloride and sodium citrate buffer (5x SSC): 3.0 M NaCl, 3.0 M sodium citrate, pH 7.0
- 1x concentrated phosphate buffered saline buffer (1x PBS): NaCl, KCl, Na_2HPO_4, KH_2PO_4, pH 7.4
- Sodium Phosphate Buffer: 100 mM, pH 7.5
- Starting Block (TBS) Blocking Buffer: Protein based blocker formulation in tris buffered saline (Thermo Fisher Scientific, Rockford, IL)
- Large Klenow Fragment Buffer (NE Buffer 2): 1x concentrated (New England Biolabs, Ipswich, MA)
- Blocking Buffer: 10 mg/ mL (component D of Tyramide Signal Amplification (TSA) Kit # 21 dissolved in 1x PBS buffer)

2.2.3 Kits

- Supersignal Enzyme-linked immunosorbent assay (ELISA) Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Rockford, IL)
  - Luminol/Enhancer Solution
  - Stable Peroxide Solution
- Tyramide Signal Amplification (TSA) Kit # 21 (Invitrogen, Eugene, OR)
  - Component A: biotin- XX tyramide
  - Component B: Dimethylsulfoxide (DMS0)
  - Component C: HRP-conjugated secondary antibody or Streptavidin
  - Component D: Blocking reagent
  - Component E: Amplification buffer
  - Component F: Hydrogen Peroxide

2.2.4 Instruments

- Omni Grid Micro (Genomic Solutions)
- Microscope (Nikon *80i*)
- Charged coupled device (CCD) camera (VersArray, Princeton Instruments, Inc.)
- Waterbath Model 1202 (VWR Scientific Products)
- Heating block

2.3 Oligonucleotides Synthesis, Deprotection and Precipitation

Oligonucleotides were synthesized on the Applied BioSystems 3400 DNA synthesizer at 1 μM scale. The probes were modified with an amino group at the 5' end to easily immobilize on the silicon chip. On the other hand, for the hybridization kinetic study, the target DNA (24mer-1)

was modified with biotin on the 5' end during the synthesis. For the other targets, there was no modification during the synthesis. After the synthesis, all oligonucleotides were de-protected by aqueous ammonium hydroxide treatment and desalted by ethanol precipitation. The purity was checked, and the samples were quantified using an UV-Vis spectrophotometer.

2.4 Microchip Activation

To activate the silicon chips (0.5 × 0.5 cm), the chips were placed in the incubation chamber and treated with methylene chloride (CH_2Cl_2) for 15 min on the shaker at 100 RPM. The chips were then air-dried at room temperature (RT), and were immersed in a solution containing 19:1 ethanol to RNase-free water with 3% 3- aminopropyltrimethoxysilane (Aldrich, St. Louis, MO). Then, the chamber was left on the shaker for 45 min at 100 RPM at RT. Subsequently, the chips were rinsed with ethanol, RNase-free water, and once again with ethanol. After air-drying the chips, they were immersed in the solution containing 10 mM 1,4-phenylene diisothiocyanate (PDITC, Fluka, Buchs, Switzerland), CH_2Cl_2, and 1% pyridine. The chamber was then again placed on the shaker for 2 hr at 100 RPM at RT and rinsed three times with methylene chloride. Then, the chips were air-dried and were stored at RT in the argon atmosphere. Shown in Figure 2 are the steps involving in the activation of the silicon microchip.

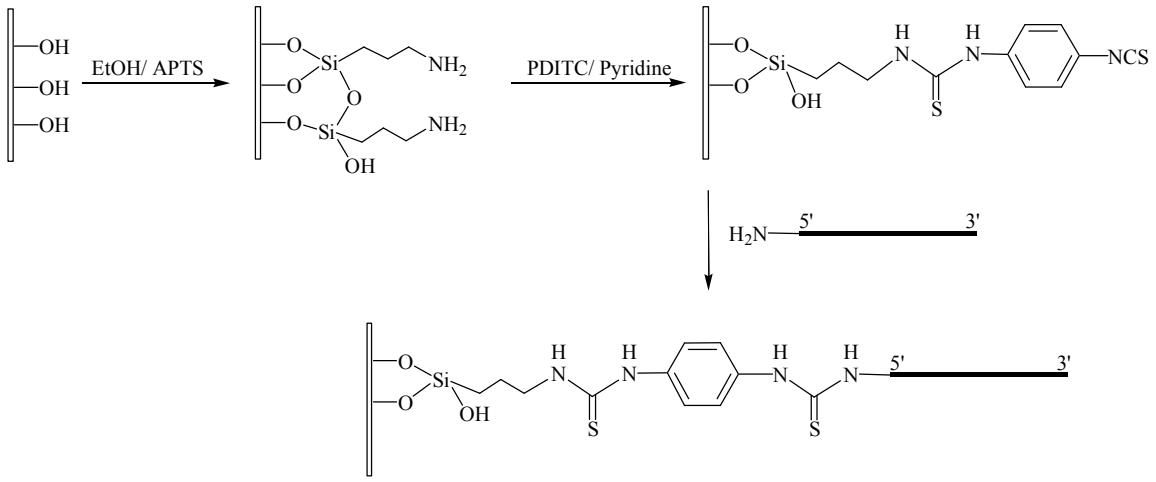

Figure 2: Microchip activation and probe immobilization.

2.5 Probe Immobilization

The probe DNA was diluted in 100 mM sodium phosphate (pH 7.5) to obtain a concentration of 250 µM. This probe DNA was then printed on the activated silicon microchip (0.5 × 0.5 cm) using the OmniGrid Micro. Each spot size is approximately 75 micron. Subsequently, the chip was incubated at 37- 40° C for 30 min.

2.6 Target Hybridization

2.6.1 Detection Using the Synthesized *lacZ E. coli* Target DNA

After the probe DNA (250 µM, 15 mer) was immobilized on the chip (15 × 15 spots), a solution containing 24mer-1 (2-500 fmol) with 5XSSC buffer (19 µL, 3.0 M NaCl, 3.0 M sodium citrate, pH 7.0) was applied on the chip. The chip was incubated for 15 min at 40-42° C in a water bath. Subsequently, the chip was rinsed with hot 1XPBS (100 µL) buffer three times to remove the unbounded DNA.

2.6.2 Detection of the *E. coli* Genomic Target DNA

After the probe DNA (250 μM, 15 mer) was fixed on the chip (15 × 15 spots), the solution containing *E. coli* genomic target DNA sample (5- 10 μg) and 5XSSC buffer (4 μL, 3.0 M NaCl, 3.0 M sodium citrate, pH 7.0), which was denatured at 95° C for 2 min, and cooled in ice for 30- 60 sec, was applied on the chip. The chip was incubated for 15 min at 45- 50° C in a water bath. Subsequently, the chip was rinsed three times with hot 1XPBS (100 μL) buffer to remove the unbounded DNA. As a positive control, 24mer-1 (10 fmol) was used as a target DNA, and as a negative control, RNase-free water was used as a target DNA.

2.6.3 Specificity in DNA Detection: Mismatched Target DNA Sequences

After the probe DNA (250 μM, 15 mer) was fixed on the chip (15 × 15 spots), the chip was divided into sections using parafilm. Then, different 24mer, mismatched-sequence samples (50 fmol) were applied on the chip for 15 min at 40- 42° C. The chip was rinsed with 100 μL of hot 1X PBS buffer three times.

2.6.4 Specificity in DNA Detection: Different DNA Probes Sequences

The activated microchip was fixed (10 × 2 spots) with four different DNA probes: 5XSSC buffer, Bird flu DNA-RNA-DNA chimeric probe (250 μM), 26mer-P (250 μM), and 15mer (250 μM). Subsequently, 24mer-1 (5 fmol) with 5XSSC buffer (19 μL, 3.0 M NaCl, 3.0 M sodium citrate, pH 7.0) was applied on the chips for 15 min at 40- 42° C. Then, the chip was rinsed with 100 μL of hot 1X PBS buffer three times.

2.6.5 Sensitivity in DNA Detection: 15mer with 24mer-1

After the probe DNA (250 μM 15 mer) was fixed on the chip (15 × 15 spots), the chip was divided into sections using parafilm. Then, different absolute quantities of 24mer-1 (2- 50 fmol) with 5XSSC buffer (2-3 μL, 3.0 M NaCl, 3.0 M sodium citrate, pH 7.0), were applied to designated sections on the chips for 15 min at 40- 42° C. The chip was rinsed three times with hot 1X PBS buffer (100 μL).

2.6.6 Combination of Specificity and Sensitivity Detection

The activated microchip was fixed (10 × 2 spots) with four probe samples: 5XSSC buffer, Bird flu DNA-RNA-DNA chimeric probe (250 μM), 26mer-P (250 μM), and 15mer (250 μM). Subsequently, 24mer-1 (2 fmol) with 5XSSC buffer (19 μL, 3.0 M NaCl, 3.0 M sodium citrate, pH 7.0), was applied on the chip for 15 min at 37- 40° C. The chip was rinsed three times with hot 1X PBS buffer (100 μL).

2.6.7 *E. coli* Genomic Target DNA Doped with Synthesized Target DNA

After the probe DNA (250 μM, 15 mer) was fixed on the chip (15 × 15 spots), the chip was divided into sections using parafilm. In the first section, 24mer-1 (2-10 fmol) doped with *E. coli* genomic target DNA (0.5 μg) was applied. In the second section, only *E. coli* genomic target DNA (0.5 μg) was applied. In the third section, only RNase-free water was applied. In the fourth section, only 24mer-1 (2-10 fmol) was applied. These target DNA samples were allowed to hybridize for 15 min at 40- 42° C. The chip was rinsed three times with hot 1X PBS buffer (100 μL).

2.7 Klenow Extension, Biotin-labeling, and Chemiluminescent Detection

After the probe is immobilized on the chip and hybridized with the target DNA, the Klenow Fragment of DNA polymerase I helps extend the probe DNA with biotin-labeled dNTPs using the target DNA as a template. In other words, after the hybridization step, the chip was incubated with Klenow buffer (2 μL, 1 X, New England Biolabs, Ipswich, MA), the Klenow fragment (0.5 μL, 5000 u mL^{-1}, New England Biolabs, Ipswich, MA), RNase-free water (16.5 μL) and biotin-14-dATP (1 μL, 0.4 mM Invitrogen, Eugene, OR) for 30 min at 37- 40° C. Then, the chip was rinsed three times with 1XPBS buffer to remove the unbounded biotin-14-dATP. Then, the solution containing HRP-conjugated Streptavidin (1 μL, component C, Invitrogen, Eugene, OR) and the blocking reagent (10 mg/ mL, component D in 1XPBS, Invitrogen, Eugene, OR) was applied on the chip. The chip was then incubated for 30 min at 37- 40° C, and was washed with 1XPBS buffer 3 times. Subsequently, the hydrogen peroxide (0.25 μL, component F, Invitrogen, Eugene, OR) and amplification buffer (50 μL, component E, 0.02% thimerosal, Invitrogen, Eugene, OR), were mixed and 1 μL of that solution is added to a mixture of amplification buffer (100 μL, component E, 0.02% thimerosal, Invitrogen, Eugene, OR) and biotin-XX-tyramide (1 μL, component A, Invitrogen, Eugene, OR). Then, this resulted solution was applied on the chip for 5 min at RT, and washed with 1XPBS buffer three times. Afterwards, Poly-HRP streptavidin (0.25 μL, Thermo Fisher Scientific, Rockford, IL) and StartingBlock (TBS) Blocking Buffer (1000 μL, Thermo Fisher Scientific, Rockford, IL) were applied on the chip for 15 min at RT. The chips were then rinsed with 1X PBS buffer 5 times, and placed in 1:1 luminol/ enhancer to stable peroxide solutions of Super Signal ELISA Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Rockford, IL). Then, the chemiluminescent signal was immediately detected using a microscope with a charged coupled device (CCD).

2.8 DNA Hybridization Kinetics

After the probe DNA (250 μM, 15 mer) was fixed on the chip (15 × 15 spots), the chip was divided into sections using parafilm. Then, Biotin-24mer-1 (50- 500 fmol) with 5XSSC buffer (2 μL, 3.0 M NaCl, 3.0 M sodium citrate, pH 7.0), was applied on the chip, and incubated at 40° C for various hybridization timings: 40, 30, 20, 15, 10, 5, 3, 2, 1 and 0 min. The chip was rinsed with 100 μL of hot 1X PBS buffer three times. Because the target DNA is already modified with biotin, polymerization using the Klenow Fragment was eliminated. Then, the solution containing HRP-conjugated Streptavidin (1 μL component C, Invitrogen, Eugene, OR) and the blocking reagent (10 mg/ mL, component D in 1XPBS, Invitrogen, Eugene, OR) was applied on the chip. The chip was then incubated for 30 min at 37- 40° C, and was washed with 1XPBS buffer 3 times. Subsequently, the hydrogen peroxide (0.25 μL, component F, Invitrogen, Eugene, OR) and amplification buffer (50 μL, component E, 0.02% thimerosal, Invitrogen, Eugene, OR), were mixed and 1 μL of that solution is added to a mixture of amplification buffer (100 μL, component E, 0.02% thimerosal, Invitrogen, Eugene, OR) and biotin-XX-tyramide (1 μL, component A, Invitrogen, Eugene, OR). Then, this resulted solution was applied on the chip for 5 min at RT, and washed with 1XPBS buffer three times. Afterwards, Poly-HRP Streptavidin (0.25 μL, Thermo Fisher Scientific, Rockford, IL) and StartingBlock (TBS) Blocking Buffer (1000 μL, Thermo Fisher Scientific, Rockford, IL) were applied on the chip for 15 min at RT. The chips were then rinsed with 1X PBS buffer 5 times, and placed in 1:1 luminol/ enhancer to stable peroxide solutions of Super Signal ELISA Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Rockford, IL). Then, the chemiluminescent signal was observed using a charged coupled device camera (CCD camera).

2.9 Multiple Pathogenic DNA Detection

Four different probes were printed on the chip (250 μM 10 × 2 spots each) in this order: BA-P, S1-P, S2-P and 15 mer. These probes were then immobilized on the chip for 30 min at 40° C. Then, one, two or three targets (50- 5pmol) were applied on the chips to hybridize with their specific probe DNA for 15 min at 45° C. The chip was then rinsed three times with 100 μL of hot 1X PBS buffer. After the hybridization step, the chip was incubated with Klenow buffer (2 μL, 1 X, New England Biolabs, Ipswich, MA), the Klenow fragment (0.5 μL, 5000 u mL^{-1}, New England Biolabs, Ipswich, MA), RNase-free water (13.5 μL) and biotin-14-dATP (1 μL, 0.4 mM, Invitrogen, Eugene, OR), biotin-14-dCTP (1 μL, 0.4 mM, Perkin Elmer, Waltham, MA), biotin-11-dGTP (1 μL, 0.4 mM Invitrogen, Eugene, OR), and biotin-11-dUTP (1 μL, 0.4 mM, Perkin Elmer, Waltham, MA), for 30 min at 37- 40° C. Then, the chip was rinsed three times with 1XPBS buffer to remove the unbounded biotin-14-dATP. Then, the solution containing HRP-conjugated Streptavidin (1 μL, component C, Invitrogen, Eugene, OR) and the blocking reagent (10 mg/ mL, component D in 1XPBS, Invitrogen, Eugene, OR) was applied on the chip. The chip was then incubated for 30 min at 37-40° C, and was washed with 1XPBS buffer 3 times. Subsequently, the hydrogen peroxide (0.25 μL, component F, Invitrogen, Eugene, OR) and amplification buffer (50 μL, component E, 0.02% thimerosal, Invitrogen, Eugene, OR), were mixed and 1 μL of that solution is added to a mixture of amplification buffer (100 μL, component E, 0.02% thimerosal, Invitrogen, Eugene, OR) and biotin-XX-tyramide (1 μL, component A, Invitrogen, Eugene, OR). Then, this resulted solution was applied on the chip for 5 min at RT, and washed with 1XPBS buffer three times. Afterwards, Poly-HRP Streptavidin (0.25 μL, Thermo Fisher Scientific, Rockford, IL) and StartingBlock (TBS) Blocking Buffer (1000 μL, Thermo Fisher Scientific, Rockford, IL) were applied on the chip for 15 min at RT.

The chips were then rinsed with 1X PBS buffer 5 times, and placed in 1:1 luminol/ enhancer to stable peroxide solutions of Super Signal ELISA Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Rockford, IL). Then, the chemiluminescent signal was observed using a charged coupled device camera (CCD camera).

3 DNA CHEMILUMINESCENCE DETECTION

3.1 Overview of DNA Detection

In this study, the 5'-amino-modified *lacZ E. coli* DNA (15mer) is synthesized and printed on the activated silicon surface. This probe DNA (15mer) is complementary to the target DNA (24mer-1) and hybridizes with 24mer-1 at a high temperature. After the hybridization, the Large Klenow Fragment of DNA polymerase I of *Escherichia coli* incorporates the biotin-labeled dNTPs using the target DNA as a template. In other words, this process extends the probe DNA (15mer). Poly-HRP streptavidin then binds to the labeled biotin, forming a streptavidin-biotin complex, and proceeds through the oxidation-reduction reaction when combined with the SuperSignal ELISA Maximum Femto Sensitivity Substrate. The chemiluminescent signal that is given out from this reaction is detected using a microscope with a charge coupled device (CCD).

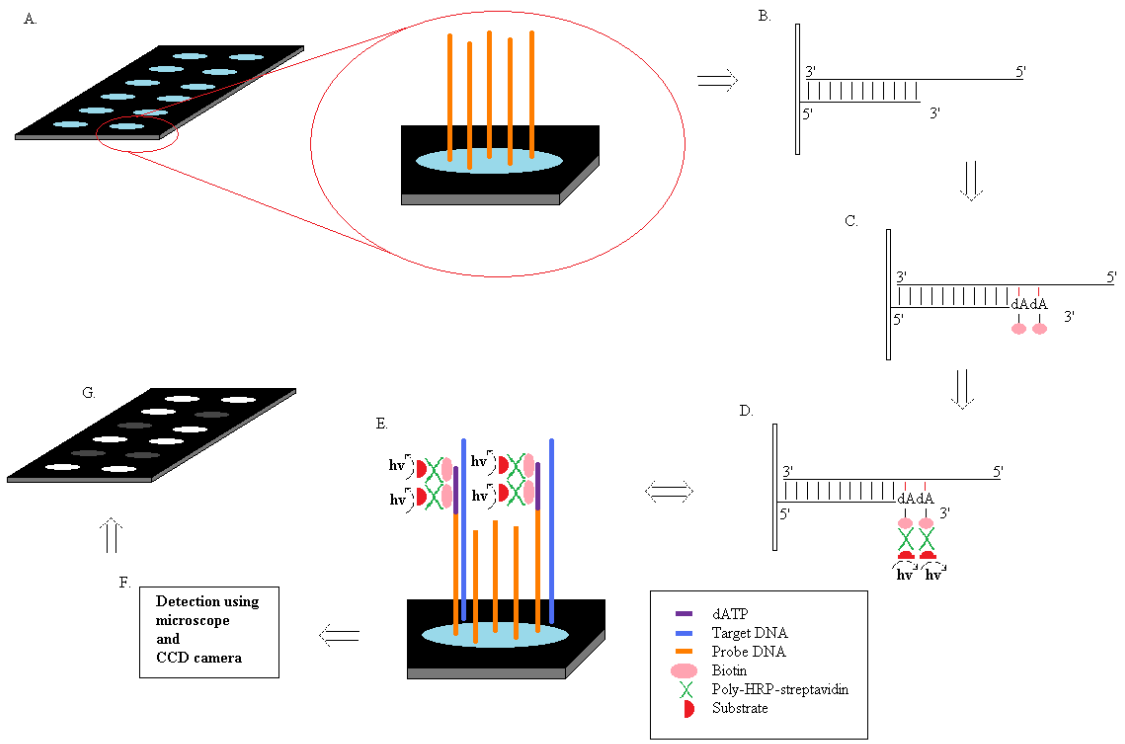

Figure 3: DNA chemiluminescent detection overview. Image A: Immobilization of DNA probes; Image B: Hybridization of the target DNA to the immobilized probes; Image C: Klenow extension using biotin-14-dATP; Image D: Poly-HRP-streptavidin binding to biotin and substrate to produce energy in a form of light; Image E: Virtual image of signal production overview; Image F: Detection using a microscope and CCD camera; Image G: Computer-generated, detectable chemiluminescent signal.

Shown in Figure 3 is an overview of the DNA chemiluminescent detection. In Image A, virtual image of the printed oligonucleotides are shown. Also, shown in Image A is the detail pictorial presentation of the immobilization of the probe DNA to the chip surface. In Image B, the hybridization of target DNA to the immobilized probes is shown. In Image C, the incorporation of the biotinylated-dATP by the Klenow Fragment using the target DNA as a template is shown. In Image D and E, the binding of Poly-HRP streptavidin to biotin and substrate are shown as well as the chemiluminescent signal that is given off when luminol is oxidized during the reaction with hydrogen peroxide (H_2O_2). This reaction is catalyzed by horseradish peroxidase

(HRP). The energy in the form of light is produced and the chemiluminescent signal is then detected using a microscope with a charge coupled device (CCD) as shown in Image F. Shown in Image G is a virtual presentation of the computer-generated result.

3.2 Processes Involved in DNA Detection

3.2.1 Microchip Activation

Activation of the silicon chip surface is necessary in order to immobilize the probe DNA on the silicon surface. In this study, the activation of chip is done by silylation, amino-functionalization and treatment with 1,4-phenylene diisothiocyanate (PDITC). PDITC is a bifunctional linker. In other words, it binds covalently to the amino groups from 8-Aminopyrene-1,3,6-trisulfonic acid, trisodium salt (APTS) and the probe DNA. To activate the microchips, the chips must be cleaned and their surfaces must be free of dust and oil. Therefore, to degrease and remove dust from the surface, the silicon-coated microchips are washed several times. These cleaned microchips then undergo silylation and amino-functionalization. Then, the chip is activated using the PDITC and it provides support for the oligonucleotides modified with an amino group at the 5'end.

3.2.2 Immobilization of DNA Probes

The amino group on the 5' end of the probe DNA is attached to the activated silicon surface. This step requires the chip to be incubated at 37° C for 30 min. Once the probe DNA is immobilized on the chip, it is ready to undergo hybridization with the target DNA. Shown in Figure 4 is the immobilized probe on the activated microchip.

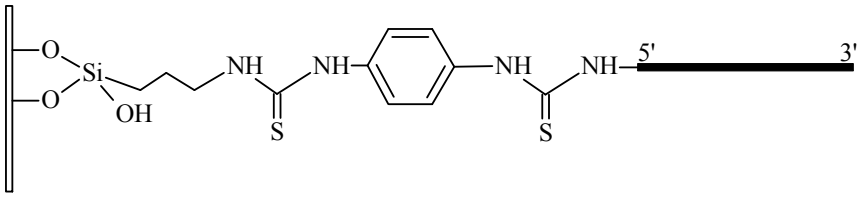

Figure 4: Probe immobilization on the activated silicon surface.

3.2.3 Target Hybridization

When the probe DNA is immobilized on the chip, the complementary DNA is applied on the chip to hybridize with the probe DNA. In this experiment, 15mer is the probe DNA and 24mer-1 is complementary to 15mer. This 24mer-1 is a single-stranded DNA (ssDNA), and therefore, it does not require denaturing. However, it is better to hybridize the two DNA stands at an optimum temperature because at a high temperature, the enzymes as well as the duplex DNA will denature whereas at a low temperature, non-specific binding increases the background noise. If the target DNA that will undergo hybridization with the probe DNA is a dsDNA, then first, it must be denatured and must be hybridized immediately with the probe DNA before the two stands of the duplex DNA hybridize again.

3.2.4 Klenow Extension, Biotin- labeling, TSA Amplification and Detection

After the target DNA hybridizes with the probe DNA, in order to have the biotinylated-dNTPs bind to the extended region of target DNA, the Large Klenow Fragment of DNA polymerase I must create a bond between the last nucleotide of the probe DNA and the biotinylated-dNTPs.

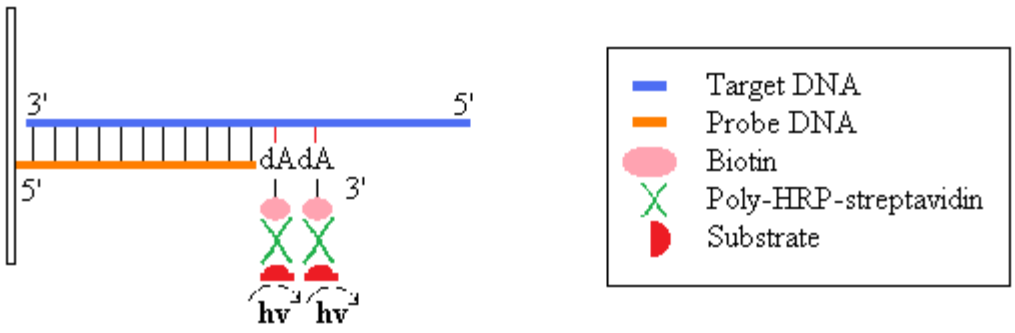

Figure 5: The virtual microchip surface during the DNA chemiluminescent detection.

Poly-HRP-streptavidin then binds to the available biotin from the biotinylated-dATP. The signal is further amplified using the tyramide signal amplification (TSA) kit. At the last step, the substrate is added to produce chemiluminescent signal. This signal is then detected using a microscope with a charge coupled device (CCD). Shown in Figure 5 is the virtual illustration of the microchip during the detection.

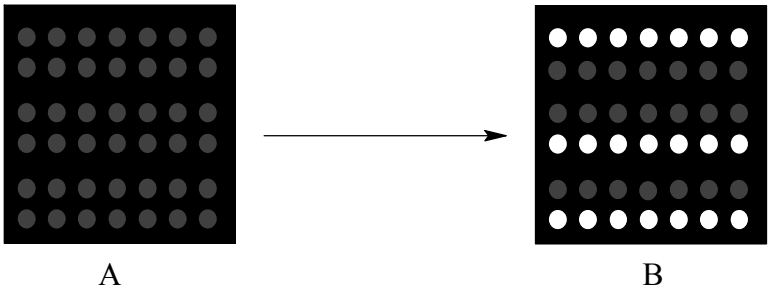

Figure 6: The virtual microchip with immobilized DNA probes (Image A), and the virtual microchip when the chemiluminescent signal is detected (Image B).

In addition, in Figure 6, the chip before and after the generation of signal, is illustrated. These images are only the virtual illustrations to show a model image of how the chip appears as a computer-generated result.

Thus, this method is a simple and efficient method to detect pathogenic DNA, and it eliminates lengthy procedures including PCR, radioactive labeling and so forth.

4 SPECIFICITY IN DNA DETECTION

4.1 Introduction

One of the requirements for any pathogenic DNA detection method is to be able to detect specifically the pathogenic sample from multiple samples. In other words, the detection method must show a good specificity. To analyze this factor, the specificity experiments are designed.

In the specificity experiments, various probes are printed on the chip to analyze how the target DNA can specifically bind to the proper probe DNA. Another type of specificity experiments is also designed so that the detection is not only specific to the correct probe, but also specific to the detail in the probe sequences. In other words, single-base mismatch discrimination should be observed. To apply this concept, several target DNA, varied in sequences by one or two nucleotides, are designed (24mer-2 to 24mer-8). It is expected that the probe DNA will bind specifically to the target DNA with the perfect match. In addition, to show the specificity using different samples, multiple pathogens will be designed and detected using the chemiluminescent detection. In other words, the experiments should provide support that this detection method allows differentiation between various pathogenic DNA samples.

4.2 Results

4.2.1 Specificity in Detection: Four Different Probes

In this experiment, four different probes were spotted on the activated silicon surface. These probes include 5XSSC buffer, Bird flu DNA-RNA-DNA chimeric probe, 26mer-P, and 15mer. Then, 24mer-1 (5 fmol) was applied on the chip to observe whether it binds specifically to 15mer discriminating the other probes. Based on the results obtained,

24mer-1 binds only to 15mer and did not bind to other probes that were immobilized on the chip. In other words, the results show a great specificity of 24mer-1 to 15mer.

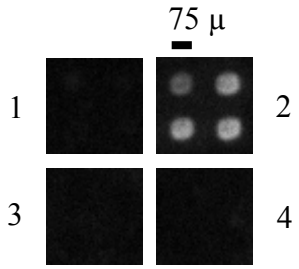

Figure 7: The specificity of 24mer-1 (5 fmol) to 15mer. Image 1: 5XSSC buffer; Image 2: 15mer; Image 3: DNA-RNA-DNA chimeric probe of rRNA *E. coli* (26mer-P), Image 4: Bird flu DNA-RNA-DNA chimeric probe. (Detection conditions: 5 sec exposure, 2×2 binning, 2X lens)

4.2.2 Specificity in Detection: Mismatched Sequences of 24mer-1

In this experiment, the probe DNA (15mer) was spotted (15× 15 spots) on the chip and different sequences of the target DNA (24mer-1) were applied on different sections of the chip. These mismatched-sequences of 24mer-1 differ in that each has one or two mismatched nucleotides. As shown in Figure 8, the results showed that 15mer binds specifically to 24mer-1, which is its perfect match.

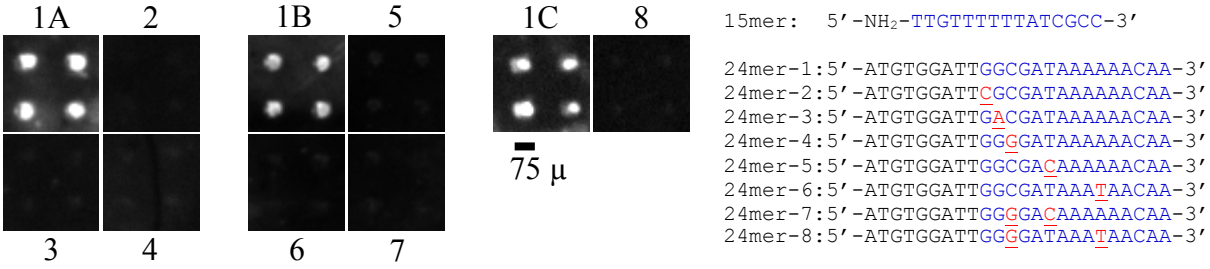

Figure 8: The specificity of probe DNA (15mer) to the target DNA (mismatched sequences of 24mer, 50 fmol). Image 1A, 1B, and 1C: 24mer-1; Image 2: 24mer-2; Image 3: 24mer-3; Image 4: 24mer-4; Image 5: 24mer-5; Image 6: 24mer-6; Image 7: 24mer-7; Image 8: 24mer-8. (Detection conditions: 5 sec exposure, 2×2 binning, 2X lens)

4.2.3 Specificity in Detection: Multiple Pathogenic DNA Detection

To observe whether the chemiluminescent DNA detection method shows specificity between different pathogens, four probes (*Bacillus anthraces,* Sample 1, Sample 2, and 15mer) were printed on the chip and one or more target DNA (50- 5 pmol) was applied on the chip. The results obtained from using 5 pmol of each target DNA, are shown in Figure 9. Although there was some background noise, it was mainly due to the chip contamination. According to the results from the multiple pathogen detection, the DNA chemiluminescent detection is highly specific. It can discriminate between different pathogenic DNA.

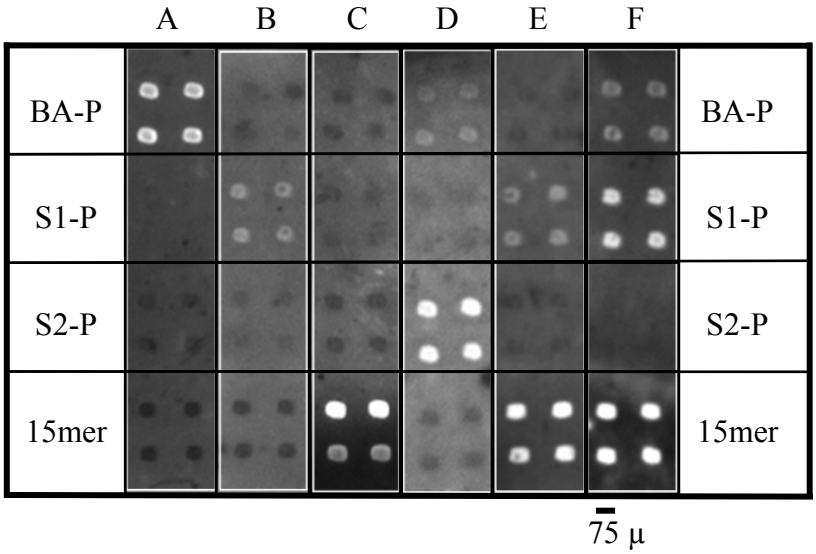

Figure 9: Multiple Pathogen Detection. Four different probes DNA were spotted on the chips (from top down): *Bacillus anthraces* (BA-P), Sample 1 (S1-P), Sample 2 (S2-P), and 15mer. Then, target DNA (5 pmol) is applied to observe specificity. Image A: *Bacillus anthraces* target DNA (BA-T); Image B: Sample 1 target DNA (S1-T); Image C: 24mer-1; Image D: *Bacillus anthraces* target DNA (BA-T) and Sample 2 target DNA (S2-T); Image E: Sample 1 target DNA (S1-T) and 24mer-1; Image F: *Bacillus anthraces* target DNA (BA-T), Sample 1 target DNA (S1-T) and 24mer-1. (Detection conditions: 5 sec exposure, 2×2 binning, 2X lens)

4.3 Conclusion

Based on the results obtained, it was observed that 24mer-1 and 15mer recognized each other well in the presence of other probes. In addition, 15mer can also discriminate between the mismatch sequences and the perfect match (24mer-1). Moreover, other probes and targets from different pathogens can also recognize each other, discriminating the non-specific pathogens, and allowing only the specific probes and targets to hybridize. This makes the multiple pathogenic DNA detection using the DNA microchip technology, highly specific. In conclusion, this DNA detection method shows a great specificity in detection not only for *lacZ* gene of *E. coli*, but also for other pathogenic DNA as well.

5 SENSITIVITY IN DNA DETECTION

5.1 Introduction

Detection of pathogenic DNA on the microchip is indeed useful for clinical studies as well as on-site detection. However, at times, the concentrations of the samples that are obtained on-site may be low. Thus, it is extremely important that the signal can be detected at low concentrations. To study sensitivity in DNA detection, the detection limit must be determined. The experiments are designed so that even when samples with a low concentration are supplied, the detection can be done. In other words, in these experiments, different absolute quantities of target DNA are used to observe the detection limit. Moreover, it is also necessary to detect different samples at low concentration. Thus, some experiments are designed to differentiate between samples at the detection limit.

5.2 Results

5.2.1 Detection Limit of the Synthesized *E. coli* Target DNA

In this experiment, 24mer-1, which is the synthesized *lacZ E. coli* target DNA, was hybridized with the probe DNA (15mer) at different absolute quantities. Shown in Figure 10 are the results of the synthesized target DNA detection. Image 1 through 8 represents the results obtained from using different absolute quantities of target DNA (24mer-1). At the absolute quantity of 5 pmol, the signal was very strong. However, as the absolute quantity decreased, the signal became weaker. The detection limit was observed to be 2 fmol.

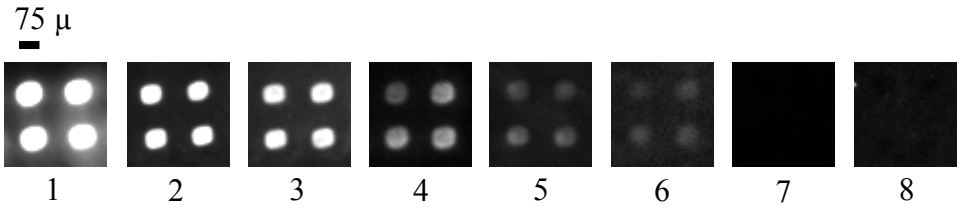

Figure 10: Synthesized target DNA detection at different absolute quantities. Image 1: 5 pmol; Image 2: 50 fmol; Image 3: 10 fmol; Image 4: 5 fmol; Image 5: 3 fmol ; Image 6 : 2 fmol ; Image 7 : 1 fmol ; Image 8: 0 fmol. (Detection conditions: 15 sec exposure, 2×2 binning, 2X lens)

5.2.2 Combination of Specificity and Sensitivity in DNA Detection

In this experiment, four different probes were spotted on the chip to show the specificity, and the target DNA (24mer-1) was applied to specifically bind to 15mer. The absolute quantity of the target DNA was at the detection limit (2 fmol). The results showed highly specific binding as well as the successful detection at a very low concentration.

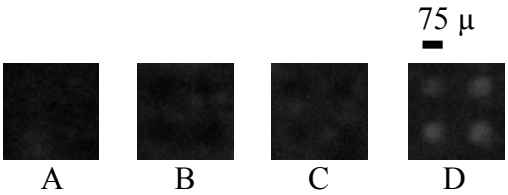

Figure 11: Combination of specificity and sensitivity experiments. Image A: 5XSSC buffer, Image B: Bird flu DNA-RNA-DNA chimeric probe; Image C: 26mer-P; Image D: 15mer. Absolute quantity of the target DNA (24mer-1) is 2 fmol. (Detection conditions: 5 sec exposure, 2×2 binning, 2X lens)

5.3 Conclusion

The results showed that the probes and target DNA can hybridize selectively even with the absolute quantity of the target DNA at the detection limit. Therefore, this DNA chemiluminescent detection method is highly specific and also, sensitive to detect at the femtomole level.

6 DNA HYBRIDIZATION KINETICS

6.1 Introduction

DNA hybridization kinetic experiments are designed to obtain the measurements of the signals' average intensity at a given time of hybridization between the target and probe. It is important to understand the kinetics of hybridization because in DNA microarray techniques, hybridization step is normally the rate- limiting step, [34, 35] and it takes a long period to hybridize the two DNA strands. It is also known that short DNA strands can hybridize quicker than long DNA strands. [36, 37] Therefore, it is expected that the signal intensity that resulted from the hybridization of the two short DNA strands (15mer and 24mer-1) used in this study, will reach the plateau at a short hybridization time. However, hybridization temperature is kept constant at all time to study mainly two variables: signal intensity and hybridization time. In this experiment, probe DNA (15mer) is immobilized on the chip and the target DNA (Biotin-24mer-1), which was newly synthesized particularly for this hybridization kinetics, was applied on the chip. Once the target DNA is applied on the chip, the two DNA strands are allowed to hybridize for various time points: 0- 40 min. Biotin-24mer-1 is labeled with biotin at the 5' end of the DNA strand. In other words, after hybridization occurs at a certain time point, biotin will then bind directly to Poly-HRP streptavidin, which, in turn, will react with the substrate. To be exact, in this process, Klenow extension and the use of biotinylated-dNTPs are eliminated. The chemiluminescent signal is then detected using a microscope with a CCD camera.

6.2 Results

6.2.1 DNA Hybridization Kinetics Curve

Shown in Figure 12 are the results obtained from the DNA hybridization kinetics experiments. The best fit curve was generated using SigmaPlot 11.0 (Systat Software, Inc. SigmaPlot for Windows) to represent the data that was obtained from the experiments. The graph showed that the intensities reached the plateau around 9-11 min.

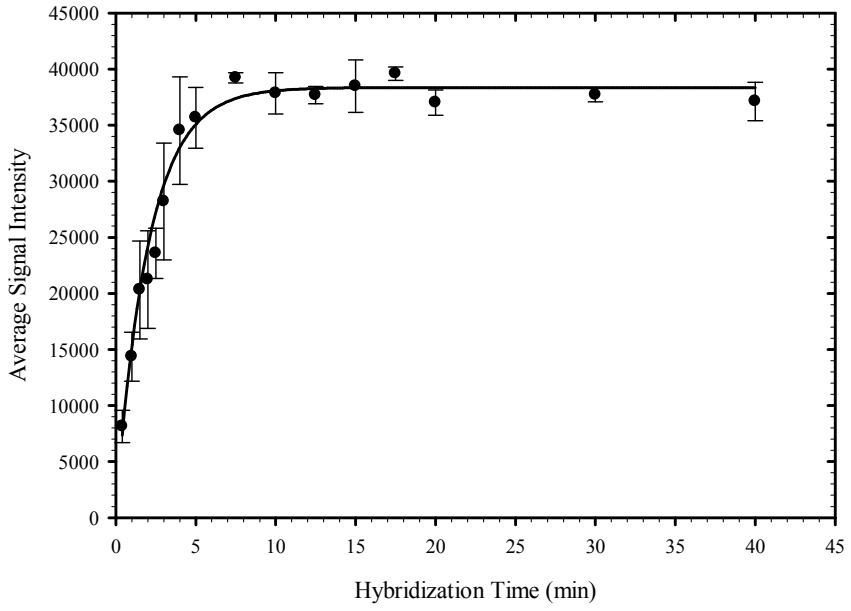

Figure 12: DNA hybridization kinetics of the interaction of the target DNA (Biotin-24mer-1: 5'-biotin-ATGTGGATTGGCGATAAAAAACAA-3') and the probe DNA (15mer: 5'-NH_2-TTGTTTTTTATCGCC-3').

6.3 Conclusion

According to the results, it is observed that the target DNA (Biotin-24mer-1) hybridizes relatively fast with the probe DNA (15mer) that is immobilized on the chip. In other words, this DNA detection method is a rapid method to detect pathogenic DNA.

7 DETECTION OF GENOMIC DNA SAMPLES

7.1 Introduction

In recent years, there have been illnesses caused by food infections by *Esherichia coli* and flu outbreaks occurring including Swine Flu, Avian Influenza and seasonal flu. These illnesses and flu outbreaks are easily spread from one continent to another because of the ease of traveling and the difficulty of containing the disease due to inefficient disease detection methods. Thus, it is necessary to be able to detect diseases rapidly on-site to prevent from spreading.

Moreover, patients diagnose with diseases, such as cancer, are increasing as well. Capability to detect such diseases at early stage is essential. Detection is available through microarray technology and PCR techniques. [1, 9, 13, 14] However, these techniques require a long processing time and are difficult to perform. In this study, a novel method to detect the pathogenic DNA is established. Instead of using longer oligonucleotides or dsDNA that needs denaturing after it is immobilized on the chip, this study uses the short and synthesized single-stranded DNA. In addition, not only the synthesized DNA, but also, the *E. coli* genomic DNA can be detected using this detection method. This detection is important because the potential to detect genomic DNA means that this chemiluminescent detection method is indeed useful in real life applications. Furthermore, this study eliminates reverse transcription, fluorescent labeling and the use of antibodies. Thus, it is an efficient and easy method to detect pathogenic DNA and could be useful for many applications. [38]

7.2 Results and Discussion

7.2.1 Detection of the *E. coli* K-12 Strain Genomic DNA

To determine the detection limit of the *E. coli* K-12 Strain Genomic Target DNA, 15 mer was printed on the chip, and the *E. coli* genomic target sample (10- 5 µg) was applied on the chip.

According to the results, it was determined that 5 µg was the detection limit for *E. coli* K-12 genomic DNA. In Figure 13, these results are shown. 24mer-1 (10 fmol) was used as a positive control and RNase-free water was used as a negative control. In between the two controls are the genomic samples with the absolute quantities of 10, 5, and 3 µg (Figure 13: Image 2, 3, and 4).

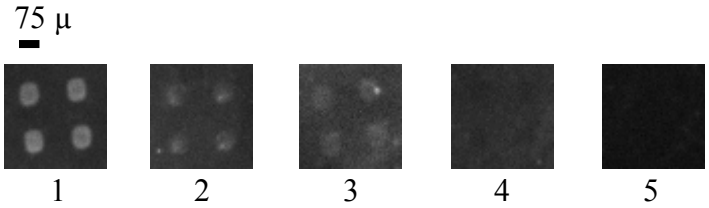

Figure 13: Detection limit of the *E. coli* K-12 strain using chemiluminescent detection method. Image 1: 10 fmol 24mer-1; Image 2: 10 µg *E. coli* genomic DNA; Image 3: 5 µg *E. coli* genomic DNA; Image 4: 3 µg *E. coli* genomic DNA; Image 5: RNase-free water. (Detection conditions: 15 sec exposure, 2×2 binning, 2X lens)

7.3 Conclusion

The *E. coli* genomic DNA was successfully detected using the DNA chemiluminescent detection method. In conclusion, this study simplifies the pathogenic and genomic DNA detection and is cost-effective and rapid. Thus, it will be useful for many applications in real life.

8 Doping of Genomic DNA with Synthesized Target DNA

8.1 Introduction

Synthesized target DNA (24mer-1) is a short oligonucleotide sequence from *lacZ* gene of *Escherichia coli.* To observe how the presence of the genomic DNA in the chemical environment during the hybridization of 24mer-1 to its complementary probe DNA (15mer) affects the detection, 24mer-1 is doped with the genomic DNA of *Escherichia coli K-12 strain*. The genomic DNA must be denatured first to participate in the hybridization process. Although both 24mer-1 and the genomic DNA have sequences complementary to 15mer, the chemiluminescent signal should be observed mainly from 24mer-1. This is due to the fact that the detection limit for *E. coli* genomic DNA using this DNA detection method is 5 μg. Thus, it is expected that using the absolute quantity lower than its detection limit of *E. coli* genomic DNA should not interfere with the detection of 24mer-1. In addition, a relatively strong signal should be observed mainly from the synthesized target DNA (24mer-1).

8.2 Results and Discussion

8.2.1 Doping of *E. coli* Genomic DNA with 24mer-1

The detection limit of the hybridization between the denatured, genomic DNA of *Escherichia coli K-12 strain* and 15mer was previously observed to be 5 μg. Thus, the absolute quantity of 0.5 μg *E. coli* genomic DNA was used for the doping experiments. The signal was observed from doping 0.5 μg *E. coli* genomic DNA with 24mer-1. This signal was produced from the hybridization of 24mer-1 to 15mer since the absolute quantity of the *E. coli* genomic DNA used in the doping experiments was lower than its detection limit. Figure 14 shows the detailed results of the doping experiments in which

0.5 μg *E. coli* genomic DNA was doped with various absolute quantities of 24mer-1 (10, 5, and 2 fmol). To serve as the negative control, 0.5 μg *E. coli* genomic DNA (Image G) and RNase-free water (Image H) were used. Since the detection limit of *E. coli* genomic DNA was 5 μg, the absolute quantity of 0.5 μg *E. coli* genomic DNA alone did not produce a detectable signal (Image G).

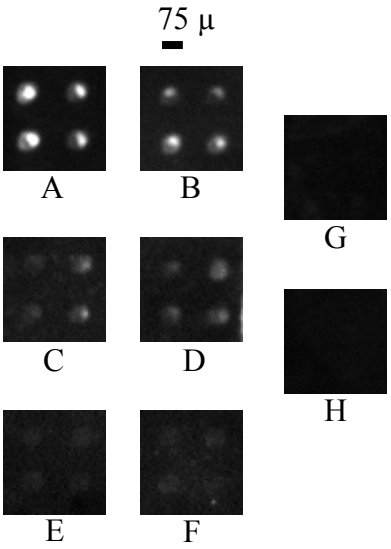

Figure 14: Doping of *E. coli* genomic DNA with 24mer-1. Image A: 0.5 μg *E. coli* genomic DNA and 10 fmol 24mer-1; Image B: 10 fmol 24mer-1; Image C: 0.5 μg *E. coli* genomic DNA and 5 fmol 24mer-1; Image D: 5 fmol 24mer-1; Image E: 0.5 μg *E. coli* genomic DNA and 2 fmol 24mer-1; Image F: 2 fmol 24mer-1; Image G: 0.5 μg *E. coli* genomic DNA; Image H: RNase-free water. (Detection conditions: 5 sec exposure, 2×2 binning, 2X lens)

8.3 Conclusion

As expected, doping of 24mer-1 with 0.5 μg *E. coli* genomic DNA produced a strong signal. Even at an absolute quantity lower than that of the detection limit of the *E. coli* genomic DNA, the signal was observed when 24mer-1 was present in the hybridization process. This shows that the presence of *E. coli* genomic DNA in the hybridization environment does not interfere with the hybridization of 24mer-1 to 15mer.

BIBLIOGRAPHY

1. Miller, M. B.; Tang, Y. W., *Clin Microbiol Rev* **2009,** *22* (4), 611-33.

2. Schulze, A.; Downward, J., *Nat Cell Biol* **2001,** *3* (8), E190-5.

3. Harrington, C. R.; Lucchini, S.; Ridgway, K. P.; Wegmann, U.; Eaton, T. J.; Hinton, J. C.; Gasson, M. J.; Narbad, A., *BMC Microbiol* **2008,** *8*, 195.

4. Bejjani, B. A.; Shaffer, L. G., *J Mol Diagn* **2006,** *8* (5), 528-33.

5. Xue, X.; Xu, W.; Wang, F.; Liu, X., *J Am Chem Soc* **2009,** *131* (33), 11668-9.

6. Yoo, S. M.; Choi, J. H.; Lee, S. Y.; Yoo, N. C., *J Microbiol Biotechnol* **2009,** *19* (7), 635-46.

7. Vana, G.; Westover, K. M., *Mol Phylogenet Evol* **2008,** *47* (3), 1100-10.

8. Potter, C. W., *J Appl Microbiol* **2001,** *91* (4), 572-9.

9. Curran, T.; Coulter, W. A.; Fairley, D. J.; McManus, T.; Kidney, J.; Larkin, M.; Moore, J. E.; Coyle, P. V., *J Microbiol Methods 80* (3), 257-61.

10. Day, I. n. m., *Microarrays and Microplates: Applications in Biomedical Sciences*. Garland Science: 2003.

11. Wittmann, C., *Immobilisation of DNA on Chips I (Topics in Current Chemistry)*. Springer: 2005.

12. Wittmann, C., *Immobilisation of DNA on Chips II (Topics in Current Chemistry)*. Springer: 2006.

13. Bowtell, D.; Sambrook, J., *DNA microarrays : a molecular cloning manual*. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, N.Y., 2003; p xxiii, 712 p.

14. Finckh, U.; Rolfs, A.; Weber-Rolfs, I., *Methods in DNA Amplification*. Springer: 1994.

15. Cheek, B. J.; Steel, A. B.; Torres, M. P.; Yu, Y. Y.; Yang, H., *Anal Chem* **2001,** *73* (24), 5777-83.

16. Russell, S.; Meadows, L. A.; Russell, R. R., *Microarray Technology in Practice*. Academic Press: 2008.

17. Dill, K.; Liu, R. H.; Grodzinsky, P., *Microarrays: Preparation, Microfluidics, Detection Methods, and Biological Applications*. Springer New York: 2009.

18. Brown, P. O.; Botstein, D., *Nat Genet* **1999,** *21* (1 Suppl), 33-7.

19. Hardiman, G., *Microarray Innovations: Technology and Experimentation*. CRC Press: 2009.

20. Duan, L.; Wang, Y.; Li, S. S.; Wan, Z.; Zhai, J., *BMC Infect Dis* **2005,** *5*, 53.

21. Freitag, S.; Le Trong, I.; Klumb, L.; Stayton, P. S.; Stenkamp, R. E., *Protein Sci* **1997,** *6* (6), 1157-66.

22. Lindqvist, Y.; Schneider, G., *Curr Opin Struct Biol* **1996,** *6* (6), 798-803.

23. Laitinen, O. H.; Nordlund, H. R.; Hytonen, V. P.; Kulomaa, M. S., *Trends Biotechnol* **2007,** *25* (6), 269-77.

24. N/a, *A Laboratory Guide to Biotin-Labeling in Biomolecule Analysis*. Birkhauser: 1996.

25. Abelson, J. N.; Simon, M. I.; Wilchek, M.; Bayer, E. A., *Avidin-Biotin Technology, Volume 184: Volume 184: Avidin-Biotin Technology*. Academic Press: 1990.

26. Blalock, E. M., *A Beginner's Guide to Microarrays*. Springer: 2003.

27. Hu, Y.; Zhang, Q.; Meitzler, J. C., *J Appl Microbiol* **1999,** *87* (6), 867-76.

28. Martins, S. A.; Prazeres, D. M.; Fonseca, L. P.; Monteiro, G. A., *Anal Bioanal Chem* **2008,** *391* (6), 2179-87.

29. Blanco, J. E.; Blanco, M.; Blanco, J.; Mora, A.; Balaguer, L.; Mourino, M.; Juarez, A.; Jansen, W. H., *J Clin Microbiol* **1996,** *34* (12), 3101-7.

30. Deisingh, A. K.; Thompson, M., *J Appl Microbiol* **2004,** *96* (3), 419-29.

31. Nam, J. M.; Stoeva, S. I.; Mirkin, C. A., *J Am Chem Soc* **2004,** *126* (19), 5932-3.

32. Huang, Z.; Alsaidi, M., *Anal Biochem* **2003,** *322* (2), 269-74.

33. Huang, Z.; Szostak, J. W., *Nucleic Acids Res* **1996,** *24* (21), 4360-1.

34. Wang, D.; Coscoy, L.; Zylberberg, M.; Avila, P. C.; Boushey, H. A.; Ganem, D.; DeRisi, J. L., *Proc Natl Acad Sci U S A* **2002,** *99* (24), 15687-92.

35. Call, D. R., *Crit Rev Microbiol* **2005,** *31* (2), 91-9.

36. Liu, R. H.; Lenigk, R.; Druyor-Sanchez, R. L.; Yang, J.; Grodzinski, P., *Anal Chem* **2003,** *75* (8), 1911-7.

37. Sakamoto, T.; Mahara, A.; Iwase, R.; Yamaoka, T.; Murakami, A., *Anal Biochem* **2005,** *340* (2), 369-72.

38. Schena, M., *DNA Microarrays: A Practical Approach*. Oxford University Press, USA: 1999.