SANDIA REPORT
SAND2006-6922
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Printed November 2006

Quantification of False Positive Reduction in Nucleic Acid Purification on Hemorrhagic Fever DNA

Conrad D. James, Komandoor Achyuthan, Jaime McClain, Kenneth Pohl, and Mark Derzon

Prepared by
Sandia National Laboratories
Albuquerque, New Mexico 87185 and Livermore, California 94550

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Conrad D. James, Mark Derzon, Kenneth Pohl
Advanced MEMS

Komandoor Achyuthan
Biosensors and Nanomaterials

Jaime McClain
MEMS Core Technologies

Sandia National Laboratories
P.O. Box 5800
Albuquerque, New Mexico 87185-MS1080

Abstract

Columbia University has developed a sensitive highly multiplexed system for genetic identification of nucleic acid targets. The primary obstacle to implementing this technology is the high rate of false positives due to high levels of unbound reporters that remain within the system after hybridization. The ability to distinguish between free reporters and reporters bound to targets limits the use of this technology. We previously demonstrated a new electrokinetic method for binary separation of kb pair long DNA molecules and oligonucleotides. The purpose of this project 99864 is to take these previous demonstrations and further develop the technique and hardware for field use. Specifically, our objective was to implement separation in a heterogeneous sample (containing target DNA and background oligo), to perform the separation in a flow-based device, and to develop all of the components necessary for field testing a breadboard prototype system.

ACKNOWLEDGMENTS

We would like to thank all of the people who were instrumental in this project, specifically our collaborators W. Ian Lipkin, Gustavo Palacios, and Omar Jabado (Columbia University) as well as Dave Norwood, and George Ludwig of USAMRIID. We also extend our gratitude to Jeb Flemming for the glass substrate device fabrication and Christian Arrington, Ron Manginell, and Sarah Rich for the microfluidic mold fabrication performed at the Microsystems and Engineering Science Applications facility. Our summer student Nigel Reuel helped with designing and performing experimental testing and troubleshooting. Sandia is a multiprogram laboratory operated by Sandia Corporation, a Lockheed Martin Company, for the United States Department of Energy under contract DE-ACO4-94-AL85000.

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NOMENCLATURE

AC	alternating current
bp	base-pair
DEP	dielectrophoresis
DNA	deoxyribonucleic acid
DOE	Department of Energy
dsDNA	double-stranded DNA
kbp	kilobase-pair
pDEP	positive dielectrophoresis
PDMS	polydimethylsiloxane
RF	radio frequency
nDEP	negative dielectrophoresis
SNL	Sandia National Laboratories
ssDNA	single-stranded DNA

1.0 INTRODUCTION

Columbia University has developed a sensitive highly multiplexed system for genetic identification of nucleic acid targets. The method has several potential applications including clinical microbiology, biodefense, and forensics. The invention is based on a strategy for enrichment of a reporter template for numerous gene amplification methods such as PCR. A reporter oligonucleotide (oligo) binds to a target DNA molecule serving as a beacon for initiating gene amplification of the target. The primary obstacle to implementing this new technology is the high rate of false positives due to high levels of unbound reporters that remain within the system after hybridization. The ability to distinguish between free reporters and reporters bound to targets limits the use of this technology. Our previous late start LDRD (C. James et al. 2005) demonstrated several basic components of a new electrokinetic method for binary separation of kb pair long DNA molecules and oligonucleotides. This previous work used static experimental conditions in that the DNA preconcentration was performed from a static droplet of sample placed on a microelectrode chip. Also, the samples were homogeneous, and we demonstrated preconcentration of target DNA in one sample, and no preconcentration on oligoneucleotides in a separate sample. The purpose of this year's project (99864, FY 2006) was to take these previous demonstrations and further develop the technique and hardware for field use. Specifically, our objective was to implement separation in a heterogeneous sample (containing target DNA and background oligo), to perform the separation in a flow-based device (permitting sample introduction and processed sample capture), and to develop all of the components necessary for field testing a breadboard prototype system. The first objective is the primary scientific objective, which is directed toward the specific application of spatially separating unbound oligos from target DNA bound to oligos. Dielectrophoresis (DEP) has been used for preconcentrating DNA in many labs, but our lab is aiming to be the first to demonstrate a functional separation of two sizes of DNA in a mixed sample. The second objective of implementing the separation in a microfluidic channel is crucial for making the device practical for sample preparation and subsequent analysis with additional techniques (PCR, etc.). The channel format enables the sample to be injected into the device, processed at the microelectrode chip, and then the effluent is released from the channel and captured for subsequent analysis. In addition, the microfluidic channel has a serpentine configuration that provides longer interaction times between the sample and the microelectrode chip, thus enhancing the separation effect. The third objective is to develop a breadboard system containing the microelectrode chip, the microfluidic channel, the MHz power supply, and the fluid pump. The fully integrated system will allow us to test the system in the field, starting with W. Ian Lipkin’s lab at Columbia University.

2.0 DEP SEPARATION OF DNA

Our previous SAND report (C. James et al. 2005) describes the phenomena of dielectrophoresis (DEP) of small molecules with the relevant literature references. Our technique relies on the size and charge disparity (dsDNA has twice the charge per bp as ssDNA) between the dsDNA

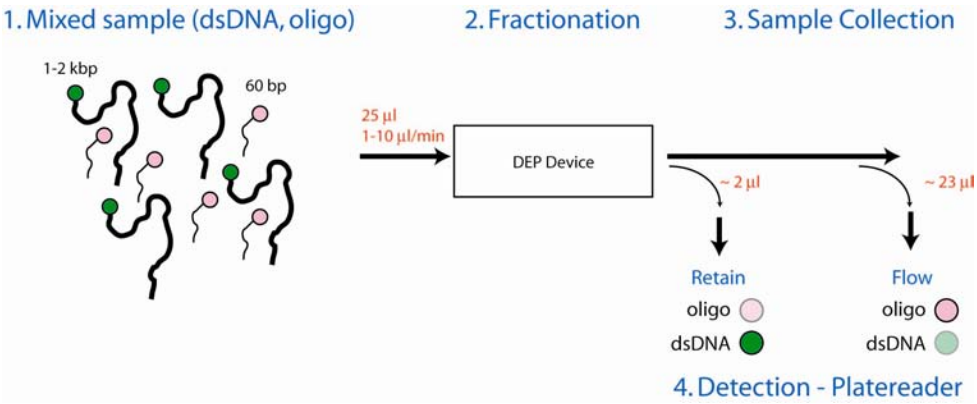

Figure 1: Experimental protocol for separating oligo from dsDNA in a flow through microfluidic system.

(1-2 kbp) targets and ssDNA probes (25-60 bp) for using DEP-based separation. This method of DEP field-flow-fractionation has been demonstrated on cells and particles in flow-through systems in the literature [Yang et al. 2000, Lapizco-Encinas et al. 2004]. We are attempting the first application of this technique to an application-based nucleic acid fractionation. The initial detection scheme is shown in Figure 1. We begin with a mixed sample containing both target dsDNA and oligo. The sample volume is approximately 25 µl. The sample is flowed through the DEP device where the dsDNA undergoes selective trapping and is impeded from flowing out of the device. The ssDNA travels through the device unhindered and should be evenly disbursed throughout the sample as it exits the device. The bulk of the sample will (~90% of the original volume) will exit the device under pressure from a syringe pump, and this is termed the "flow" portion of the sample. A small remnant of the sample will be left behind in the device, unable to exit under normal pressure, and this portion of the sample is termed the "retain" component of the sample. This last remnant of the sample is then removed using washes of DI water to remove residual sample.

3.0 DEVICE FABRICATION

3.1 Interdigitated Microelectrode Array

The microelectrode chip for this project utilizes a simple single pronged interdigitated design as shown in Figure 2. Two designs were made, one with 4 µm wide electrodes separated by 4 µm spaces, and then a 2 µm wide electrode, 2 µm space design. Glass chips were prepared using a chrome mask to pattern photoresist into the inverse pattern of the microelectrode array. Twenty nm of titanium and then 120 nm of gold were then evaporated onto the chips. The resist was then removed with sonication in acetone, leaving the metal microelectrodes on the chip. Wires were soldered onto the bond pads of the chip. The microelectrode features produce large field gradients ($\nabla E^2 \sim 10^{18}$ V^2/m^3), with the smaller electrodes producing a larger gradient than larger electrodes. This design also differs from the previous device used in the previous work. In that case, the IDT was double pronged, which led to DNA collection in every other electrode gap. The current design will allow DNA to concentrate at every electrode gap on the device, increasing our ability to handle larger amounts of DNA.

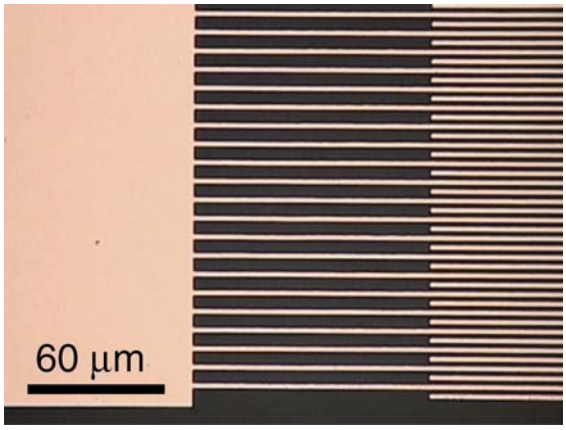

Figure 2: Interdigitated (IDT) comb microelectrode device. The device contains single pronged microelectrodes 2 µm in width and spaced by 2 µm.

3.2 Microfluidic Channel

One of the drawbacks to the previous LDRD on DNA separation using DEP was the lack of integrated sample injection and collection of processed sample. This made it impossible to isolate ssDNA concentrated to the IDT chip from oligo that was freely floating above the chip. The strategy taken here is to perform a field-flow fractionation using DEP as the separation force. In this layout, target dsDNA would undergo more interactions with the IDT chip at the floor of the channel, thus slowing its transport through the channel. The ssDNA, which will undergo minimal interaction with the DEP force generated at the floor of the channel, will remain evenly distributed throughout the channel under fluid flow. At the outlet of the channel, we will increase the ratio of [dsDNA]/[ssDNA], with larger ratios for longer channel lengths, larger DEP forces, and reduced flow velocities. This will reduce the false positive rate of detection events when implemented into Columbia's nucleic acid detection scheme.

Figure 3 shows the design and fabrication of molding masters for microfluidic channels. We have designed different iterations of serpentine channels, varying the width (25, 50 and 100 µm) of the channels and the spacing (200, 600 µm) of each leg. The first designs were patterned with SU8, a negative tone photoresist. The height of the structures was 150 µm. Master structures were also made using Bosch-etching of silicon substrates. In this instance, silicon wafers were coated with JSR resist (3 µm thick) and exposed. Wafers were then etched to a depth of 50 µm, leaving elevated channel structures for molding once the remaining resist was stripped. The silicon masters were then coated with hexamethyldisilazane to reduce adherence between the mold and the cured elastomer. Fluid channels were then molded using polydimethylsiloxane (PDMS) elastomeric molding. PDMS monomer was mixed with its curing agent at a 10:1 ratio, mixed, and placed under vacuum to remove bubbles. The mixture was then poured over the SU8 master and cured either overnight at room temperature, or for 2 hours at 60 ° C. Once cured, the PDMS mold was cut out using a razor blade, and connections through the top of the channel to the inlet/outlet ports of the channel were made using a needle.

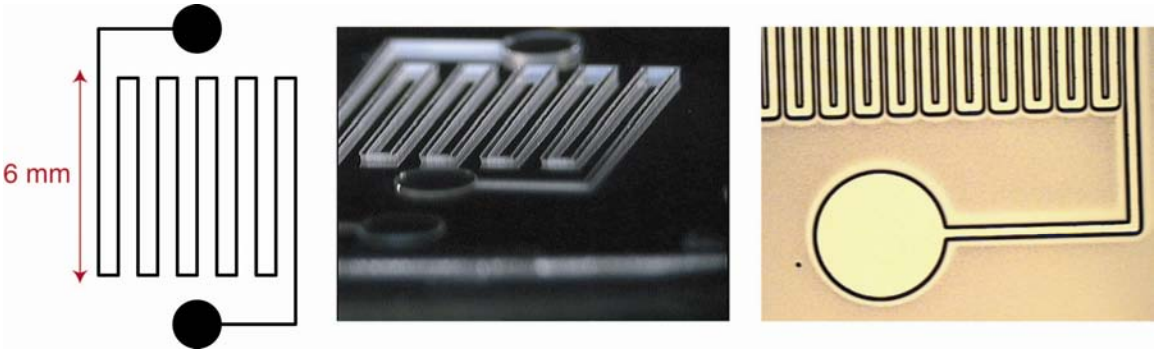

Figure 3: Serpentine channel for microfluidic separation of DNA (left). Skewed angle optical image of a serpentine channel fabricated in SU8 (middle). Bosch-etched silicon master (right).

The next step is to bond the PDMS fluid channel to the IDT electrode chip. This was accomplished using an RF plasma treatment. Both the IDT chip and the PDMS channel were treated with an RF plasma (Harrick Scientific PDC-32) for approximately 1 minute at high power with an air feed-gas. A drop of methanol was then placed on the IDT chip under a microscope, and the PDMS channel was placed on the droplet. The methanol allows the channel to be aligned precisely to the IDT chip to place the IDT electrodes perpendicular to the axis of fluid flow (Figure 4, left). Once the alignment was finished, the hybrid chip was placed on a hot plate with a 100 gram weight on top to facilitate bonding. After bonding, the edges of the PDMS slab were reinforced with PDMS around the interface and cured on a hotplate. A 340 µm outer diameter capillary tube was placed into the outlet port, while a 29 gauge dispenser tip was placed in the inlet (Figure 4). PDMS was again used to seal the capillary/tip interfaces. Five minute epoxy was used to reinforce the structural rigidity of the dispenser tip and capillary.

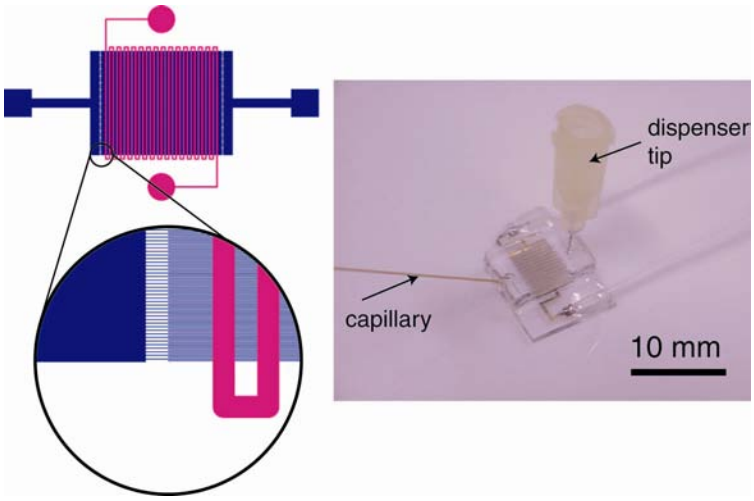

Figure 4: Schematic showing the assembly of the IDT chip (blue) with the PDMS microfluidic channel (pink, left). Assembled device containing the IDT chip and PDMS microfluidic channel (right).

The next step for the device fabrication is to passivate the surface to reduce non-specific adsorption of DNA to the IDT chip and the PDMS channel walls. Our initial experiments with flowing oligo through the uncoated device resulted in nearly all the oligo being adsorbed inside the device with no detectable signal on the effluent. Initially, we used various concentrations of bovine serum albumin (1, 5, and 10%) at various temperatures (room temperature and 60 ° C) to

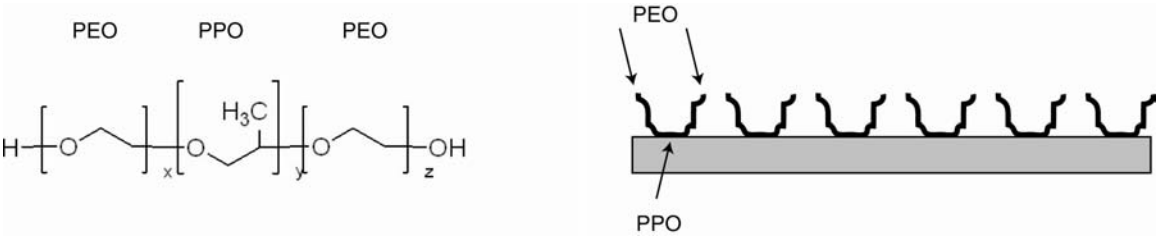

Figure 5: The molecular structure of Pluronic (left). Pluronic as it adsorbs to a surface at low concentrations (right). Adapted from Boxshall et al. 2006.

coat the inside of the device. We found improved results in terms of less DNA loss, with recovery of DNA (oligo and dsDNA simply flowed through the device with the DEP off) typically around 40-80%. Devices still required BSA treatment immediately prior to use, and while the BSA treatment significantly reduced the adsorption of the oligo to the device, we still had lots of dsDNA loss even with the BSA treatment (40-60% loss). We then experimented with coating devices with Pluronic, a surfactant/detergent, due to evidence in the literature of improved protein/cell resistance [Boxshall et al. 2006]. When Pluronic is adsorbed to a hydrophobic surface, the hydrophobic PPO portion of the molecule preferentially adsorbs to the surface while the hydrophilic PEO arms extend up into the channel (Figure 5). These PEO arms are hydrophilic and uncharged, which reduces both hydrophobic and electrostatic interactions with proteins and DNA. There is also an osmotic pressure and steric hindrance caused by the PEO arms that promote protein/nucleic acid fouling resistance. An additional benefit of the Pluronic coating is that it renders the PDMS more hydrophilic, which aids in sample injection and flow through the device, allowing the separation experiments to be performed at lower flow-rates. The procedure for coating a device with Pluronic is to make a 5% solution (w/v) of the Pluronic in water, fill the device with the solution, and let it adsorb at room temperature over night. The device is then flushed with DI water and dried at 60 ° C at room temperature. We've found the most consistently high recovery rates with Pluronic, with some experiments achieving >90% recovery of dsDNA. Typically, we recoat the device with Pluronic before each use/reuse.

4.0 PORTABLE ELECTRONICS

One of the main objectives of this project was to develop hardware that was portable for field testing of a bread-board system. The electronics required for dielectrophoresis is a function generator, and for our applications, the system must be capable of 10 V p-p AC sinusoidal signals, with frequencies up to 10 MHz. A low power hand held function generator printed circuit board (PCB) was designed to be powered by a 9 volt battery and produce and output signal with a maximum of 10 volts peak to peak. This PCB is a high frequency, precision function generator producing accurate, high

frequency triangle, saw tooth, sine, square, and pulse wave forms with a minimum of external components. The output frequency can be controlled over a frequency range of 0.1Hz to 20 MHz by an internal 2.5V band gap voltage reference and an external resistor /capacitor combination. All desired variables such as amplitude, frequency, pulse width and function can be set manually. The duty cycle can be varied over a wide range by applying a +/- 2.3 volt control signal, facilitating pulse-width modulation and the generation of saw tooth waveforms. The duty cycle and frequency controls are independent. Sine, square or triangular waveforms can be selected at the output

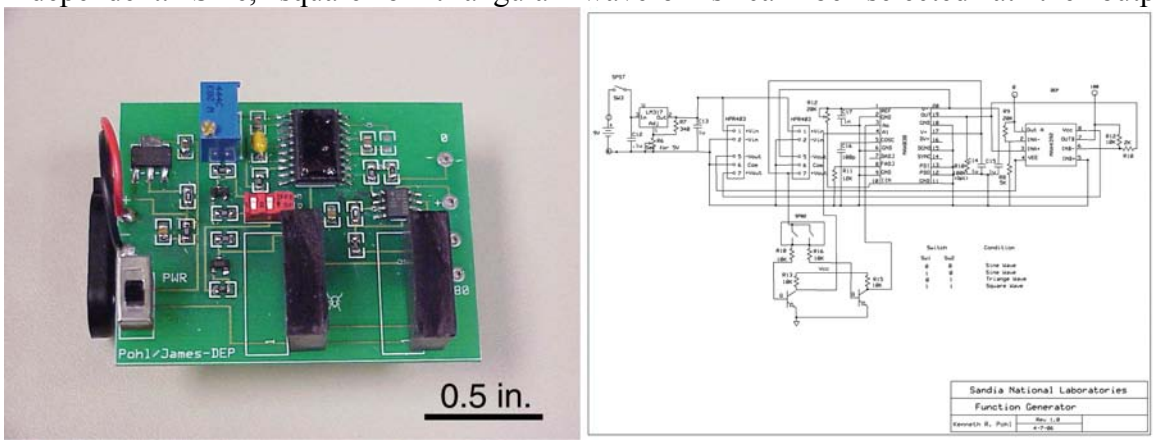

Figure 6: Mini function generator powered by a 9V battery (left). Schematic of the circuit layout for the function generator (right). Design and fabrication courtesy of Ken Pohl.

by setting the appropriate binary code at two TTL compatible select pins. The maximum output current is on the order of 20mA. In addition, we incorporated two signal outputs, with 180 ° phase difference between the signals to generate larger DEP forces.

5.0 SYSTEM OPERATION

The separation experiments are performed as following. The mixed sample of DNA (target dsDNA and background ssDNA) is placed into the dispenser tip with a pipette. A luer-lock Upchurch Scientific fitting connected to a Teflon tube interfaced to a syringe is connected to the dispenser tip (Figure 7). Care is taken to place the sample at the bottom of the dispenser tip to minimize sample loss. The syringe and tube are filled with air for pressurized delivery of the sample to the device. A limit on the sample volume of 25 microliters was set to avoid touching the sample with the Luer-lock fitting, which is another route towards sample loss. Connections from the function generator to the soldered leads on the IDT chip were made with alligator clips. A standard syringe pump was used to flow the sample through the device (5-25 µl/min).

The device is first flushed with two separate rinses of DI water to provide a pre-experiment control for background fluor detection. The DEP function generator is turned on before the sample is injected into the device. The sample then begins to enter the channel, and after approximately 10-20 minutes the sample begins exiting the outlet capillary. The first liquid to exit the device is termed the “flow21111”, as this portion of the sample is performed using constant flow using the syringe pump. This portion of the

sample should contain the native concentration of ssDNA (as the DEP doesn't affect the ssDNA) and should be deficient in dsDNA as it is restricted from flowing through the device by the DEP force generated by the electrodes at the bottom of the channel. After the sample runs its course through the device, there is usually remnant sample left behind. This remnant should be rich in the concentrated dsDNA. This is removed with two separate washes of the device with 12.5 µl of DI water. After the washes, the device is washed tow more times with 12.5 µl of DI water for monitoring post-experiment background or residual DNA left behind. A plate reader (Berthold, Mithras Model) was used to analyze the fluorescent signals of the samples after processing in the DEP system. DI water controls were used to subtract background signals from the samples.

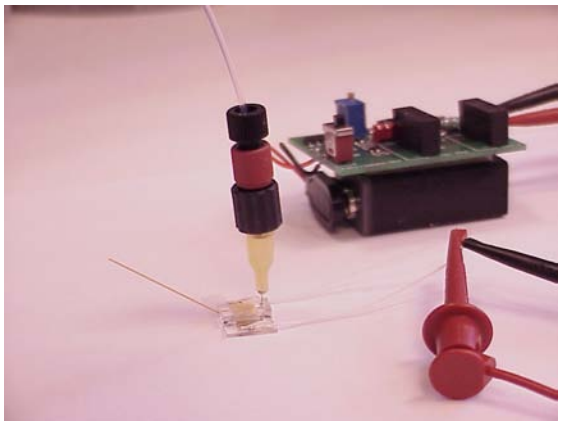

Figure 7: DEP separation system fully assembled with function generator connected.

6.0 RESULTS

Samples used in experiments are detailed here. The oligo chosen was a fluorescein (485 nm ex., 535 nm em.) labeled 60-mer (5'-AAC AGA TAC AAA CTC ATC ACG AAC GTC AGA AGC AGC CTT ATG GCC GTC AAC ATA CAT ATC-3'). The sample concentrations used in experiments was 1.2 and 3.6 pmol. Typical samples processed in the system were 15 µl, giving at total number of $7.2x10^8$ or $2.1x10^9$ molecules of DNA per sample. The 1500 bp dsDNA was labeled with Cy5 (650 nm ex., 685 nm em.) dye and consisted of a proprietary sequence. Concentrations of the dsDNA were the same as for the oligo.

6.1 Single Component Samples

In last year's project (C. James et al. 2005), we found that the optimum frequency for capturing DNA to the IDT chip was 1 MHz. Above that frequency, we found that gold electrodes electrochemically dissolved (15 MHz), while at lower frequencies, electrothermally-induced fluid currents are produced (~100 kHz). The first set of experiments utilized single component samples made of either pure oligo or pure dsDNA.

	Total	Flow	Retain	Recovery (%)	Retain/Flow (%)
200 kHz (dsDNA)	7290	7320	300	~100	4.1
1 MHz (dsDNA)	13880	5000	1560	47	31.2
1 MHz (oligo)	51320	46330	1960	94	4.2

Table 1: Dataset for experiments using different frequencies for manipulating dsDNA targets and oligos.

The first set of experiments is shown in Table 1. In the first row, we show data for a condition in which the IDT was activated with 20 Vpp at 200 kHz. The sample consisted of pure dsDNA labeled with Cy5. The total concentration was 240 fmol/µl, with 10 µl at that concentration yielding a 7290 fluorescent unit count on the PlateReader (with background subtraction using a separate identical sample as a standard). The flow column refers to the number of counts that first exit the device, and in this instance the total counts in the flow was 7320 (with background subtraction). A small number of counts were found in the retained portion of the sample that was washed out with DI water. The total sample recovery from the device was ~100%, meaning no sample was lost irreversibly to the device. The ratio of counts between the retain and flow portions of the sample was 4.1%. For an experiment using dsDNA at 1 MHz, the ratio of retain-to-flow was 31.2%, indicating more counts were located in the remained portion of the sample at this frequency. However, the recovery from this experiment was only 47%, meaning nearly half of the sample was lost irreversibly to the device. This could be due to irreversible binding of the DNA to the actuated IDT electrodes. In a third experiment (third row), a sample of oligo was tested using a 1 MHz frequency. In this case, the retain-to-flow ratio was low (4.2%), similar to the case of the dsDNA using 200 kHz.

6.2 Two Component Samples

The next set of experiments used mixed samples containing both oligo and dsDNA. We found inconsistent results in terms of two particular measurable quantities: the recovery rate and the DEP-induced redistribution of DNA. The recovery rate is the amount of DNA from the sample that is injected into the device and recovered afterward in either the flow output or the retain output. Ideally, we want no sample to be lost irreversibly to the device, and so recovery

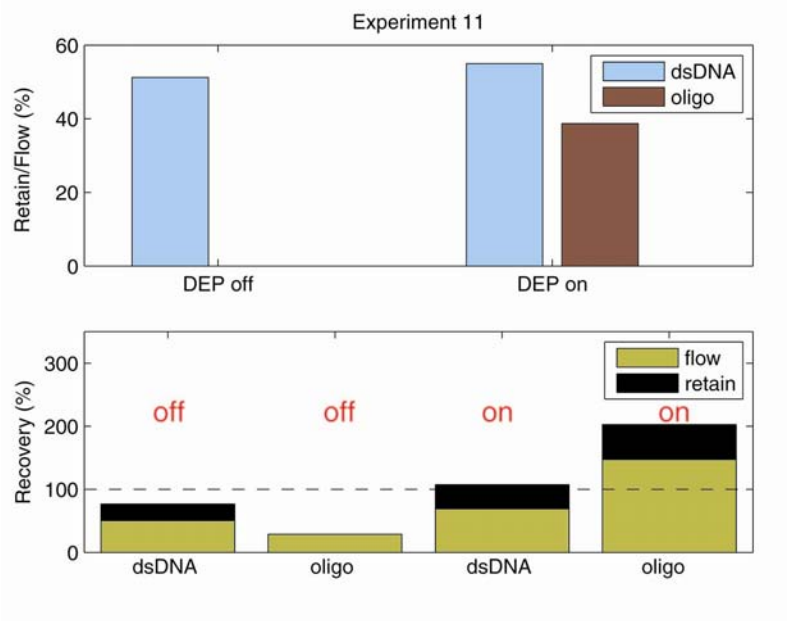

Figure 8: Set of experiments examining the DEP-induced redistribution of oligo and dsDNA (top) and recovery rates (bottom) as a function of DEP actuation.

rates should be near 100% when combining the signal from the two portions (flow and retain) of each processed sample. The DEP-induced redistribution of DNA should show a reduced dsDNA signal in the flow portion and an increase in the retain:flow ratio for dsDNA signal when the DEP is on. The retain:flow ratio from the oligo (which is presumably unaffected by DEP) should not change whether the DEP is on or off. Figure 8 shows two graphs of an experiment using a mixed sample. The top portion of the figure shows the retain:flow ratios for the dsDNA and the oligo in separate cases in which the DEP signal was either on or off. The first point to note is that the ratio for the dsDNA increases only slightly when the DEP is on. A second point to note is the lack of oligo signal in the case where the DEP was off, and the large retain:flow ratio for the oligo when the DEP is on. Our objective here is to increase the ratio for the dsDNA when the DEP is on, and to maintain or reduce the oligo retain:flow ratio when the DEP is turned on. Here, we see only a slight increase in the dsDNA ratio under the DEP. A second figure of merit is the recovery rate of the sample as it is processed through the device. The bottom of Figure 8 shows the recovery rate as a percentage of the original fluorescent signal from the oligo and the dsDNA for a sample. In the case where the DEP is off, we see that the recovery rate of the dsDNA from the flow and retain combined is around 75%, while the recovery rate for the oligo is ~25%. Ideally, we want these rates to be 100%, and this data demonstrates that we are irreversibly losing DNA to the device. The recovery rate for the case where the DEP is on improved: ~100% for the dsDNA, and then ~200% for the oligo (meaning oligo from the previous experiment with the DEP off was eluted in the following experiment when the DEP was on). The inconsistency of the DEP-induced redistribution of DNA and the recovery rates was typical for most of the experiments.

Figure 9 shows another set of experiments in which the recovery rates were good, with all being near 100%. In this set of data, the oligo retain:flow ratio was reduced when the DEP was on (which is a desirable outcome), but the dsDNA ratio dropped when the

DEP was on (which is an undesirable outcome). No experiments demonstrated the preferred outcome of simultaneously increasing the dsDNA ratio (significantly) while maintaining or reducing the oligo ratio.

7.0 DISCUSSION AND CONCLUSIONS

In summary, we have designed and built a bread-board system with a portable foot-print that can be used in DNA sample processing. The active device, the sample loading and collection

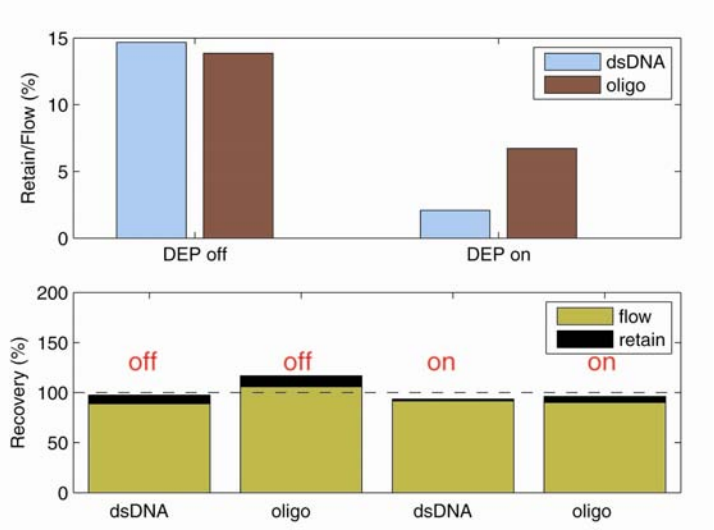

Figure 9: Another set of experiments examining the DEP-induced redistribution of oligo and dsDNA (top) and recovery rates (bottom) as a function of DEP actuation.

of processed sample, and the MHz frequency function generator have all been developed and demonstrated in operation. As the system is currently configured, the largest component is the brick-sized syringe pump for actively flowing fluids into the device. Future work is aimed at optimizing a pump system with a smaller footprint (coin size) that can operate at low flow rates (1-10 µl/min).

The DNA separation of oligo from dsDNA targets was inconsistent and not reproducible. We believe part of that difficulty has been resolved through optimization of the coating used to passivate the device surface from non-specific adsorption of oligo and dsDNA. We are able to achieve high recovery rates with the Pluronic coating when applied prior to each use. A second issue to resolve is to modify the buffer in which the dsDNA is suspended. The native buffer (Tris- EDTA) has a relatively high conductivity (1000 µS/cm) which is 1000 times more conductive than DI water. We believed that through dilutions the conductivity could be reduced enough to permit pDEP of the dsDNA. That may not be the case, and as such, our future effort is to resuspend the dsDNA in DI water immediately prior to separation.

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