Development of the first generation in-situ pathogen detection system (Gen1-IPDS) based on NanoGene assay for near real-time *E. coli* O157:H7 detection

by

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Abstract

We developed the 1st generation in-situ pathogen detection system (Gen1-IPDS) based on the NanoGene assay for detecting and quantifying *E. coli* O157:H7 gene. The NanoGene assay employs the hybridization of target DNA with quantum dot labeled magnetic beads and probe DNA to detect and quantify the target bacterial gene. The Gen1-IPDS is currently capable of executing three key steps required in the NanoGene assay: sample and reagents introduction, DNA hybridization, and magnetic separation of complexes. Operational parameters such as magnet position, hybridization buffer composition, hybridization flow rate, and hybridization temperature were investigated. Using the experimentally determined operational parameters, the target gene was successfully quantified (R^2=0.97) over a range of six orders of magnitude (10^{-12} to 10^{-6} mol L^{-1}). The limit of detection (LOD) was determined to be 49×10^{-15} mol L^{-1}. The specificity was also demonstrated by the differential discrimination of mismatched target DNAs. The NanoGene assay quantification results via Gen1-IPDS were validated by correlation with its laboratory version (R^2=0.97).

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List of Abbreviations

bp	Base-pair
BSA	Bovine serum albumin
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EDC	Ethylcarbodimide hydrochloride
Gen1-IPDS	First-generation in-situ pathogen detection system
LCD	Liquid crystal display
LOD	Limit of detection
MB	Magnetic bead
NHS	*N*-Hydroxysulfosuccinimide
PCR	Polymerase chain reaction
PMMA	Poly-methyl methacrylate
PTFE	Polytetra-fluoroethylene
PWM	Pulse-width modulation
QD	Quantum dot
SDS	Sodium dodecyl sulfate
SSC	Saline-sodium citrate
ssDNA	Single stranded DNA

Chapter 1: Introduction

1.1 *Escherichia coli* O157:H7 Contamination

Public waterways are extremely vulnerable to contamination from organisms such as *Escherichia coli* [1, 2], *Cryptosporidium* [3], and *Giardia lamblia* [4]. *E. coli* O157:H7, a pathogenic strain, is the center of an abundance of research endeavors. This strain is harmful to public health and can cause severe symptoms including fever, diarrhea, stomach cramps, nausea, and vomiting [5]. If *E. coli* O157:H7 contamination is severe enough, it can even cause death. There have been many reported cases of *E. coli* O157:H7 related deaths in the modern developed world. For example, *E. coli* O157:H7 was responsible for the deaths of 20 people in Lanarkshire, UK in 1996 [6]. There were also a reported seven deaths in Ontario, Canada in 2000 caused by the consumption of *E. coli* O157:H7 contaminated water [7]. Unfortunately, public officials knew the water source in Ontario was susceptible to pathogenic contamination. Had this waterway been continuously monitored for *E. coli* O157:H7, perhaps these deaths could have been prevented.

E. coli O157:H7 is often found in fecal matter and can cause public waterways to be contaminated by a number of different pathways. Some of these pathways include contaminated cattle feces transported to waterways via surface runoff [8], overflow of sewage systems, and leaking septic systems [9].

1.2 Need for In-Situ Monitoring of *E. coli*

Given its potential catastrophic impact on public health, it would be beneficial to monitor waterways on a near real time basis for *E. coli* contamination. This will require an in-situ pathogen detection system (IPDS) that is capable of executing a pathogen detection method under field instead of laboratory conditions. Field conditions will include the lack of means to use existing laboratory equipment and its associated kits. Therefore, the selection of the appropriate pathogen detection method is critical to the success of the IPDS.

1.3 Traditional *E. coli* Detection Methods

Currently, the most widely used pathogen detection methods are culture based counting and polymerase chain reaction (PCR) [10]. Culture-based methods are labor intensive, time consuming, and not sensitive. PCR yields fast results but requires a large footprint, clean environment, and is prone to inhibition [11, 12]. Therefore, both culture-based methods and PCR in their present form are not yet suitable to be employed by the IPDS.

1.4 Novel *E. coli* Detection Methods

Fortunately, new pathogen detection methods based on nanoparticles (including quantum dots, QD) have been developed in recent years [13-15]. They can be more robust and yield faster results than culture-based methods or PCR [16, 17].

Quantum dots are nanoparticles with an average diameter of 15 to 20 (nanometers) nm. They were first discovered in the early 1980s by Alexander Efros and

Alexei Ekimov. Quantum dots are semiconducting nanocrystals with a Cadmium-Selenium core and a Zinc-Sulfur shell [18]. These relatively new nanoparticles are often selected as fluorescent labels over organic dyes because they are photo-stable and have a broad adsorption spectrum, allowing for the detection of a range of different colored particles [19]. Quantum dots have numerous applications including neuroscience [20] and oncology [21]. Also, because of their comparable size with DNA, they are often used to fluorescently label DNA for various quantification and detection methods.

A new nanoparticle detection method is the magnetic bead-quantum dot based NanoGene assay that was previously developed for the quantification and detection of *E. coli* O157:H7 gene [22]. A schematic of the NanoGene assay can be seen in Figure 1. This approach employs quantum dot labeled magnetic beads (MB) tethered with probe DNA and quantum dot labeled signaling DNA. The probe and signaling DNAs have sequences complementary to that of *E. coli* O157:H7 gene. When the MB-QD-DNA complexes and the signaling QD-DNA complexes are hybridized with the target DNA (*E. coli* O157:H7 *eaeA* gene), a sandwich configuration is formed and can be magnetically separated. The fluorescence of the magnetically separated signaling QD-DNA complexes is measured and normalized against the fluorescence intensity of the MB-QD-DNA complexes to quantity *E. coli* O157:H7. NanoGene assay is selected as the pathogen detection method for the IPDS because it appears to be capable of working with inhibitor-laden samples [23] and has the potential to be performed under field conditions.

1.5 Experimental Objectives

The long term objective of this effort is to develop a portable briefcase size IPDS, based on the NanoGene assay, where the human user or robotic siphon can introduce a raw water sample into the IPDS and obtain near real time (within 15 minutes) detection and quantification results of the target *E. coli* O157:H7 gene. This data can be transmitted wirelessly immediately to the relevant agencies or used to trigger an alarm or response. As a part of this effort, we developed the 1st generation IPDS (Gen1-IPDS) to perform three key steps in the NanoGene assay with minimal human intervention with potential for eventual autonomous operation. The keys steps are: *(i) sample and reagents introduction, (ii) DNA hybridization, and (iii) magnetic separation of complexes*. To achieve this purpose, an assembly of components such as miniature peristaltic pumps, magnet positioner, microfluidic chip, and microcontroller were employed. The microfluidic chip was selected to facilitate rapid mixing and hybridization. The peristaltic pumps were selected for autonomous implementation and the minimization of contamination to reagents and samples. Hybridization buffer composition that minimized bubble formation in the microfluidic chip and operational parameters such as hybridization flow rate and magnet positioning were investigated. In addition, the viability of performing hybridization at ambient temperature was also studied. The linearity, sensitivity, range of quantification, and specificity of the NanoGene assay via Gen1-IPDS (Gen1-IPDS NanoGene assay) were also obtained. Finally, the results were compared with that from the laboratory version of the NanoGene assay (laboratory NanoGene assay).

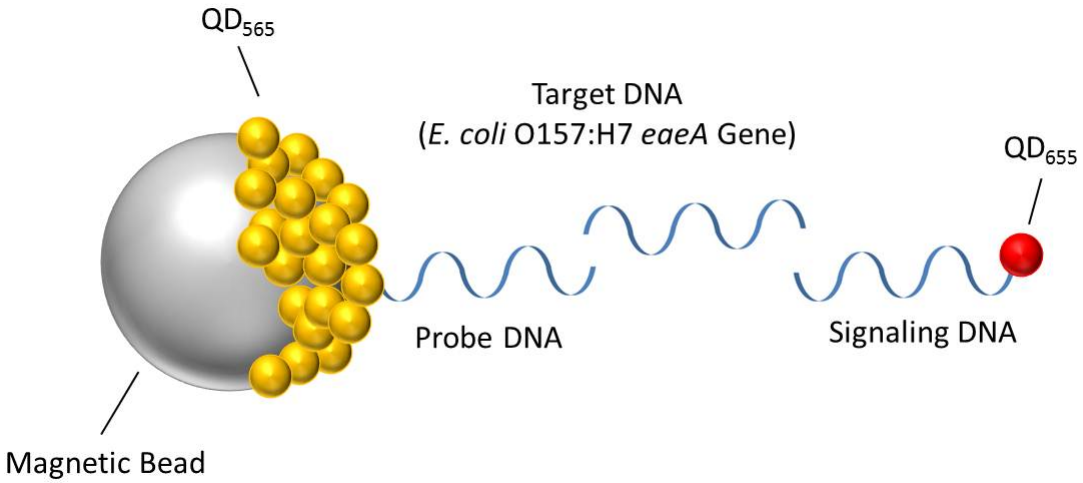


Figure 1. The schematic diagram of the NanoGene assay used in the proposed microfluidic NanoGene assay. QD depicts quantum dots.

Chapter 2: Materials and Methods

2.1 Materials and Apparatus

Single-stranded DNA oligonucleotides were commercially synthesized in accordance with the sequence of *Escherichia coli* O157:H7 gene (Integrated DNA Technologies, Coralville, IA). The DNA sequences can be seen in Table 1. Aminated magnetic beads (MB, Invitrogen, Carlsbad, CA) were coated with carboxyl quantum dots (QD_{565}, Invitrogen) and the signaling DNA was labeled with carboxyl quantum dots (QD_{655}, Invitrogen). Complexes were stored in centrifuge tubes which had been pretreated with a 0.5% bovine serum albumin (BSA, New England BioLabs, Ipswich, MA) in phosphate buffer saline (0.1 M, pH 7.4) solution to prevent nonspecific binding [24, 25]. Tris-EDTA buffer, ethylcarbodimide hydrochloride (EDC), *N*-Hydroxysulfosuccinimide (NHS), monobasic sodium phosphate ($H_2NaO_4P{\cdot}2H_2O$), and dibasic sodium phosphate (Na_2HPO_4) were purchased from Sigma-Aldrich (St. Louis, MO). Sodium borohydride ($NaBH_4$) and sodium dodecyl sulfate (SDS) were purchased from MP Biomedicals, LLC (Solon, OH). Saline-sodium citrate (SSC, 20×) was purchased from Fisher Scientific (Fair Lawn, NJ). DIG easy hybridization buffer (Roche Diagnostic, Basel, Switzerland) and a hybridization oven (UVP, HB-500 Minidizer Hybridization Oven) were used for DNA hybridization in the laboratory NanoGene assay. A magnet was used for the magnetic separation and washing of MB-QD-DNA complexes (Invitrogen, DynaMag™-2). A water bath (Fisher Scientific, ISOTEMP 202S)

was used for the passivation of and a centrifuge was used to wash the DNA-QD conjugations (Eppendorf, Centrifuge 5418).

Fluidic, electromechanical, and electronic components of the Gen1-IPDS were purchased from various retailers and assembled. Fluidic components include microfluidic chips, tubing, and connectors. Electromechanical components include a magnet positioner (in the form of a linear actuator) and miniature peristaltic pumps. Electronic components include a microcontroller, switches, wires, liquid crystal display (LCD), and breadboards. Other components include grade N52 neodymium magnets (K&J Magnetics, Jamison, PA) [26] and laser cut acrylic plates (Pololu Laser Cutting, Las Vegas, NV). One hundred twenty μL poly-methyl methacrylate (PMMA) rhombic chamber microfluidic chips, male mini luer fluid connectors, male mini luer plugs, and a microfluidic support kit including silicon tubing, forceps, and syringes were purchased from the Microfluidic ChipShop (Jena, Germany). Miniature peristaltic pumps, a peristaltic pump tubing pack, polytetra-fluoroethylene (PTFE) tubing (1/16 inch outer diameter, 0.5 mm inner diameter), and a PTFE tube cutter were purchased from Dolomite (Royston, UK). The magnet positioner used for positioning the magnet as well as alternating waste and sample collection was purchased from Firgelli (British Columbia, Canada). The microcontroller used is an Arduino Uno which is based on ATmega328 and has 14 digital input/output pins as well as 6 analog input pins (Italy).

2.2 NanoGene Assay

The laboratory version of the NanoGene assay suitable for quantification and detection of *E. coli* O157:H7 has been previously developed [22]. The NanoGene assay

employs magnetic beads encapsulated with QD_{565} and tethered with probe DNA and signaling DNA labeled with QD_{655}. These particles are then hybridized with target DNA. The target DNA is captured during hybridization. A schematic of the assay is shown in Figure 1. Fluorescence measurements can allow for the quantification and detection of the target.

Preparation of MB-QD particle complexes and DNA-QD conjugation. A suspension of MBs (2×10^7 MB) and QD_{565} (16 moles) were added to a BSA treated centrifuge tube and thoroughly mixed. A solution of EDC-NHS (1:1 molar ratio) was prepared immediately prior to use and 10 μL was added to the MB-QD solution to promote covalent bonding. The tube was then placed in a mix plate (eppendorf, MixMate), protected from photobleaching, and incubated at 1500 rpm for 2 hours at ambient temperature. The complexes were then washed 3 times with phosphate buffer saline (0.1 M, pH 7.4) in conjunction with magnetic separation and supernatant disposal. 500 picomoles of aminated probe DNA was added to the MB-QD complexes along with 10 μL of EDC-NHS solution. The tubes were incubated in accordance with the previously described procedure. Following incubation, the complexes were washed 3 times with phosphate buffer (0.1 M, pH 7.4) using magnetic separation and supernatant disposal. The complexes were stored in PB. A suspension of QD_{655} (16 moles), 160 picomoles of signaling DNA, and 10 μL of EDC-NHS solution were added to a BSA treated centrifuge tube and thoroughly mixed. The tube was incubated in accordance with the previously described procedure. Following incubation, the complexes were passivated to prevent non-specific binding by inactivating the remaining functional groups. A passivation solution was prepared using 10 mL 20× SSC, 0.5 mL 10% SDS, and 90 mL

autoclaved deionized water. Immediately prior to passivation, 5 mg $NaBH_4$ was dissolved in 1 mL of the passivation solution; 100 µL of the $NaBH_4$-passivation solution was added the QD_{655} labeled signaling probe DNA complexes. The complexes were passivated in a 42°C water bath for 20 minutes. The signaling probe DNA labeled with QD_{655} were washed twice using 1× SSC and 0.2× SSC in conjunction with centrifugal separation and supernatant disposal. The complexes were stored in phosphate buffer.

Quantification of E. coli using MB-QD particle complexes based on DNA hybridization. In order to quantify the ssDNA target gene using the laboratory method, 10 µL of the prepared MB-QD-DNA complexes, 1.6 µL of the prepared signaling DNA complexes, and target DNA was suspended in 400 µL DIG easy hybridization buffer. The complexes were placed in the hybridization oven on a slow rotation at 37°C for 8 hours. After DNA hybridization, a magnet was used to hold the tethered particles while the untethered particles were removed. The tethered particles were released from the magnet and washed with PB; this process was repeated three times. The sample was transferred to a 96-well plate and the fluorescent intensity was measured.

Fluorescence measurement. To quantify the target DNA post-hybridization, the fluorescent intensity of the internal standard of QD_{565} and the labeled probe of QD_{655} were measured using a bench-top spectrofluorometer (Molecular Devices, SpecraMax M2, Sunnyvale, CA). An excitation of 360 nm was used for both signals, while an emission of 560 nm was measured for QD_{565} and 650 nm for QD_{655}. The intensity of the signaling probe was normalized (QD_{655}/QD_{565}) to adjust for the possibility of varying amounts of MB-QD complexes.

2.3 Design and Construction of the Gen1-IPDS

The Gen1-IPDS was designed to implement the three earlier mentioned key steps using miniature components instead of laboratory bench top equipment. Sample and reagent introduction was performed via a miniature peristaltic pump (also referred to as injection pump) instead of manual pipetting. Hybridization was performed in a microfluidic chip instead of a vial. A second miniature peristaltic pump (also referred to as recirculation pump) was used to provide agitation during hybridization instead of a rotator in the hybridization oven. It was also used to control retention time during hybridization. Finally, a magnet positioner and magnet replaced a handheld magnet during magnetic separation.

The components were mounted on a laser cut acrylic platform (Figure 2). Holes were laser cut to hold the vials and the miniature peristaltic pumps were held down by a washer and screw. Similarly, the microfluidic chip was secured to the platform via screws and washers to allow easy attachment and adjustment. The microfluidic chip contained two chambers and each chamber has two inlets and two outlets. The chamber and channels have a depth of 500 μm and each chamber is capable of holding a volume of 120 μL. Two separate acrylic pieces were custom cut, joined with epoxy, and attached to the magnet positioner in order to hold the magnet. The magnet positioner moved the magnet onto and off of the microfluidic chip in accordance with the programmed sequence. The Arduino Uno microcontroller board was wired to both a 16 × 2 LCD screen as well as to the electromechanical components via two breadboards. A toggle switch controlled the power supply to the microcontroller from a 9 V battery. Pictures of the assumed Gen1-IPDS are shown in Figure 3a to 3c. The assembled Gen1-IPDS was

sufficiently small and fit inside a briefcase (with dimensions of 37 × 20 × 12 cm and weight of 2.25 kilograms) as shown in Figures 3a and 3c.

After the microcontroller was powered up and the start button was pressed, the injection pump drew the sample and reagents from its vial into the fluidic chamber at a programmed flow rate. The injection pump was turned off and the recirculation pump was turned on to initiate the hybridization cycle at a programmed flow rate and retention time. At the end of the hybridization cycle, the recirculation pump was turned off. The inlet tubing was manually connected to the washing buffer vial and the outlet tubing was manually directed to the waste vial. Magnetic separation and washing were performed by turning on the injection pump. Finally, the magnet positioner was retracted and the outlet tubing was manually directed to the sample collection vial to collect the washed MB-QD-DNA complexes. Figures 4a to 4d show the 4-step sequence schematic of the Gen1-IPDS performing the three key steps and the sample collection with washing.

2.4 Software Control of Peristaltic Pumps and Magnet Positioner

The Arduino Uno microcontroller was programmed with the provided Arduino 1.0.3 software. Pulse-width modulation (PWM) was used to control the magnet positioner extension and the flow rates of the pumps. PWM is a method of delivering electrical power in pulses instead of a continuous analog signal [27]. PWM provides a series of on-off analog patterns, using analog signals to simulate signals between full on and full off [28]. The percentage of time the pulse is “on” is referred to as the duty cycle. The PWM settings for the Arduino Uno microcontroller output pins ranged from 0 to 255, with 255 representing a 100% duty cycle and an output voltage of 5 V. For example, a PWM

setting for the Arduino Uno of 127 (or a 50% duty cycle) would provide 2.5 V at the designated output pin. This feature was used to control the voltage supplied to the pumps which in turned controlled the flow rate.

The magnet positioner has a built-in position feedback feature that uses PWM to determine its position. A 100% duty cycle will give a full extension of the magnetic positioner at 50 mm. At full retraction, the magnet was approximately 30 mm away from the center of the fluidic chamber. To determine the appropriate PWM setting for the magnet positioner in order to trap the MB-QD complex effectively, PWM settings ranging from 125 to 155 (in increments of 5) were investigated. These values represent the range of settings that placed the magnet over the microfluidic mixer chip. Values outside of this range placed the magnet off of the microfluidic chip. For each data point, the magnet positioner was extended in accordance with a PWM setting. One hundred μL of MB-QD-DNA complexes were flushed through the microfluidic chip and the waste was collected. Since the complexes were fluorescently labeled, the fluorescence of the complexes held could be correlated to the percent of complexes held by the magnet. The magnet positioner was retracted and the microfluidic chip was washed with 200 μL of phosphate buffer to collect the complexes held by the magnet. The fluorescence intensity of the sample was measured. Duplicate samples were used for each setting and the average fluorescence intensity was calculated. The highest average fluorescence intensity was assumed to correspond to one-hundred percent of complexes held by the magnet. All other samples were normalized to this data point.

2.5 Identification of Suitable Hybridization Buffer Composition, Flow Rate, and Temperature

In order to identify the suitable hybridization buffer for the Gen1-IPDS NanoGene assay, a variety of hybridization buffers were tested including the formulation used in the laboratory NanoGene assay. The composition of the buffers included: (1) 50% DIG easy hybridization buffer (similar to that used for the laboratory version of NanoGene assay), (2) 0.00% SDS + 0.01% BSA in 10× SSC, (3) 0.05% SDS + 0.01% BSA in 10× SSC, (4) 0.10% SDS + 0.01% BSA in 10× SSC, and (5) 0.20% SDS + 0.01% BSA in 10× SSC [29, 30]. The DNA hybridization performance by each buffer was evaluated by the fluorescence intensity from both the laboratory and Gen1-IPDS NanoGene assays with 10^{-12} mol L^{-1} target ssDNA. No ssDNA (negative control) was also used to evaluate the hybridization performance of each buffer from the laboratory assay. In the Gen1-IPDS NanoGene assay, the hybridization was performed at varying flow rates (50, 90, 150, 210, and 270 μL min^{-1}). The bubble generation mainly from the surfactant (e.g., SDS) of the buffer was also monitored since the substantial amount of bubbles can be a significant inhibition for the hybridization in the microfluidic chip. In order to determine the flow rate to be used in the Gen1-IPDS, hybridization was performed in a separate microfluidic chip using the determined hybridization buffer composition at flow rates of 50, 90, 150, and 210 μL min^{-1}. The fluorescence intensity was measured at 0, 2.5, 5, 10, 20, 30, and 45 min. To determine the appropriate hybridization temperature for the Gen1-IPDS NanoGene assay, hybridization was performed at 10, 23, 37, and 45°C. Experiments were conducted using the previously determined buffer composition and flow rate. The fluorescence intensity was measured at 0, 15, 30, 45, and 60 min.

2.6 Implementing Gen1-IPDS NanoGene Assay

All investigated parameters were combined to finalize the protocol of the assay used in the Gen1-IPDS. These parameters were implemented and hybridization experiments were conducted in order to quantify *E. coli* O157:H7 gene. To allow for hybridization using the in-situ pathogen detection system, 5 μL of the MB-QD-DNA complexes, 2 μL of the DNA-QD signaling complexes, 92 μL of hybridization buffer, and target ssDNA were thoroughly mixed in a centrifuge tube and placed in the device. The device was turned on and the magnet positioner was extended. The injection pump was turned on to load the sample into the microfluidic chip. The injection pump was turned off and the recirculation pump was turned on. The recirculation pump circulated the sample throughout the chip until satisfactory hybridization was achieved. The recirculation pump was turned off and the injection pump was turned on. The sample was washed with 405 μL of phosphate buffer for 1.5 min. The linear actuator was retracted and the sample was collected. The sample was transferred to a 96-well plate and fluorescence was measured using the spectrofluorometer.

These data were then used to generate a calibration curve that allowed for the determination of the range of quantification and limit of detection (LOD) of the assay. Target concentrations used were 0, 10^{-12}, 10^{-10}, 10^{-8}, and 10^{-6} mol L^{-1}. The LOD was determined by adding three standard deviations to the averaged fluorescence intensity of 7 blank samples [31].

Additional experiments were also conducted to prove the specificity of the assay based on discrimination of mismatched target DNA. Perfect match target DNA, target

DNA with 1 and 2 mismatched basepair (bp) sequences (out of a total 55 bp), and non-match target DNA were selected [32]; the sequences of all DNA nucleotides can be found in Table 1. Mismatches of 1 and 2 bp were selected because they can be phylogenetically similar to the target gene sequence. Non-match target DNA represents a completely different bacterium. The results from each data point were compared with that of perfect match DNA and a student's two-sample *t-test* was performed to determine if there was a significant difference in the data.

Table 1. Sequences and modifications of ssDNA nucleotides. The boldface basepairs represent complementary sequences while the underlined basepairs represent mismatched sequences.

	Sequence (5' → 3') and modification
Probe DNA	NH_2- CGG **AT AAGAC TTCCG CTAAA**
Signaling DNA	**CTTAT ACCGC GACGG T**GA AA -NH_2
Target ssDNA	**ACCGT CGCGG TATAA G**TAAT GGTAT CGGCG TTATC CGC**TT TAGCC GAAGT CTTAT**
1 bp mismatched target DNA	**ACCGT CGCGG TATAA G**TAAT GGTAT CGGCG TTATC CGC**TT TAGCC GAA<u>C</u>T CTTAT**
2 bp mismatched target DNA	**ACCGT CGCGG TATAA G**TAAT GGTAT CGGCG TTATC CGC**TT TA<u>C</u>CC GAA<u>C</u>T CTTAT**
Non-matched target DNA	**A**<u>T</u>AAG <u>A</u>CTTC GGCT**A** <u>A</u>AGCG G<u>A</u>TA<u>A</u> CG<u>C</u>CG <u>A</u>TA<u>C</u>C <u>A</u>TTA<u>C</u> **T**<u>T</u>ATA CCGCG A<u>C</u>G**T**

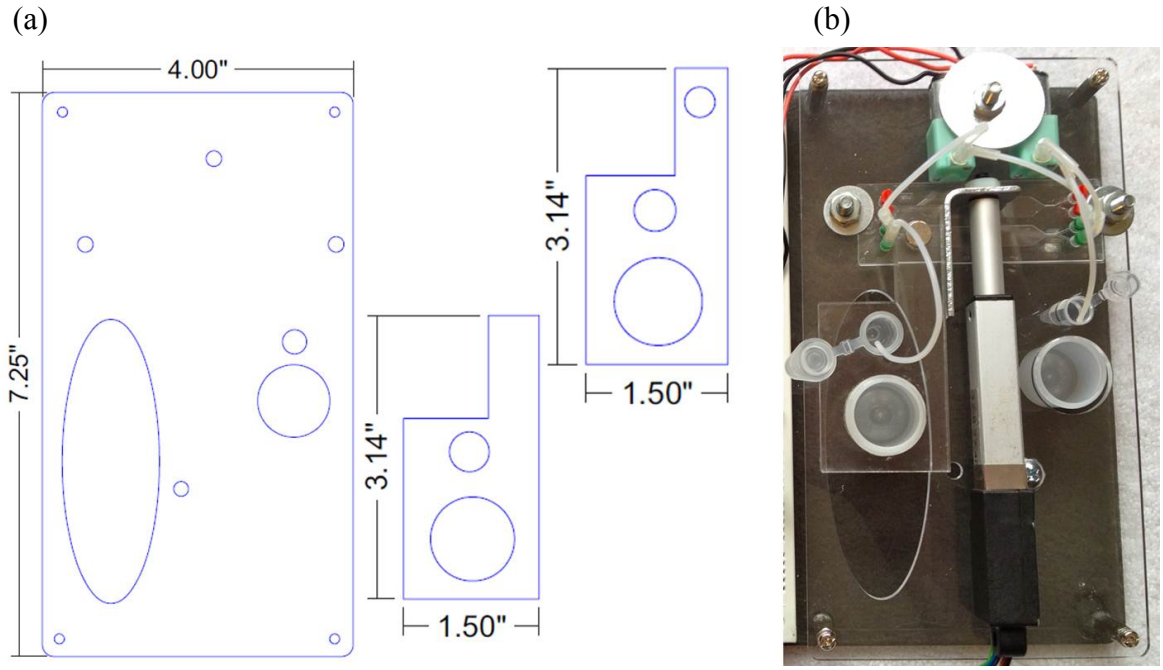
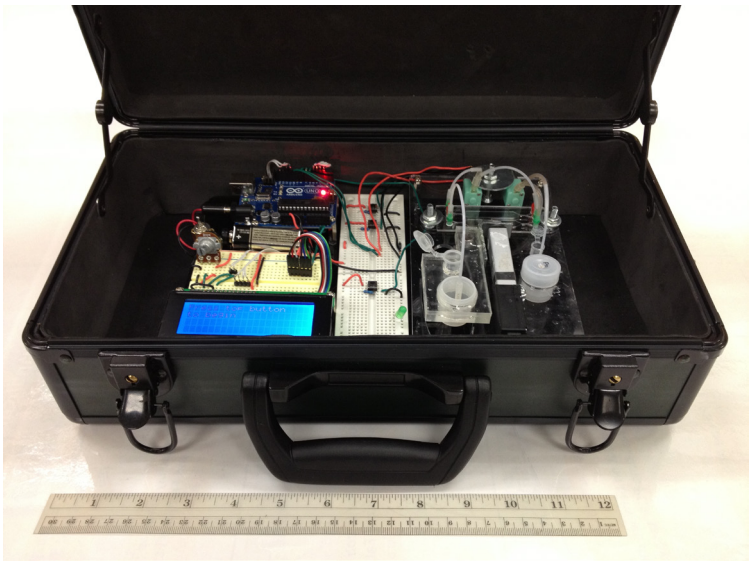


Figure 2. Acrylic cut pieces for the Gen1-IPDS. (a) The computer aided drawings for the acrylic platform and magnet/effluent vial holder; (b) Assembled acrylic pieces in the Gen1-IPDS.

(a)



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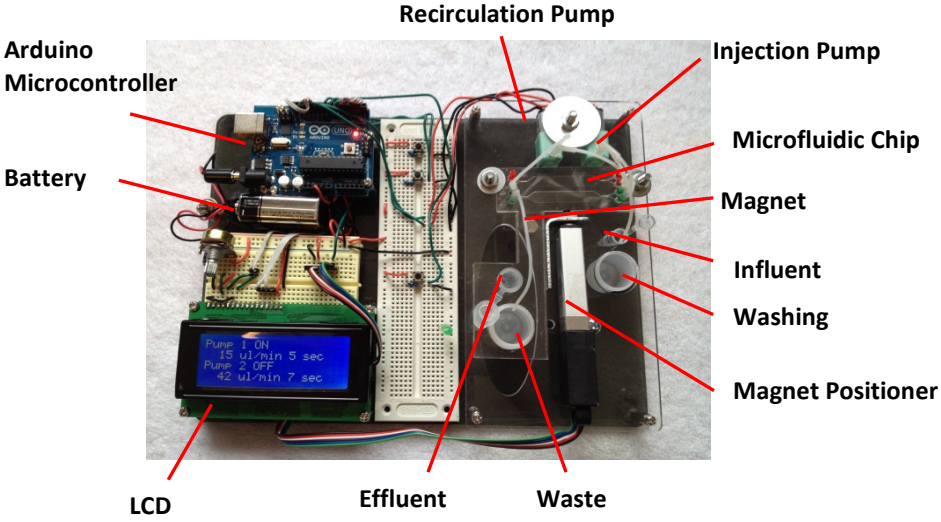

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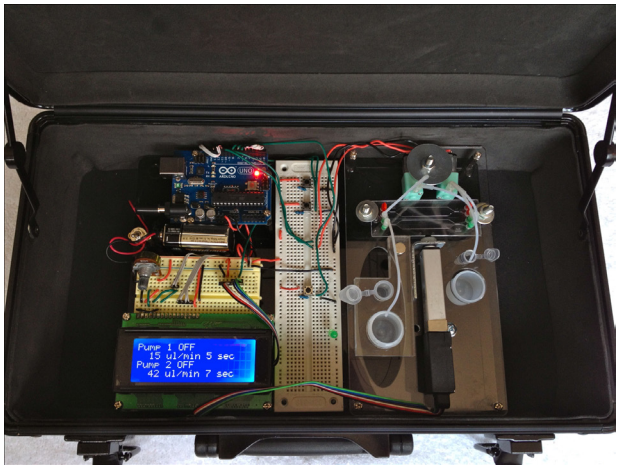


Figure 3. Pictures of the Gen1-IPDS showing: (a) a size comparison of Gen1-IPDS, (b) the labeled hardware of the Gen1-IPDS, and (c) Gen1-IPDS in its portable briefcase.

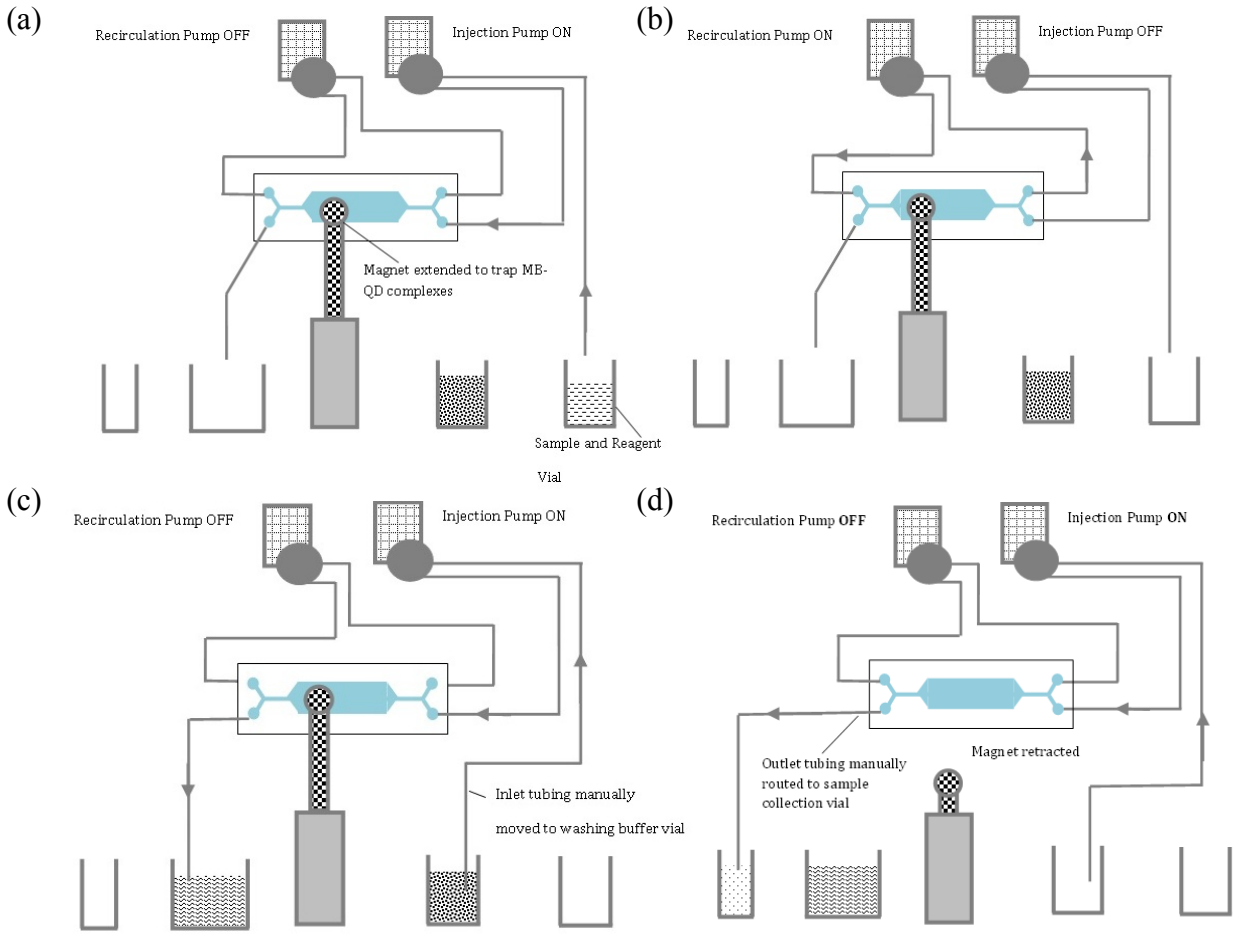


Figure 4. Schematics of the Gen1-IPDS that show (a) sample and reagents injection step (The injection pump is timed such that the sample and reagents do not exit from the microfluidic chip), (b) the hybridization step where the recirculation pump agitates the sample and reagents within the microfluidic, (c) the magnetic separation step where the MB-QD complexes are held in the fluidic chamber by the magnet extended with the magnet position, and (d) the sample collection step after magnetic separation and washing.

Chapter 3: Results and Discussion

3.1 PWM Setting for Magnet Positioner and Pump Flow Rates

The PWM setting for the magnet positioner extension length was manipulated to determine the appropriate settings for use in the in-situ pathogen detection system (Figure 5a and 5b). Since the magnet is connected to the magnet positioner, determining the extension length was imperative to determine the appropriate position of the magnet relative to the microfluidic chip. A schematic of the position of the magnet relative to the microfluidic chip for settings of 130 to 155 can be seen in Figure 5a. Approximate magnetic field lines have been drawn to show the relative magnetic strength felt in the center of the chip at each magnet location. Figure 5a shows that the most concentrated magnetic field lines are placed in the middle of the chip at settings of 135 and 150. It is important to note that the two PWM settings of 135 and 150 are optimum for the system based on the estimated magnetic field lines. Therefore, the ideal magnet position is not being at the center of the chip, but being placed at some identical distance to the opposite direction from the center of the chip.

At each PWM setting, the normalized percent of MB-QD-DNA complexes held by the magnet was determined (Figure 5b) to confirm the ideal magnet position. At each setting, the fluorescence intensity of the complexes held by the magnet was measured. To correlate the fluorescence intensity of the complexes to the percent of complexes held by the magnet, fluorescence at each PWM setting was normalized using equation 1 below.

$$N = \frac{F}{F_0} \times 100 \qquad \text{Eq. 1}$$

Where N is the normalized fluorescence, F is the fluorescence intensity at each PWM setting, and F_0 is the highest fluorescence intensity of all PWM settings. Emphasized by the dashed oval (Figure 5b), the settings corresponding to nearly 100 percent of complexes held by the magnet are at PWM settings of 135 and 150. Both the schematic of the magnetic field lines (Figure 5a) and the fluorescence results (Figure 5b) indicate the appropriate setting for the linear actuator extension length is either 135 or 150. To allow for the shortest extension length (and thus reducing wear on the actuator) and to avoid potentially displacing the peristaltic pumps, a setting of 135 was determined to be the suitable PWM setting for all subsequent experiments.

The range of possible flow rates for the Gen1-IPDS was investigated by varying the PWM setting for the pumps from 115 to 205 (in increments of 5). A sample of known volume of MB-QD-DNA complexes was weighed. The mass of the complexes was divided by the volume, giving the unit weight (in g μL^{-1}). For each data point, the complexes were flushed through the system in accordance with a PWM setting. At each setting, a sample was collected for 1 minute and then weighed. The mass of the sample was divided by the unit weight to yield the flow rate (in $\mu L\ min^{-1}$). The flow rates corresponding to each PWM setting are nearly linear (R^2=0.99) and range from 50 to 270 $\mu L\ min^{-1}$ (Figure 6).

3.2 Hybridization Buffer Composition

In order to determine the appropriate hybridization buffer composition for use in the microfluidic NanoGene assay, various buffer compositions were investigated. A 50

percent dilution of DIG easy hybridization buffer was selected because of its proven performance in the laboratory method. The hybridization buffers containing SDS, SSC, and BSA were selected because they are common constituents of hybridization buffers. SDS (a surfactant) helps to overcome repulsive forces between the probe and target DNA [33], while SSC (a salt) helps to improve hybridization stringency, and BSA (a protein) helps to prevent non-specific binding.

The hybridization buffers were first investigated using the laboratory method as a preliminary indication of their performance (Figure 7a). Hybridization buffer compositions of 0.10% SDS + 0.01% BSA in 10× SSC (d in Figure 7a) and 0.20% SDS + 0.01% BSA in 10× SSC (e in Figure 7a) yielded the greatest fluorescence intensities when hybridized with 10^{-12} mol L^{-1} target DNA and the greatest difference when compared to the fluorescence of the negative control. These two buffer compositions yielded at least two times larger fluorescence than that of the negative control. The direct relationship between SDS and fluorescence intensity indicates that the repulsive forces between the probe and target DNA may be weakened with an increase in SDS.

Selected from the above result, the two most efficient hybridization buffer compositions (SSC and BSA containing 0.10% and 0.20% SDS) were used for another hybridization with varying flow rate settings (Figure 7b). The two buffer compositions performed similarly at flow rates of 50, 90, and 270 μL min^{-1}. However, at flow rates of 150 and 210 μL min^{-1}, the buffer composition containing 0.10% SDS yielded a greater fluorescence intensity. While the buffer containing 0.20% SDS performed well in the laboratory method, it is hypothesized that this caused the viscosity of the hybridization buffer to increase beyond what is optimum for use in the microfluidic NanoGene assay

[34]. Therefore, the hybridization buffer composition selected for use in the microfluidic NanoGene assay was 0.10% SDS + 0.01% BSA in 10× SSC. Figure 7b also gives foresight into selecting the appropriate flow rate for use in the microfluidic NanoGene assay. The bell-shaped curve of the fluorescent intensities of complexes hybridized with the hybridization buffer containing 0.10% SDS shows fluorescence intensity increasing from 50 to 150 μL min^{-1} and then decreasing through flow rates of 270 μL min^{-1}, with a fluorescence peak at 150 μL min^{-1}. Confirmation of the elimination of bubble generation with the custom hybridization buffer can be seen in Figure 7c.

3.3 Hybridization Flow Rate

A range of flow rates was investigated to determine appropriate setting for use in the Gen1-IPDS. As shown in the Figure 8, all four flow rates yielded similar fluorescent intensities at times from 0 to 20 min. However, at recirculation times of 30 and 45 min, the fluorescent intensities from flow rates of 50, 90, and 210 μL min^{-1} plateaued while the fluorescence intensity from a flow rate of 150 μL min^{-1} substantially increased. It is hypothesized that flow rates of 50 and 90 μL min^{-1} are too slow to keep the particles in suspension during hybridization. At these low flow rates, particles may be precipitating and not recirculating through the chip for hybridization. A flow rate of 210 μL min^{-1} may be too swift to allow for adequate contact time between the DNAs. A flow rate of 150 μL min^{-1} is swift enough to keep the particles in suspension during hybridization, but slow enough to allow adequate contact time. Therefore a flow rate of 150 μL min^{-1} was selected for use in the in-situ pathogen detection system. Also, because it yielded the greatest fluorescence intensity, a recirculation time of 45 min was selected.

Supplementary and subsequent experiments confirmed that recirculation times greater than 45 min cause a decrease in fluorescence intensity.

3.4 Hybridization Temperature

Hybridization temperatures of 10, 23, 37, and 45°C were investigated to determine the suitable temperature setting as well as to examine the viability of ambient temperature setting in the Gen1-IPDS (Figure 9a). Fluorescence intensity was measured at 0, 15, 30, 45, and 60 min. It was expected that the highest temperature would yield the largest amount of hybridized DNA and therefore the highest fluorescence intensity. Contrary to what was expected, a hybridization temperature of 45°C yielded the lowest fluorescence intensity. It is hypothesized that this high temperature denatured the BSA protein and therefore inhibited hybridization. A hybridization temperature of 45°C is not recommended for use in the proposed assay.

In order to more easily visually compare the data, the fluorescent intensity results from Figure 9a were plotted as normalized hybridized amount of DNA (Figure 9b) using Equation 1 (where F_0 corresponded to the fluorescent intensity results from hybridization at 37 °C and a retention time of 45 min). The data points were then overlaid with lines of logarithmic regression using SigmaPlot regression software. The normalized hybridized amount of DNA data also illustrate that a hybridization temperature of 37 °C yields the highest fluorescent intensity. However, the results also indicate that hybridization temperatures of 10 and 23°C allow for partial hybridization

As has been presented in Figures 9a and 9b, a hybridization temperature of 37°C consistently yielded the highest fluorescence intensity. The decrease in fluorescence

intensity at 30 min was attributed to inherent error caused by using discrete samples for each data point. While the fluorescent intensities resulting from hybridization temperatures of 10°C and 23°C were not as intense, the results did indicate that they allowed for partial hybridization. Because 23°C is approximately ambient temperature, it was selected as the hybridization temperature for use in this study. The trade off in partial hybridization was chosen because it allowed for a simpler design for the first generation of in-situ pathogen detection system.

3.5 Quantification of *E. coli* O157:H7 Gene Using Gen1-IPDS

Linearity/Sensitivity. To determine the quantification range and the LOD of the Gen1-IPDS NanoGene assay, hybridization experiments were performed using a range of target DNA concentrations, the configuration illustrated in Figure 1, and the parameters determined in the previous experiments. The quantification results can be seen in Table 2 and Figure 10. A linear trend was observed and the data was well correlated (R^2=0.97). The range of quantification for the assay was proven to span six orders of magnitude (10^{-12} to 10^{-6} mol L^{-1}). The LOD for the assay was determined to be 49×10^{-15} mol L^{-1}. This LOD is significantly lower than those reported using similar technology (for commercially synthesized ssDNA): 0.1×10^{-12} mol L^{-1} for 22 bp ssDNA [35] and 14×10^{-12} mol L^{-1} for 24 bp ssDNA [29].

Specificity. To determine the specificity of the Gen1-IPDS NanoGene assay, hybridization experiments were performed using target ssDNAs with 1 and 2-bp mismatch and non-matched ssDNA. The results were then plotted against perfect match DNA (Figure 11). The figure shows a slight increase in fluorescence intensity with an

increase in the concentrations for 1 and 2 bp mismatched DNAs. However, for a range of concentration of non-match DNA (i.e., total 41 bp mismatch out of 55 bp), the fluorescence intensity was approximately zero. A student's *t-test* was performed to confirm a significant difference in the fluorescent intensities of mismatched and perfect-match DNA. All *p-values* (Table 3) were less than 0.05, indicating that there was a significant difference (at a 95% confidence level) in all mismatched and perfect-match DNA. Therefore, it can be concluded that the Gen1-IPDS NanoGene assay is sensitive enough to discriminate between both phylogenetically similar and completely different bacterial species. In other words, our method is capable of discriminating a pathogenic strain from non-pathogenic bacterial species.

3.6 Validation of Gen1-IPDS Assay by the Correlation with Laboratory Method

Hybridization experiments were conducted to determine a correlation between hybridization results of the Gen1-IPDS NanoGene assay and results of the laboratory method (Figure 12). Target concentrations of 0, 10^{-12}, 10^{-10}, 10^{-8}, and 10^{-6} mol L^{-1} were used. Identical DNA samples were prepared and hybridization was allowed using each method. The fluorescence intensity results for the laboratory method were then plotted on the x-axis while the results for the Gen1-IPDS NanoGene assay were plotted on the y-axis. The results follow a linear trend and were well correlated (R^2=0.97). The data was best fit with the equation $y = 0.35\,x + 1.3$. Based on the slope, the fluorescent intensities of the Gen1-IPDS NanoGene assay are approximately one-third of that of the laboratory method, indicating that the samples are partially hybridized. For the proposed assay, rapidity was selected over sensitivity. To improve sensitivity of the assay, recirculation

time would need to be extended past 45 min. However, based on the results in Figures 10 and 12, partial hybridization still allowed for good correlation.

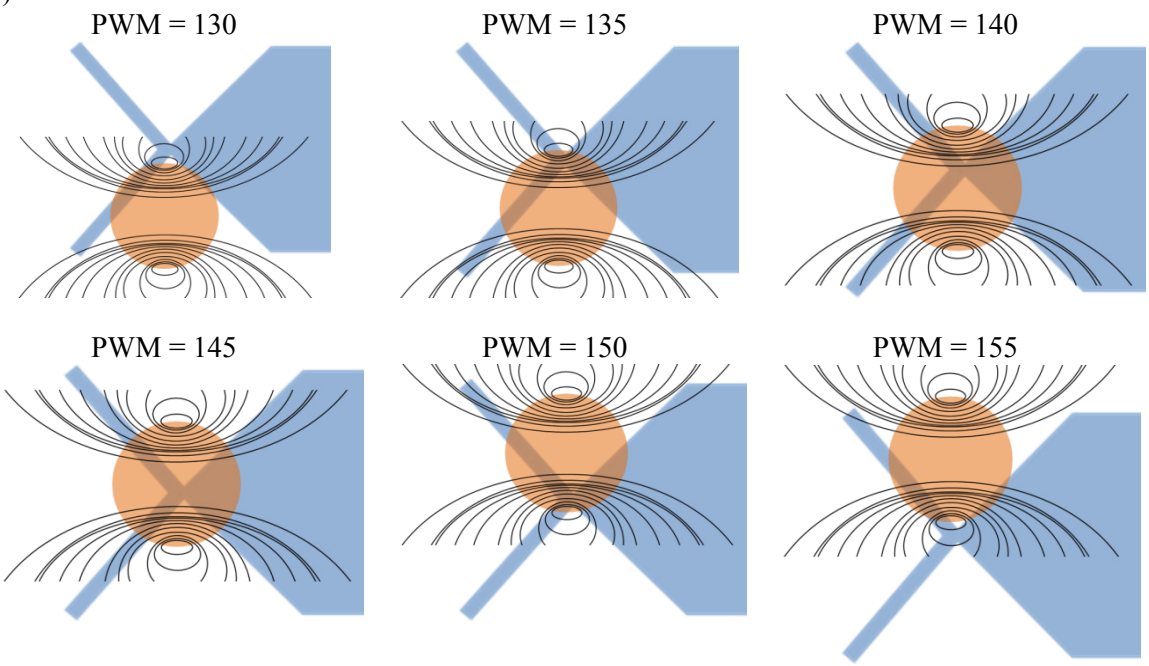
Table 2. Regression equation, correlation coefficient (R2), range of quantification, and limit of detection (LOD) for *E. coli* O157:H7 quantification using the Gen1-IPDS

Quantification result	
Regression equation	$y = 1.84\ ln\ x + 59.85$
Correlation coefficient (R2)	0.97
Range of quantification	10^{-12} to 10^{-6} mol L^{-1}
Limit of detection (LOD)	49×10^{-15} mol L^{-1}

Table 3. Statistical comparison of perfect match and mismatched target ssDNA based on the fluorescent intensity of hybridized particles

	Perfect Match	**1 bp-mismatch**		**2 bp-mismatch**		**41 bp-mismatch**	
	Mean ± SD	Mean ± SD	p-value	Mean ± SD	p-value	Mean ± SD	p-value
10^{-12}	2.00 ±0.16	0.06 ± 0.01	3.59E-08	0.03 ± 0.00	3.13E-08	0.06 ± 0.01	3.18E-08
10^{-10}	3.67 ± 0.21	0.16 ± 0.02	7.34E-08	1.45 ± 0.07	4.94E-07	0.04 ± 0.01	7.85E-08
10^{-8}	5.06 ± 0.74	2.80 ± 0.11	3.83E-04	2.73 ± 0.65	2.69E-04	0.14 ± 0.02	1.01E-05
10^{-6}	10.03 ± 0.72	4.01 ± 0.25	1.26E-05	3.36 ± 0.04	2.46E-05	0.05 ± 0.01	1.28E-06

(a)



(b)

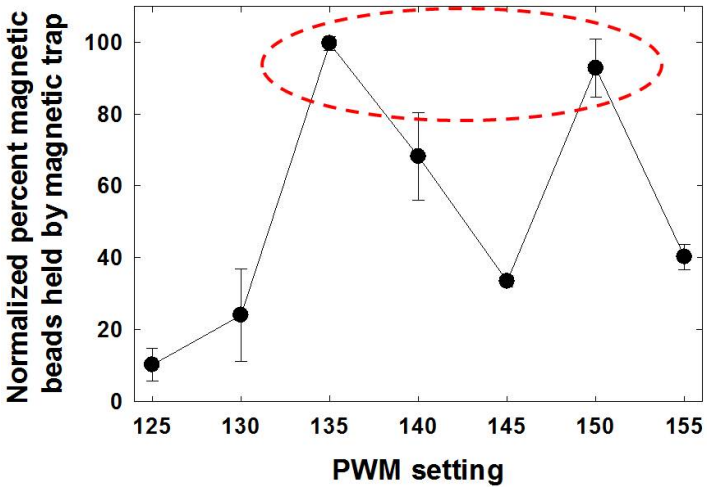


Figure 5. The effect of the manipulation of the PWM setting for the magnet positioner extension length on the position of the magnet. (a) Illustrates the position of the magnetic trap and the magnetic field lines relative the microfluidic chip at a range of PWM. The blue shape represents the microfluidic chip, the orange circle represents the magnet, and the black lines represent the magnetic field lines. (b) Normalized percent of magnetic beads held by the magnetic trap at a range of pulse-width modulation settings. Dots and error bars indicate the mean and standard deviation of duplicate samples.

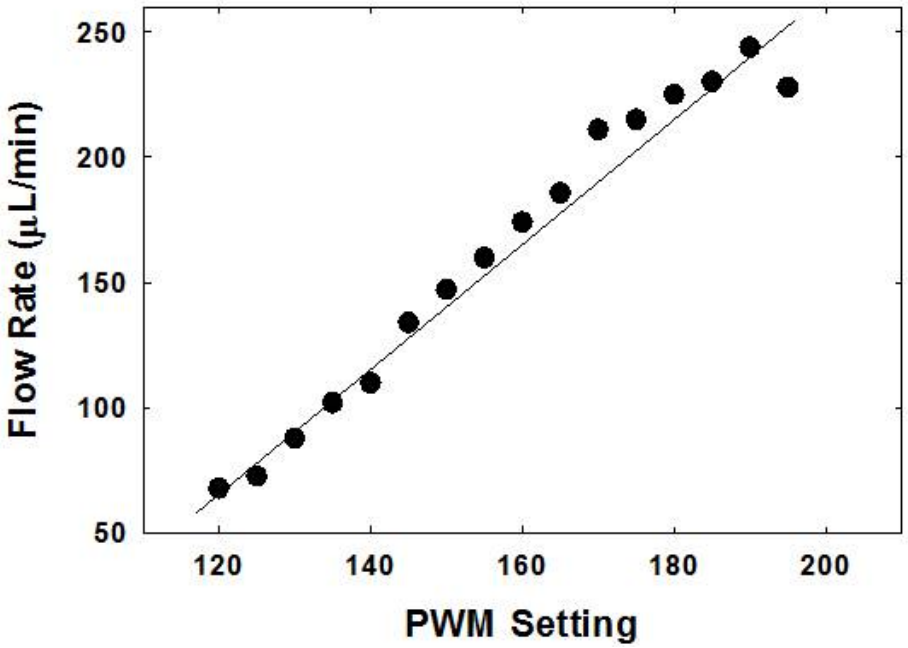
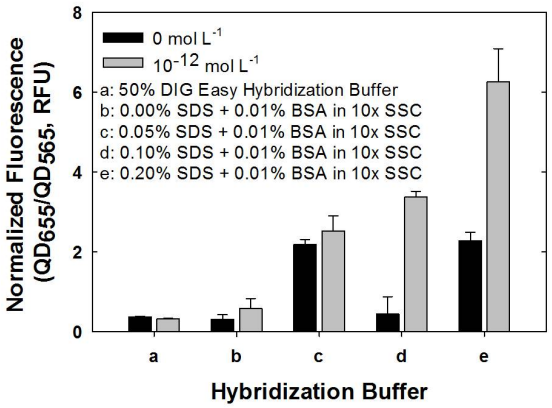
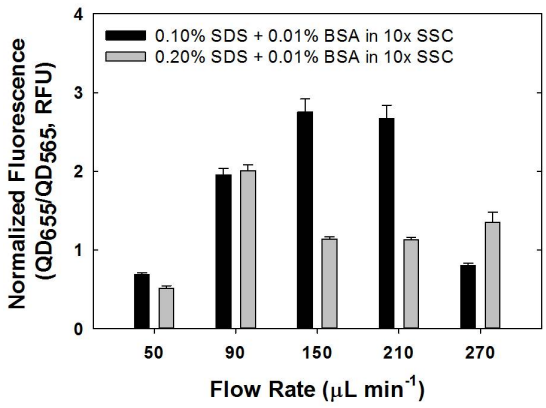


Figure 6. The effect of the manipulation of the pulse-width modulation setting for the miniature peristaltic pumps on the flow rate through the system.

(a)



(b)



(c)

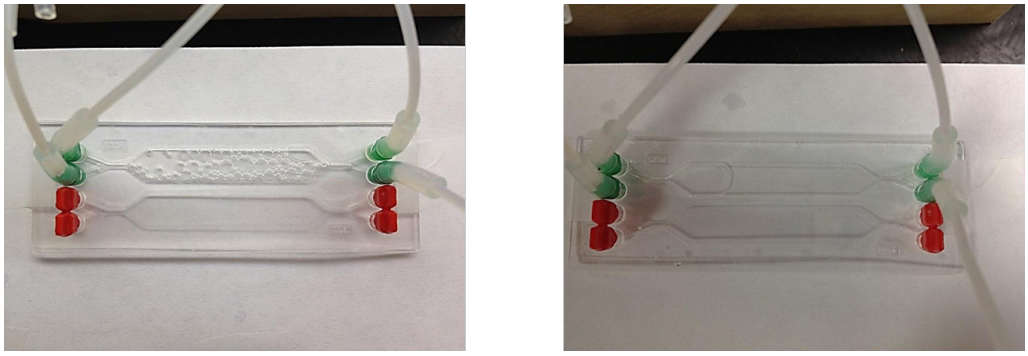


Figure 7. The investigation of: (a) Five different hybridization buffer compositions on the normalized fluorescence of hybridized complexes using the laboratory method. (b) The normalized fluorescence in relative fluorescence units (RFU) of two hybridization buffer compositions (labeled as 'd' and 'e' in Figure 3a) at a range of flow rates using Gen1-IPDS. Signal and error bars represent average and standard deviation based on seven measurements of fluorescence intensity with a spectrofluorometer. This is consistent for Figures 3.6 to 3.8. (c) Confirmation that bubble generation caused by the DIG Easy Hybridization buffer (left) was eliminated with our custom hybridization buffer (right).

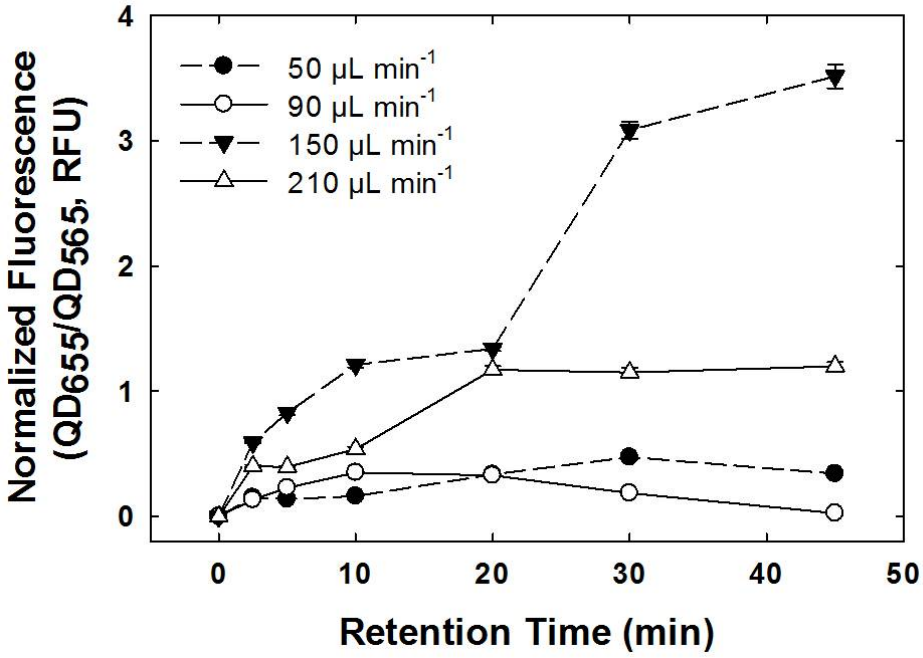


Figure 8. The effects of flow rate and on the normalized fluorescence of hybridized particles after DNA hybridization.

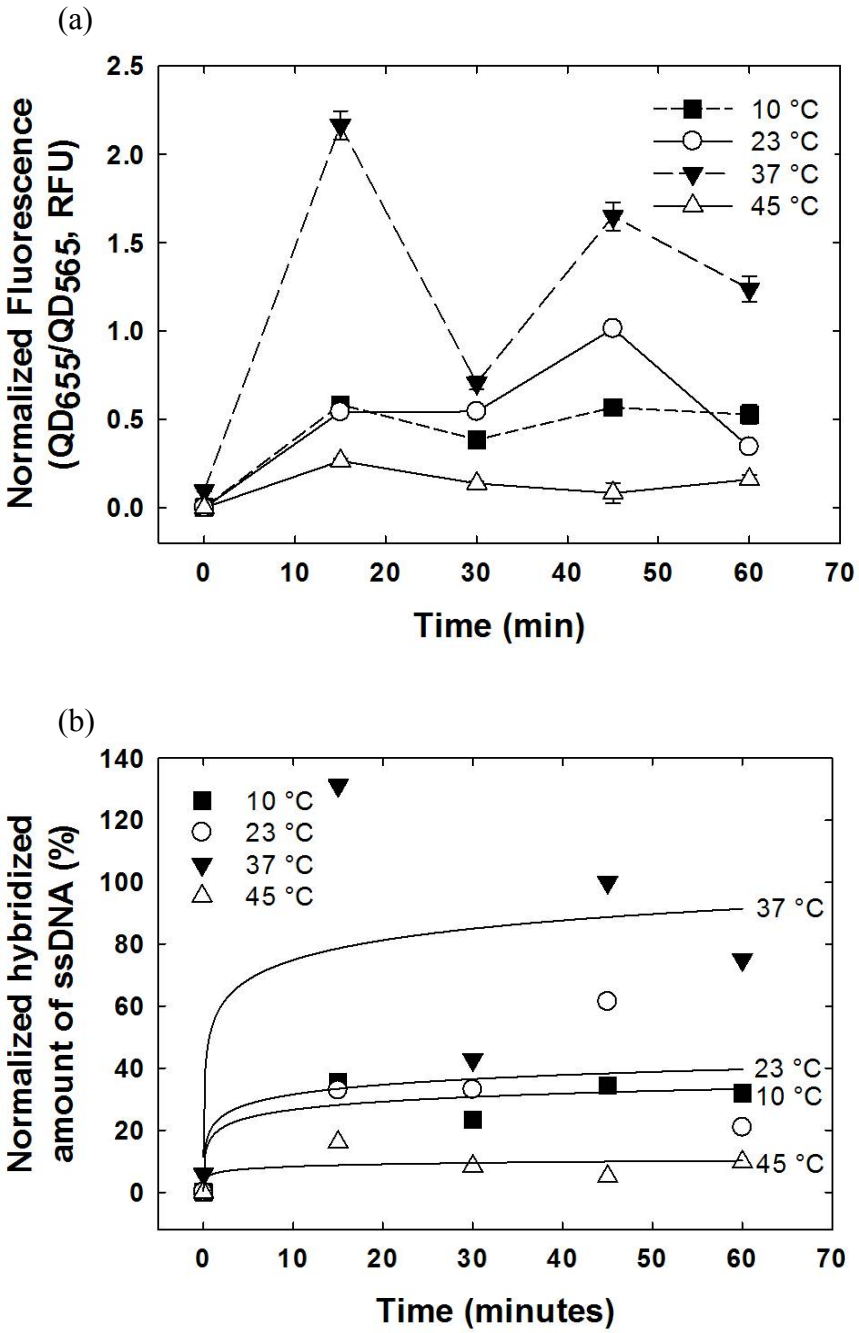


Figure 9. The effect of temperature on DNA hybridization: (a) The normalized fluorescence of hybridized particles at a range of temperatures; (b) The normalized hybridized amount of DNA overlaid with lines of logarithmic regression.

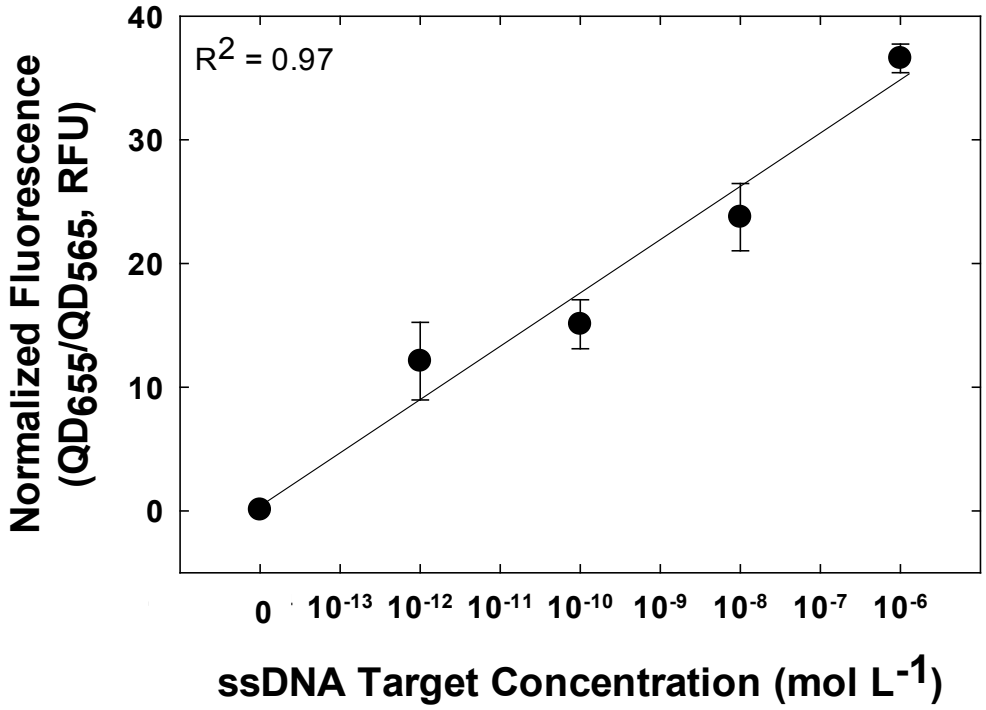


Figure 10. *E. coli* O157:H7 gene quantification using the Gen1-IPDS NanoGene assay. Signal and error bars represent average and standard deviation based on triplicate samples.

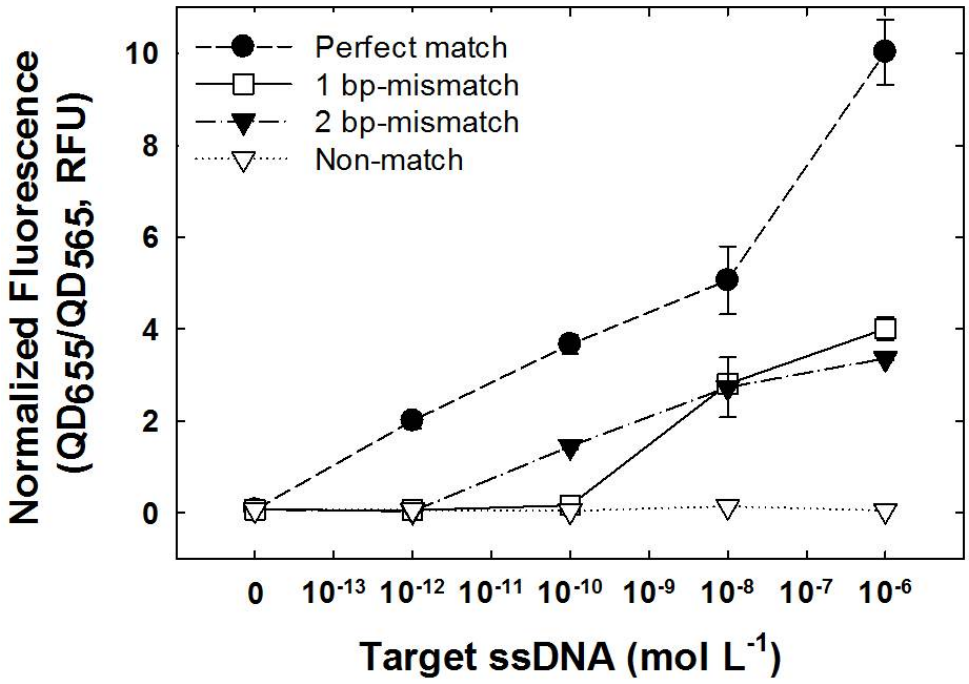


Figure 11. Specificity of assay based on discrimination of mismatched DNAs. Perfect match and 1-, 2-, and 41-bp mismatched target ssDNA were hybridized with MB-QD and probe complexes. Signal and error bars represent average and standard deviation based on seven measurements of fluorescence intensity with a spectrofluorometer. This is consistent for Figures 3.10 and 3.11.

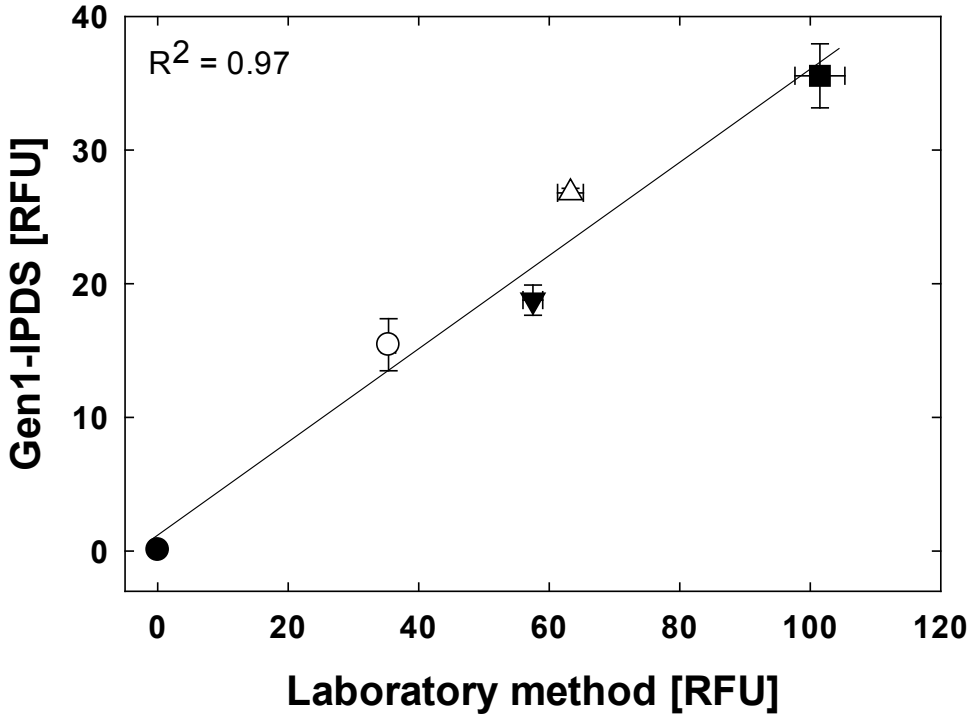


Figure 12. Correlation between the Gen1-IPDS NanoGene assay and the laboratory NanoGene assay method for target ssDNA concentrations of 0 (closed circle), 10^{-12} (open circle), 10^{-10} (inversed triangle), 10^{-8} (open triangle), and 10^{-6} (closed square) mol L^{-1}.

Chapter 4: Conclusions and Future Work

4.1 Conclusions

The Gen1-IPDS was proven capable of performing the hybridization and magnetic separation components of the NanoGene assay.

1. The Gen1-IPDS was constructed using commercially available components that were assembled in a portable device. The final device had dimensions of approximately 37 × 20 × 12 cm and weighed approximately 2.25 kilograms.
2. The Gen1-IPDS was programmed to run automatically with the press of a button. The software used to control the device was the software provided with the Arduino microcontroller, Arduino 1.0.3.
3. The PWM setting for the magnet positioner was manipulated so that the magnet would be placed in a position to retain the greatest amount of magnetic particles during hybridization. A PWM setting of 135 was selected for this device.
4. A custom hybridization buffer composition was designed the Gen1-IPDS NanoGene assay. The constituents of the final buffer were 0.10% SDS + 0.01% BSA in 10× SSC.
5. A hybridization flow rate of 150 μL min^{-1}, a retention time of 45 min, and a hybridization temperature of 23°C were selected.

6. All selected parameters were combined to successfully quantify *E. coli* O157:H7 gene ($R^2 = 0.97$). The LOD was found to be 49×10^{-15} mol L^{-1} and the range of quantification was found to span six orders of magnitude (10^{-12} to 10^{-6} mol L^{-1}).
7. The Gen1-IPDS was found to be capable of successfully discriminating between perfect match target DNA and mis-matched target DNA. The system was able to determine a significant difference between perfect match target DNA and both phylogenetically similar species (1 or 2-bp mismatch out of 55 bp) and completely different species (non-matched target DNA).
8. To confirm the success of the Gen1-IPDS, hybridization results were correlated with results from the laboratory method. The results were well correlated (R^2=0.97), but the slope of the correlation was less than one, indicating that only partial hybridization was achieved with the Gen1-IPDS. However, since quantification results were well correlated, specificity was sacrificed for improved rapidity in the Gen1-IPDS.

4.2 Future Work

The ultimate goal of this project is to create a device capable of *in-situ* detection of pathogens, including components for sample preparation, hybridization, magnetic separation, and detection. The scope of this portion of the project focused on creating a device capable of performing the hybridization and magnetic separation components (Gen1-IPDS).

The next phase in this project will be in creating the second generation in-situ pathogen detection system, or Gen2-IPDS. The Gen2-IPDS will still feature components

for hybridization and magnetic separation, but will also allow for the implementation of a portable spectrofluorometer. This will allow the device to measure the fluorescence of the hybridized particles and to ultimately compare the fluorescence against a known standard curve in order to relay contamination levels back to the user.

The final phase in this project will be in creating a third generation in-situ pathogen detection system, or Gen3-IPDS. The Gen3-IPDS will be capable preparing a raw environmental sample for use in hybridization. The device will extract the genomic DNA from the sample, and then denature and fragment the DNA before sending it the hybridization component of the device. The Gen3-IPDS will be the complete device, capable of performing all aspects of the NanoGene assay. This device will be capable of either being transported to the field where a sample can be manually introduced or will be instrumented with robotic sample introduction and wireless capabilities to relay real-time information off site.

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Appendix A: Arduino 1.0.3 Code to Control Gen1-IPDS

```
//Automatic SequenceTest run for pump and actuator
//Initial June 13, 2012
//include the library code:
#include <LiquidCrystal.h>
//initialize the library with number of interface pins for display
LiquidCrystal lcd(12, 11, 5, 4, 3, 2);
void setup(){
  //set up the LCD number of columns and rows:
  lcd.begin(20,4);//This is a 20 column 4 row LCD
  //LCD Display Code for Initial Start Up
  lcd.setCursor(0,0); //Set cursor to column 0 row 0
  lcd.print("Auburn University");
  lcd.setCursor(0,1); //Set cursor to column 0 row 1
  lcd.print("Son Laboratory");
  lcd.setCursor(0,3); //Set cursor to column 0 row 3
  lcd.print("NanoGene Assay Kit");
  lcd.setCursor(0,2);
  for(int Timer1=0;Timer1<20;Timer1++){
    lcd.print("-");
    delay(200);
  }
  lcd.clear();
  //Declaring functions of individual pins
 pinMode(7,INPUT); //Automatic sequence start switch. Press this and sequence will start
          automatically.
 pinMode(6,OUTPUT); // This drives the blue wire of the actuator in PWM mode
 pinMode(9, OUTPUT);//This drives Pump 1
 pinMode(10,OUTPUT); // This drive Pump2
lcd.setCursor(0,0);
lcd.print("Press top button");
lcd.setCursor(0,1);
lcd.print("to begin");
}
void loop (){
  //Assigning variables to respective pins
int autostart = digitalRead(7);
//Assigning Pin 7 to Switch for starting automatic sequence.
//Make sure there is a hardware switch connection for it.
```

```
//Note: Pin 9 is analogWrite output for Pump 1, Pin 10 is analogWrite output for Pump2,
//Note: Pin 6 is analogWrite output for actuator PWM (blue wire). Make sure wires are
        connected correctly
//User Input for Pump Speed
int pump1value = 152; //User input to assign speed of Pump 1 to 152
int pump2value = 255; //User input to assign speed of Pump 2 with values 0 to 255
int pump1time = 30000; //User input to assign pumping time for Pump 1.
int pump2time = 30000; //User input to assign pumping time for Pump 2.
int ActLength = 135;
int ActTime = 5000;
// When autostart button (Pin 7) is pressed
if (autostart == HIGH ){
//Extending Actuator
  lcd.clear();
  lcd.setCursor(0,0);
  lcd.print("Actuator Extending");
analogWrite(6, ActLength); //Extending actuator arm
delay(5000);
//Switching ON and OFF Pump 2
  lcd.clear();
  lcd.setCursor(0,0);
  lcd.print("Injecting sample");
  lcd.setCursor(2,1);
  lcd.print(270);
  lcd.print(" ul/min ");
analogWrite(10, pump2value); //Using Pin 10 to drive Pump 2 at the user input speed.
        User input values go from 0 to 255
delay(pump2time); //User input -> 5000 = 5 seconds
analogWrite(10,0); //Using Pin 10  to stop Pump 2
delay(2500);
//Switching ON and OFF Pump 1
  lcd.clear();
  lcd.setCursor(0,0);
  lcd.print("Recirculation");
  lcd.setCursor(2,1);
  lcd.print(150);
  lcd.print(" ul/min ");
  lcd.setCursor(2,2);
  lcd.print("t=45min");
for(int Timer2=0;Timer2<90;Timer2++){//Set Timer2 < number of 30 sec increments for
        recirc
analogWrite(9, pump1value); //Using Pin 9 to drive Pump 1 at the user input speed. User
        input vlaues go from 0 to 255
delay(pump1time); //User input -> 5000 = 5 seconds
analogWrite(9,0); //Using Pin 9 to stop Pump 1
}
```

```
delay(2500);
 lcd.clear();
  lcd.setCursor(0,0);
  lcd.print("Washing");
for(int Timer3=0;Timer3<3;Timer3++){//Set Timer2 < number of 30 sec increments for
        washing
analogWrite(10, pump2value); //Using Pin 10 to drive Pump 2 at the user input speed.
        User input vlaues go from 0 to 255
delay(pump2time); //User input -> 5000 = 5 seconds
analogWrite(10,0); //Using Pin 1- to stop Pump 2
}
//Retracting Actuator
  lcd.clear();
  lcd.setCursor(0,0);
  lcd.print("Actuator Retracting");
analogWrite(6, 0); //Retracting actuator arm
delay(5000);
//Switching ON and OFF Pump 2
  lcd.clear();
  lcd.setCursor(0,0);
  lcd.print("Sample Collection");
analogWrite(10, pump2value); //Using Pin 10 to drive Pump 2 at the user input speed.
        User input vlaues go from 0 to 255
delay(pump2time); //User input -> 5000 = 5 seconds
analogWrite(10,0); //Using Pin 1- to stop Pump 2
  lcd.clear();
  lcd.setCursor(0,0);
  lcd.print("Finished");
}
}
```