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Fluorescent Labeling of Antibiotic Resistant Bacteria Model DNA

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A thesis submitted to the faculty of
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in partial fulfillment of the requirements for the degree of

Master of Science

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ABSTRACT

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Global threats to treatment of bacterial infections due to antibiotic resistance (AR) have been on the rise in recent years. Current diagnostic tests identify bacteria by using blood culture, which takes more than 24 hours. This study focuses on the fluorescent labeling of DNA derived from bacterial AR genes (KPC & VIM) and other model DNAs using oligreen dye (OG) and molecular beacons (MB). A NanoDrop 3300 fluorospectrometer was used to take fluorescence measurements. Linear dynamic range and labeling efficiency were dependent on the following optimized conditions: dilution factor of OG (200 fold), buffer (20 mM Tris HCl, pH 8), and heat treatment of 95 °C for 15 min.

Fluorescence analysis of a target DNA with a designed MB showed signal-to-background of 10 with our buffer only and 20 with our buffer and 25% ethanol. I also demonstrated a simple microfluidic device capable of detecting AR genes using model DNAs, magnetic beads, and designed MBs for assays of 50 µL volume. This study provides a first step towards detecting MB-DNA complexes by a simple, low cost, and fast non-amplified method, which may be used to detect AR genes in clinical samples in the future.

Keywords: antibiotic resistance. fluorescence labeling. oligreen dye. molecular beacon

ACKNOWLEDGEMENTS

There is an old African saying that "the fly that has nobody to advise it, follows the corpse into the grave" and "with patience, even a hot plate of soup can be licked." With that in mind, I would like to express my sincere gratitude to my supervisor, Dr. Adam Woolley, for having patience and confidence in my abilities and for guiding and providing me the opportunity to work in his lab and on this project with freedom. I would also like to thank Dr. Steven Goates and Dr. Richard Robison for all their positivity, encouragement, and feedback in making this project a success. Like the old African saying goes, "the lips have to come together before they can whistle" they were indeed a part of the lip that came together to make this thesis project happen. I am thankful to all my lab members, friends, and colleagues for their help and support. I would also like to thank all the faculty and the staff at the Department of Chemistry and Biochemistry for giving me the opportunity as a graduate student to achieve my aim. In addition to this, I would also like to thank the National Institutes of Health (R01 AI116989) for funding this project.

Finally, my utmost gratitude goes to my family, especially my parents and husband who helped take care of my children while I was in school. I am grateful to my heavenly father for giving me the health and strength to make this possible. As long as "success is not a doorway, but a stairway" (Dottie Walters), and I know that "if I wish to move mountains tomorrow, I must start by lifting stones today" (African proverb), I will continue to pursue my dream of making differences in peoples' lives with all this knowledge I have gained from my masters' degree in Analytical Chemistry. I know the journey will not be smooth because “smooth seas do not make skillful sailors” (African proverb); thus I can only learn by making mistakes and correcting them and then moving on.

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Chapter 1

Introduction

1.1 Background and Motivation

According to the Centers for Disease Control and Prevention, antibiotic resistance (AR) causes over 2 million illnesses and results in over 23,000 related deaths annually (1). There has been an increase in the number of bacteria becoming resistant to antibiotic drugs, thus resulting in longer hospital stays, spread of more of these AR diseases, increase in healthcare costs, etc. This calls for rapid diagnostic tests that are sensitive, effective, and reliable to enable specific diagnosis of AR pathogens to aid in prescribing the proper drugs for patients. Current clinical tests, as shown in Table 1, identify bacteria with limits of detection (LOD) of far more than 10 CFU/mL (2). Some of these methods use blood cultures; thus, their results take over 24 hours.

One of the common laboratory techniques employed to detect disease causing pathogens is an amplification method called polymerase chain reaction (PCR), in which small amounts of nucleic acids (NAs) are amplified enzymatically within a short time enabling NA detection (3). The limitations to the use of PCR are that it requires blood sample purification, and contamination during NA purification can cause false negative results due to PCR inhibitors in blood samples or false positive results due to its extreme sensitivity (4-6). This is where the use of non-amplified NA detection methods come to play. One of these methods is the use of hybridization, which involves labeling one or both strands of two complementary sequences that form double-stranded deoxyribonucleic acid (dsDNA), indicating positive species specific relationship (7). Direct, fast analysis with more linear quantitative signals are achieved due to assay simplicity and robustness to contamination (8-9).

In this study, the use of the hybridization method was employed because of the above mentioned advantages. I fluorescently labeled DNA derived from bacterial AR genes (KPC and VIM) and other model DNAs using oligreen dye (OG), molecular beacons (MB), and magnetic beads.

1.2 DNA Quantitation

The three most common laboratory methods used to quantify DNA concentrations are absorbance, gel electrophoresis, and fluorescent DNA-binding dyes (10). In this study, the use of fluorescent DNA-binding dyes was used because of its advantages over the other methods, such as speed, low LODs, and insensitivity to contaminants (11-12).

Fluorescence in certain dissolved molecules occurs when they absorb light at specific wavelengths causing electron transitions to higher energy levels (13). These excited electrons return to a slightly lower energy level first before finally falling to the ground electronic state resulting in fluorescence emission at a longer wavelength for the emitted light than excited light. DNA concentrations can be determined by taking fluorescence measurements of samples and comparison to standards also labeled with fluorescent dyes. The hydrogen bonding capabilities of the DNA base pairs and the outer edges of the nitrogenous bases provide means to bind to other molecules such as fluorophores or dyes (14). Dyes such as oligreen and molecular beacons, and solid supports such as magnetic beads have been employed over the years to aid with the identification and quantitation of various disease causing genes (15-17).

Table 1. Time required for pathogen identification using various diagnostic methods (2)

Diagnostic Method	Time for Pathogen Identification
Culture and phenotypic biochemistry on/in artificial media (bacterial, mycobacterial, fungal)	Days to weeks
In vitro antimicrobial susceptibility	Days to weeks
Real-time PCR for microorganisms and drug resistance genes	One to several hours

1.2.1 Oligreen

OG is a fluorescent, unsymmetrical cyanine nucleic acid stain for the quantitation of oligonucleotides and ssDNA in solution (Fig. 1). OG exhibits more than 1000-fold fluorescence enhancement upon binding to ssDNA, with excitation and emission maxima of 480 and 520 nm, respectively (18-20). For proprietary reasons, the structure, mode of action, and initial concentration of OG from the manufacturer are unknown. I used OG to fluorescently label complementary sequences of ssDNA and dsDNA derived from bacterial AR genes, initially using synthetic oligonucleotides as mimics of nucleic acid sequences found in bacterial lysates.

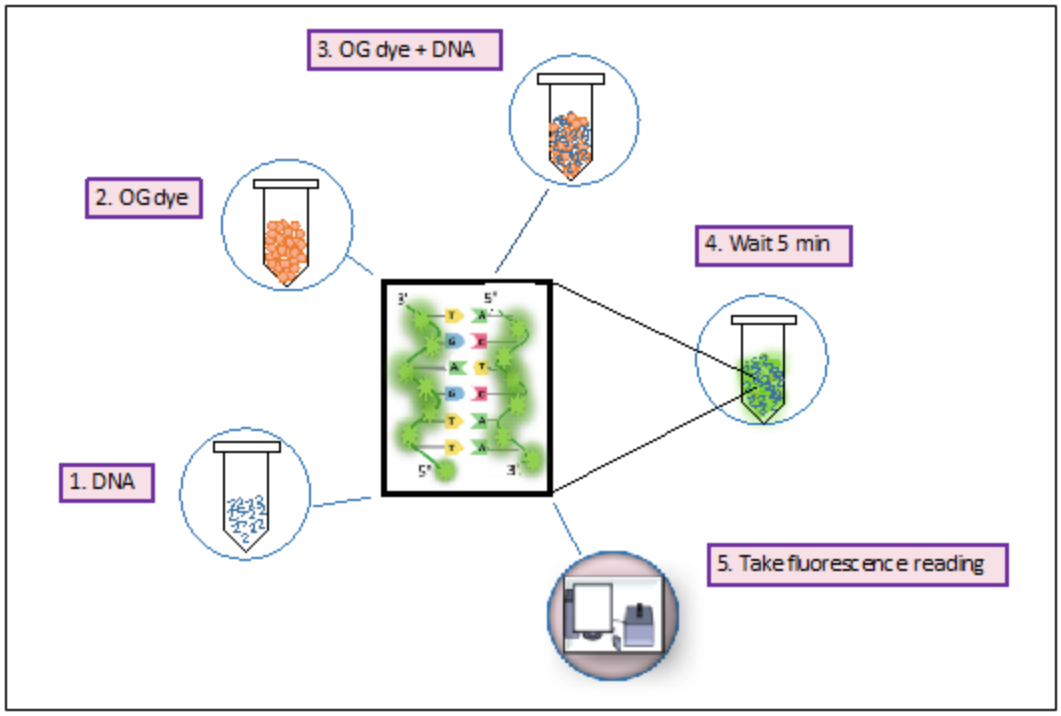


Fig 1. *Schematic of how OG is used to label DNA.*

1.2.2 Molecular Beacons

MBs are short oligonucleotide, dual-labeled probes with stem-loop structures that fluoresce upon hybridization with complementary DNA target sequences (Fig. 2) (21).

MBs have low fluorescence on their own because of the close proximity of their stem hybrid labels (one end a fluorophore and the other end a quencher). MBs are designed with a loop portion (15–30 nucleotides long) matching a complementary target DNA sequence and the stem containing matching complementary short (5-7 base pair) sequences.

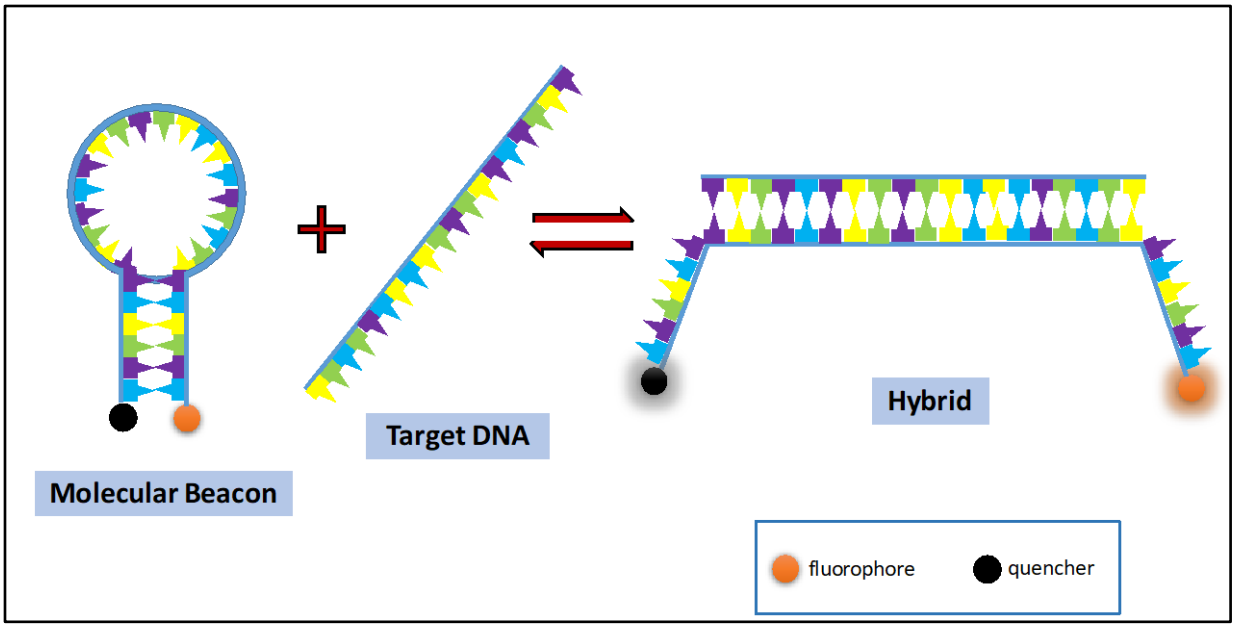


Fig 2. *Illustration of how a MB hybridizes with a target DNA sequence to form a fluorescent product.*

When MBs are mixed with their target complementary DNA, they form a hybrid which separates the fluorophore from the quencher, thus increasing fluorescence intensity by as much as 200-fold (21). MBs have been used in many biological and biochemical applications in detecting various RNA and DNA target sequences that have been amplified using PCR (21,22). MB-target hybrid fluorescence signal is significantly impacted by various factors which include how well MBs are designed, hybridization buffer, denaturing temperature, and hybridization time (23). I optimized conditions to improve the signal to background ratio (S/B) for designed MBs for *Klebsiella pneumoniae carbapenemase* (KPC), *Escherichia coli* (E. coli), and Verona Integron-Mediated Metallo-β-lactamase (VIM) DNA.

1.2.3 Magnetic Beads

Magnetic beads are micro particles (of sizes ranging from 1 - 120 μm in diameter) made of a magnetic core of paramagnetic/ferromagnetic material surrounded by a nonmagnetic coating, which is usually functionalized with proteins or a DNA sequence (24). Magnetic beads have been used for several clinical applications, such as monitoring tumor cells in lung cancer patients, cell isolation studies, drug delivery, and hyperthermia-causing agents for cancer therapy (25-26). Magnetic beads offer better interaction with analyte solution due to their functionalized surface and ability to be manipulated with a magnet (27). Magnetic beads combined with specific ligands allow separation and purification of cells, proteins, nucleic acids, and other molecules in a highly efficient and specific manner (28-29). When magnetic force is applied to the solution, modified magnetic beads separate hybridized ssDNA (those with magnetic beads attached) from unhybridized ones, encouraging specific analysis of targeted DNA (30), as depicted in Fig. 3. A general overview of how magnetic beads are used in this experiment is as follows: (1) addition of beads, (2) specific binding occurs with desired analyte, (3) analyte-bound beads are captured with a magnet; (4) crude sample components are washed away, and (5) analyte is eluted from beads.

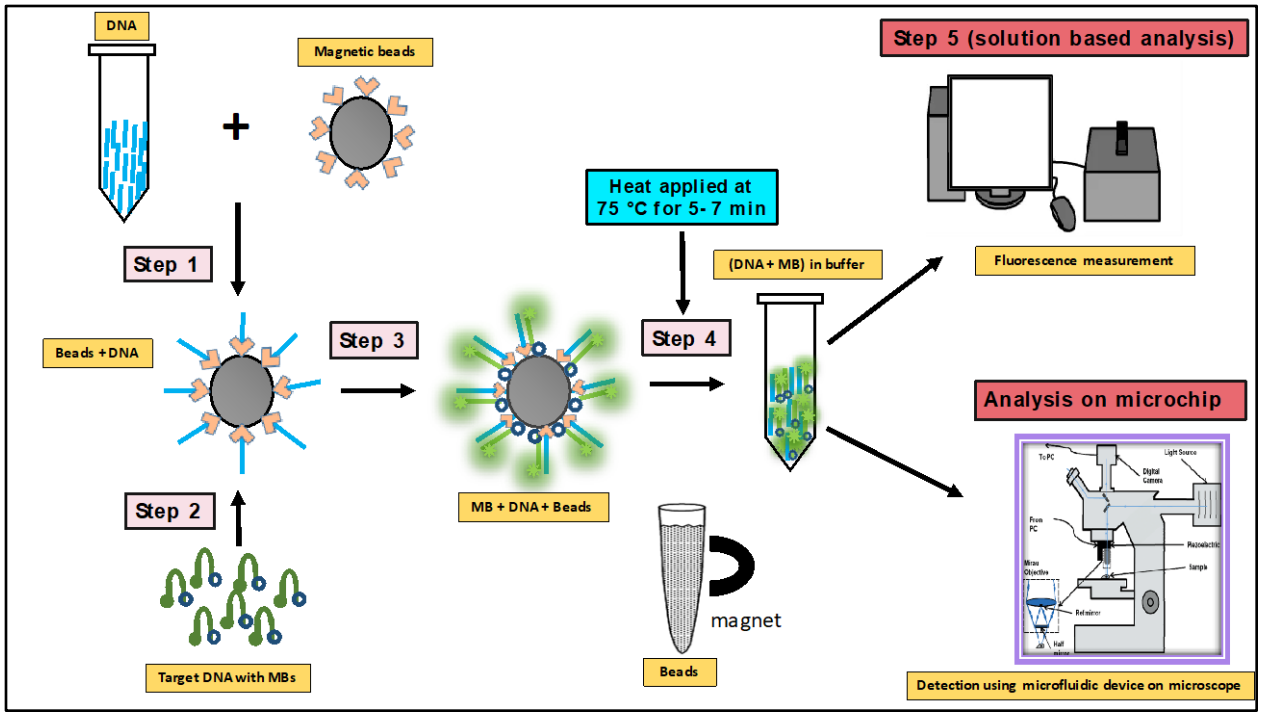


Fig 3. *Magnetic beads for labeling target DNA in solution and on chip. (1) addition of beads, (2) specific binding occurs with desired analyte, (3) analyte-bound beads are captured with a magnet, (4) crude sample components are washed away, and (5) analyte is eluted from beads.*

1.3 Microfluidic Chips and Current Approaches

Microfluidics is the use of small amounts fluids within the ranges of nanoliters to attoliters (10^{-9} to 10^{-18} L) confined within micrometer channels (31). For the past three decades, microfluidic technology has become increasingly popular in the analytical and diagnostic areas of science owing to its advantages of portability, low cost, low reagent and sample consumption, and rapid turnaround time (32-33). Microfluidic chips have been used in the rapid detection of several pathogenic disease causing agents such as *E. coli*, HIV RNA, influenza virus H5N1, and Dengue virus using samples such as whole blood, serum, urine, throat swab, and serum respectively (34-39). In my research, I used microchannels to successfully capture, detect, and label minute

amounts of model AR DNA. This reduced experimental times and the amount of reagents needed for the experiments, allowing cost reduction in the future.

1.4 Aims and Objectives

My research is part of an ongoing collaborative project whose major goal is to develop rapid, miniaturized nucleic acid diagnostic tests for drug-resistant blood-borne pathogens. The overall approach involves taking 7 mL of blood from patients and detecting any of 3 *Enterobacteriaceae* species and 4 common AR genes at levels as low as 10 CFU/mL, using a microchip device in 60 min. The overall goal for my thesis was to demonstrate effective labeling of species-specific DNA derived from bacterial AR genes (KPC & VIM) using OG, MBs, and magnetic beads, first in a tube-based method and then in a microfluidic device.

1.5 Thesis Overview

My thesis has the following sections: Chapter 1 serves as an introduction to this thesis, giving the background, motivation, aims and objectives of the project. In chapter 2, I review the various materials and methods that were employed for this work. DNA sequence designs, device fabrication and operation, instrument set-up, and various DNA labeling methods are described. Chapter 3 describes the results of the different labeling agents used for the model DNAs in-tube and on-chip methods. I demonstrated fluorescent labeling of DNA derived from bacterial AR genes and other model DNA using OG and MBs. I also used a simple microfluidic device to detect model AR gene sequences with magnetic bead capture and MBs for labeling. Chapter 4 discusses the conclusions and future work that should follow this thesis.

Chapter 2

Materials and Methods

2.1 Chemicals and Reagents

All chemicals were of analytical grade purity or higher. Tris hydrochloride, Tris base, sulfuric acid, sodium chloride, magnesium chloride, and ethanol were obtained from Sigma-Aldrich (St. Louis, MO), Merck (Darmstadt, Germany) or Fisher Scientific (Pittsburgh, PA). Sodium hydroxide was from Mallinckrodt Baker (Paris, KY). OG was obtained from Molecular Probes (Portland, OR). Since OG concentration is proprietary, the initial concentration was defined as 1x. Hybridization/capture buffer (20 mM Tris-HCl pH 8 with 500 mM NaCl, 50 mM $MgCl_2$, and 20% ethanol (v/v)) was used for diluting OG (200X, 400X, and 600X) and oligonucleotides prior to use. This buffer was prepared using deionized water (18.3 MΩ) purified by a Barnstead EASYpure UV/UF system (Dubuque, IA).

2.1.1 DNA Sequence Design

Oligonucleotides used in these experiments (see Table 2) were obtained from Eurofins Operon (Huntsville, AL).

Table 2. DNA oligonucleotide sequences used. Sequences are given 5′–3′.

KPC1 Length: 90 mer Sequence: CATTCAAGGGCATCTTTCCGAGATGGGTGACCACGGAACCAGCGGATGCCCATGCCCTAT CAGTCAAGACAGCAGAACTAGACGGCGATA Capture sequence: TATCGCCGTCTAGTTCTGCTGCTGTCTTG
Fl KPC1 Length: 90 mer Sequence: [Fl]CATTCAAGGGCATCTTTCCGAGATGGGTGACCACGGAACCAGCGGATGCCCATGCCCT ATCAGTCAAGACAGCAGAACTAGACGGCGATA
KPC2 Length: 120 mer Sequence: ATGTCACTGTATCGCCGTCTAGTTCTGCTGTCTTGTCTCTCATGGCCGCTGGCTGGCTTTTC TGCCACCGCGCTGACCAACCTCGTCGCGCTAAACTCGAACAGGACTTTGGCGGCTCCA Capture sequence: TATCGCCGTCTAGTTCTGCTGCTGTCTTG
KPC MB Length: 90 mer Sequence: CATTCAAGGGCATCTTTCCGAGATGGGTGACCACGGAACCAGCGGATGCCCATGCCCTAT CAGTCAAGACAGCAGAACTAGACGGCGATA Capture sequence: TATCGCCGTCTAGTTCTGCTGCTGTCTTG
E. coli Length: 108 mer Sequence: GCTACACAAGCCGAAAGAACTGTACAGCGAAGAGGCAGTGTAGCGCATAACCGTAGCAT G GACCTAGAGCGAAGCTTGCAATCGAAACYCAGCARGCGCACTTACAGG
VIM MB New Length: 24 mer with bolded stem part Sequence: [6-FAM] GCGAGCTACCCGTCCAATGGTCTCATTGTCCGTGATGGTGATGCTCGC[BHQ1a-Q]
VIM MB New target Length: 36 mer Sequence: ATCACCATCACGGACAATGAGACCATTGGACGGGTA

Fl VIM Length: 90 mer Sequence: [Fl]TGCCGCTGGTGTTTGGACGCATATCTCATCGCAGTCGTTTGATGGCNCGG TCTTCCCGTATTCCTGTCTGGTAAAGTCGACCTCTCCGAC
VIM Length: 90 mer Target sequence: TGCCGCTGGTGTTTGGACGCATATCTCATCGCAGTCGTTTGATGGCNCGG TCTTCCCGTATTCCTGTCTGGTAAAGTCGACCTCTCCGAC
VIM Length: 90 mer Complement sequence: GTCGGAGAGGTCGACTTTACCAGACAGGAATACGGGAAGACCGNGCCATCAAACGACTG CGATGAGATATGCGTCCAAACACCAGCGGCA
VIM Bio Length: 53 mer Sequence: [BioTEG]ACCCAGGTTTAGTGAGTGTTGAGCTGTCGGTCGGAGAGGTCGACTTTACCAGA
Model MB Length: 32 mer Sequence: FAM-GCGAGCCAGGTTCTCTTCACAGATGCGCTCGC-BHQ1
Model MB target Length: 24 mer Sequence: ACGCATCTGTGAAGAGAACCTGGG

2.2 Instruments

2.2.1 NanoDrop 3300 Spectrophotometer

The concentrations of labeled solutions were measured by a Nanodrop ND-3300 UV spectrophotometer (Wilmington, DE). A NanoDrop 3300 fluorospectrometer instrument was also used to take fluorescence measurements on volumes as small as 1 µL to evaluate the DNA labeling efficiency. The top and bottom sample pedestals were first thoroughly cleaned with sterile deionized water before beginning DNA quantification. About 2 µL of sample mixtures were carefully transferred by pipette onto the bottom sample pedestal, and the quantity of DNA present was calculated by the NanoDrop 3300 collection software. Standard curves were created

by serial dilutions of known concentrations of the DNA strands being quantified according to the manufacturer's instructions (Wilmington, DE). Blank readings made of only buffer or only DNA were also taken. Fluorescence measurements were performed at room temperature. DNA-containing samples were mixed with equal volumes of OG at different dilutions and fluorescence was measured within 5 min at 515 nm (with excitation at 480 nm).

2.2.2. Device Fabrication

Microfluidic devices were made from polypropylene obtained from Great Basin Corporation (Salt Lake City, Utah) and had channels with cross-sections 500 μm wide and 500 μm deep.

2.2.3. Device Operation

The microfluidic device was connected to a Fluigent (Lowell, MA) pressure driven pump and valve system for introduction of buffer, sample, MBs, and beads through various arms as depicted in Fig 4. Flow was typically done between 0.3 to 0.5 bar yielding a 0.4 to 20 μL/ min flow rate. First, the device was filled with hybridization buffer and then 100 μL of modified beads (0.5 μg/μL) were passed through channel arm 1 (Fig. 4). Then, the channel was rinsed with 20 μL of buffer and flow was stopped. About 100 μL of DNA samples of different concentrations were passed through channel arm 2. The heating component, which was set up similar to the one described in a previous paper (40), was then heated to 95 °C for 5 min to denature DNA as depicted in Fig. 5. Next, this same channel (arm 2) was rinsed with 20 μL of buffer. Then the heater near the magnet was heated to 70 °C for 5-7 min and then rinsed with about 20 μL of buffer. The eluted nucleic acid band was detected using a confocal laser-induced fluorescence setup probing a point 2 mm beyond the magnet as described in prior publications (41-42).

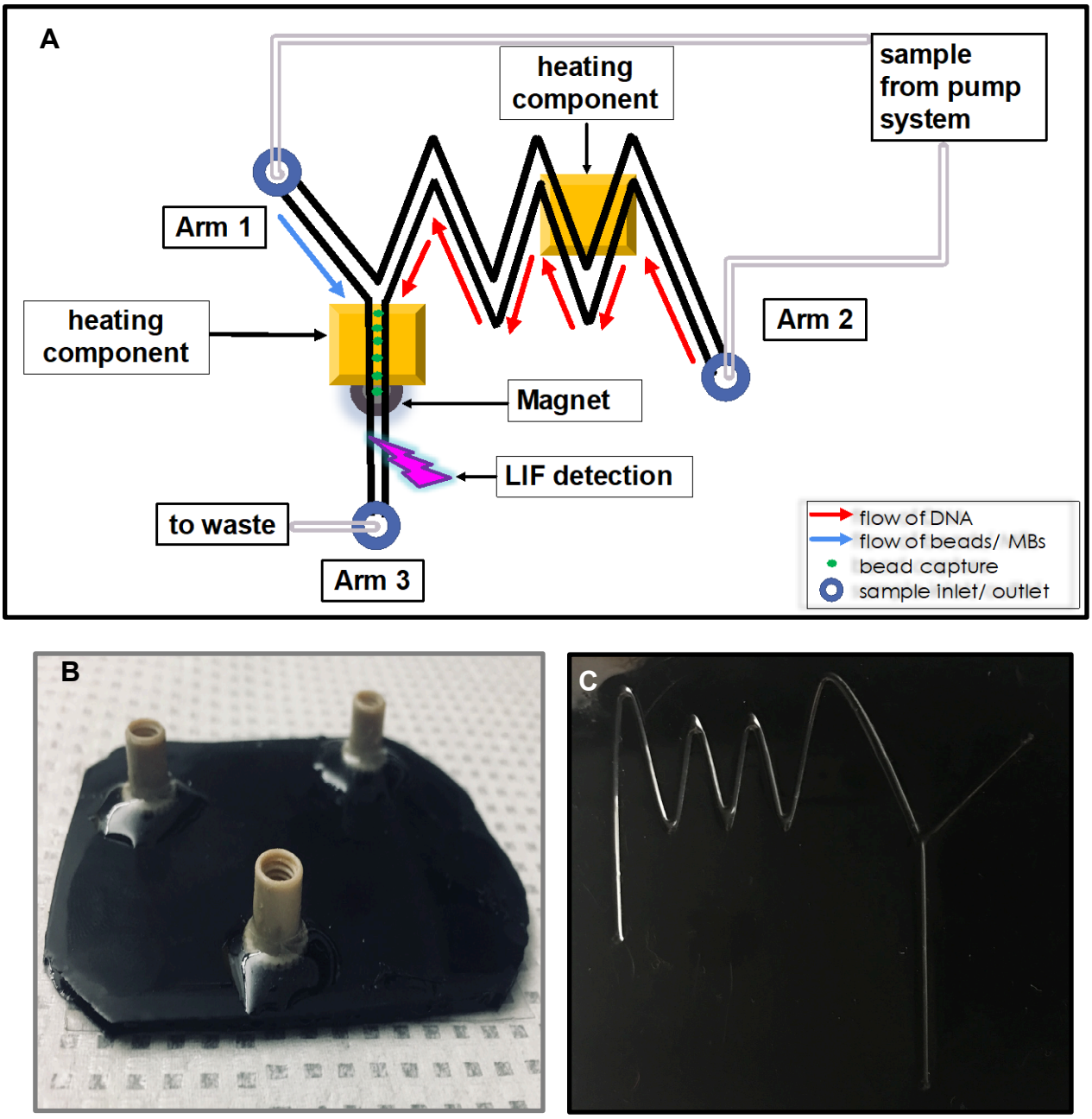


Fig 4. *Microdevice. (A) Schematic representation of design of microchip, set up, and operation. (B) Photograph of the microchip, top view with 3 connectors that lead to the pump system; (C) Photograph of a microchip bottom view showing serpentine channels.*

2.3 DNA Quantitation

2.3.1 Oligreen ssDNA Quantitation

Oligonucleotides (up to 1 μM) were diluted with 1x hybridization buffer. A 50-μL sample of every concentration was mixed well with 50 μL of 200-, 400-, and 600-fold diluted OG and incubated for 5 min at room temperature. After incubation, 2 μL of the sample was measured by means of a NanoDrop 3300 fluorospectrometer (excitation at 480 nm, emission at 515 nm) and a standard calibration curve was plotted.

2.3.2 Molecular Beacon Quantitation

The fluorescence signals of the oligonucleotide (up to 1 μM) in hybridization buffer were measured by adding them to either of 50 or 100 nM MBs with sequences shown in Table 2. The DNA was mixed with the MB and denatured at 65 °C, then cooled to room temperature; fluorescence readings were taken after 15 min.

2.3.3 Magnetic Bead Quantitation

Streptavidin-coated magnetic beads were modified with biotinylated DNA sequences shown in Table 2. First 50 μL of magnetic beads (0.25 mg/mL) were added into 500 μL microcentrifuge tubes and rinsed 3 times with 200 μL of buffer. Next, 2 μL of 100-μM biotinylated DNA capture sequence was added to the beads and incubated for 30 min at room temperature with agitation. The beads were then rinsed with 250 μL of buffer and stored at 4 °C, if not to be used on that same day. About 50 μL of the magnetic beads suspension was then measured and rinsed 1-3 times with 200 μL of buffer. Then, 200 μL of 1-10 nM of the target DNA sequence (Table 2) was added, vortexed to mix, and incubated for 10 min at room temperature on the agitator.

Captured oligonucleotides were then heat denatured at 70 °C for 5-7 min, beads were immobilized, supernatant was transferred, and fluorescence measurements were taken using the NanoDrop 3300 fluorospectrometer (excitation at 480 nm, emission at 515 nm) to confirm successful attachment. For on-chip experiments, the beads were used after capture sequence attachment, and the process described in section 2.2.3 was followed.

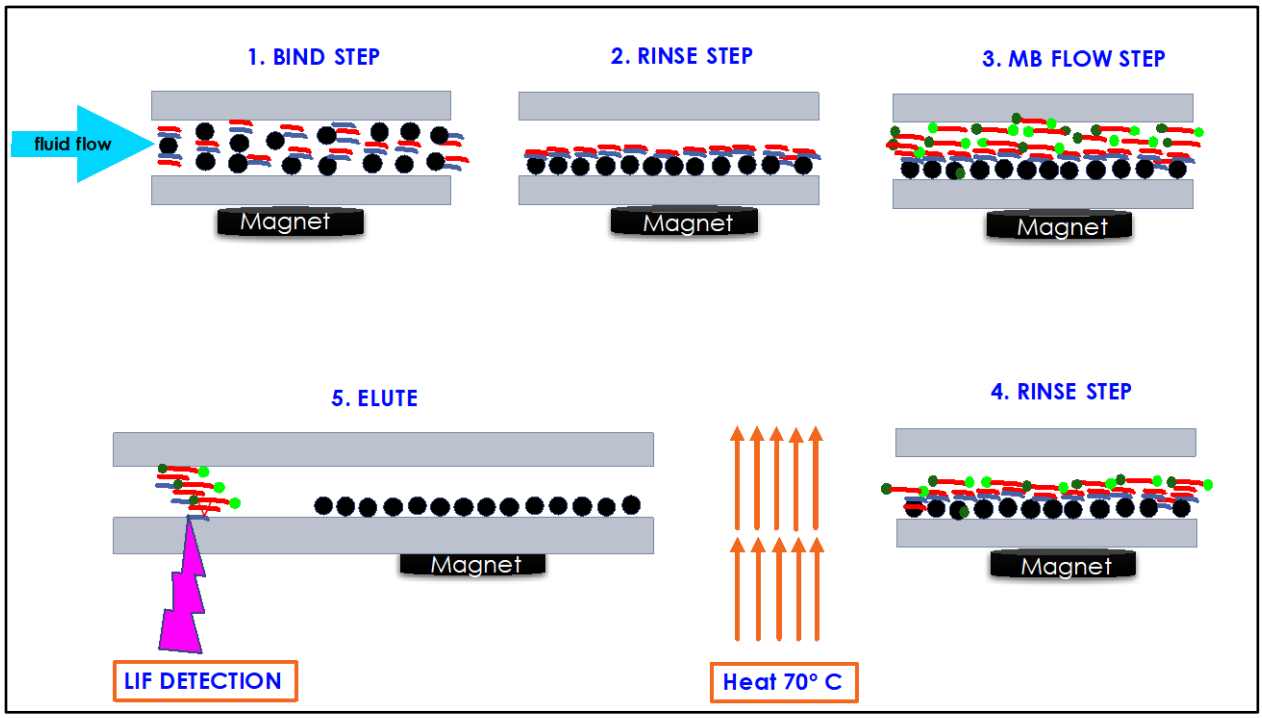


Fig 5. *Illustration of DNA hybridization detection using magnetic beads and MBs in a chip device. 1: Streptavidin magnetic beads functionalized with biotinylated capture strands bind to target DNA strands 2. Buffer is used to wash off excess unbound target DNA. 3. MBs are passed over captured-target DNA functionalized beads at 45℃. 4. Channel is rinsed with buffer to take off excess unbound MBs after cooling to room temperature. 5. After heat of 70℃ is applied, captured-target DNA - MB complex bound strands are released from magnetic beads and detected using LIF system.*

Chapter 3

Results and Discussion

My initial study which was in-tube based using OG and MBs was to find out whether I would be able to effectively label ssDNA using these dyes. However even though the results were promising when I conducted them in-tube, I ran into challenges. These issues included the fact that the Nanodrop device could not detect DNA concentrations below 1 nM, and that when I used longer DNA strands (>250 mer). Therefore, I chose to move to microfluidic devices to overcome these issues. On-chip analysis is able to detect DNA concentrations below 1 nM and longer DNA strands are able to be separated from their complement when captured during flow-through channels.

3.1 Initial OG Testing

Fig. 6 shows fluorescence as a function of concentration of ssDNA (KPC 120 mer, *E. coli* 108 mer), and ssM13mp18+ DNA for different dilution factors of OG dye from 200 to 1000 fold. The results indicate that the signal was linear with increasing concentration of DNA. The results also showed that the more I diluted the OG dye, the less fluorescence was obtained. For all dilutions of oligonucleotides, I was able to obtain a linear plot of fluorescence with DNA concentration. OG dye diluted 200-fold (the dilution recommended by the manufacturer) gave the best signal in Fig. 6. OG dye could be successfully used for labeling and determining unknown ssDNA concentrations quantitatively, which agrees with previous findings (15,17). OG is able to achieve efficient labeling of ssDNA because it has an overall positive charge (15) and thus is able to bind through electrostatics to the negatively charged phosphate backbone of DNA. Fig. 7 shows fluorescence with 200X OG of ssM13mp18+ DNA against dsM13mp18 DNA denatured at 95 °C for 5 min. The magnitude of fluorescence signal doubled for denatured

dsDNA when compared to ssDNA, because the heat denatured the dsDNA into two ssDNA molecules, thus allowing OG dye to complex with the ssDNA to give twice as much signal. Heat treatment of dsDNA (95 °C) can be used to prepare ssDNA for labeling. These OG tube experiments confirmed the properties of OG dye as being able to label ssDNA. However, for this to be used in sepsis diagnosis, I needed to address the issue that OG dye labels all ssDNA due to nonspecific interactions. Thus I studied the use of specific labeling agents such as MBs.

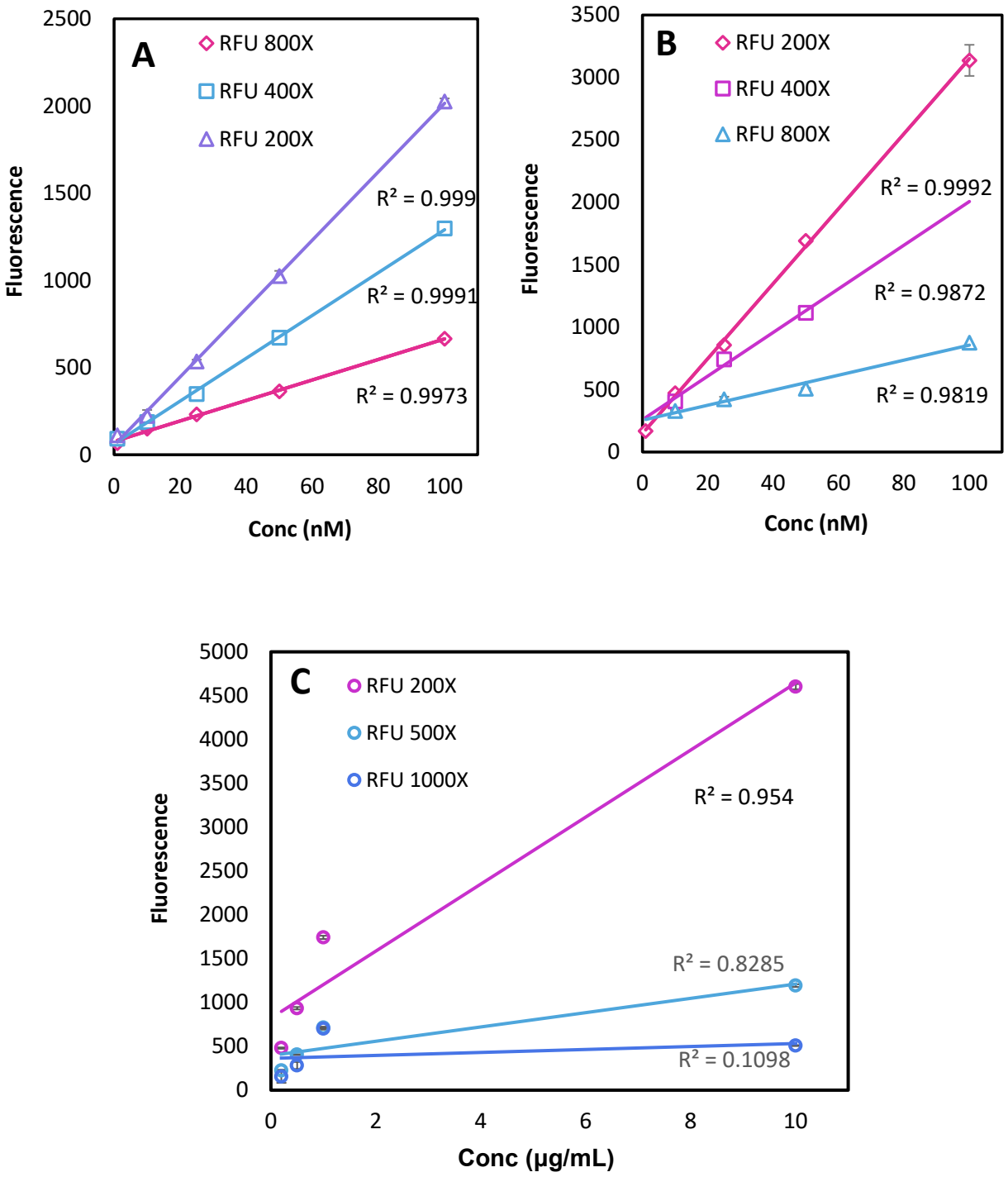


Fig 6. *Effect of different dilution factors of OG dye with different concentrations of ssDNA on fluorescence; (A) KPC 120 mer (B) E. coli 108 mer and (C) ssM13mp18+ DNA in 20 mM Tris HCl, pH 8 on the fluorescence of DNA-dye complex.*

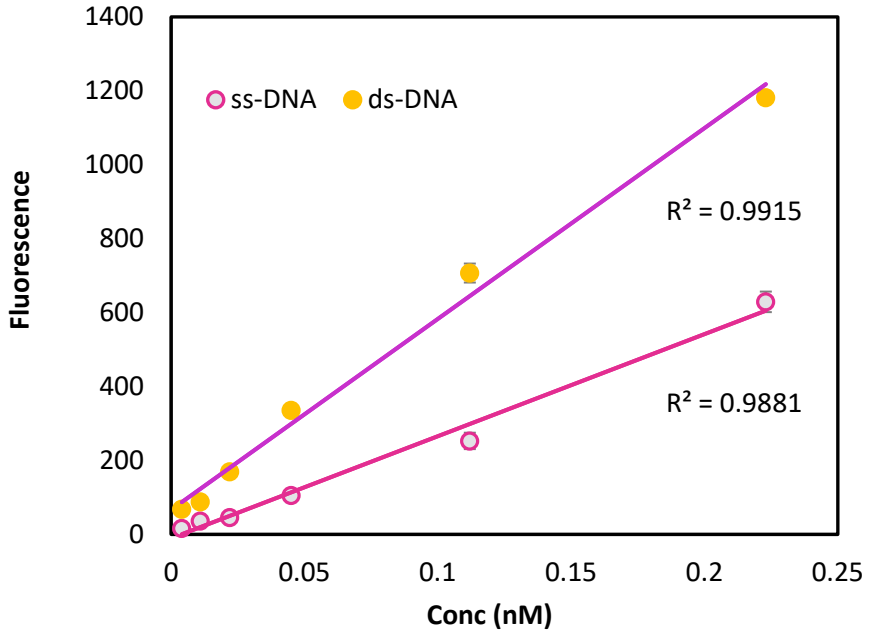


Fig 7. *Comparison of OG fluorescence of ssM13mp18+ DNA against dsM13mp18 DNA denatured at 95 ℃ for 5 min. Labeling was with 200-fold diluted OG in 20 mM Tris HCl, pH 8.*

3.2 Initial MB Testing

For these experiments, I studied various MBs for DNA (KPC and VIM). I measured the fluorescence after hybridizing MBs to their target ssDNA in 20 mM Tris HCl, pH 8. The initially designed MBs had high background signals with little or no measurable difference when they were mixed with their target sequences as shown in Fig.8. These high background signals of MBs could have been caused by contamination by free fluorophores, oligonucleotides that contain the fluorophore but not the quencher, suboptimal salt content of the buffer solution, or incorrect folding of MBs into alternate conformations that result in a sub-population that was not quenched well (43-44).

3.2.1 Initial Assay Conditions

Initially, I investigated various experimental parameters to see if these low signal to background (S/B) ratios were caused by suboptimal conditions or design problems. I pretreated the MBs by heating them to 95 °C for 15 min and waited 30 min to take readings to eliminate the possibility of having some of them fold into alternate conformations that did not quench well. I also changed the buffer composition to include higher salt concentrations (50 mM NaCl and 500 mM $MgCl_2$ in the 20 mM Tris HCl pH 8) to aid with the MB-target hybrid formation. Increased cation concentrations have more interaction with the negatively charged phosphate backbone of the ssDNA, thus screening the negative charge, which could aid with the MB-DNA duplex formation and increase fluorescence signal (14,45). In the end, no difference in fluorescence signal was obtained for MB or MB with target.

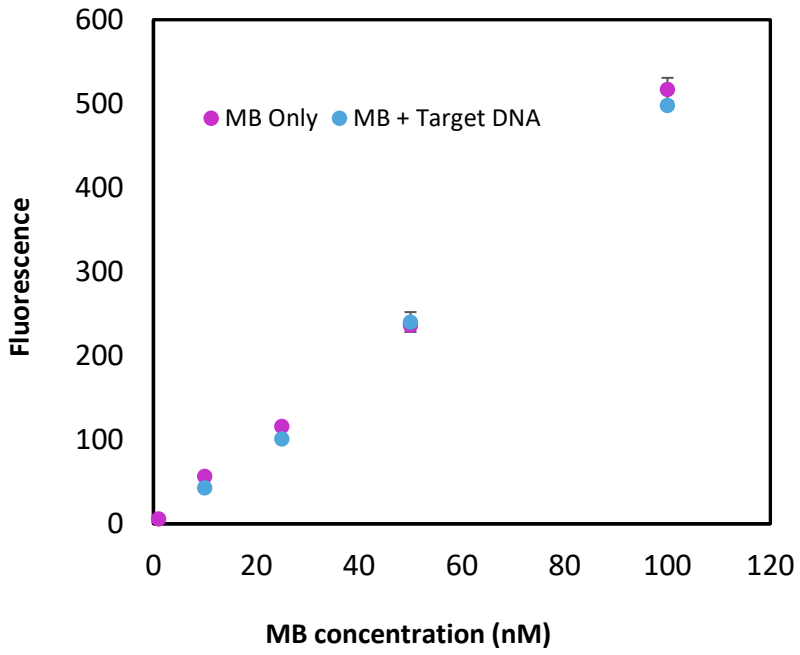


Fig 8. *Fluorescence of VIM 250-mer labeled with VIM MB. Ratio of VIM MB to VIM 250-mer concentration was 1:2 in 50 mM NaCl 500 mM $MgCl_2$ in 20 mM Tris HCl, pH 8.*

3.2.2 Design of MB Stem

Because of these results, I decided to obtain a different MB and DNA from a published paper that achieved high S/B ratio (45). This paper used a MB with a six-base-pair stem and an 18-nucleotide loop. This paper also used ethanol to increase the fluorescence signal, therefore 25% ethanol was used as well as part of my buffer. The sequences for the model MB and model target are given in Table 2. My results for the model MB and target DNA showed a clear difference between fluorescence for MB alone and for MB plus target (Fig. 9). The S/B ratio was 10 (Fig. 9A) using the same conditions as in Fig. 5 and the S/B was 20 with 25% ethanol in the hybridization buffer (Fig. 9B). These results confirmed that it is possible to detect MB-target binding, likely indicating some design flaw in our initially chosen MB. Fluorescence is also linear with concentration, and using a buffer that has some ethanol increases S/B ratio over ethanol-free buffer.

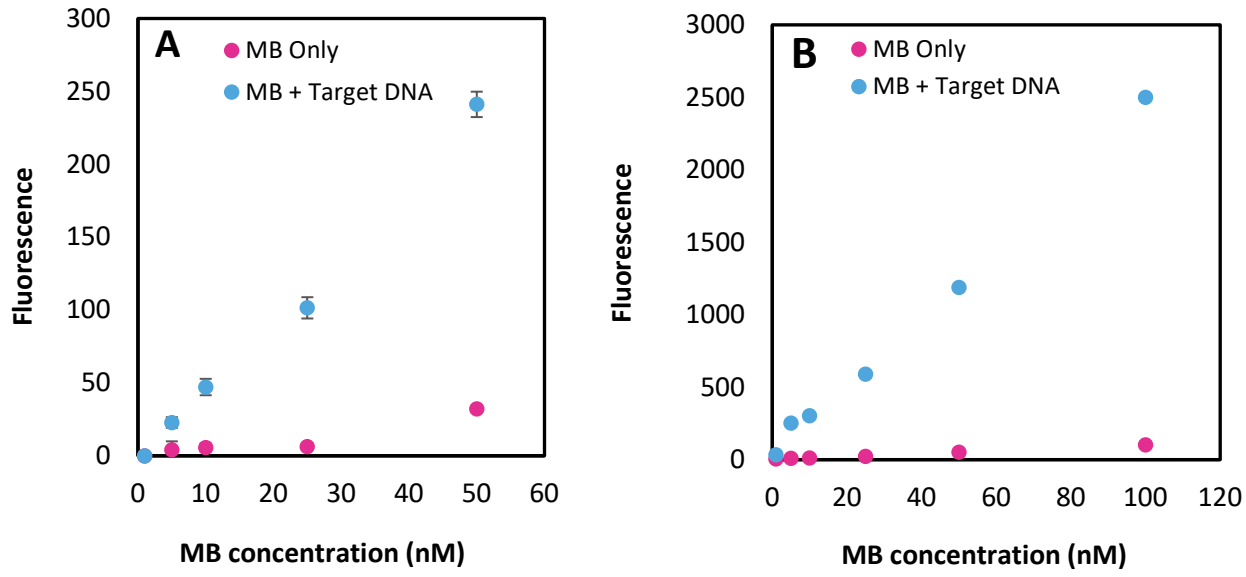


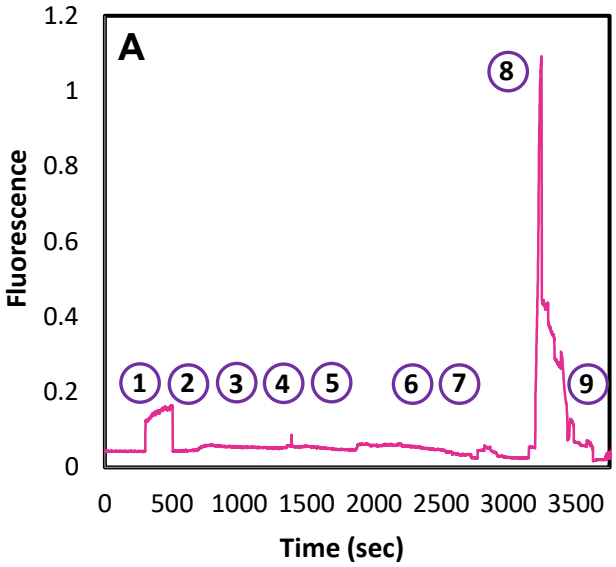
Fig 9. *Model target labeled with model MB denatured at 65 °C. (A) Fluorescence versus MB concentration in buffer without ethanol. (B) Fluorescence versus MB concentration in buffer with 25% ethanol. MB to target ratio is constant at 1:2.*

3.3 Analysis of Model DNA on-chip Using Beads

Model DNA for VIM (90-mer) and KPC (120-mer) was used for on-chip analysis with their corresponding designed MBs. I used the same stem sequence from the model MB as shown in Table 2 to design these new MBs. Streptavidin-coated beads were modified with biotinylated capture DNA sequence, after which DNA target sequences were added to the beads and then labeled by mixing in their complementary target DNA sequence MBs. The captured target DNA with hybridized MBs (sandwich-like structure) were then released from the beads by heating to 70 °C, and supernatant fluorescence was detected.

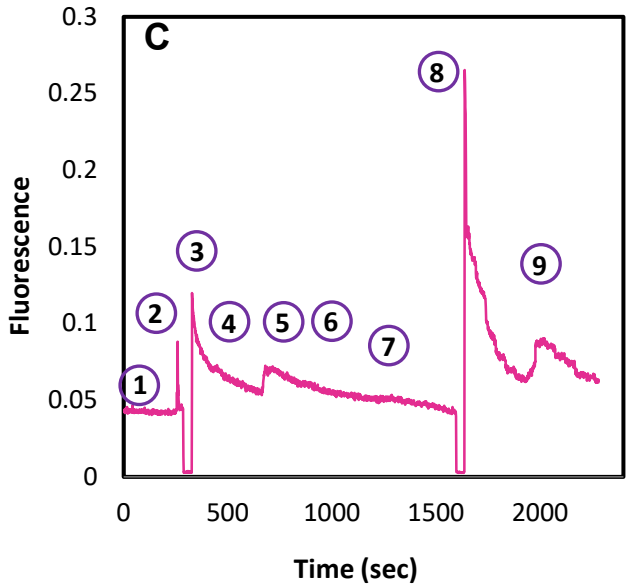
3.3.1 VIM 90 mer Analysis on-chip Using Beads

On-chip fluorescence analysis of model target DNA (VIM 90 mer) was studied. As shown in Fig. 10, two concentrations (10 and 100 pM) were studied using a constant VIM MB concentration of 10 nM. The buffer used was 50 mM NaCl and 500 mM $MgCl_2$ in 20 mM Tris HCl pH 8, and the volume ratio of VIM MB:DNA was 1:1. Event tables for each run are also presented in Fig. 10 to show the sequence of events that took place during on-chip analysis. The results from Fig. 10 show that the main peak occurs after DNA and bead loading, rinsing, MB loading, rinsing, and finally heating for elution. It can be seen that, as the concentration of loaded VIM 90 mer increased from 10 pM to 100 pM, the eluted peak height also increased by about four-fold. This reflects increased capture and labeling of the higher loaded DNA concentration. As shown in Fig. 10, I was able to detect 10 pM of VIM 90 mer with a S/N of 230, so I estimate the limit of detection (LOD) for this system to be about 0.13 pM. The results from Fig. 10 also showed that bead capture worked in this chip-based system for VIM 90 mer using 10 nM VIM MB.



B

Flow	Event	Time (sec)
On	**1.** Beads and DNA loading	0 - 525
On	**2.** Buffer wash	525 - 870
Off	**3.** Heat to 45 deg	870 - 1380
On	**4.** MB flow	1395 - 1530
Off	**5.** Cool to RT	1530 - 1875
On	**6.** Buffer wash	1900 - 2460
Off	**7.** Heat to 70 deg	2470 - 2950
On	**8.** Elute	2950 - 3510
On	**9.** Rinse	3520 - 3710



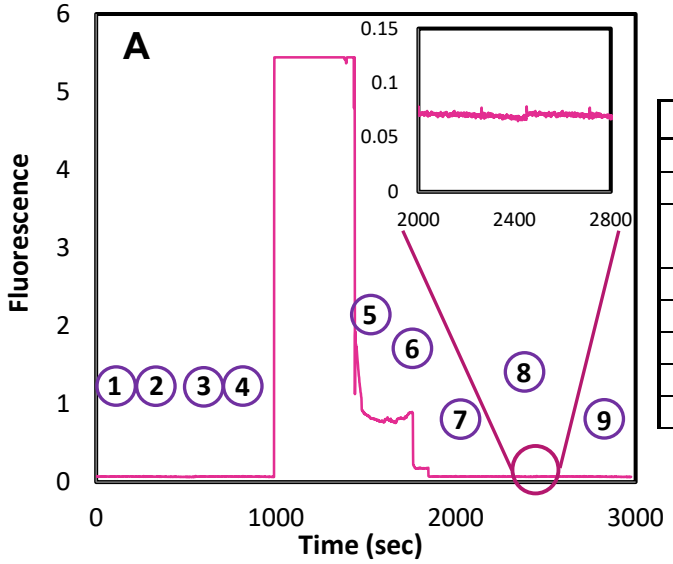
D

Flow	Event	Time (sec)
On	**1.** Beads and DNA loading	0 - 120
On	**2.** Buffer wash	133 - 220
Off	**3.** Heat to 45 deg	266 - 320
On	**4.** MB flow	320 - 620
Off	**5.** Cool to RT	620 - 1092
On	**6.** Buffer wash	1092 - 1200
Off	**7.** Heat to 70 deg	1200 - 1506
On	**8.** Elute	1506 - 1930
On	**9.** Rinse	1930 - 2274

Fig 10. *On-chip fluorescence analysis of VIM 90 mer.* ***(A)*** *100 pM, with VIM MB (10 nM).* ***(B)*** *Event table for (A).* ***(C)*** *10 pM VIM 90 mer with 10 nM VIM MB. (D) Event table for data in (C). Flow rate was 0.4 μL/min.*

3.3.2 VIM 90 mer and KPC MB Stem Analysis on-chip using Beads.

On-chip fluorescence analysis of model target DNA (VIM 90 mer, 100 pM) was studied in labeling with KPC MB (50 nM) as shown in Fig. 11. The buffer used was 50 mM NaCl and 500 mM $MgCl_2$ in 20 mM Tris HCl pH 8 and the flow rate was 0.5 µL/ min. The volume ratio of KPC MB:DNA was 1:1. The event table is presented as Fig. 11B to show the sequence of events that took place during on-chip analysis. The results from Fig. 11 showed no elution peak, which meant that labeling of DNA was not feasible for VIM 90 mer using KPC MB due to lack of specificity. This experiment confirms that fluorescence labeling with MBs in these chips is sequence specific.



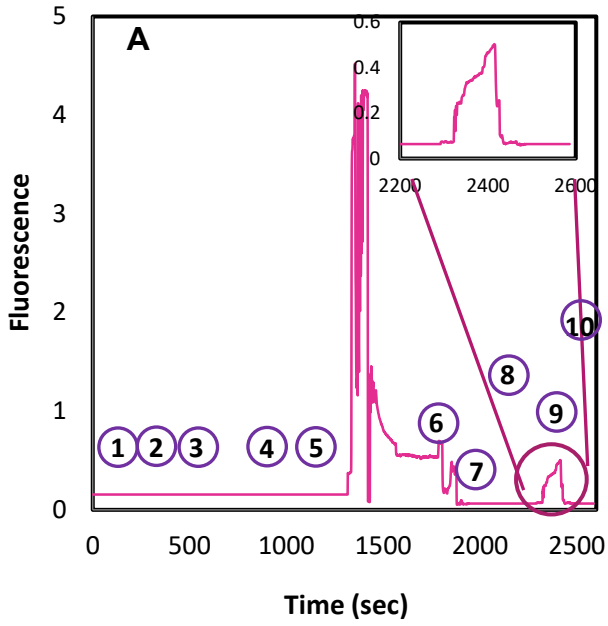
B

Flow	Event	Time (sec)
On	**1.** Beads and DNA loading	0 - 205
On	**2.** Buffer wash	205 - 412
Off	**3.** Heat to 45 deg	473 - 757
On	**4.** MB flow	798 - 1567
Off	**5.** Cool to RT	1567 - 1702
On	**6.** Buffer wash	1702 - 2001
Off	**7.** Heat to 70 deg	2001 - 2291
On	**8.** Elute	2356 - 2655
On	**9.** Rinse	2655 - 2961

Fig 11. *On-chip fluorescence analysis of VIM 90 mer (100 pM) with KPC MB (50 nM) at eluting temperature of 70 ºC and flow rate of 0.5 µL/min.*

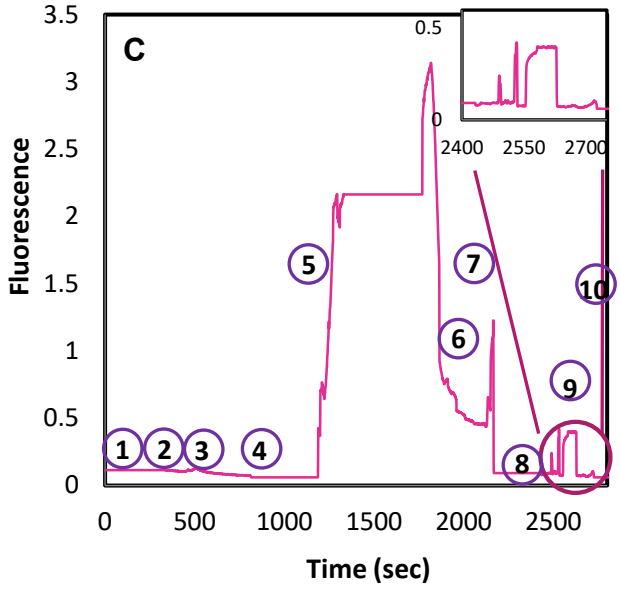
3.3.3 VIM 250 mer Amplicon Analysis on-chip Using Beads

On-chip fluorescence analysis of VIM 250 mer amplicon was also studied. As shown in Fig. 12, two concentrations of amplicon, 50 and 100 nM, were captured and labeled using 25 nM VIM MB. The buffer used was 50 mM NaCl and 500 mM $MgCl_2$ in 20 mM Tris HCl, pH 8 and the flow rate was 2 μL/min. The volume ratio of VIM MB: DNA was 1:1. Event tables for the runs are presented in Fig. 12B and 12D to show the sequence of events that took place during on-chip analysis. The results from Fig. 12 show that bead capture worked for VIM 250 mer amplicon, with labeling using VIM MB and on-chip elution and fluorescence detection. The eluted peak height for 100 nM amplicon was about 40% higher than for 50 nM amplicon showing a concentration dependent trend.



B

Flow	Event	Time (sec)
Off	**1.** Heat to 95 deg	0 - 275
On	**2.** Beads and DNA loading	275 - 467
On	**3.** Buffer wash	467 - 644
Off	**4.** Heat to 45 deg	705 - 1174
On	**5.** MB flow	1215 - 1744
Off	**6.** Cool to RT	1744 - 1874
On	**7.** Buffer wash	1874 - 2014
Off	**8.** Heat to 70 deg	2014 - 2234
On	**9.** Elute	2234 - 2477
On	**10.** Rinse	2477 - 2585



D

Flow	Event	Time (sec)
Off	**1.** Heat to 95 deg	0 - 239
On	**2.** Beads and DNA loading	239 - 416
On	**3.** Buffer wash	416 - 534
Off	**4.** Heat to 45 deg	534 - 1041
On	**5**. MB flow	1041 - 1882
Off	**6.** Cool to RT	1882 - 2124
On	**7.** Buffer wash	2124 - 2259
Off	**8.** Heat to 70 deg	2259 - 2415
On	**9.** Elute	2415 - 2676
On	**10.** Rinse	2676 - 2800

Fig 12. *On chip fluorescent labeling using VIM 250 bp amplicon and VIM MB 53 mer (25 nM) with beads. (A) 100 nM VIM 250 mer with VIM MB; (B) Event table for the run in (A). (C) 50 nM VIM 250 mer with VIM MB. (D) Event table for the run in (C). Flow rate was 2 μL/min.*

3.4 Discussion

In this preliminary study, I was able to fluorescently label, detect and capture model DNAs derived from bacterial AR genes (KPC and VIM) and other model DNA (M13 ssDNA) using OG or MBs, both in-tube and on-chip. I was also able to detect DNA concentrations <1 nM on-chip, improving over the in-tube where the NanoDrop instrument had detection limit issues. My method has potential to be used to label and detect AR genes with simple sample preparation. To have effective labeling of DNA requires good MB design and microchip analysis methods. Microchip analysis can reduce human from sampling and manipulation, while increasing automation; thus, integration of on-chip DNA analysis methods could be effective. The on-chip analysis could involve a disposable single-use device. A future option will be to perform two phases of detecting bacterial AR genes from patients. The first step would involve detecting the lowest amount of DNA that is of potential harm to the patient within some few minutes (as used in this study). The second step would involve more detailed analyses to determine concentrations and could involve qPCR, enzyme assisted ligation, single molecule counting, etc.

Finally, the small amounts of reagents and DNA needed for on-chip analysis in this study make this promising as a low cost assay method that could be commercially viable. Ongoing studies will further examine better ways for denaturing DNA by enzymatic means to avoid the use of heat, which leads to device failure. Also further studies addressing system parameters, such as capture-target-MB incubation time, flow speed, and channel length to select optimal assay conditions, will be needed. Another area that can be studied further is getting the hybrid products to the detection area and preventing liquid movement during heating and cooling. Also, the use of alternative methods of amplification to PCR can be employed (46).

Chapter 4

Conclusions and Future Implications

I have demonstrated fluorescent labeling of DNA derived from bacterial antibiotic resistance genes and other model DNAs using oligreen dye and molecular beacons. Successful labeling with oligreen dye was achieved under the following conditions: 200-fold OG dilution factor, 20 mM Tris HCl, pH 8 buffer, and heat treatment at 95 °C for 15 min. I also used a microfluidic device to detect model AR gene sequences with magnetic bead capture and MB labeling. This study provides a step towards detecting MB-DNA complexes using an automated method which can eventually be applied to detect AR genes in real samples. This preliminary study provides the basis for future work on detecting low levels of non-amplified AR genes at concentrations <10 pM. Additionally, future efforts will focus on labeling different AR genes in the same sample with different MBs (multiplexing) and on refining DNA extraction methods, to simplify sample inputs to devices. The eventual development of such an integrated diagnostic would aid in detecting other pathogenic DNAs, enabling rapid treatments to be administered to patients.

At present, the results from this bead-based capture, on-chip device will serve as the basis to be integrated with a single molecule optical detection system being developed by collaborators (47) for detection of very low DNA concentrations (<10 pM). Through this and other improvements, I expect that bead-based microfluidic devices of this sort will ultimately have significantly lower LODs and faster analysis times.

References

1) Center for Disease Control and Prevention. Antibiotic/Antimicrobial Resistance. https://www.cdc.gov/drugresistance/about.html (Accessed Jan 30, 2017).

2) Caliendo, A.M.; Gilbert, D. N.; Ginocchio, C.C.; Hanson, K.E.; May, L.; Quinn, T.C.; Tenover, F.C.; Alland, D.; Blaschke, A. J.; Bonomo, R.A.; Carroll, K.C.; Ferraro, M.J.; Hirschhorn, L.R.; Joseph, W.P.; Karchmer, T.; MacIntyre, A.T.; Reller, L.B.; Jackson, A.F. Better tests, better care: improved diagnostics for infectious diseases. *Clin Infect Dis.* **2013**, 57, 3, S139-S170.

3) Mackay, I.M.; Arden, K.E.; Nitsche, A. Real-time PCR in virology. *Nucleic Acids Res.*, **2002**, 30, 1290-1305.

4) Ignatiadis, M.; Perraki, M.; Apostolaki, S.; Politaki, E.; Xenidis, N.; Kafousi, M.; Stathopoulos, E.; Lianidou, E.; Sotiriou, C.; Georgoulias, V.; Mavroudis, D. Molecular detection and prognostic value of circulating cytokeratin-19 messenger RNA-positive and HER2 messenger RNA-positive cells in the peripheral blood of women with early-stage breast cancer. *Clin Breast Cancer*. **2007**, 7, 11, 883–889.

5) Maggi, R.G.; Birkenheuer, A. J.; Hegarty, B.C.; Bradley,J.M.; Levy, M.G.; Breitschwerdt, E.B. Comparison of serological and molecular panels for diagnosis of vector-borne diseases in dogs. *Parasites & Vectors.* **2014**, 7, 127.

6) Fredricks, D.N.; & Relman, D. A. Application of Polymerase Chain Reaction to the Diagnosis of Infectious Diseases. *Clinical Infectious Diseases,* **1999**, *29*, 3, 475-486.

7) Arnold, L. J Jr.; Hammond, P. W.; Wiese, W. A.; Nelson, N. C. Assay formats involving acridinium-ester-labeled DNA probes. *Clin Chem*. **1989**, 35, 1588-1594.

8) Forbes, B.A.; Sham, D. F.; Weissfeld, A. S. Nucleic acid based analytic methods for microbial identification and characterisation. Bailey and Scott's Diagnostic Microbiology, 12th ed, **2002.**

9) Malhotra, S.; Sharma, S.; Bhatia, N. J. K.; Kumar, P.; Hans, C. Molecular Methods in Microbiology and their Clinical Application. *J Mol Genet Med*. **2014**, 8, 142.

10) Shokere, L.A.; Holden, M.J.; Jenkins, G.R: Comparison of fluorometric and spectrophotometric DNA quantification for real-time quantitative PCR of degraded DNA. *Food Control* **2009**, 20:391–401.

11) Singer, V.L.; Jones, L.J.; Yue, S.T.; Haugland, R.P: Characterization of PicoGreen reagent and development of a fluorescence-based solution assay for double-stranded DNA quantitation. *Anal Biochem* **1997**, 249:228–238.

12) Georgiou CD, Papapostolou I: Assay for the quantification of intact/ fragmented genomic DNA. *Anal Biochem* **2006**, 358:247–256.

13) Johnson, I.; Spence, M.T.Z. The Molecular Probes Handbook: A Guide to Fluorescent Probes and Labeling Technologies, 11th ed.; Life Technologies, Inc.: Carlsbad, CA, USA, **2010**.

14) Watson, J. D.; Crick, F. H. C. A structure for deoxyribose nucleic acid. *Nature* **1953**, 171, 737–738.

15) Reyderman, L.; Stavchansky, S. Determination of single-stranded oligodeoxynucleotides by capillary gel electrophoresis with laser induced fluorescence and on column derivatization. *J. Chromatogr. A.* **1996**, 755:271–280.

16) Reyderman, L.; Stavchansky, S. Quantitative Determination of Short Single-Stranded Oligonucleotides from Blood Plasma Using Capillary Electrophoresis with Laser-Induced Fluorescence. *Anal. Chem*. **1997**, 69, 3218–3222.

17) Zhang, J.; Liang, D.; He, W.; Wan, F.; Ying, Q.; Chu, B. Fast separation of single-stranded oligonucleotides by capillary electrophoresis using OliGreen® as fluorescence inducing agent. *Electrophoresis*. **2005**, 26, 4449–4455.

18) Fang, X.; Mi, Y.; Li, J. J.; Beck, T.; Schuster, S.; Tan, W. Molecular beacons: fluorogenic probes for living cell study. *Cell Biochem Biophys* **2002**, 37, 71-81.

19) https://www.antibodies-online.com/resources/17/5035/magnetic-beads/ (Accessed March 04, 2018)

20) Kularatne, B.Y.; Lorigan, P.; Browne, S.; Suvarna, S.K.; Smith, M.O.; Lawry, J. Monitoring tumour cells in the peripheral blood of small cell lung cancer patients. *Cytometry,* **2002**, 50, 3, 160–167.

21) Tyagi, S.; Kramer, F.R. Molecular beacons: probes that fluoresce upon hybridization. *Nat. Biotechnol*. **1996**,14, 303-308.

22) Tyagi, S.; Bratu, D. P.; Kramer, F. R. Multicolor molecular beacons for allele discrimination. *Nat. Biotechnol.* **1998**, *16*, 49-53.

23) Fang, X.H.; Liu, X.J.; Schuster, S.; Tan, W.H. Designing a novel molecular beacon for surface-immobilized DNA hybridization studies. *J. Am. Chem. Soc.* **1999**, *121*, 2921-2922.

24) Zigeuner, R.E.; Riesenberg, R.; Pohla, H.; Hofstetter, A.; Oberneder, R. Isolation of circulating cancer cells from whole blood by immunomagnetic cell enrichment and unenriched immunocytochemistry in vitro. *J. of Urology***, 2003***,* 169, 2, 701–705.

25) Matsunaga, T.; Higashi, Y.; Tsujimura, N: Drug delivery by magnetoliposomes containing bacterial magnetic particles. *Cell Eng* **1997**, 2:7-11.

26) Jordan, A.; Scholz, R.; Wust, P.; Fahling, H.; Krause, J.; Wlodarczyk, W.; Sander, B.; Vogl, T.; Felix, R: Effects of magnetic fluid hyperther- mia (MFH) on C3H mammary carcinoma *in vivo***.** *Int J Hyperthermia* **1997**, 13,6,587-605.

27) Plouffe, B. D.; Murthy, S. K.; Lewis, L. H. Fundamentals and Application of Magnetic Particles in Cell Isolation and Enrichment. *Reports on Progress in Physics,* **2015**, *78*, 016601.

28) Alphonse, R. S.; Vadivel, A.; Zong, S.; McConaghy, S.; Ohls, R.; Yoder, M. C.; Thébaud, B. The isolation and culture of endothelial colony-forming cells from human and rat lungs. *Nat. Protoc,* **2015,** 10, 11, 1697–1708.

29) Joshi, P.; Williams, P. S.; Moore, L. R.; Caralla, T.; Boehm, C.; Muschler, G.; Zborowski, M. Circular Halbach Array for Fast Magnetic Separation of Hyaluronan-Expressing Tissue Progenitors. *Anal. Chem,* **2015**, 87, 19, 9908–9915.

30) How does magnetic separation work? https://www.sepmag.eu/blog/bid/333680/how-does-magnetic-bead-separation-work (accessed March 14, 2018).

31) Whitesides, G. The origins and the future of microfluidics *Nature,* **2006**, 442 ,7101, 368-373.

32) Mark, D.; Haeberle, S.; Roth, G.; Stetten, F.; Zengerle, R. Microfluidic lab-on-a-chip platforms: requirements, characteristics and applications. *Chem Soc Rev* **2010**, 39, 1153–1182.

33) Mabey, D.; Peeling, R.W.; Ustianowski, A.; Perkins, M. D. Tropical infectious diseases: diagnostics for the developing world. *Nat Rev Microbiol* **2004,** 2, 231–240.

34) Liu, Y.; Rauch, C.B.; Stevens, R.L.; Lenigk, R.; Yang, J.; Rhine, D.B.; Grodzinski, P. DNA amplification and hybridization assays in integrated plastic monolithic devices. *Anal Chem,* **2002**, 74, 13, 3063–3070.

35) Juergen, P.; Yi, Z.; Pavel, N.; Tseng-Ming, H. Clockwork PCR including sample preparation. *Angew Chem,* **2008**, 47, 3900–3904.

36) Pipper, J.; Inoue, M.; Ng, L.F.P.; Neuzil, P.; Zhang, Y.; Novak, L. Catching bird flu in a droplet. *Nat Med,* **2007**, 13, 1259–1263.

37) Anderson, R.C.; Su, X.; Bogdan, G. J.; Fenton, J. A miniature integrated device for automated multistep genetic assays. *Nucleic Acids Res,* **2000**, 28, e60.

38) Govind, V.K.; Ryan, J. H.; Jutta, P.; Xiao-Li, P.; Linda, M. P.; Christopher, J. B. Automated screening using microfluidic chip-based PCR and product detection to assess risk of BK virus-associated nephropathy in renal transplant recipients. *Electrophoresis*, **2006**, 27, 3753–3763.

39) Lee, Y-F.; Lien, K-Y.; Lei, H-Y.; Lee, G-B. An integrated microfluidic system for rapid diagnosis of dengue virus infection. *Biosens Bioelectron*, **2009**, 25, 745–752.

40) Knob, R.; Nelson, D.B.; Robison, R.A.; Woolley, A. T. Sequence-specific DNA solid-phase extraction in an on-chip monolith: Towards detection of antibiotic resistance genes*, J. Chromatogr. A*. **2017**, 1523, 309-315.

41) *Sun*, X.; Yang, W.; Pan. T.; Woolley, A.T. Affinity Monolith-Integrated Poly (methyl methacrylate) Microchips for On-Line Protein Extraction and Capillary Electrophoresis. *Anal. Chem.,* **2008**, 80, 5126-5130.

42) Yang, W.; Yu, M.; Sun, X.; Woolley, A. T. Microdevices integrating affinity columns and capillary electrophoresis for multi-biomarker analysis in human serum. *Lab Chip*, **2010**, 10, 2527-2533.

43) Vet, J.A.M.; Marras, S.A.E. *Design and optimization of molecular beacon real-time polymerase chain reaction assays. In Herdewijn, P. (ed.), Oligonucleotide synthesis: Methods and Applications*. Humana Press, Totowa, NJ, **2004**, 288, 273-290.

44) Porschke, D.; Uhlenbec, O.; Martin, F. H. Thermodynamics and kinetics of the helix-coil transition of oligomers containing GC base pairs. *Biopolymers* **1973**, *12*, 1313.

45) Dave, N.; Liu, J. Fast molecular beacon hybridization in organic solvents with improved target specificity. *J. Phys. Chem. B.* **2010***,* 114, 15694–15699.

46) Fakruddin, M.; Mannan, K. S. B.; Chowdhury, A.; Mazumdar, R. M.; Hossain, M. N.; Islam, S.; Chowdhury, M. A. Nucleic acid amplification: Alternative methods of polymerase chain reaction. *Journal of Pharmacy & Bioallied Sciences***, 2013**, *5*, 4, 245–252.

47) Ozcelik, D.; Parks, J. W.; Wall, T. A.; Stott, M. A.; Cai, H.; Parks, J. W.; Hawkins, A. R.; Schmidt, H. Optofluidic wavelength division multiplexing for single-virus detection. *Proc. Natl. Acad. Sci,* **2015**, 112, 12933–12937.