A Rapid Genotyping Device for Complex DNA Samples

Final Report

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Abstract

The objective of this work is to develop an automated methodology for DNA extraction, purification, concentration, and quantification from a complex sample. The notional sample used to test the instrument during construction was a dirty buccal swab, or a buccal swab completely covered in soil. The resulting system is based on gradient elution isotachophoresis, a novel capillary electrophoretic focusing and extraction method that employs buffers of two different conductivities. The tabletop device is fully automated, employs two different detectors for detection of the DNA plug as well as detection of the focusing interface, delivers the DNA extract into a fresh tube for downstream analysis, and extracts DNA from both clean and dirty buccal swabs in approximately 5 min. The device can be deployed in field lab settings and operated by minimally trained personnel using an easy-to-use software package. Further experiments performed using the device indicate that the extraction efficiency can be increased by incorporating a negatively-charged coating to the capillary, performing longer extractions, and extracting DNA from the same sample multiple times. Further, the device can extract DNA from sample volumes as low as 5 μL. GEITP data indicates that DNA has been extracted from samples such as bone, gum, and plant material matrices.

Summary

The purpose of this work is to design, develop and build a device that will extract DNA from crude matrices. The DNA extraction device developed for this project is significantly different from conventional DNA extraction devices or methods in that it uses electric fields to remove DNA molecules from a sample solution rather than expose the sample to a solid-phase capture matrix. The methods developed in this work are based on gradient elution isotachophoresis (GEITP). GEITP is a combination of gradient elution moving boundary electrophoresis (GEMBE) and isotachophoresis (ITP). In GEMBE, separation of charged species, such as DNA, occurs electrophoretically and against a pressure-based counterflow. In ITP, an interface between an electrophoretically fast and an electrophoretically slow buffer is created. Analytes, such as DNA, with electrophoretic mobilities between these two buffers will focus at the interface. In GEITP, a combination of GEMBE and ITP, a dual electrolyte system is used for the extraction of analytes with a specific mobility; pressure is used to control the current within the capillary and facilitate the incorporation of the analyte plug into the capillary. Positive pressure is also used in this system to push the plug of extracted analytes out of the capillary into a clean vessel.

The device designed and built for the extraction and delivery of DNA is based on the GEITP principle. The device possesses an autosampling stage for automated extraction and delivery of the DNA, a laser-induced fluorescence (LIF) detector for the detection and quantification of the DNA plug, a capacitively-coupled contactless conductivity detector for the detection of the electrolyte interface, and a method for regulating the current in the capillary, thereby increasing the extraction time of the DNA within the sample. The technique is fast (5 minutes); it works with particulate- and inhibitor-laden samples with minimal sample pre-preparation; it isolates and collects DNA from crude matrices; it delivers clean and quantifiable DNA into a small volume (1-5 μL) ready for PCR amplification; and it requires simple hardware, making it a promising new technology for stand-alone DNA extraction and for integration into complete DNA analysis systems.

The notional sample that was used during the design, development, and construction of the device was a dirty buccal swab. Every variation of the device was tested with standard DNA samples as well as clean and dirty buccal swabs. DNA extracted from these samples was quantified with quantitative polymerase chain reaction (qPCR) analysis; the extraction efficiency of the device was determined to be approximately 16% based on a known amount of DNA incorporated into a standard sample. Once the direct rapid analysis generating extracted nucleotides (DRAGEN) device and method was implemented in our laboratory, the extraction of DNA from matrices such as bone, chewing gum, and plant material was performed. DNA was extracted and quantified from fresh bone; extractions from ancient bones did not yield quantifiable DNA and data indicated the presence of inhibitors in extracted samples. For these reasons, alternative buffer systems utilizing higher conductivity leading electrolyte and a trailing electrolyte that is only slightly electrophoretically slower than DNA was explored. DNA was extracted and quantified from chewing gum matrices. Chewing gum is a very complex matrix; experiments showed that there is some incorporation of PCR inhibitors with DNA after extraction. As a result, trailing electrolytes that are electrophoretically only slightly slower than DNA were utilized. Finally, DNA was extracted and quantified from both fresh and dried plant material.

Recommendations for future research include a thorough, systematic, GEMBE-based examination of trailing electrolytes, and research into modifiers for DNA that will slow its electrophoretic mobility for further exclusion of contaminants that possess similar electrophoretic mobilities to DNA. In addition, future work will include DNA extraction from a wide array of complex matrices, trace DNA extraction, development of a field-portable DNA extraction device, extraction of DNA from biothreats for identification purposes, and integration of the extraction device into a complete forensics system.

1. Introduction

Several laboratory methods exist that are aimed at extracting DNA from crude matrices. The oldest and most notable method of extracting DNA from such samples is by first filtering the sample and then adding an organic phase to salt out the DNA species. While this method has been used for many years, it is a time-intensive process that requires centrifugation; thus, this method is not appropriate for field use. Silica columns are commercially available as kits and they have been used for the purification of DNA in dirty samples. However, these columns require a centrifuge, are time-intensive, and yield dilute concentrations of DNA. The use of magnetic beads is a very popular method for purifying DNA. These methods yield pure DNA in a relatively short time but the instrumentation required is complicated and necessitates a trained user. Newer methods of analysis, such as the synchronous coefficient of drag alteration (SCODA) technique developed by scientists at Boreal, utilize electrophoretic approaches to yield pure DNA after extraction. This technique, however, is complicated, requires complicated instrumentation and significant amounts of time to perform an analysis.

Clearly, there is a significant need for relatively simple instrumentation and a relatively simple technique that can be used for the extraction and purification of DNA from crude samples. An ideal technique is one that requires very little sample preparation, will extract the DNA very quickly, and will deliver clean, pure DNA for human identity purposes.

The goal of the present project is to provide an automated, rapid, and fieldable DNA extraction device and method that can be paired with almost any DNA amplification and analysis equipment to provide field-portable DNA forensic analysis capabilities. The approach that will be used is based on a novel and unique separation methodology, gradient elution moving boundary electrophoresis (GEMBE).[1] The GEMBE technique is a rigorous, robust separation method that provides analysis capabilities for crude samples with minimal sample preparation.[2] With GEMBE, a combination of an electric field and a variable, pressure-driven counterflow is used to control the separation and quantitative detection of a variety of ionic and molecular species from an aqueous or slurry sample. Accordingly, GEMBE has a number of advantages over conventional capillary electrophoretic separation methods:

- Short separation times: Separations are performed with relatively short channels; the GEMBE device is thus smaller, simpler, less expensive, and more robust compared to other electrophoretic approaches.
- No sample injection required: Because complex samples can be introduced into the capillary without traditional injection using an applied electric field and voltage switching, the GEMBE device can be fabricated without moving parts. This trait makes the GEMBE device far more robust than other separation methods and makes it an ideal choice for field portable applications.
- Facile parameter optimization: Separation optimization is simplified through software controlled parameter changes; accordingly, a diverse range of molecular target assays can be accommodated on the same GEMBE platform simply by changing the reagent components employed.
- Interferent-free separation: The run buffer counterflow can be used to exclude matrix interferents including proteins, particulates, or other polymerase chain reaction (PCR) inhibitors from entering the GEMBE separation channel; thus, crude DNA samples such as blood or soil can be analyzed with little to no sample preparation.

These advantages make GEMBE a perfect platform for benchtop, hand-held, or remotely emplaced/extended service detection systems.

For this project, we utilized a combination of GEMBE with isotachophoresis, coined GEITP (gradient elution isotachophoresis),[3] as an extraction mechanism for genomic DNA in complex samples. The GEITP approach allowed us to facilitate execution of four required sample preparation steps: DNA extraction, purification, concentration, and quantification. These sample preparation steps are included in an automated platform that accepts crude samples and delivers clean, quantifiable genomic DNA for further analysis via short tandem repeat (STR) or other genetic analysis.

The GEITP method has been described prior to this work for the analysis of dye molecules, amino acids, proteins, and nucleic acids.[3] In the GEITP method, the buffer filling the capillary or microchannel is composed of an electrophoretically fast ion, such as chloride, while the sample, containing DNA, is prepared in a solution of an electrophoretically slow ion such as HEPES. When an electric field is applied, the DNA from the sample is focused at the interface between the two different electrolyte solutions. In the case of conventional ITP, this interface is formed only inside the capillary or microchannel and transport of DNA to the interface is limited by the small cross-sectional dimensions of the capillary or microchannel. With GEITP, however, this interface first forms outside the entrance of the capillary, facilitating a larger mass flux of DNA from the sample to be focused on the interface. As the counterflow used for GEITP is reduced, the interface (and the focused DNA) moves into the channel for detection and quantification.

The following sections of this document describe the tasks performed and the data collected in support of implementing the GEITP methodology for the extraction of human DNA from crude samples. Our notional sample during instrument construction is a dirty buccal swab, or a buccal swab that has been exposed to soil and thus shows soils particles adhered to it. The DNA from the samples is detected using fluorescence; an intercalating fluorescent dye that is compatible with PCR amplification of the DNA after the extraction is utilized to detect the DNA. In the initial stages of this project, work was performed using a fluorescence microscope for visualization and detection. However, in the final direct rapid analysis generating extracted nucleotides (DRAGEN) instrument, designed and built by our team, the DNA detection and quantification is performed using a laser-induced fluorescence (LIF) detector at a single point along a capillary. In addition to the fluorescence detection for quantification of the DNA, a capacitively coupled, contactless conductivity detector has been integrated into the DRAGEN instrument to determine the position of the ionic interface. This secondary mode of detection provides a useful diagnostic to indicate the proper functioning of the extraction device and has also proved useful in the control of the device for collection and delivery of the extracted DNA. The interface between the two electrolytes also provides a useful "trigger"—once the low-conductivity buffer passes the conductivity detector, the software that controls the DRAGEN device automatically sets itself to a DNA dispensing step. The DNA is then dispensed into a clean tube containing 10 μL of 10 mM TRIS EDTA buffer. To determine the amount of DNA extracted from the sample, quantitative PCR (qPCR) was performed after every experiment involving real samples. Further, qPCR with an internal DNA standard was performed to determine the presence of inhibitors potentially extracted into the device. Finally, to underscore the utility of the device for forensic applications for crude samples, we obtained short tandem repeat (STR) profiles of human DNA extracted from clean and dirty buccal swabs. Note that for this study, the terminology "sample" or "input sample" refers to the material that is loaded into the device and the term "extract" is the DNA solution that is delivered from the DRAGEN device.

Concurrent with the development of the GEITP methods for simultaneous DNA extraction, purification, concentration, and quantitation, several iterations of hardware were built to suit the needs of the expected user. The final version of the DRAGEN hardware includes a self-aligning fluorescence excitation and detection system, a capacitively coupled conductivity detection system, integrated components for application of the GEITP pressure and high voltage, a three-axis stage for DNA extraction and delivery, and an easy-to-use software control package.

In addition to performing DNA extractions on the notional samples, dirty buccal swabs, DNA extractions were performed on bone matrices, chewing gum, and plant material. During the course of developing these extraction protocols, the efficiency of the technique was increased after coating the capillary walls with a negatively-charged species, extracting for a longer period of time, and performing multiple extractions on the same sample. Further, extractions were performed on samples with volumes as low as 5 μL. The use of high-conductivity leading electrolyte as well as various trailing electrolytes for optimum extraction was explored. DNA was extracted from bone matrices, chewing gum, and plant material.

2. Methods, Assumptions, and Procedures

2.1. Chemicals and reagents

All chemicals and reagents, unless noted, were used as provided from the manufacturer. The leading and trailing electrolyte concentrations were varied and are presented in the body of the document. Materials and manufacturers utilized in this work are as follows: Tris (Sigma), HCl (Fluka), oxalic acid (Sigma), acetic acid (Sigma), lactic acid (Fluka), formic acid (Sigma), phosphoric acid (Fluka), ethylenediaminetetraaceticacid (EDTA, pH 8, 500 mM, Sigma), poly(vinylpyrrolidone) (40000 amu, Sigma), Triton-X 100 (Sigma), 1X SYBR Green I (Molecular Probes). Triton-X 100 was included in the leading and trailing electrolytes due to its ability to degrade cell membranes during cell lysis. Poly(vinylpyrrolidone) was used to coat the capillary surfaces to eliminate carry-over of DNA between samples. The fluorescent dye SYBR Green I allowed on-line DNA quantification using LIF during GEITP. After extraction, DNA molecules were delivered into a buffer solution that consisted of 10 mM Tris 0.1 mM EDTA (Sigma).

2.2. Apparatus

The device constructed for this project will be described in Sections 3.1-3.7. Briefly, the fused silica capillary was purchased with a transparent coating. The coating did not hinder the fluorescence of the DNA and, thus, the capillary did not require a window for LIF detection. The capillary was 8.8 cm in length and the inner diameter of the capillaries used for this device are 75 um. The leading electrolyre reservoir was fabricated from poly(etherimide) and was machined such that a threaded tap was emplaced in the center bottom of the reservoir. The reservoir contains 1 mL of leading electrolyte solution that was prepared fresh daily. The capillary is affixed to the leading electrolyte buffer reservoir using a LabSmith threaded fitting. A custom 3-axis stage controlled the placement of the tip of the capillary and the electrode into the sample under investigation. The headspace pressure in the leading electrolyte solution reservoir was controlled using a custom syringe pump. Platinum electrodes were used to apply a high voltage of +2000 V dc (EMCO High Voltage) at the leading electrolyte solution reservoir and ground the solution electrically at the lower end of the capillary. The capillary passed through a custom laser induced fluorescence (LIF) detector and a custom capacitively-coupled contactless conductivity (C4D) detector.[4] The LIF and conductivity detection points were approximately 58 mm and 44 mm from the lower end of the capillary, respectively. The apparatus was controlled and the data recorded using custom software (LabView, National Instruments).

2.3. DNA Samples

NIST Human DNA Standard at a concentration of 52.45 ng/μL was used as the control DNA standard. The control DNA solutions were diluted in trailing electrolyte buffer to concentrations described in the body of the document.

Buccal swabs were collected from anonymous human donors. Buccal swabs were placed into a 1.5 mL microcentrifuge tube containing 485 μL trailing electrolyte solution and 15 μL Proteinase K solution (20

mg/mL, Ambion) for cell lysis. The cotton swab tip was submerged, and the swab stick was cut to allow the tube to close. The tube was then inverted by hand gently for approximately 30 sec and placed into a water bath at 56 °C for 15 min for cell lysis. After vortexing briefly, fluid from the tube containing the swab was placed into 200-µL microcentrifuge tubes for analysis.

Soiled buccal swabs were prepared to mimic crude environmental samples and will be described in the body of the document. Briefly, approximately 50 mg to 100 mg of soil was placed into 1.5 mL microcentrifuge tubes. A buccal swab containing a human buccal sample was rehydrated with trailing electrolyte buffer just to the point of wetness. The buccal swab was then placed into the tube containing soil, the stick was cut to allow the tube to close securely, and the tube was agitated by hand to coat the swab with soil thoroughly. The soiled swab was then prepared for analysis similarly to a clean buccal swab.

DNA collected from bone matrices, chewing gum, and plant material is described in Sections 3.8-3.10.

2.4. qPCR

The amount of human DNA delivered from each sample was quantified using qPCR. Analyses were performed using a 7500 Real-Time PCR System (Life Technologies) and Quantifiler Human DNA Quantification Kit (Life Technologies). The analysis required 9.8 µL Quantifiler Human PCR Reaction Mix, 8.2 µL Quantifiler Human Primer Mix, and 2 µL of DNA solution in each sample well. Amplification proceeded according to the manufacturer's recommended program of 95 °C for 10 min, followed by 40 cycles of 15 sec at 95 °C and 1 min at 60 °C. A standard curve was constructed using NIST Human Standard DNA (Reference Material 2372; Component A) at concentrations ranging from 10.49 ng/µL to 0.03 ng/µL. All samples were measured in duplicate wells on the same plate.

The amount of pig DNA delivered from pig-based samples was quantified using the same instrument as above. Pig primers were obtained from SA Biosciences and PCR was performed using a SYBR Green master mix and the pig primer mix. The analysis required 12.5 μL SYBR Green Master Mix (Invitrogen), 1 μL pig primer mix, 10.5 μL water, and 1 μL of DNA solution in each sample well. Pig genomic DNA (Zyagen) was used to construct the standard curve. The concentrations of the standard curve range from 10.65 ng/µL to 0.04 ng/µL.

Spinach DNA was quantified using the same qPCR instrument described above. Spinach primers were custom-ordered from IDT; the primers are applicable to the 18S mRNA gene.[5] The primers were supplied at concentrations of 200 μM and diluted to 750 nM prior to incorporation into each well for qPCR. The analysis required 10 μL SYBR Green Master Mix (Invitrogen), 4.5 μL spinach primer mix, 3.5 μL water, and 2 µL of DNA solution in each sample well. Spinach genomic DNA (Zyagen) was used to construct the standard curve. The concentrations of the standard curve range from 10 ng/µL to 0.03 ng/µL.

2.5. STR Analysis

STR analysis was performed to demonstrate human identification from DNA delivered from a crude sample using the GEITP method. STR analysis used the Promega PowerPlex 16 HS STR Amplification Kit. The 16 locus multiplex PCR kit contained primers to type the 13 core STR markers, the sex-typing marker amelogenin, and the two additional STR markers Penta E and Penta D. The multiplex PCR reaction used a 12.5 µL total reaction volume containing 2.5 µL PowerPlex HS 5X Master Mix, 1.25 µL PowerPlex 16 HS Primer Set, 5 µL of the delivered DNA solution with a target DNA amount of 0.5 ng, and 3.75 µL deionized water. Thermal cycling was performed in a GeneAmp PCR System 9700 (Life Technologies) operating in the 9600 emulation mode with the following cycling parameters: 2 min incubation at 96 °C; 10 cycles of ramp 100% to 94 °C for 30 sec, ramp 29% to 60 °C for 30 sec, and ramp 23% to 70 °C for 45 sec; 20 cycles of ramp 100% to 90 °C for 30 sec, ramp 29% to 60 °C for 30 sec, and ramp 23% to 70 °C for 45 sec; and a 30 min incubation at 60 °C. The temperature was subsequently held at 4 °C until the

samples were removed. Following multiplex PCR analysis, 1 μL of amplified product was diluted in 10 μL Hi-Di formamide (Life Technologies) and 1 μL Internal Lane Standard 600 (Promega) and analyzed with an ABI PRISM 3130xl Genetic Analyzer using Data Collection v3.0 software (Life Technologies), POP-4 polymer (Life Technologies), and a 36 cm capillary array. All genotyping was performed with GeneMapper ID v3.2 software (Life Technologies) using allelic ladders, bins, and panels provided by the manufacturer and a peak detection threshold of 50 relative fluorescent units (RFU).

3. Results and Discussion

The results of this work are presented below by task. Each task is highlighted at the beginning of each section and the data required to demonstrate that the task was completed successfully are provided.

3.1. Extract Genomic DNA from Simple Mixtures Using GEITP

To determine the appropriate reagents and parameters for DNA extraction using GEITP, several buffers were evaluated on the basis of their conductivities and electrophoretic properties. Ideally, the two-electrolyte system consists of two buffers that are slightly different in pH but are significantly different in electrophoretic mobility. Further, the difference in electrophoretic mobility must envelope the mobility of the DNA and ideally, must be significantly different from any inhibitors that may be present in a dirty buccal swab. The buffers that met these initial criteria were TRIS-Cl^- coupled to TRIS-HEPES, TRIS-ACES, and TRIS-tricine. We decided on the TRIS-HEPES buffer system because the difference in the mobilities of the two buffers was greater than that for the other buffer systems. The final concentrations of the leading (TRIS-Cl^-) and trailing (TRIS-HEPES) electrolytes are summarized in the table below. Note that all experiments use these electrolyte concentrations.

Table 1: Concentrations of the leading and trailing electrolytes used in the initial GEITP experiments.

Leading Electrolyte	Trailing Electrolyte
50 mM TRIS	25 mM TRIS
25 mM Cl^-	25 mM HEPES

In addition to the buffers noted, 0.1% TRITON X was added because it is used in the lysing process. Poly(vinylpyrrolidone) (PVP) was added at a concentration of 0.1% to prevent surface interactions of the DNA with particles. Fluorescence detection was utilized throughout this project; the dye that was used for fluorescence detection was SYBR© Green I, available from Molecular Probes. This dye was chosen based on its optical properties and its ability to act as an intercalator; the free dye exhibits little to no fluorescence but displays significant fluorescence when intercalated into the minor grooves of double-stranded DNA. Into every DNA/trailing electrolyte mixture was added SYBR Green I at a concentration ratio of 1:10000 of stock solution. The human DNA that we utilized in our first proof-of concept experiments was isolated from the buffy coat fraction of a centrifuged human blood sample. However, in all subsequent experiments, NIST human DNA standard was utilized. DNA at different concentrations was diluted from the stock genomic DNA NIST standard.

Displayed in Figures 1 and 2 are still micrographs of a GEITP DNA focusing experiment. In the first image (Figure 1), the brown capillary is evident inside the sample cup. The background beyond the capillary appears green due to the presence of DNA into which SYBR Green I has intercalated. At the tip of the capillary is a dark area surrounded by a green ring. The dark area is indicative of the leading electrolyte slowly flowing into the sample reservoir by pressure (induced, in this case, using a syringe). The bright area is indicative of the DNA focusing, upon turning on the electric field to 1000 V, at the conductivity junction between the leading and trailing electrolytes. In a typical GEITP experiment, the pressure is

then decreased such that the focused DNA is drawn into the capillary for detection. Figure 2 is a still micrograph of the DNA plug as it appears after pressure is utilized to expel the DNA from the capillary. In this case, the DNA plug is simply expelled, using positive pressure, into the sample solution from which it was extracted. As is readily noted, the DNA plug is nicely shaped and the image indicates that the DNA is present in a reasonable concentration.

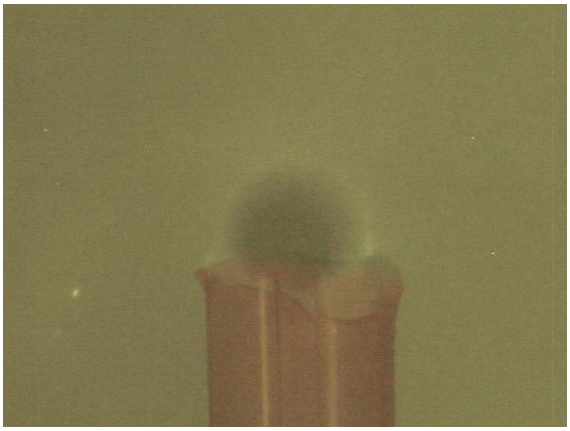


Figure 1: Still micrograph of the beginning stages of a GEITP experiment. The capillary is the brown feature in the bottom of the image and the background is colored green due to the presence of the DNA/SYBR Green I. The dark portion at the tip of the capillary indicates that there is fluid flow from the capillary into the sample reservoir—the leading electrolyte (pressure supplied by syringe). The bright ring that frames the dark portion is the focused DNA. The DNA focuses in this region when the electric field (1000 V) is applied.

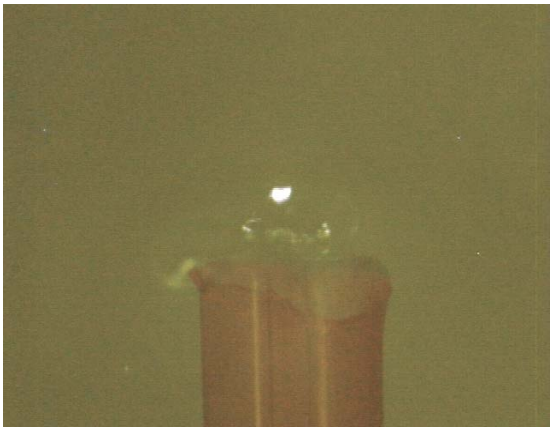


Figure 2: Still micrograph of the focused DNA after it has been focused into the capillary and expelled, using pressure, into the original sample solution. The electric field is not necessary at this point of the experiment; the DNA is expelled from the capillary using pressure supplied by a syringe.

Our first experiments indicate that the DNA is focused into a tight plug after GEITP extraction. Further experiments were required to determine the shape of the plug within the capillary; these experiments were necessary to aid in the development of the LIF detection module as well as determine the physical nature of the plug within the capillary.These experiments were performed using a GEITP instrument that was interfaced with an upright microscope for plug detection and visualization; a light-emitting diode (LED) was used as the excitation source. Displayed in Figure 3 is a still micrograph of NIST human DNA standard in buffer during extraction. In this case, a 180 μm capillary was used to visualize the plug; several diameter capillaries were used in experiments to determine the appropriate diameter capillary for the final device. Note that the plug is highly fluorescent and thus significantly concentrated. Figure 4 shows a fluorescence micrograph of NIST human DNA standard in soil. The plug is significantly different in shape possibly due to the presence of bacterial DNA or charged species in the soil. However, the DNA can still be quantified and delivered to clean buffer for further analysis.

Figure 3: Fluorescence micrograph of NIST human DNA standard in buffer during extraction using GEITP-ITP with a 180 µm inner diameter fused silica capillary. A concentrated plug of DNA moves from right to left. The conductivity interface between the leading and trailing electrolyte solutions is seen as a sharp boundary to the left edge of the DNA plug. DNA from the sample continues to stream in towards the interface from the right.

Figure 4: Fluorescence micrograph of NIST human DNA standard and dirt in buffer during extraction using GEITP-ITP. Experimental conditions are otherwise identical to Figure 2A. Although the plug of DNA has a different shape, this does not prevent effective DNA concentration, quantification, and delivery for further analysis.

During the course of our experimentation, it was necessary to manipulate the applied voltage and pressure ramp to ensure adequate extraction of DNA. Note that these experiments were performed after each iteration of experimentation and it was sometimes necessary to further manipulate the parameters on a day-by-day basis until the final instrument was utilized for all experiments. With respect to applied voltage optimization, displayed in Figures 5 and 6 are GEITP electropherograms detailing the fluorescence intensity with respect to time of DNA plugs detected in a microchannel in addition to schematics of the detectors placement along the capillary. In Figure 5, the applied voltage across a 180 mm capillary is 175 V. The peak is broad, indicating that the DNA is not well focused. In Figure 6, the applied voltage is 1000 V and the peak corresponding to the fluorescence of the DNA plug is sharp and well-defined, indicating that the DNA is well focused into a tight plug.

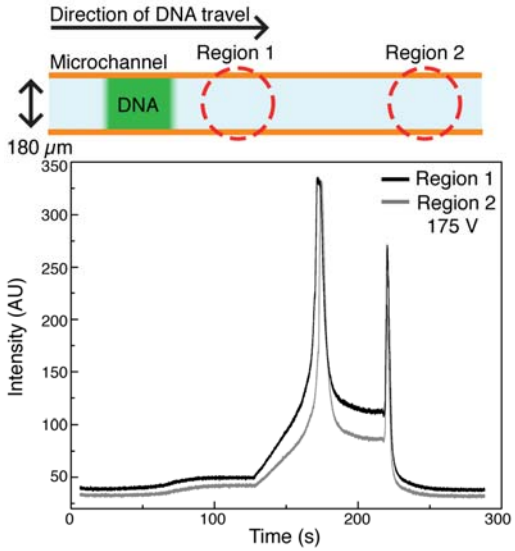


Figure 5: GEITP electropherogram of NIST standard human DNA at an applied voltage of 175 V. The fluorescence was collected at two points along the channel. The DNA peak is broad and indicates that the DNA is not well focused.

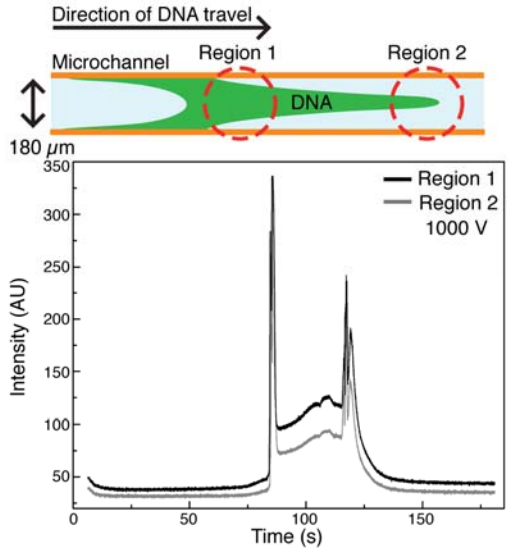


Figure 6: GEITP electropherogram of NIST standard human DNA at an applied voltage of 1000 V. The fluorescence was collected at two points along the channel. The DNA peak is sharp and nicely-shaped; indicating that the DNA is well-focused.

In addition to optimizing the applied electric field, it is also possible to optimize the pressure ramp to better focus the DNA plug. Displayed in Figure 7 is a comparison plot of fluorescence intensity of the

DNA plug with respect to the pressure gradient utilized for the separation in -Pa/sec. The fluorescence intensity of the DNA plug is highest, and the DNA plug is thus best focused, at no applied pressure. However, it is necessary to apply a positive pressure in the beginning of the extraction in order to ensure that particulates, globular proteins, and other large molecules are confined to the sample reservoir. It is necessary to apply a negative pressure during the separation to incorporate the plug into the capillary in a timely fashion and then detect it. The data indicate that the fluorescence intensity is greatest, and the DNA plug is best focused, at low pressure gradients.

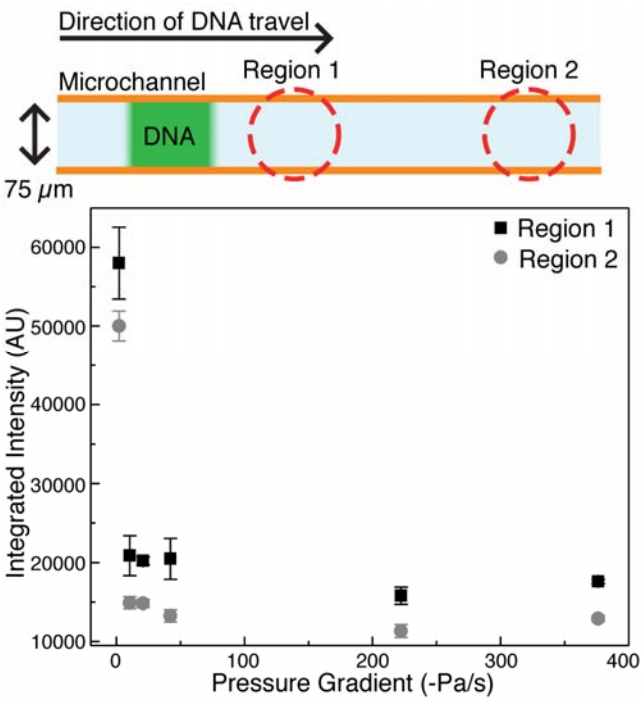


Figure 7: Data collected after experiments measuring fluorescence intensity of the focused DNA plug with respect to applied pressure gradient. The fluorescence intensity is highest with no applied pressure gradient; however, it is necessary to apply the pressure gradient to extract the DNA in a timely fashion. The DNA plug becomes less focused as the pressure gradient increases.

The data presented show that the DNA extraction program can be modified by changing the applied voltage and pressure gradient parameters of the GEITP method. Modifying these parameters allows us to extract various amounts of DNA. Further, modifying these parameters allows us to further exclude inhibitors.

In addition to the parameter determination experiments performed with NIST standard DNA, we also performed plug visualization experimentation with human DNA collected from a buccal swab using the same GEITP instrument with an upright microscope for detection. Displayed in Figure 8 is a GEITP electropherogram of human DNA extracted from a buccal swab. The DNA peak shows several spikes, indicating large globules are present in the sample plug. However, the DNA peak is extremely well-defined and quantification and delivery of this plug can be performed.

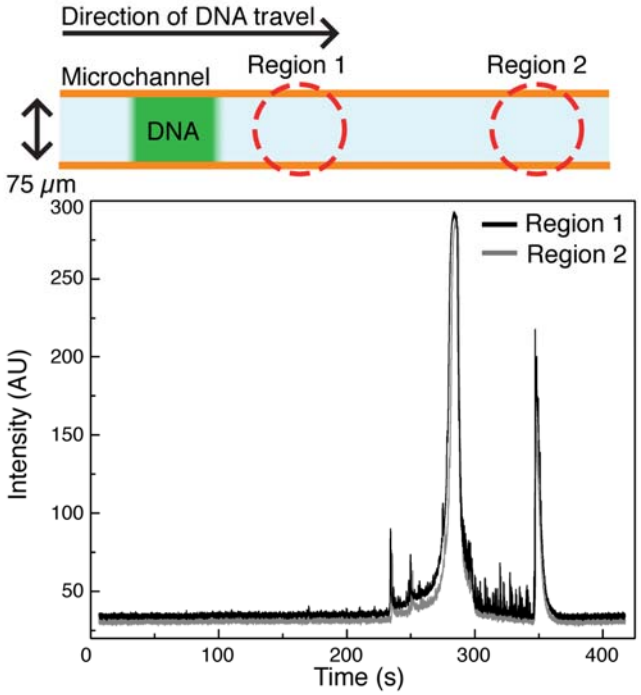


Figure 8: GEITP electropherogram of DNA collected from a human buccal swab. The fluorescence peaks, and thus the DNA plug, is sharp and well-defined.

The experiments described in support of this task allowed us to visualize the DNA plug within the sample capillary and develop our extraction methodology in a controlled manner. The resultant data from these experiments indicate that we have successfully extracted genomic DNA from simple matrices—both human DNA present as a NIST standard and human DNA extracted from a buccal swab.

3.2. Collect DNA Fraction after Extraction

The experiments presented above were performed to prove that the GEITP methodology was appropriate for DNA collection into a capillary. However, collecting the extracted DNA from an instrument positioned horizontally proved challenging. Essentially, the DNA is extracted from a sample reservoir; in order to collect the DNA after extraction, it is necessary to remove the sample from the reservoir, rinse the reservoir with buffer, and then collect the DNA plug into the same reservoir as the original crude sample. Clearly, contamination is a significant issue with this method. In addition, the configuration of the sample reservoir in the horizontal instrument with respect to capillary placement within the sample reservoir is such that at least 150 μL of fluid must be present in the sample reservoir for collection. Thus, any DNA collected from the horizontal instrument is present in very low concentrations. While we did perform some qPCR experiments using the horizontal apparatus, it was obvious that a vertical configuration was necessary.

The experiments performed in support of Task 1 described previously were performed, in most cases, concurrently with the vertical DNA extraction device that was built, DNA Extraction Device Version 0.1. Displayed in Figure 9 is a schematic and photograph of the device. The pressure control was afforded using a Mensor Series 600 pressure calibrator, the high voltage power supply used was a Stanford Research Systems Model PS350 power supply, and the LIF detector was home-built and will be described in Section 3.4.

This instrument, Version 0.1, allowed us to extract DNA from various solutions and dispense the extracted DNA into clean buffer in a tube. The DNA was extracted from a sample vessel, typically a 150-μL tube used for PCR samples, and then dispensed into 10 μL of clean TE-4 buffer present in a clean 150-μL PCR tube. This method of extracting from a dirty sample and then dispensing the clean DNA into a clean vessel allows for the analysis of the DNA by virtually any forensic DNA analysis system; that is, while the DRAGEN methodology was designed for potential integration into a total forensic DNA analysis system, the potential for use with any analysis product is far greater with the more generic approach of simply dispensing the clean DNA into a clean tube that can be handed off for forensic analysis.

We experimented, using a GEITP device in its horizontal configuration, with capillaries of various inner diameters to determine if a larger inner-diameter capillary would allow us to extract more DNA. However, after building device Version 0.1, it was determined that the 75 μm inner diameter capillaries were the best fit for the device because capillaries of larger diameter would leak leading electrolyte (LE) while the instrument was not in use. The DNA extraction program for Version 0.1 of the device consisted of applying 2.4 kV across a 10-cm capillary as the pressure ramped down at 2.5 Pa/sec. Collection was performed by visual inspection of the trace, after the LIF peak completed, the pressure was set to -780 Pa while the capillary was moved to the delivery tube. Delivery consisted of applying 100 Pa for 104 seconds to the delivery tube, resulting in 1 μL of delivery solution.

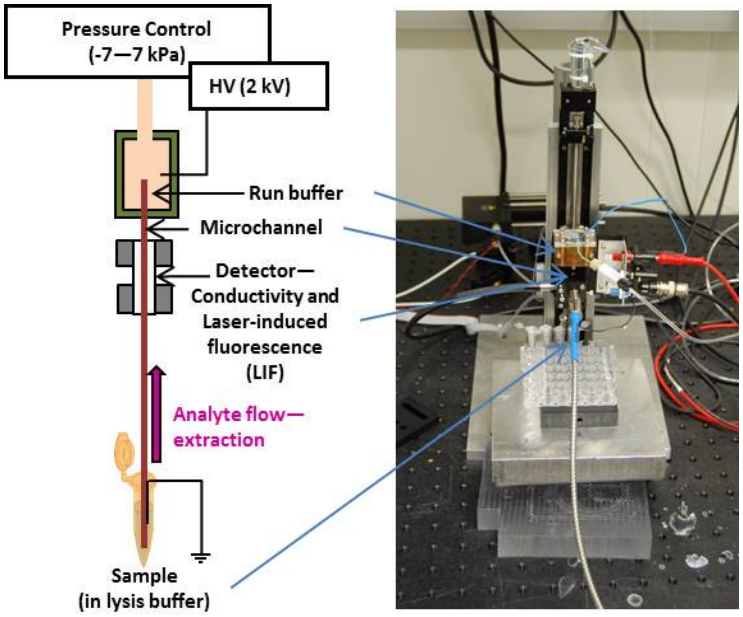


Figure 9: Schematic and photograph of the vertical DNA extraction device that was first built in support of the collection and quantification task of this project. This device was used for the initial collections of DNA. The schematic for the device is located on the left of the image. The pressure control was afforded using a Mensor pressure regulator, the high voltage power supply used was a Stanford Research Systems power supply, the LIF detector was home-built and will be described in Section 3.4.. Note that the schematic shows analyte flow vertically from the sample vial. After the DNA has entered the channel and is detected using the LIF detector, a clean vial containing 10 μL TE-4 is placed under the capillary and pressure is used to dispense the plug of DNA into the clean solution.

To determine the amount of DNA that was extracted from the sample and dispensed into the clean solution, qPCR analysis was performed. The thermocycler and its associated software used was an Applied Biosystems 7500 Real-Time PCR System. Our initial experiments were performed using primers that were designed to amplify the TH01 gene; the amplification was detected using fluorescence of the

SYBR Green I intercalating dye. A master mix kit made specifically for use with the SYBR Green I intercalating dye was supplied by Applied Biosystems. Sample volume was 20 μL and a standard curve of DNA (NIST standard DNA) concentrations from 10.49 ng/μL to 0.033 ng/μL was constructed for every qPCR run. The samples and standards were each analyzed in duplicate. The threshold Ct values were determined by the Applied Biosystems software.

Displayed in Figure 10 is typical optical data that was obtained from the extraction of DNA from a solution of DNA prepared from a NIST standard. The shape of the extraction (first peak) and delivery (second peak) peaks are almost identical to those collected in Figure 8.

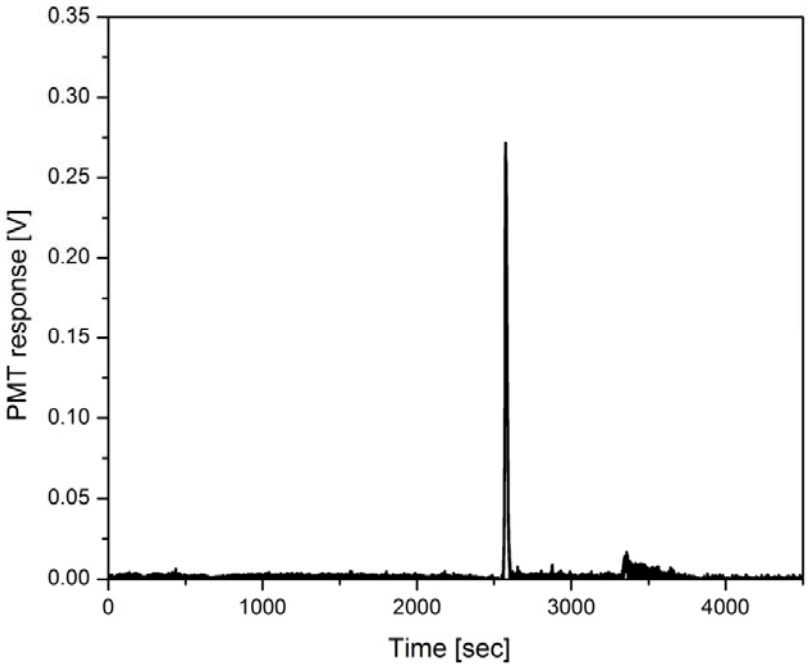


Figure 10: GEITP electropherogram of the extraction and subsequent delivery of DNA extracted from a solution of 536 pg/μL NIST standard DNA. After extraction and subsequent delivery, the DNA was subject to qPCR analysis.

Displayed in Figure 11 are qPCR amplification plots of the DNA collected in Figure 10. The amount of DNA collected from this particular run, as determined by qPCR, is 0.240 ng. This amount of DNA extracted is typical for the early experiments.

The work performed in support of this task, collect DNA fraction after extraction, was intended to prove that our methodology of extracting DNA from a sample vial into a capillary and then using pressure to dispense the DNA from the capillary into a clean vessel is a viable one. These proof-of-method experiments allowed us to determine that DNA carryover, while not significant, could be mitigated using a simple 0.100 mM NaOH rinse step between extractions. Further, these experiments allowed us to further optimize the extraction program for clean DNA from a clean sample. Because the overall goal of the project is to extract DNA from a dirty buccal swab, we continued to optimize the extraction parameters while concentrating on determining the most appropriate methods for extracting DNA from a buccal swab.

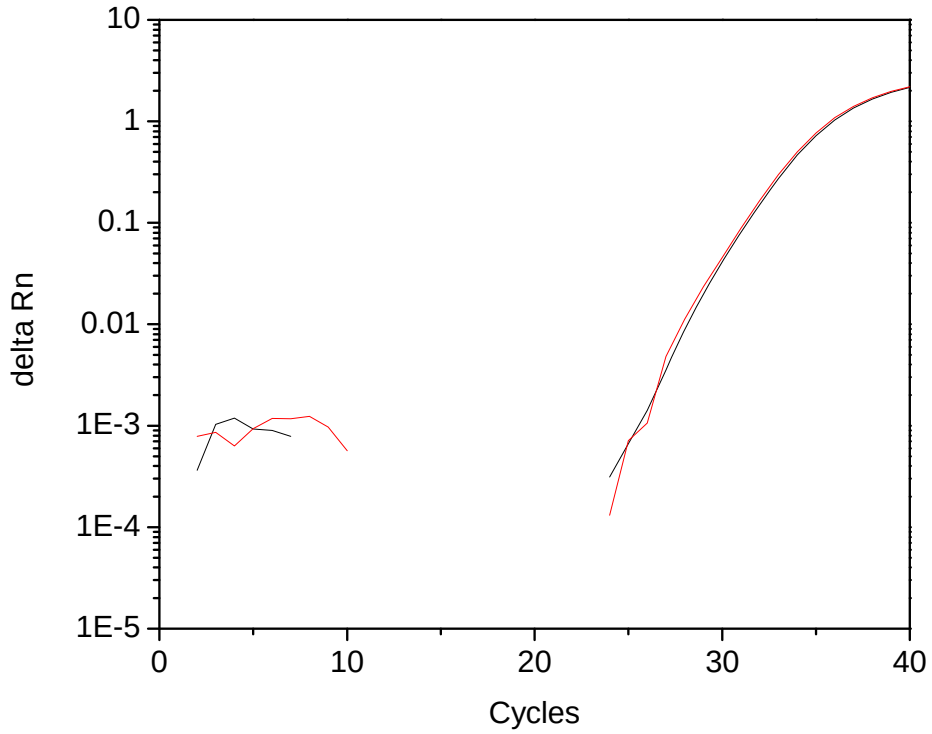


Figure 11: qPCR amplification plots of DNA extracted from a solution of DNA prepared from a NIST standard. The amount of DNA collected in this example is 0.240 ng and is typical for these early experiments.

3.3. Extract Genomic DNA from a Dirty Buccal Swab

Our notional sample for this project is a dirty buccal swab. To determine a semi-quantitative method for making a notional dirty buccal swab sample, several buccal swabs were obtained and weighed. The buccal swabs were exposed to soil collected from under an oak grove on the NIST campus. After exposure, each buccal swab was reweighed and an average soil exposure amount per buccal swab was obtained. To better mimic an environmental sample, approximately 20 mg of soil in addition to the average amount of soil required to coat a buccal swab was used for dirty buccal swab preparation. All dirty buccal swabs prepared during this project contain approximately 50-100 mg soil/sample. A typical dirty buccal swab was prepared by first weighing approximately 50-100 mg soil into a 1.5 mL tube. The buccal swab was sampled; the stick was cut just above the cotton bud to allow for inclusion into the sample tube. The tube was then gently rolled on a desktop or between fingers to thoroughly coat the swab with the dirt particles. Displayed in Figure 12 is an image of a dirty buccal swab.

After the dirty buccal swabs were prepared, cell lysis was performed. The lysis buffer consisted of 485 μL 25 mM TRIS-HEPES (note that this is also the trailing electrolyte, TE), and 15 μL Proteinase K (Ambion; used as received). The swab/soil/lysis buffer slurry was then exposed to a water bath held at a constant temperature of 56 ^{o}C for 10 min. The mixture was then gently vortexed prior to aliquotting 99 μL of solution into each of three 150 μL tubes. SYBR Green I (1 μL) was added to each tube to a final concentration of 1:10000. At this point, the tubes were ready for further experimentation. Figure 13 depicts a dirty buccal swab in a tube into which the lysis buffer has been added to make a slurry.

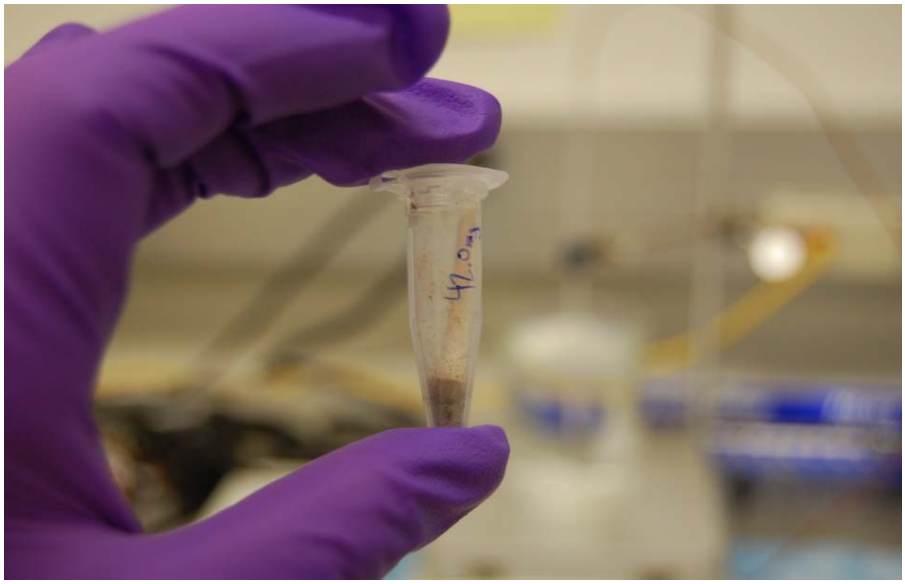


Figure 12: Image of a representative dirty buccal swab. Swabs are sampled and then exposed to 50-100 mg soil in a 1.5 mL tube. The tubes are gently rolled and inverted to thoroughly coat the swab with the soil.

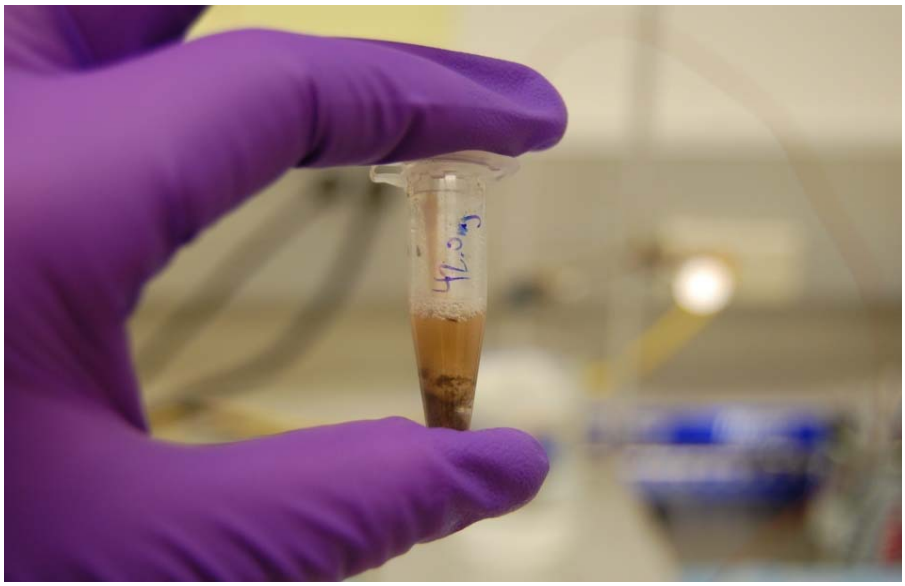


Figure 13: Representative image of a dirty buccal swab exposed to lysis solution consisting of 485 μL 25 mM TRIS-HEPES and 15 μL Proteinase K. The sample was allowed to incubate in a water bath at 56 ^{o}C for 10 min prior to extraction.

After preparation, the samples were subjected to DNA extraction using the GEITP methodology. Displayed in Figure 14 is a representative GEITP electropherogram of an extraction of DNA from a clean human buccal swab. The electropherogram contains more peaks and shoulders than the data representative for a GEITP extraction from standard DNA because it is less uniform than the standard DNA. Figure 15 shows corresponding qPCR amplification plots for the human DNA extracted from a clean buccal swab described above. The average amount of DNA extracted from this swab was 0.911 ng.

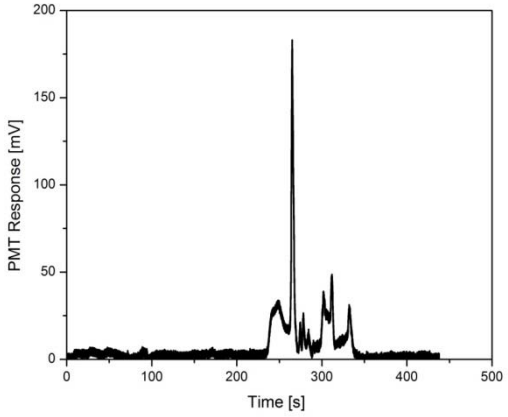


Figure 14: Representative GEITP electropherogram of DNA extracted from a clean human buccal swab and subsequently delivered into a clean tube for qPCR analysis.

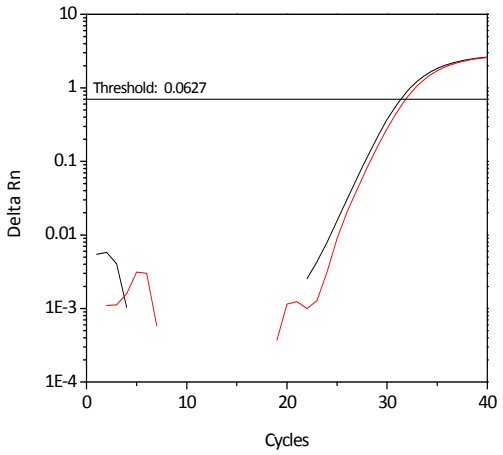


Figure 15: qPCR amplification plot of DNA extracted from a clean buccal swab. The average amount of DNA extracted was 0.911 ng.

The GEITP electropherogram shown in Figure 16 is representative of DNA extracted from a dirty human buccal swab and subsequently delivered to a clean buffer solution for subsequent qPCR analysis. The extraction peak appears wide and large due to the potential presence of bacterial DNA from the soil extracted into the apparatus. Additionally, there may be some charge species in the soil that are also being extracted; these species will add to the overall width of the peak. The qPCR amplification plots shown in Figure 17 correspond to the human DNA that was extracted from this dirty buccal swab. The average amount of DNA extracted from this dirty buccal swab sample was 0.623 ng.

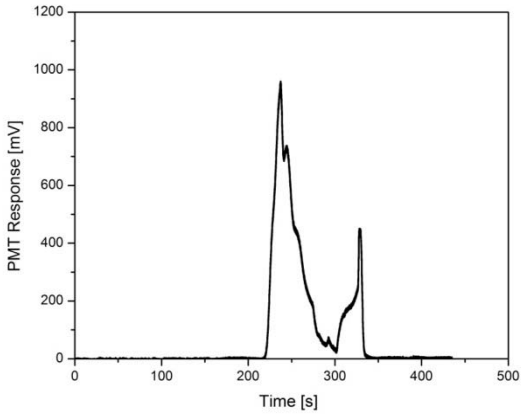


Figure 16: Representative GEITP electropherogram of DNA extracted from a dirty human buccal swab and subsequently delivered into a clean tube for qPCR analysis.

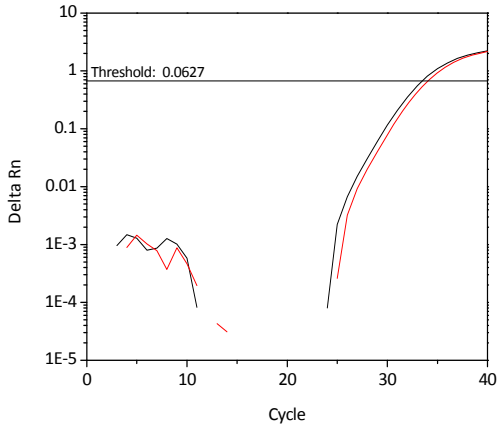


Figure 17: qPCR amplification plot of DNA extracted from a dirty buccal swab. The average amount of DNA extracted was 0.623 ng.

Because our overall goal is to demonstrate the extraction of human DNA from a dirty buccal swab, we researched some of the impurities that can be present in soil and the potential for qPCR inhibition of

these materials. Many of the reports in the literature could not quantitatively state the degree of inhibition exhibited by materials present in soil. Because the GEITP method is an extraction as well as a concentration process, we undertook a careful study to determine the degree of inhibition of various inhibitors. Note that many of these inhibitors are not present in soil; we studied these inhibitors because the data will be useful for other types of samples present in environmental matrices (i.e., blood in soil, etc.). Shown in Figure 18 is a chart showing the ratio of the mass of inhibitor to mass of DNA that is required for inhibition. Clearly, the majority of the inhibitors studied are large globular molecules (such as IgG) that will not be present in the extracted DNA plug because of their exclusion from the capillary due the counterflow that is omnipresent in the GEITP technique. However, even if these molecules were present, they must be present in masses that are orders of magnitude more than the DNA. Melanin, glycogen, and pectin were studied in addition to those present in the chart and they exhibited no inhibition.

Humic acid is the inhibitor that is most often present in soils and it inhibits qPCR amplification at an approximately 1:1 mass ratio to DNA. We performed several experiments with pure humic acid from Sigma-Aldrich that were aimed at determining the electrophoretic mobility of the humic acid with respect to the DNA. The electropherograms displayed several steps, indicating that there are several species within the humic acid mixture that display different electrophoretic mobilities. While many of these species eluted after the DNA, one particular analyte within the humic acid matrix eluted similarly to DNA. Thus, we continued to optimize the instrument to exclude from the extraction process any species with similar electrophoretic properties to DNA.

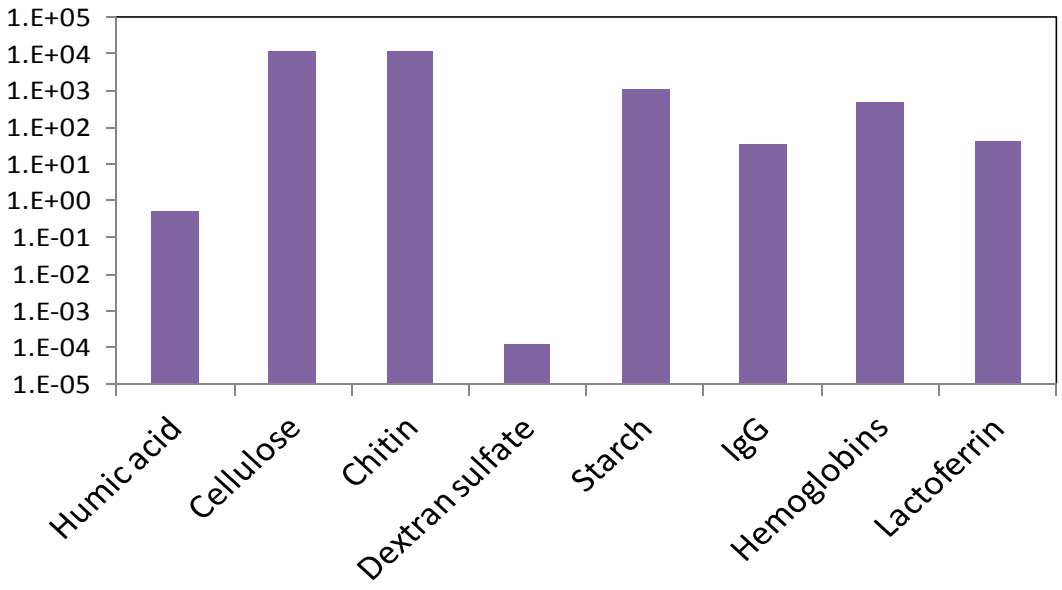


Figure 18: Ratios of the mass of inhibitor:mass of DNA required to inhibit PCR amplification.

3.4. Build Fluorescence Detector

The goal of this task included building a fluorescence detection unit capable of detecting the extracted DNA within the capillary prior to collection. The components of this system were selected to meet the limit of detection requirement of 100 pg while minimizing the system complexity and footprint. Note that the data described in Sections 3.1-3.3 were collected with the detector described in this section.

The first decision involved selecting the light source and detection components complimentary to the dye, SYBR Green I. Upon binding to double stranded DNA, the molecule becomes fluorescent. SYBR

Green I possesses a λ_{ex} at 490 nm and λ_{em} at 520 nm, Figure 19. While the goal was to develop this instrument towards field-portability, on the timeframe of this project we chose to focus on ensuring that we purchased components that would allow us to quantify and detect small amounts of DNA. To do that, we needed maximum stability with a relatively high power light source, and a low-noise, high gain detector.

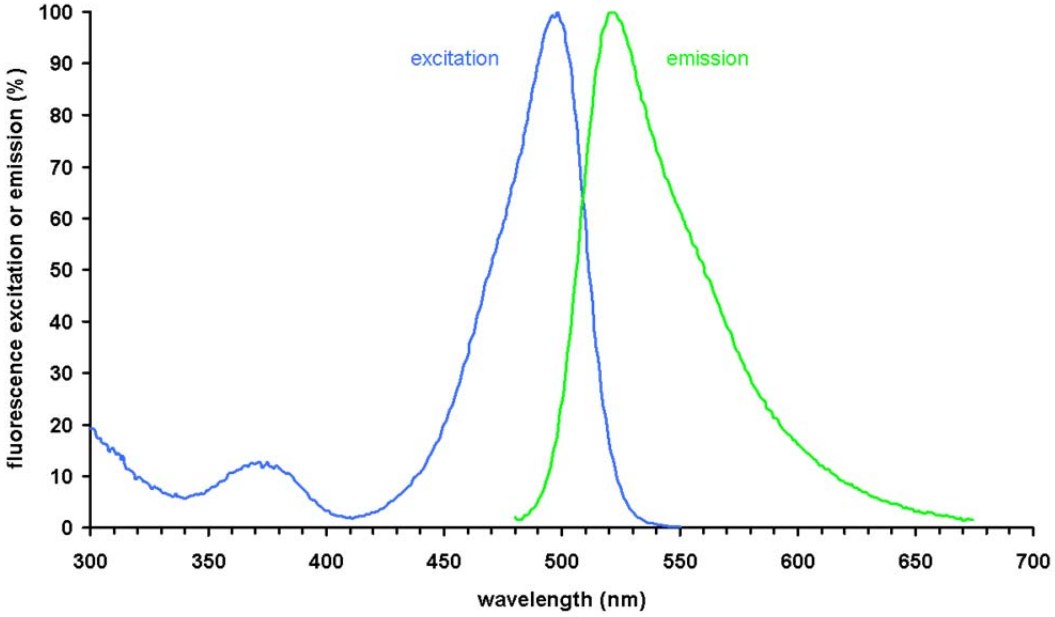


Figure 19: The excitation and emission spectra of SYBR Green I dye.

The laser induced fluorescence system is driven by a Coherent Cube laser at 488 nm. This is a continuous wave, fiber-pigtailed laser (model number Cube 488-30FP) producing a 1-mm-diameter collimated beam with maximum output power of 30 mW. The light is incident on the fused silica capillary, and the resulting light is collected via a liquid light guide (LLG) 4 feet long, with a core diameter of 3 mm (Thorlabs LLG0338-4) placed a few millimeters from the capillary. The other end of the LLG is mounted in a custom-built adapter for the Thorlabs SM1 cage optics system, which is then placed in a 2" lens tube. The LLG has a NA = 0.68, and this output light is focused by two aspheric condenser lenses with a working distance of 12.1 mm (Thorlabs ACL2520-A). The light is passed through a 488-nm notch filter (Thorlabs NF488-15), two 505-nm longpass filters (Chroma Technology Corp., HQ505LP), and a 500-nm longpass filter (Thorlabs FEL-500) all contained within a 50-mm lens tube. The light is then incident on a photomultiplier tube (Thorlabs PMM02) with a sensitivity of 0.55 A/W at 520 nm, 10^6 gain, and dark current 3 nA with a 22-μm diameter photocathode diameter. The maximum gain of the phototube is 3.1×10^6, and the sensitivity at 520 nm is 0.055 A/W. High voltage is driven by output from a National Instruments (NI) data acquisition (DAQ) board (NI-USB-6211) at 500 mV, and the transformer in the PMT module converts this to a voltage across the tube of 5 kV. Output from the PMT is transferred to the same DAQ board.

The optics assembly is photographed and diagrammed in the Figures 20-22. A head-on photograph is shown in Figure 20, and the assembly, mounted via the Thorlabs cage optics system, to the PMT is shown in Figure 21. The LLG is mounted in a light-tight custom holder which screws into the cage system, which then screws into the PMT. Figure 22 diagrams the optics in the lens tube, drawn to scale except for the PMT.

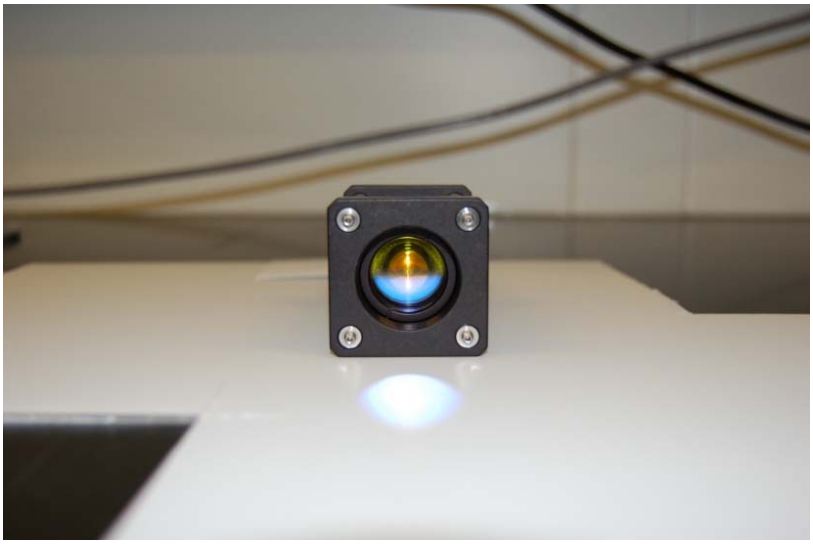


Figure 20: An end-on view of the lens tube.

Figure 21: The lens tube integrated with the LLG, the LLG holder, and the PMT.

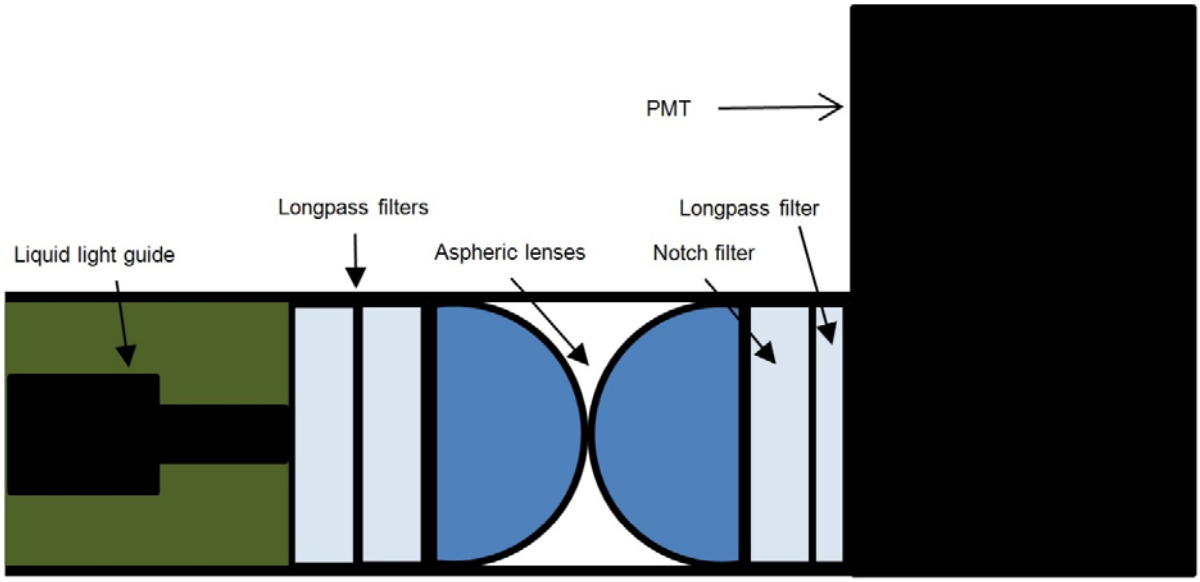


Figure 22: A diagram of the optics in the lens tube. The light is emitted from the LLG, and passes through two fast aspheric lens, collimating the light. The collimated light then passes through the appropriate filters and onto the PMT photocathode.

While the final optics are shown above, initial research and proof-of-concept experiments were performed with the apparatus shown below in Figure 23. The horizontal setup has a sample reservoir on the left, with the appropriate capillary fittings (LabSmith) holding the capillary. The LIF block, in the middle, holds the laser (not shown) and the LLG off at a 90^{o} angle. A syringe for applying pressure is attached for applying a negative pressure to the sample reservoir, moving the sample into the capillary. Figure 24 shows the entire setup, including the laser in the LIF block, and the oscilloscope used to read the PMT signal.

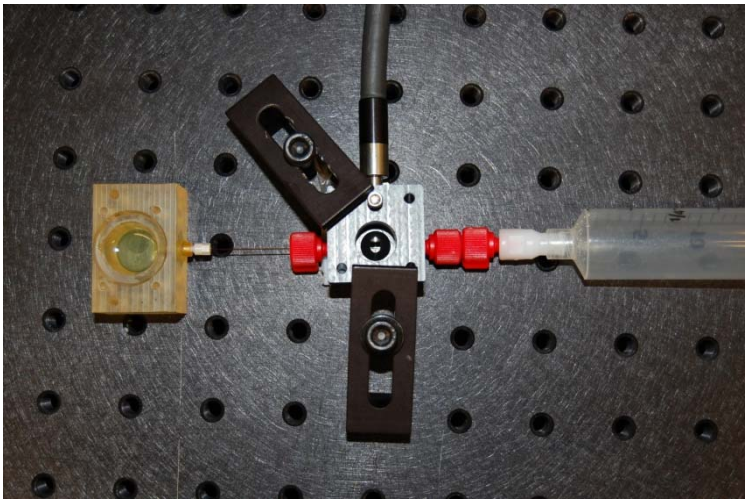


Figure 23: Initial experiments were performed using a syringe to pull fluid through a capillary in a horizontal geometry. The laser was positioned normal to the lab plane, and collection at a 90^{o} angle from that plane. The PMT read out onto the oscilloscope. This image shows a close-up of the LIF block

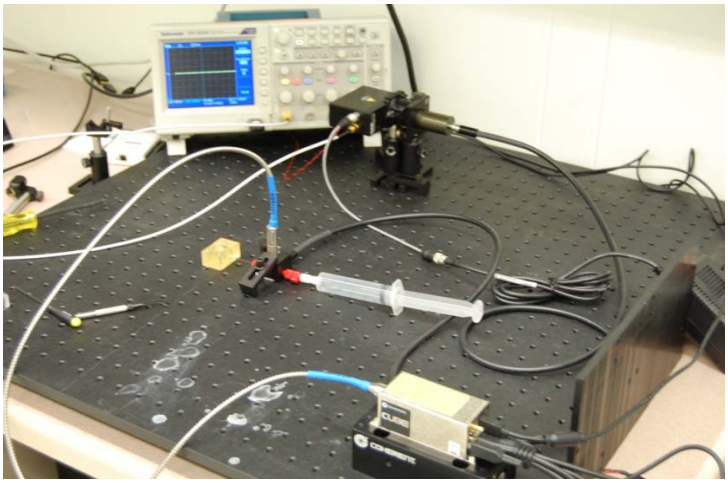


Figure 24: Initial experiments were performed using a syringe to pull fluid through a capillary in a horizontal geometry. The laser was positioned normal to the lab plane, and collection at a 90° angle from that plane. The PMT read out onto the oscilloscope.

Initial experiments used the dye molecule carboxyfluorescein. Carboxyfluorescein has excitation and emission wavelengths similar to SYBR Green I, but does not require binding to another molecule. Subsequent tests with SYBR Green I and λ DNA showed that the instrument could detect SYBR Green I as well; traces from both are shown in Figure 25. Several differences are readily apparent from these traces. Carboxyfluorescein shows a more stable signal with a greater increase above background. This indicates that carboxyfluorescein is more impervious to the photobleaching that occurs as the sample sits in front of the laser. The SYBR Green I trace reaches a maximum value, and signal quickly decreases due to photobleaching.

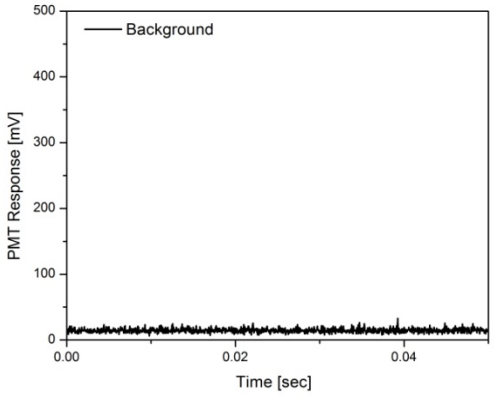


Figure 25: Background signal trace.

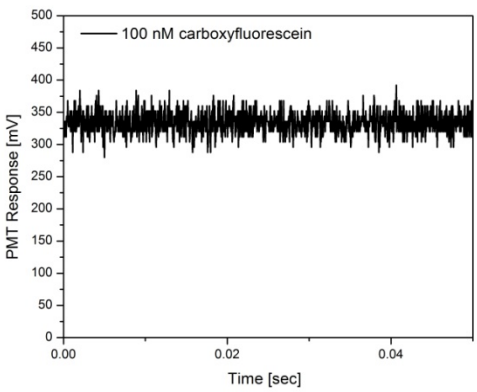


Figure 26: Signal level after carboxyfluorescein is injected into the capillary.

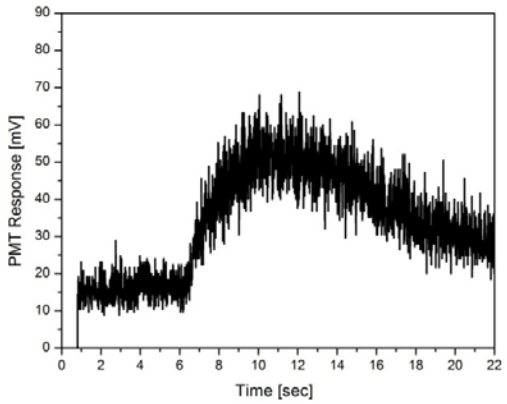


Figure 27: SYBR Green I with DNA trace utilizing 400 μg/mL DNA, laser power at 20 mW and PMT gain set to 0.6 kV.

After performing the proof-of-concept experiments with carboxyfluorescein, it was necessary to move to a setup which allowed us to collect the resulting DNA into a vial for qPCR analysis. The vertical jig is shown in Figure 28 and is identical to the one shown in Figure 9. The LIF block uses the same 90^{o} angle between laser and collection as in the previous setup; however, for the setup shown in Figure 28, the PMT data is read off of the NI DAQ. The run buffer reservoir and LIF block are mounted onto a motorized, hand-controlled translational stage.

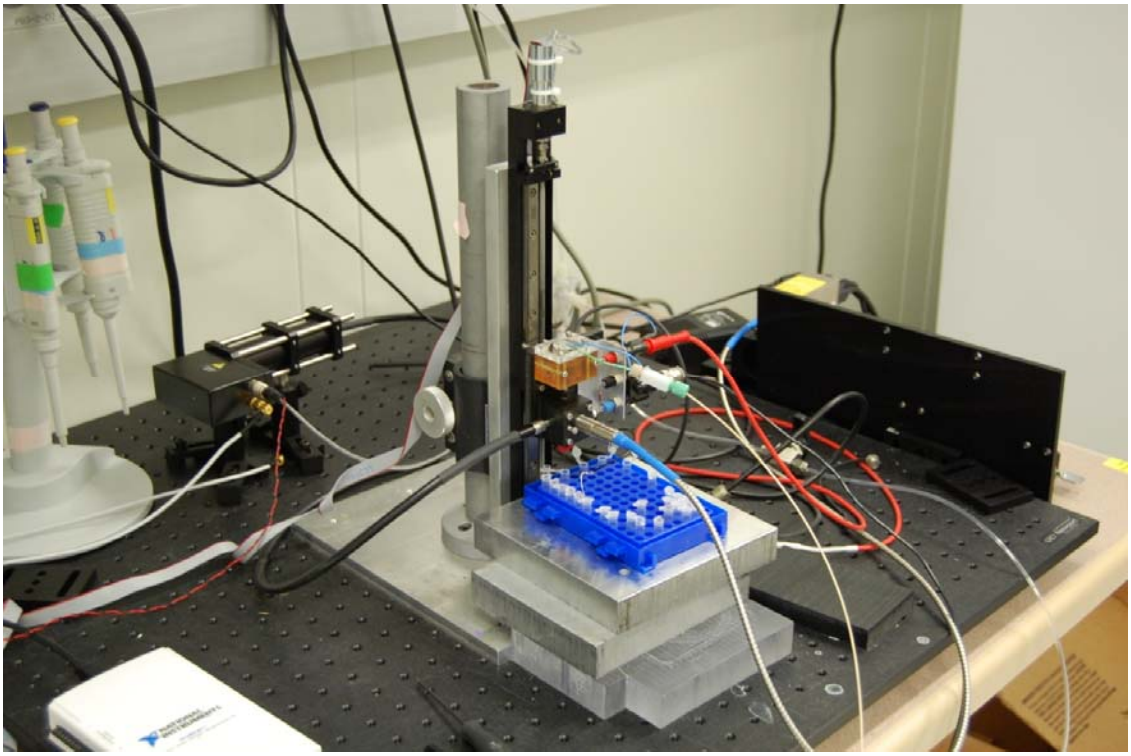


Figure 28: Vertical jig used for sample collection.

We further improved the optical setup by changing the geometry of the LIF/capillary housing block. Performing stability experiments demonstrated that the noise in the background was due to minor fluctuations of the laser itself. Because the laser itself was quite stable, this indicated that significant

amounts of reflected fundamental laser light penetrated the filters. The scattered light and minor fluctuations were significantly amplified by the PMT, leading to an unstable background. Redesigning the LIF block to take advantage of spatial filtering of the laser light led to a large increase in signal to noise. The diagrams of the initial and new LIF blocks are shown in Figures 29 and 30, and a comparison of the optical traces from the different blocks is shown in Figures 31 and 32.

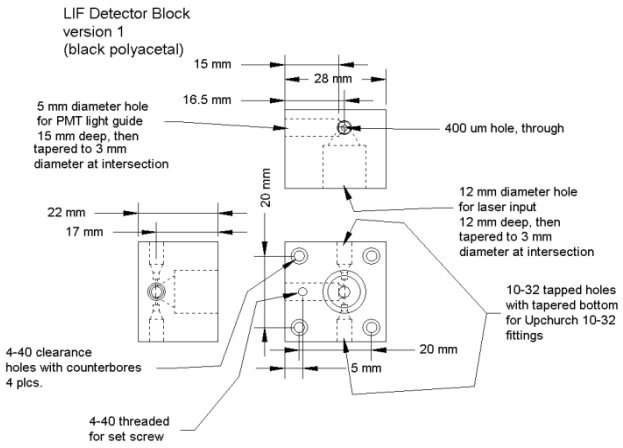


Figure 29: Detailed schematic of first version of LIF detector block.

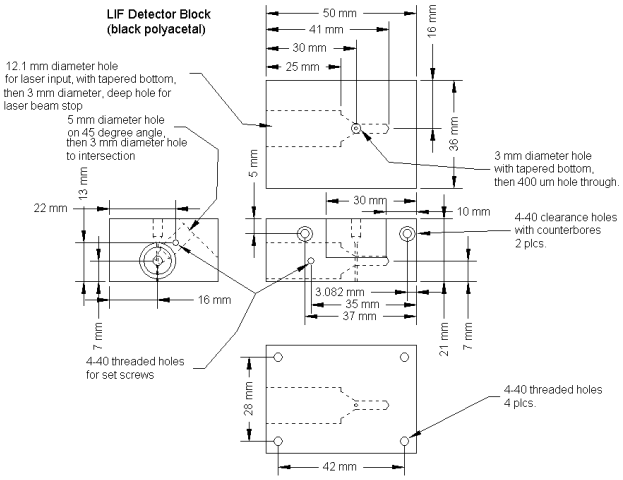


Figure 30: Detailed schematic of final version of LIF detector block.

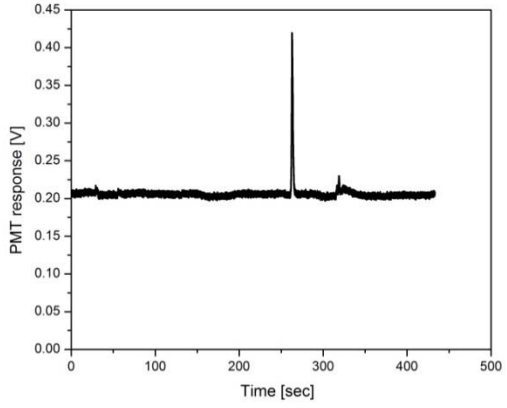


Figure 31: LIF trace with collection at 90° from incidence. Both traces Figures 20A-B) are completely raw – no smoothing and no background subtraction

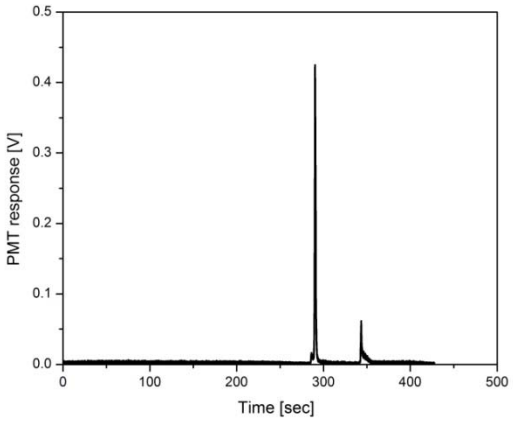


Figure 32: LIF trace with collection at 45° from incidence. Both traces (Figures 20A-B) are completely raw – no smoothing and no background subtraction

A comparison of the two traces shown in Figures 31 and 32 shows the decrease in background noise, along with a more stable background. For a relatively simple optical system, such as the one for this LIF system, it should be straightforward to ensure that the optical trace is limited by either shot noise or bit noise from the DAQ board. The trace in Figure 29 is not; there is noise due to variations in the amount of scattered laser light. The baseline is at 0.206 V with a standard deviation of 0.00247. The peak height is 0.219 V. This indicates that the signal to noise in the system is so low that it was necessary to increase the gain such that the scattered background laser light is a significant contribution. By spatially filtering

the laser light, the noise level is now limited by the DAQ and the RMS stability of the integrated high voltage power supply in Figure 30. In this graph, the baseline is at 0.00283 V with a standard deviation of $7.2x10^{-4}$ with a peak height of 0.437 V. Figure 31 uses a basic extraction procedure, and PCR results show 98 pg of DNA were collected. Figure 32 uses a current control extraction procedure (described in Section 3.7), and collected 213 pg of DNA.

3.5. Provide Limit of Detection of 100 pg DNA from Fluorescence Detection Unit

There are two main components to this deliverable that were proposed; a limit of detection of 100 pg DNA, and reproducibility within 10% for identical samples. . To ensure quantification to 10% reproducibility, we performed a series of repeat extractions on NIST standard DNA samples of the same concentration. Buccal swabs can vary in concentration from approximately 500 ng/mL to 5 μg/mL.[6] This is dependent on the amount of DNA on the swab itself as well as the amount of DNA extracted. In this context, we are referring to the amount of DNA on the swab. We performed these experiments at the low end of this range. Seven samples using NIST Standard DNA Component A at 524 ng/mL (524 pg/μL) were extracted according to the standard procedure developed during this project. The results are shown in Table 2. The mean is 5.06 with a standard deviation of 0.240. Approximately 10% error is within two standard deviations for these samples.

Table 2: Results of repeat extractions on samples of the same concentration. The mean is 5.06 with a standard deviation of 0.240. Approximately 10% error is within two standard deviations for these samples.

Repeat Number	Peak Area
1	5.360
2	5.177
3	4.910
4	5.117
5	5.080
6	5.191
7	4.615

Peak areas were calculated in Origin. After plotting the PMT trace, we determined the baseline by averaging the data between 50 seconds and 100 seconds. That was subtracted from the entire trace, and then Origin's integrate function was used to find the area for the peak. The current peak area returned by the software is based on this procedure, but integrates the entire trace after the 100-sec mark. Day-to-day reproducibility, as well as reproducibility between capillaries, was determined. At this point, different capillaries show variation of up to a factor of 3. The best practice is to run a daily calibration curve, or a calibration point for 524 ng DNA/mL. This reproducibility must also be shown over a wide dynamic range; Figure 33 shows a plot of peak area against sample DNA concentration from 113 pg/μL to 543 pg/μL. Error bars at 10% have been placed on the data points.

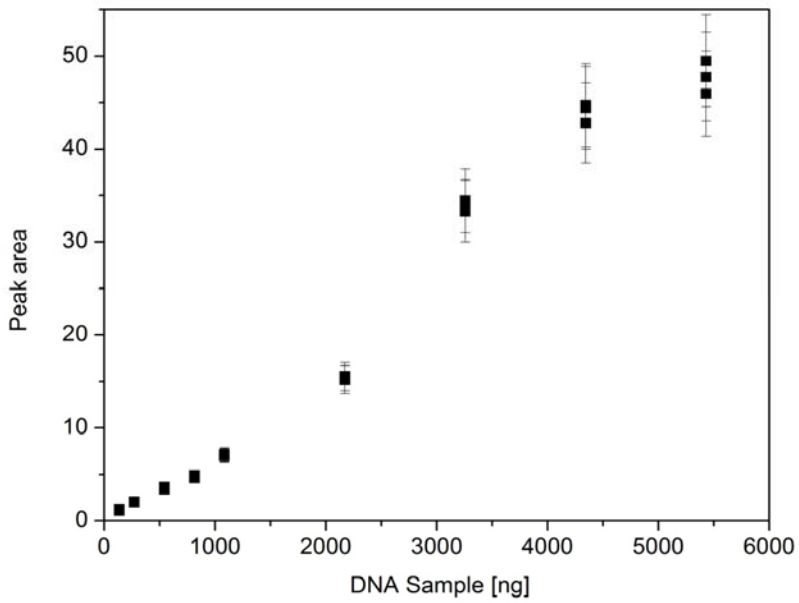


Figure 33. A plot of peak area against the amount of DNA originally in the sample. This presents a maximum error (and not a true error) from the LIF measurement due to the fact that the quantity of DNA extracted is not constant.

The critical metric, however, is comparison against the best-known method for quantifying DNA at this concentration range–qPCR. We plotted the peak areas from the samples above against results from qPCR, shown in Figure 34.

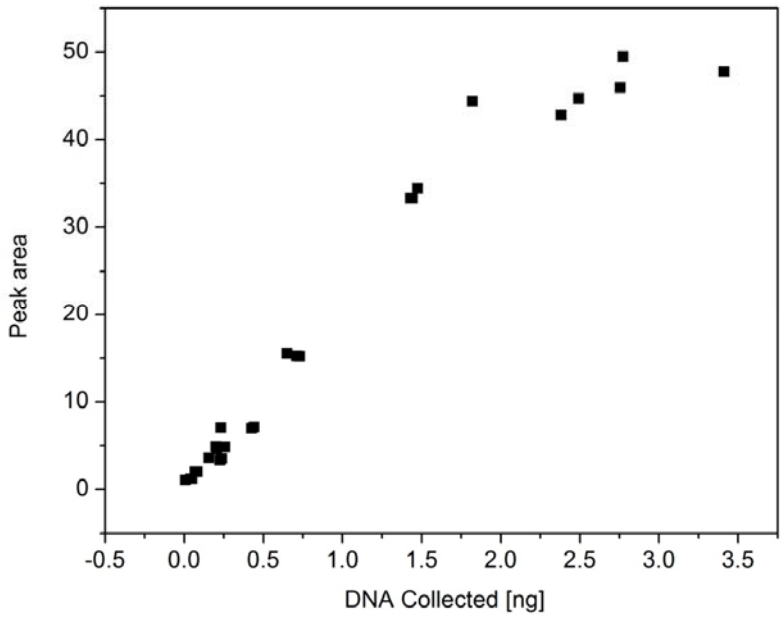


Figure 34: Plot of peak area again the DNA collected and quantified by qPCR.

As can be seen in Figure 34, there appears to be larger error in the x-axis than the y-axis, especially at higher concentrations. Common estimates for qPCR error are in the 20-30% range,[7] greater than we observed with the LIF peaks; thus, this is a reasonable result. While both these graphs show that we can

detect less than 100 pg of DNA, we performed experiments at lower DNA concentrations to explore the limit of detection for this system.

Shown in Figure 35 are peak areas from extractions down to 1.3 pg/μL. At this concentration, no signal was observed. The extractions from 27 pg/μL samples resulted in an average collection of 11 pg of DNA, and at this concentration, peak areas were still an order of magnitude greater than the background. This then serves as a reasonable limit of detection. However, it must be noted that if the peak areas can vary by a factor of three, an appropriate *range* is 11 pg to 33 pg. However, 11 pg suffices as the low-level detection limit.

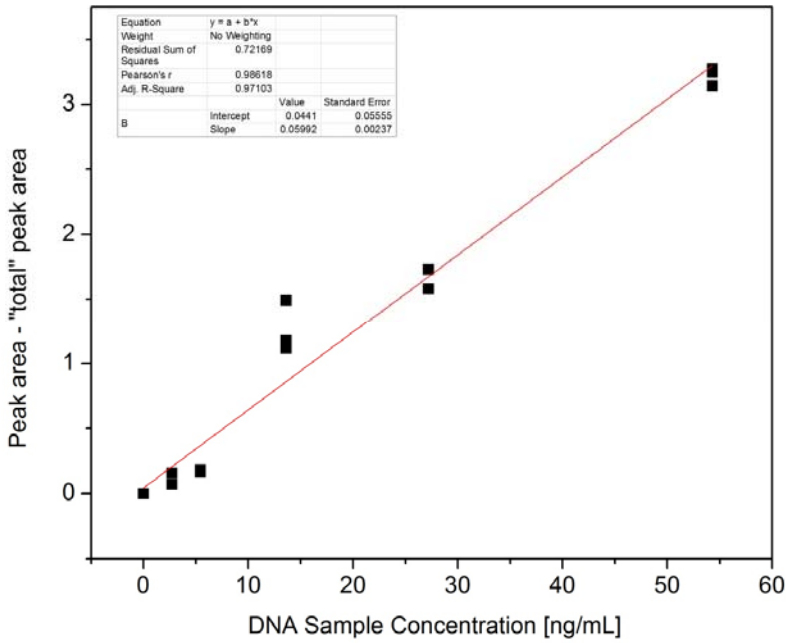


Figure 35: Determination of limit of detection. Points represent peak areas of extractions from various concentrations of DNA. DNA was quantified using qPCR.

Further development of this device would likely require replacing some components with smaller, more robust pieces. There are many photodetectors which are more rugged than photomultiplier tubes, including photodiodes, avalanche photodiodes, and CCD chips. The 488 nm laser could be replaced with high power light emitting diodes. Furthermore, instead of using liquid light guides (or fiber optics), these smaller components could be more deeply integrated with the capillary holder in such a way as to eliminate or minimize the need for light guides. Eliminating these pieces would allow less light to be lost to reflections, and further minimize alignment concerns.

3.6. Detector Hardware Integration

The fluorescence detection unit described above was integrated into a conductivity detection unit in the final device to allow for a means of detection for the two buffers that are used for the GEITP separation. Additionally, we use the conductivity detector as a triggering device for the delivery of the DNA in the DRAGEN unit; when the low conductivity buffer passes the conductivity detector, the software that controls the DRAGEN system triggers a step in the program that starts the delivery of the DNA.

It was necessary to design the conductivity detector such that it could be built around the LIF detector that was described in Section 3.4. The conductivity detector is based on the design described in Reference 4. Briefly, two tubular electrodes are threaded on the outside of a capillary. A high frequency

(100 kHz to 1 MHz) sine wave with an amplitude between 10 V and 100 V is applied to one electrode (the drive electrode). A current is induced to flow in the other electrode (the pickup electrode) via the coupling between the two electrodes. With an appropriately designed detector, this coupling is primarily through the solution in the capillary, and the amplitude of the current induced at the pickup electrode is proportional to the conductivity of that solution. For the GEITP extraction of DNA, the conductivity detector is used to determine when the interface between the LE and TE crosses the detection region. This transition between the high conductivity LE and the low conductivity TE is used as a trigger to control the end of the DNA extraction step. This triggering step is important to ensure that the applied pressure does not drop too low, potentially pulling particulate matter and/or inhibitors from the crude sample into the capillary.

The electronics for the conductivity detector head used in the DRAGEN instrument (Version 2.0) are fabricated on three small (1" x 1.15") printed circuit boards (PCBs). Each of the PCBs were 4-layer boards fabricated by ExpressPCB (www.expresspcb.com). In place of the tubular electrodes used in designs described in the literature, vias fabricated into the PCBs are used as the drive and pickup electrodes. The three PCBs are assembled in a stack.

The bottom-most board in the stack is the drive board, which contains the drive electrode (via) and a connection between the drive via and a coaxial cable for connection to an external function generator. The top and bottom layers of the drive board are shown in Figure 36. The two internal layers were not used.

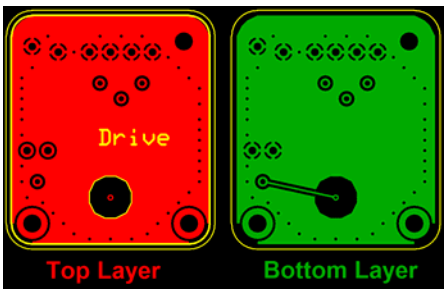


Figure 36: Top and bottom layers of the drive board (the bottom-most board in the stack) used for the conductivity detector.

The top board in the stack is the pickup board (Figure 37), which contains the pickup electrode (via) and the surface mount electronics for the first stage detector amplifier (a transimpedance amplifier circuit). The top and bottom layers of the pickup board are shown in the figure. The two internal layers were used as a ground plane and a power plane.

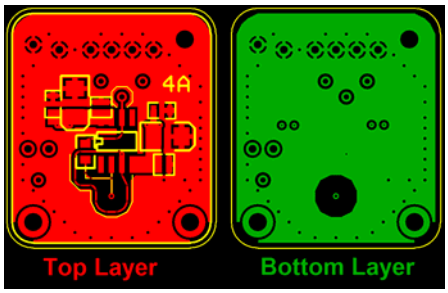


Figure 37: Top and bottom layers of the pickup board (the top board in the stack) used for the conductivity detector.

The circuit diagram for the drive layer board is shown in Figure 38. A model OPA827 op-amp (Texas Instruments) was used. The value of the feedback resistor was 1 MOhm. Not shown in the circuit diagram are the bypass capacitors used at the power connections to the op-amp to reduce noise.

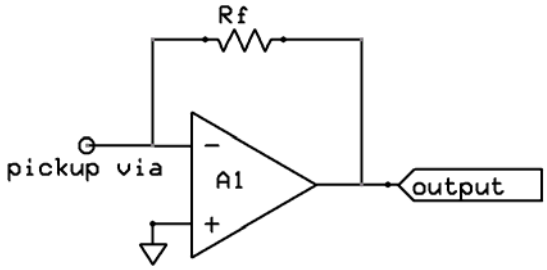


Figure 38: Circuit diagram for the drive layer board used for the conductivity detector.

The middle layer board was a spacer board to provide a ground plane to block direct coupling between the drive and pickup electrodes (vias). It was a blank board (with solid copper internal layers) except for vias and pads to match the drive signal and power couplings to the drive and pickup boards.

The drive voltage used for the conductivity detector was chosen to be at a frequency of 1 MHz, and amplitude of 5 V. A custom-built function generator, based upon an XR-2206 monolithic function generator chip (EXAR Corporation), was used.

The rectifier circuit to convert the pickup signal from a 1 MHz sine wave to a DC voltage was based on the circuit described in Reference 4. The rectifier circuit was constructed with surface mount components on a 4-layer PCB. The top layer of the PCB (Figure 39) and the circuit diagram (Figure 39) are shown. The bottom layer and the first internal layer of the PCB were used as ground planes. The second internal layer of the PCB was a power distribution layer.

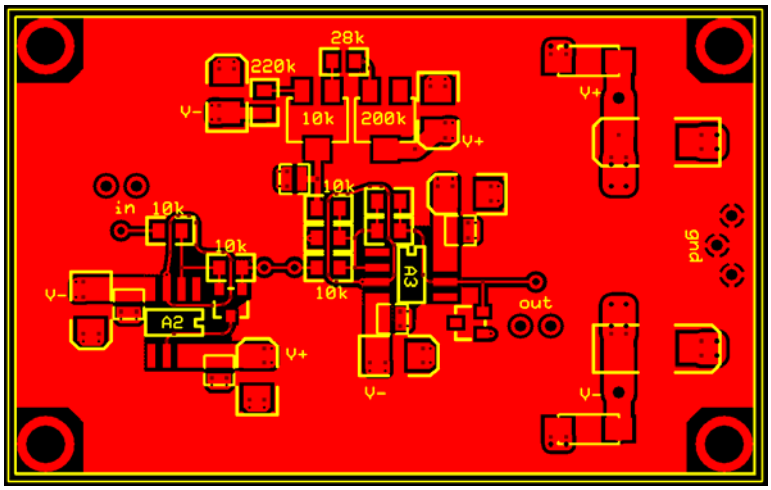


Figure 39: The top layer of the PCB used to fabricate the rectifier circuit.

Not shown in the circuit diagram in Figure 40 are the bypass capacitors used at the power connections to the op-amps and the input to the board to reduce noise. The rectifier circuit was housed in an extruded aluminum enclosure with a +-15 V linear power supply (Polytron Devices Inc.).

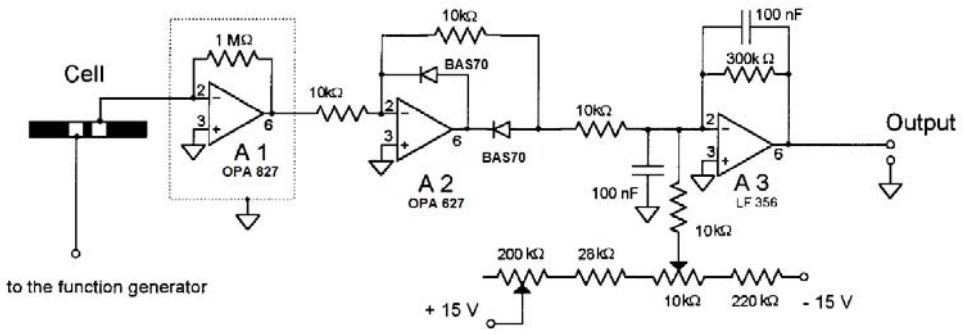


Figure 40: Circuit diagram for rectifier circuit.

3.7. Detector Hardware Integration to GEITP Module; DNA Extraction from a Buccal Swab

During the course of the project, it was determined that the best way to eliminate error, improve reproducibility, and selectively extract DNA from crude samples efficiently was to incorporate an autosampling functionality to the system. This allows us to minimize sample handling, perform extractions precisely, and more precisely deliver DNA into the final delivery vessel. The autosampling capabilities of the DRAGEN 2.0 instrument are built on the components of a 3-axis CNC milling machine kit purchased from Zen Toolworks. Limit switches, also from Zen Toolworks, were installed on both ends of each motion axis. The Z-axis stage was modified to fit the GEITP extraction unit, including the LE reservoir, LIF detector block, and conductivity detector, as well as connections for high voltage control, pressure control, and electrical current measurement. A schematic of the integrated detector and reservoir assembly that is attached to the Z-axis is shown in Figure 41.

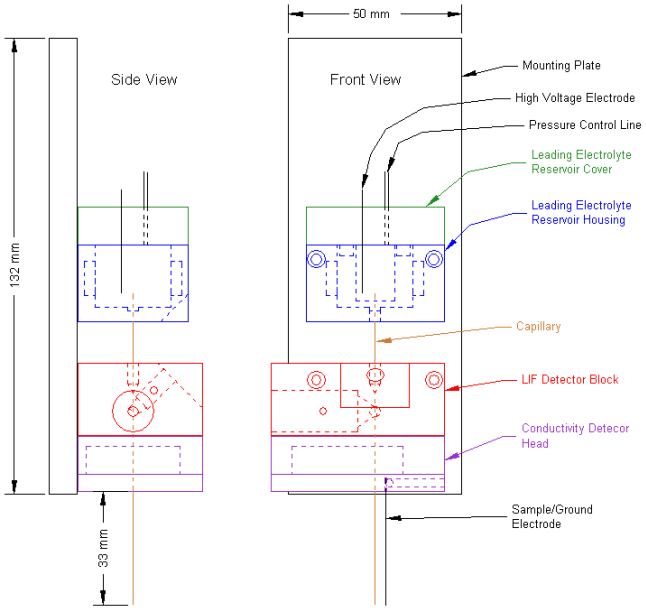


Figure 41: Schematic of the integrated detector and reservoir assembly that is part of the DRAGEN instrument.

Detailed schematics for each part in the integrated assembly are shown below in Figures 42-49. The LE Reservoir Housing and the LIF detector block are attached directly to the mounting plate, which is

connected to the Z-axis. The LE reservoir cover is screwed into the LE reservoir housing. The high voltage electrode and the pressure control line are glued into the LE reservoir cover with Bondit B45TH epoxy. The conductivity detector is screwed into the bottom of the LIF detector block. The sample/ground electrode is held in place by a set screw threaded into the side of the conductivity detector lid. Electrical connections to the high voltage and ground electrodes are made via banana plug receptacles mounted on the z-axis to the left of the mounting plate.

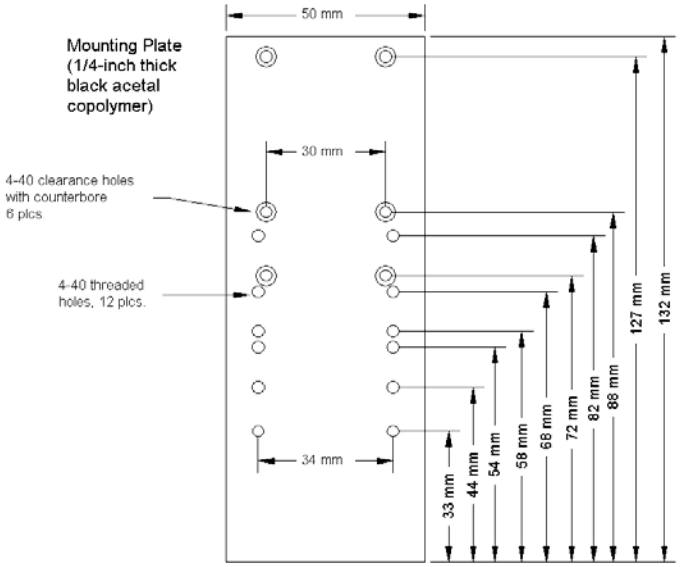


Figure 42: Schematic of the DRAGEN mounting plate.

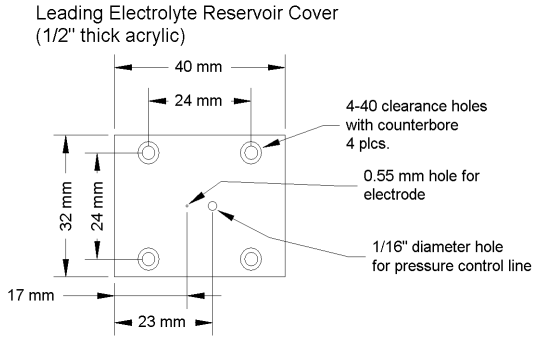


Figure 43: Detailed schematic of the leading electrolyte reservoir cover.

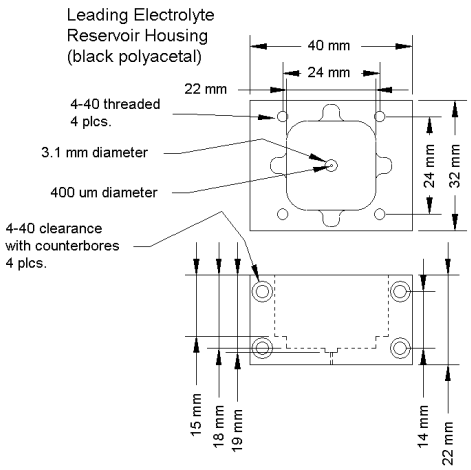


Figure 44: Detailed schematic of the leading electrolyte reservoir housing.

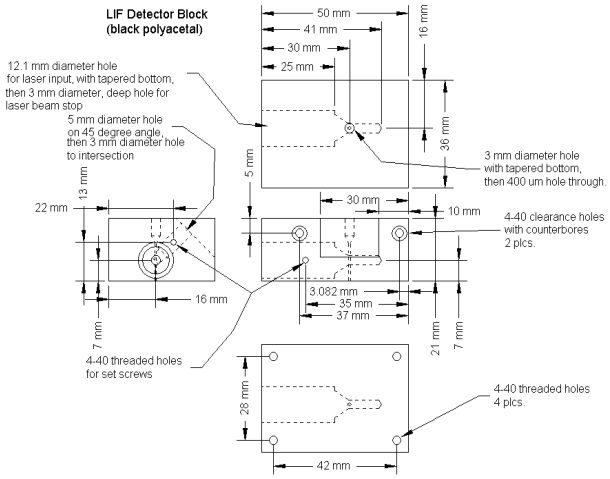


Figure 45: Detailed schematic of the LIF detector block.

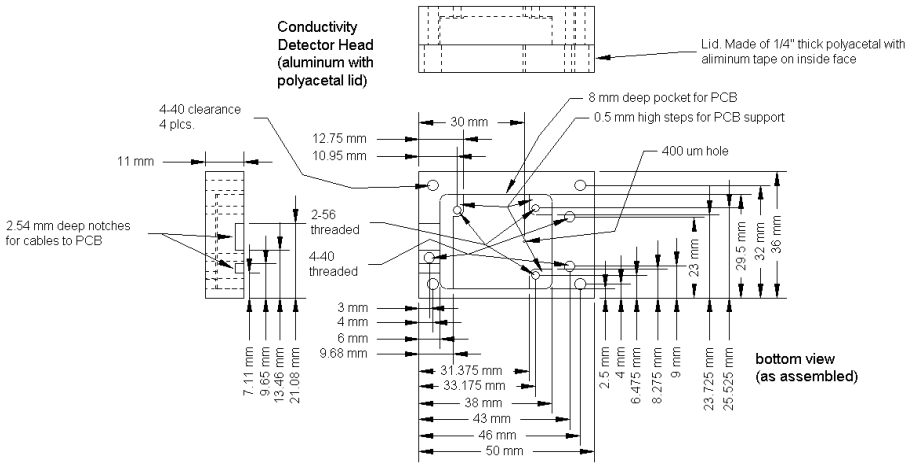


Figure 46: Detailed schematic of the conductivity detector head.

The integrated hardware that we originally proposed included a self-aligning LIF/conductivity detector module. For the beginning experiments of this project, the leading electrolyte buffer reservoir and extraction capillary as a semi-disposable part. These semi-disposable capillary assemblies were made by gluing a length of 75 μm inner diameter fused silica capillary from Polymicro Technologies (www.polymicro.com) into the bottom of the LE reservoir. The LE reservoir is machined from polyetherimide (Ultem). A detailed schematic is shown below. The capillary was glued to the reservoir using Bondit B45TH epoxy. After the epoxy was fully cured, the capillary was trimmed to length and the window for LIF detection is burned through the outer coating of the capillary. The length of the capillary from the base of the LE reservoir is 84 mm, and the distance from the base of the LE reservoir to the center of the window is 30 mm, as shown in the drawing below.

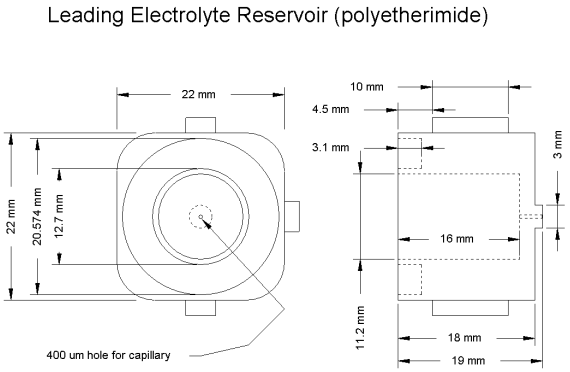


Figure 47: Detailed schematic of semi-disposable leading electrolyte reservoir.

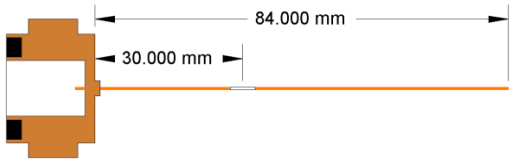


Figure 48: Diagram of semi-disposable capillary assembly.

Leading electrolyte reservoirs and capillaries were optimized after initial experiments using the DRAGEN device. The primary change to the leading electrolyte reservoir was the addition of a threaded tap into which a capillary threaded into a ferrule can be emplaced. Further, we currently use capillaries coated with a transparent coating rather than burning a window into an amice-coated capillary.

Stepper motors allowed reproducible and accurate positioning of the DRAGEN extraction unit. The stepper motors were purchased from Applied Motion Products. To simplify construction and communication between the stepper motors and the computer, stepper motors with integrated drivers and RS-232 communications were used (model number STM17S-3AN). The power supply for the stepper motors (Applied Motion, model no. PS150A24) is housed in the aluminum enclosure beneath the XYZ motion stage. Power connections to the X-, Y-, and Z-axis stepper motors are made with 4-pin mini-DIN connectors and cables.

While not a part of our original proposed project, it was determined that the pressure controllers that we purchased were not as precise as what is required for a GEITP extraction. As a result, we built more precise pressure controllers for this application and integrated one into the DRAGEN instrumentation. The custom pressure controller (Figure 49) is contained in the aluminum enclosure that also contains the stepper motors. It is based upon a Z-axis unit bought from Zen Toolworks, and is also driven by an Applied Motion integrated stepper motor. A plastic 20-mL syringe is mounted on the modified Z-axis unit, so that motion of the axis will pull or push the syringe plunger to control the pressure at the leading electrolyte reservoir. The syringe outlet is connected to both a +- 7000 Pa bidirectional pressure gauge (Omega Engineering, www.omega.com) and to the pressure control port on the back of the aluminum enclosure. The pressure control port is connected via 1/16 inch diameter tubing to the cover for the leading electrolyte reservoir. When the cover is sealed to the reservoir, motion of the pressure control stepper motor causes either a decrease or increase of the pressure. During a DNA extraction, the

pressure is regulated at the desired value through a PID control loop programmed into the DRAGEN DNA Extraction program in LabView.

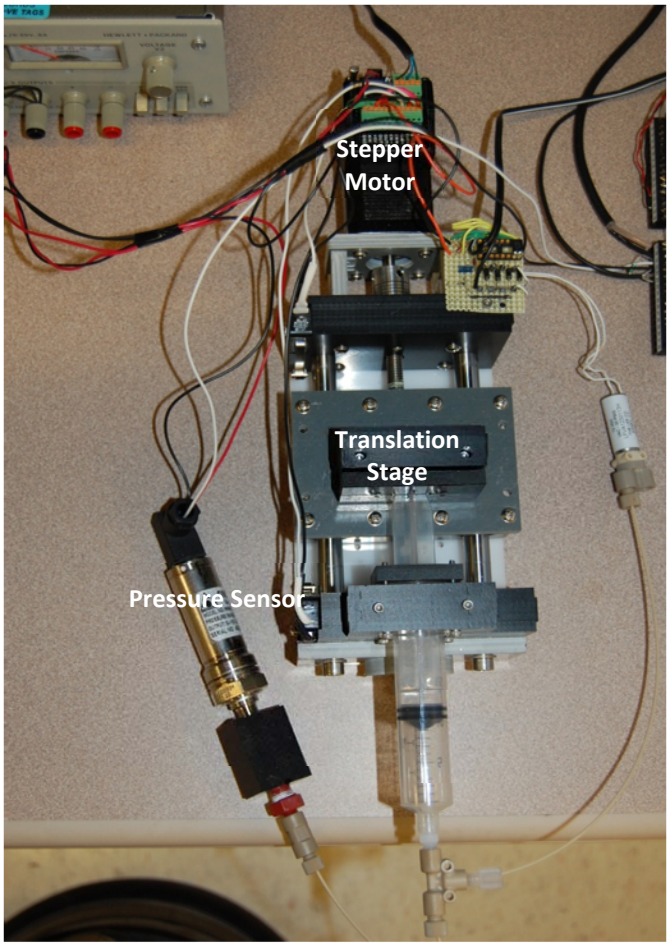


Figure 49: Custom built DPC pressure controller (without enclosure). The syringe is used to generate either positive or negative pressure as it is pushed or pulled by the translation stage. The pressure sensor is used to measure the pressure applied to the GEITP system (connection not shown in figure) and to provide a signal for feedback control of the system.

The stepper motors for XYZ motion and pressure regulation are controlled by the computer via an RS-232 interface. Each stepper motor is connected to an RS-232 communication cable (the communication cable for the pressure control unit is threaded through a hole in the back of the large aluminum enclosure). These RS-232 cables should be plugged into the serial port hub, with the X-axis, y-axis, z-axis, and pressure controller each plugged into the serial ports numbered 1, 2, 3, and 4, respectively on the serial port hub.

Displayed in Figure 50 is a photograph of the DRAGEN Version 2.0 instrument. The DRAGEN 2.0 instrument is built around the components of a 3-axis motion stage. The Z-axis stage was modified to fit the GEITP extraction unit, including the LE reservoir, LIF detector block, and conductivity detector, as well as connections for high voltage control, pressure control, and electrical current measurement. The power supply for the 3-axis motion stage and the pressure controller for the system are housed in a large aluminum enclosure located underneath the other system components.

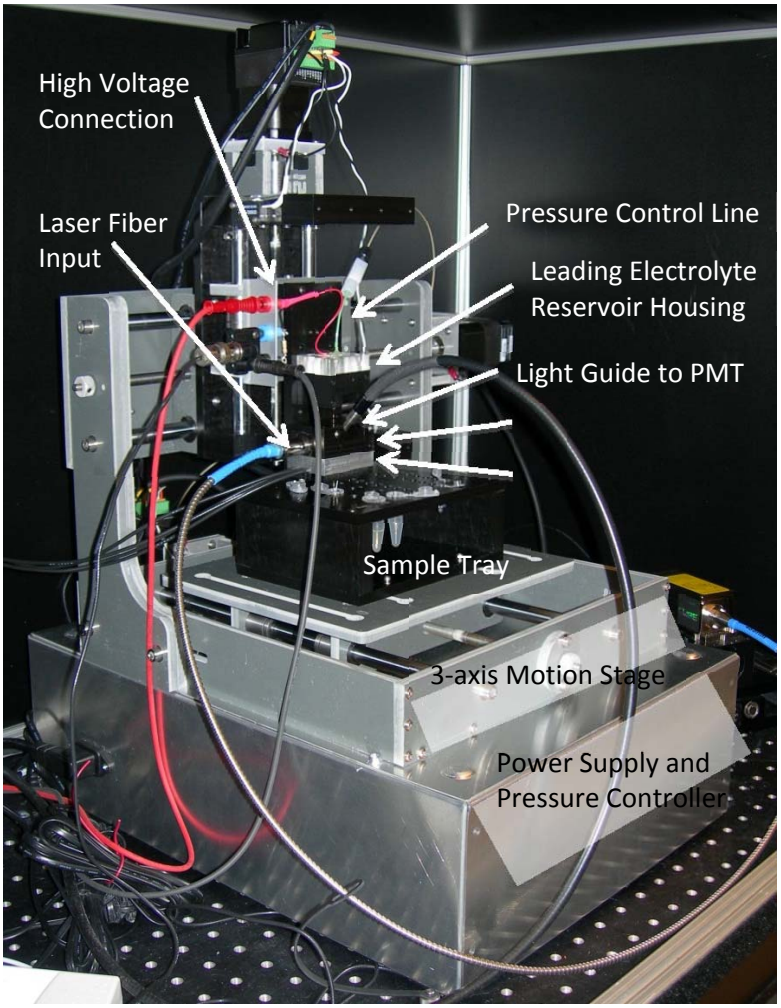


Figure 50: DRAGEN instrument labeled with all components required for DNA extraction from a dirty buccal swab.

The integrated DRAGEN Version 2.0 system has been used to extract DNA from dirty human buccal swabs at a >95% success rate, and this 95% success rate is a lower limit. Typically, we will use the device and extract DNA from samples for weeks on end without any issues with the device. Further, we routinely extract approximately 1-5 ng DNA every extraction. The final DRAGEN DNA extraction program includes 9 steps; in the first step we move the capillary to the sample while keeping a positive pressure on the capillary. Step 2 initiates the high voltage and provides time for the power supply to reach the setpoint voltage. At this stage, the DNA is focusing outside the capillary. Step 3 initiates the first pressure ramp step. The current is at approximately 26 μA for the TRIS-Cl^- buffer with SYBR Green I and 0.1% PVP and 0.1% Triton-X. The current begins to drop below 26 μA, indicating the interface, including the focused DNA, has been pulled into the capillary. During this step, the pressure gradually ramps down at -5 Pa/second from -240 Pa until the current reaches 22 μA. At this point, the program switches to step 4, and the pressure continues to ramp down until the current reaches 18 μA, and DNA continues to focus in the plug. This current is held for 1 minute by regulating the pressure. The second pressure ramp begins in step 5, pulling the DNA plug up past the conductivity detector, and then the LIF detector. The conductivity trace drops when ions are pulled past it; in this case we see multiple steps, indicating multiple ions. The last step is due to the DNA. The conductivity detector is used to trigger the

changeover to step 6 when the conductivity measurement drops below 1.66. Step 6 moves the capillary to the dip reservoir, and then immediately moves the capillary to the delivery vial (Step 7). A pressure of 1000 Pa is used to eject the DNA plug in Step 8. Delivery is executed by applying 1000 Pa of pressure to the capillary for 60 sec and adds approximately 1.1 μL to the delivery vial, and then the capillary moves back to the "home" vial filled with water.

Displayed in Figure 51 is a combination current and pressure trace for a dirty buccal swab color coded to match the steps noted above. In Figure 52 are the electropherograms showing the output of the conductivity and LIF detectors upon DNA extraction from a dirty buccal swab. In this sample, the mass of DNA extracted, as determined by qPCR, was 4.714 ng.

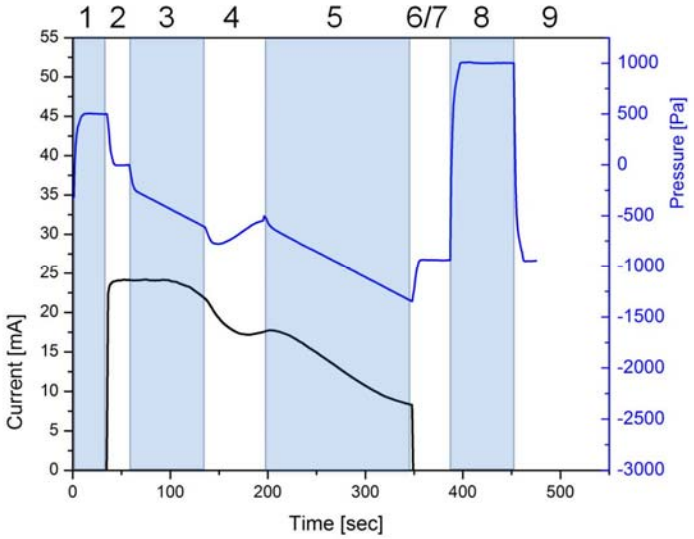


Figure 51: Current and pressure traces collected during an extraction of human DNA from a dirty buccal swab. The steps are color coded to match the extraction/delivery program noted in the text.

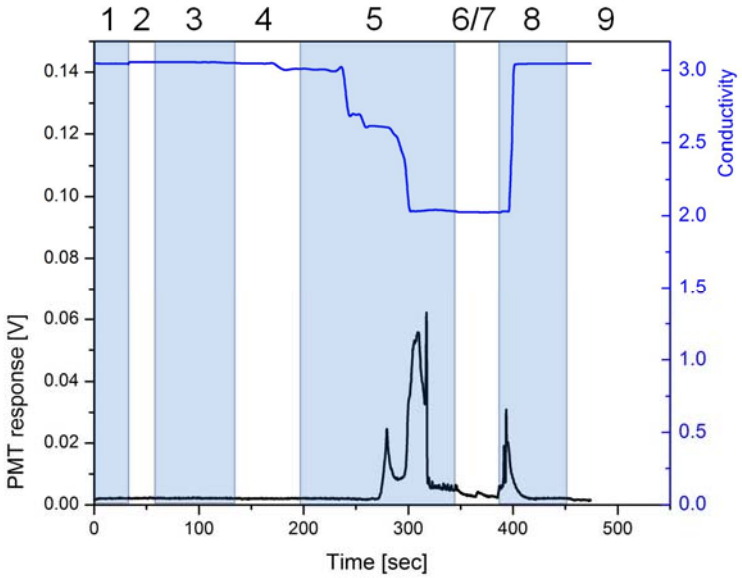


Figure 52: GEITP electropherograms, using both conductivity and LIF detection, of the extraction and delivery of human DNA collected from a dirty buccal swab. The steps are color coded to match the extraction/delivery program noted in the text.

The most useful aspect of this device is that it collects concentrated DNA that can be used for human identification purposes. We have shown that qPCR analysis is viable, and displayed in Figure 53 is a short tandem repeat (STR) DNA identity profile. In this case, the DNA was collected from a clean human buccal swab and it was determined that the quantity extracted was 1.279 ng. The data was collected using a Promega PowerPlex 16 kit and allele calling was performed using GeneMapper 3.2. Of 16 loci analyzed for this sample, 16 loci were detected. This data can be used for human identification.

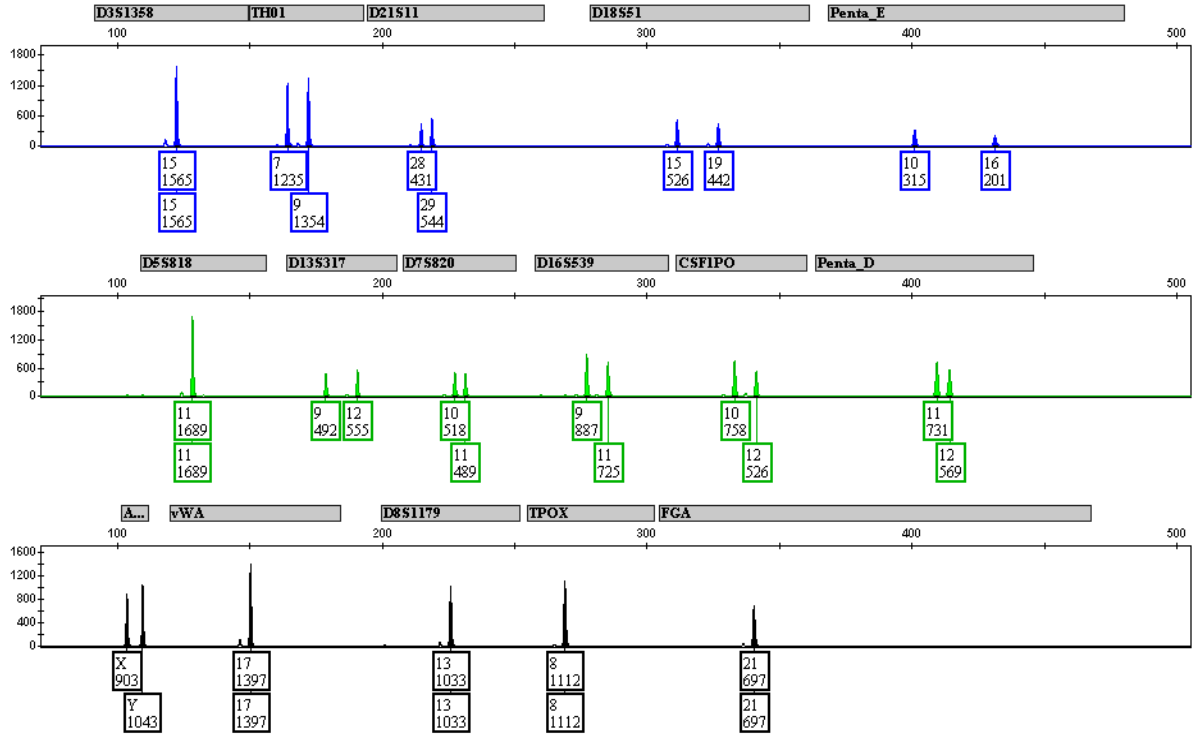


Figure 53: STR profile of human DNA extracted from a clean buccal swab.

Displayed in Figure 54 is an STR analysis profile for human DNA extracted from a dirty buccal swab. The current and pressure traces for this extraction as well as the GEITP electropherograms for this extraction and delivery are shown in Figures 50 and 51. The STR indicates that 16/16 loci were detected and analyzed. These data indicate that we have successfully extracted human DNA from a dirty buccal swab and that we were able to collect the data that can lead to human identification.

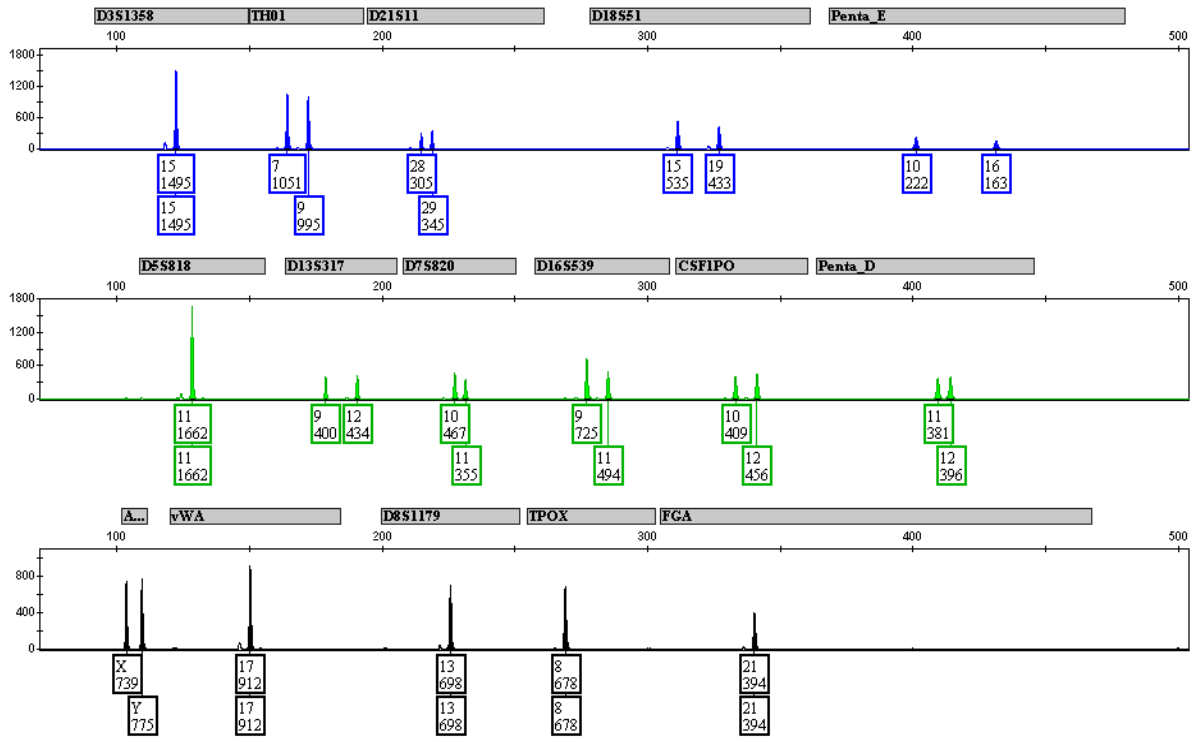


Figure 54: STR profile of human DNA extracted from a dirty buccal swab.

3.8. Increase Extraction Efficiency of DRAGEN Extraction Procedure

Human buccal swabs contain a significant amount of DNA—approximately 500-5000 pg starting material. However, many other real-world samples, such as used chewing gum and human bone, contain significantly less DNA. The first step in determining whether the GEITP technique will be useful for extracting DNA from these samples is to examine the efficiency of the technique. Experiments performed using NIST human standard DNA dissolved in buffer as the extraction matrix showed extraction efficiencies of approximately 1%. To increase the extraction efficiency, we performed experiments with surface coatings in hopes of making the DNA more available in the capillary. We investigated buffers at low pH to potentially exclude charged inhibitors. We increased the time for extraction by holding the current within the capillary constant for a long period of time, thereby allowing the DNA a longer time to focus at the trailing electrolyte-leading electrolyte interface. We investigated the use of multiple extractions to the same delivery tube. Finally, we performed extractions on small volumes of liquid.

It has been hypothesized that the extraction efficiency could be increased by simply stirring the sample; that is, if the DNA extracted from the sample solution is within a very short proximity to the capillary, we need only stir the sample to “replenish” the DNA lost after extraction. Experiments that focused on stirring the sample and, later, extracting the sample for long periods of time resulted in no marked increase in DNA extraction efficiency. However, experiments that focused on extracting dye molecules using a longer extraction time (both with and without stirring) did result in an increase in dye molecule extraction efficiency. Further, there is evidence that small amounts of DNA may be entering the channel and immediately flowing to the walls of the channel. The DNA molecules then flow slowly toward the buffer reservoir containing the leading electrolyte and eventually pass into the leading electrolyte reservoir. The DNA molecules are thus never flushed back out of the capillary into the fresh delivery

tube that is ultimately analyzed by qPCR. As a result, some of the DNA that has been extracted into the capillary is never recovered and quantified.

Displayed in Figures 55-57 are GEITP electropherograms of human standard DNA extracted from a 500 pg/uL solution. In all cases, the leading electrolyte (LE) is 15 mM histidine, 5 mM citrate with 0.1% poly(vinylpyrrolidone) (PVP) and 0.1% Triton X added as a wall coating and lysis surfactant, respectively. In addition, 1X SYBR Green 1 was added to the LE for optical visualization of the DNA. The trailing electrolyte consists of 15 mM gallic acid, 0.1% PVP, and 0.1% Triton X. This low-pH LE-TE pair was used in these experiments to facilitate DNA extraction from materials that regularly incur a negative charge at higher pH, such as humic acid. At low pH values, materials that are rich in carboxylic acid moieties, such as humic acids, are neutral.

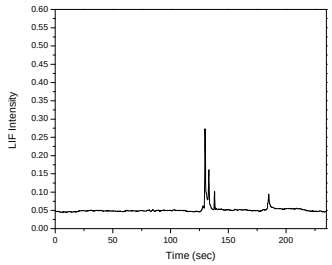


Figure 55: GEITP extraction electropherogram of human DNA, with conditions noted in the text. Time for DNA extraction: 1 min.

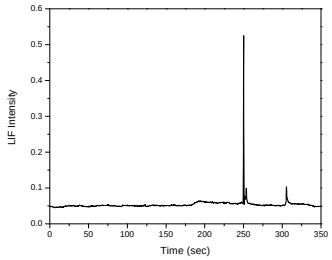


Figure 56: GEITP extraction electropherogram of human DNA, with conditions noted in the text. Time for DNA extraction: 3 min.

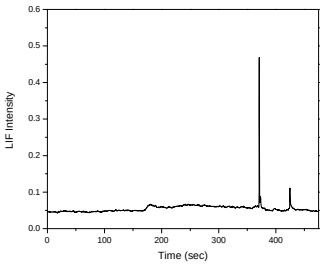


Figure 57: GEITP extraction electropherogram of human DNA, with conditions noted in the text. Time for DNA extraction: 5 min.

Figure 55 shows data collected during a 1 min extraction, Figure 56 shows data collected after a 3 min extraction, and Figure 57 shows data collected after a 5 min extraction. There are several points to note regarding this data. To begin, in the case of the 3 min and 5 min extraction times, there is a slight step increase in the LIF baseline intensity at approximately 200 sec, indicating that DNA molecules have moved through the capillary to the LIF detection point, which is “up-stream” of the GEITP focusing point. Further, there is no detectable increase in DNA extracted (determined by the area of the second peak) as the extraction time increases. In Figures 58 and 59 are GEITP data collected while the DNA solution was stirred. In both cases, the results are the same as without stirring (Figures 55 and 57), indicating that the increase in extraction time did not result in an appreciable increase in quantity of DNA extracted and delivered, even with DNA replenishment while stirring.

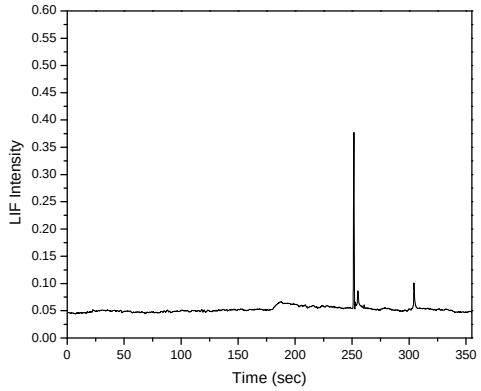


Figure 58: GEITP extraction electropherogram of human DNA, stirred. Time for DNA extraction: 3 min.

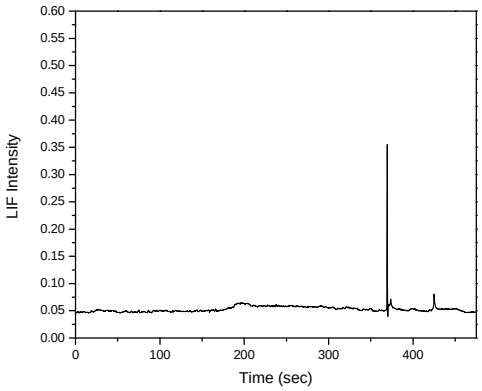


Figure 59: GEITP extraction electropherogram of human DNA, stirred. Time for DNA extraction: 5 min.

The small step increase shown in the data of Figures 56, 57, 58, and 59 occurs at a time when the interface between the leading and trailing electrolytes is located near the capillary entrance. This indicates that some of the DNA is "escaping" from the focused plug and migrating up-stream in the capillary to the LIF detection point (located approximately 6 cm from the capillary entrance). Presumably, the DNA continues to migrate past the LIF detector to the LE reservoir, and once it enters the LE reservoir it will be diluted into the 1 mL volume and will no longer be recoverable. Similar data to that shown in Figures 58 and 59 (with the slight upward step) was also obtained using the higher pH buffer system (50 mM Tris 25 mM Cl- leading electrolyte, 25 mM Tris HEPES trailing electrolyte). Also, movies collected during a GEITP run visualized with a fluorescence microscope indicated that a small amount of fluorescent material near the walls of the capillary seemed to migrate up-stream of the ITP interface. These observations are counter to the theory used to explain the operation of this method and the focusing of the DNA by isotachophoresis (ITP). According to that theory (which is just the standard 1D theory used for ITP), the DNA should be focused at the interface between the LE and TE, and should be pushed back into that interface from both directions. Measurements with fluorescent dye molecules support this standard ITP theory. Our supposition regarding why the measurements with DNA disagree with the predictions of the theory is that there are two important factors that both contribute to the reason the measurements with DNA disagree with theoretical predictions, which are not accounted for in the theory: 1. The real system is 3-dimensional whereas the theory is only 1-dimensional; and 2. The DNA molecules are high molecular weight polyelectrolytes and they do not have the same simple behavior in an electric field as a small molecular weight molecule such as a fluorescent dye. The first factor is important because in the real system, with pressure-driven counterflow, the velocity fields are different at the walls of the capillary than in the middle (pressure driven flow is parabolic across the capillary and constrained to be zero at the walls). The second factor is important because the high molecular weight DNA will form "clumps" when subjected to an electric field (because of possible induced dipole-dipole interactions between DNA molecules) and the resulting "clumps" will not freely diffuse between the walls and the center of the capillary like a small molecular weight dye would. We postulate that the DNA is focusing at the ITP interface, and subsequently "clumping". Clumps that form near the capillary wall do not experience the buffer counterflow that is required to keep them focused at the ITP interface, thus they migrate in the electric field toward the LE reservoir. Because the clumps do not diffuse back into the middle of the capillary, they remain at the walls, and continue to migrate until they exit the capillary and are lost in the LE reservoir. During the GEITP extraction, DNA is continuously pulled from the sample solution and focused at the ITP interface. However, a situation is quickly reached where the new DNA entering the capillary from the sample is offset by "clumped" DNA leaving the capillary and being lost into the LE reservoir.

In an effort to circumvent this problem, we performed experiments that utilize a dynamic coating that has been designed to increase the negative surface charge groups on the surface of the capillary. This helps mitigate the problem in two ways: 1. The negatively-charged coating will repel the negatively-charged DNA molecules and keep them from accumulating near the walls; and 2. The negatively-charged coating will provide for electroosmotic counterflow in the capillary in addition to the pressure-driven counterflow. The electroosmotic counterflow, since it is driven by charges at the walls, is uniform across the capillary from the walls to the center. Electroosmotic counterflow will provide for ITP focusing of DNA even if it does form a clump near the wall.

The initial experiment focusing on wall coatings centered on EOTrol HN, a dynamic coating. EOTrol HN was added to a leading electrolyte consisting of 15 mM histidine, 5 mM citrate with 200X EOTrol HN dynamic wall coating. In addition, 1X SYBR Green 1 was added to the leading electrolyte for optical visualization of the DNA. The trailing electrolyte consisted of 15 mM gallic acid, 0.1% Triton X, and 200X EOTrol HN. Human DNA, at a concentration of 500 pg/μL, was dissolved in the TE. Displayed in Figures

60-62 are representative GEITP DNA extraction electropherograms with extraction times of 1 min, 3 min, and 5 min, respectively, utilizing the negatively-charged dynamic wall coating, EOTrol HN. In all cases, the small step increase in LIF baseline intensity that was observed with no EOTrol HN dynamic coating is not present, possibly indicating that the DNA remains with the bulk DNA plug rather than migrating up-stream along the walls of the capillary. In addition, the area of the second peak, or the DNA delivery peak, increases with extraction time. This qualitative increase in LIF intensity of the DNA delivery peak may be indicative of the DNA remaining in the bulk extracted plug rather than migrating to the walls of the capillary and ultimately into the LE buffer reservoir. Because the delivery peak is more reproducible than the extraction (first) peak, the delivery (second) peak is always used for quantification.

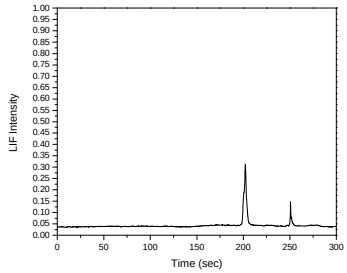


Figure 60: GEITP extraction electropherogram of human DNA, using EOTrol HN as a dynamic coating. Time for DNA extraction: 1 min.

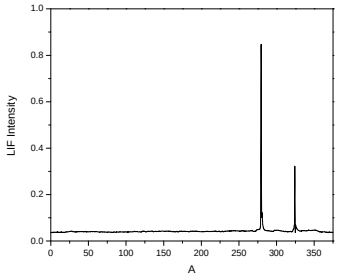


Figure 61: GEITP extraction electropherogram of human DNA, using EOTrol HN as a dynamic coating. Time for DNA extraction: 3 min.

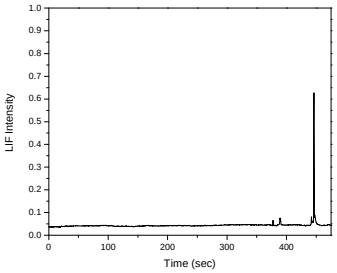


Figure 62: GEITP extraction electropherogram of human DNA, using EOTrol HN as a dynamic coating. Time for DNA extraction: 5 min. Note that the first peak at 380 sec, or the DNA extraction peak, is low in intensity and not reproducible run-to-run. It is for this reason that the second, or delivery, peak is always used for quantification.

Displayed in Figure 63 is a more quantitative comparison of the DNA delivery peak areas. Data represented by the black squares was collected without EOTrol HN; data represented by the red circles was collected using EOTrol HN as a dynamic coating. The data collected without EOTrol HN were repeated 2 times each, though the data collected with EOTRol HN are represented as averages of triplicates, except in the case of the 5-min extractions. For the 5-min extractions, the data was collected in quadruplicate. In the case of the DNA collected without EOTrol HN, the amount of DNA collected does not vary significantly with extraction time; however, the DNA collected increases with extraction time when EOTrol HN is added to the buffer solutions. At 5 min extraction times using EOTrol HN as a dynamic coating, the peak area varies greatly.

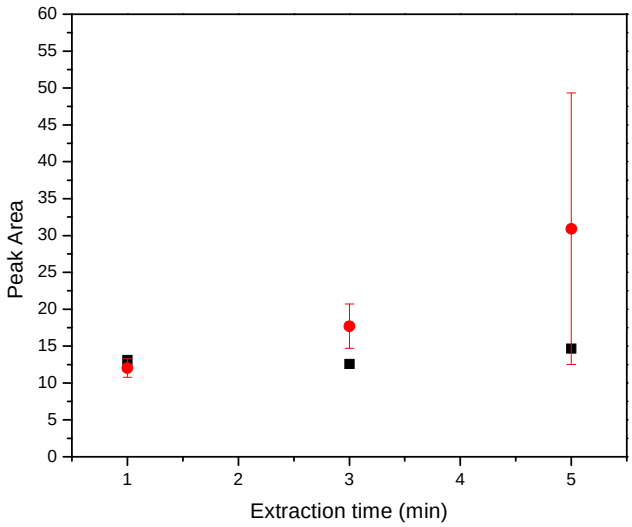


Figure 63: DNA delivery peak areas analyzed at various DNA extraction times.

Further experiments on synthetic wall coatings performed during the course of this work indicated that the data we collected was not reproducible over a given day or week because the capillary would clog or plug. The dynamic and static coatings used are poly(acrylamide)-based and, according to the manufacturer's MSDS, contains approximately 0.1-10% free acrylamide. The leading and trailing electrolytes utilized during the course of the synthetic wall coating work were 75 mM histidine 25 mM citrate as the leading electrolyte and 15 mM gallic acid as the trailing electrolyte (pH~3.4). Because acrylamide will polymerize in acidic media, we hypothesized that the clogging was due to small clusters of polymerized acrylamide entering the capillary and eventually accumulating at the channel walls until the capillary eventually closed. To test this hypothesis, we switched back to the 50 mM Tris 25 mM Cl- leading electrolyte and 25 mM Tris 25 mM HEPES TE buffer system (pH~8). The capillary continued to clog and we continued to experience irreproducibility in our data.

We believe that the best solution to this issue is to determine a surface synthetic procedure or a robust surface adhesion procedure that will result in a capillary surface that is populated in, for example, sulfonate groups, or any functionality that will remain charged at low pH values. However, we have determined that the surface of the capillary can be coated with DNA. Our first experiments utilized human DNA as the surface coating. We coated the capillary with human DNA; this coating included washing the capillary with 500 pg/μL human DNA standard in 50 mM Tris 25 mM Cl- leading electrolyte buffer for 5 min. Fresh 50 mM Tris 25 mM Cl- leading electrolyte buffer was then flowed through the channel at a pressure of 2000 Pa for 2 min. Prior to data collection, three control runs were performed; in these runs, the 25 mM Tris 25 mM HEPES trailing electrolyte buffer without DNA was exposed to a typical GEITP extraction. We did not observe carryover of human DNA after this coating step; further, we have not seen any carryover of human DNA between typical runs. However, it is important to note that this is based on qPCR data that targets nuclear DNA and is less sensitive than some of the more sensitive "next generation" STR kits and mitochondrial DNA (mtDNA) analysis.

The DNA-coated capillary increases the negative charge on the walls of the capillary, thereby allowing the DNA to accumulate with increasing time during an extraction. Shown in Figure 64 is the relationship of laser-induced fluorescence (LIF) peak area to DNA extraction time. As is clearly noted, the peak area increases with increased extraction time, indicating that a greater amount of DNA accumulates at the GEITP interface as the extraction time increases. This result is expected and is quite similar to the results

we obtained with synthetic surface coating; however, the DNA coating method results in a surface that is far more reproducible than the EOTrol HN or the Ultratrol HN coating.

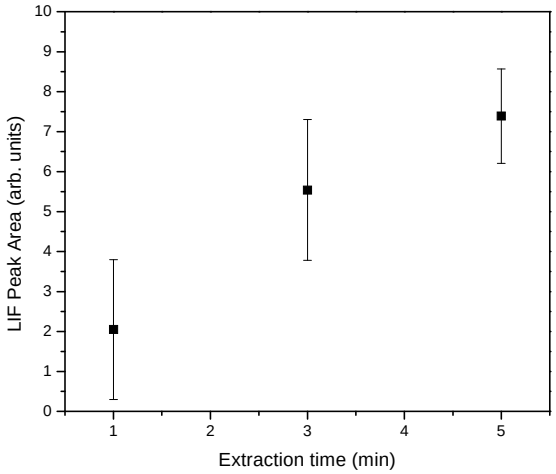


Figure 64: DNA delivery peak areas analyzed at different DNA extraction times. The extraction solution was 50 pg/μL human DNA standard solution. The data indicates that the DNA extraction efficiency increases linearly with extraction time.

To prevent human DNA carryover, we switched from utilizing human DNA as a surface coating to DNA isolated from salmon sperm. Before a day's worth of extraction experiments, we coat the surface of the capillary with 1.8 ng/μL salmon sperm DNA; the first extraction after coating is that of a control (not containing DNA) buffer. In every blank extraction performed to date, there has been no evidence of salmon sperm DNA carryover (as evidenced by the lack of fluorescence signal after each blank extraction).

Similar to experiments performed after first coating the capillary with human DNA, the efficiency of the DNA extraction increases with time of extraction. Displayed in Figure 65 is the correlation of LIF intensity with extraction time for three extractions at each of 1, 3, 5, and 10 min. In addition, mass of DNA extracted with respect to extraction time (as obtained with qPCR analysis) is reported on the same graph. Increasing extraction time yields more DNA extracted; thus, efficiency is increased with extraction time.

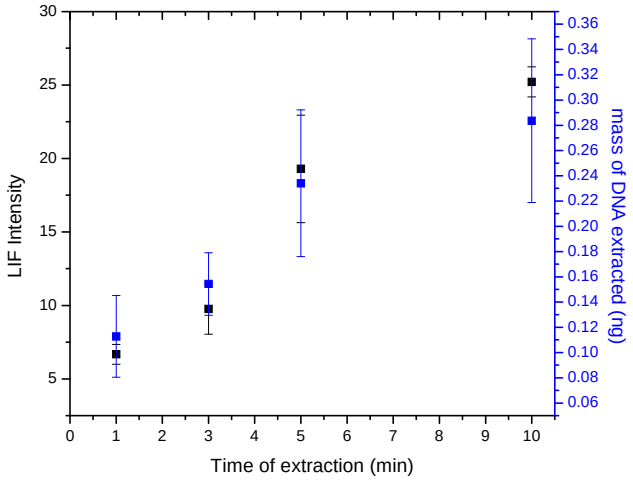


Figure 65: DNA delivery peak areas analyzed at different DNA extraction times. The extraction solution was 50 pg/μL human DNA standard solution. The data indicates that the DNA extraction efficiency increases with extraction time.

Table 3, below, shows the efficiency of DRAGEN extraction of DNA calculated using the mass of DNA extracted (as determined by qPCR analysis) and the mass of DNA in a standard DNA solution of a known concentration. The extraction efficiency increases with increased extraction time. Note that efficiency per extraction can be greatly enhanced by the addition of more than one capillary during the extraction process (a multibore capillary setup).

Table 3: Efficiency of DNA extraction with respect to time for extraction.

Time for extraction (min)	Mass of DNA extracted (ng)	Efficiency (%)
1	0.11275	2.26
3	0.15425	3.08
5	0.23405	4.68
10	0.28359	5.67

To assess the effect of pH on extraction efficiency, we performed experiments using leading electrolyte and trailing electrolyte pairs that were approximately pH 8 and approximately pH 3.4. We performed the experiments at low pH in hopes of removing negative charge from inhibitors that can be present in the sample. Data presented above in Figure 64 was collected using the pH 8 buffer system (50 mM Tris Cl- leading electrolyte, 50 mM Tris 25 mM HEPES). We experienced irreproducibility due to clogging during extractions that were performed using the pH 3.5 buffer system (75 mM histidine 25 mM citrate leading electrolyte, 15 mM gallic acid trailing electrolyte). Because histidine was difficult to dissolve, we performed the low-pH experiments with sodium as the basic counterion in this buffer system. The capillary still experienced clogging and general irreproducibility. The clogging is very likely due to DNA precipitating out of solution and onto the walls of the capillary. While working at low pH is not an issue with RNA molecules, DNA molecules will tend to precipitate at pH < 5.5. At pH 5.5, many weakly acidic materials, including materials that may be PCR inhibitors, will be charged and will thus be susceptible to GEITP extraction in addition to DNA.

Our work reported in previous paragraphs indicates that an increase in the extraction efficiency of human DNA from clean buffer samples occurs by increasing the time for extraction. However, experiments performed on real-world samples have shown that increasing the extraction time in this manner also increases the amount of contaminants (PCR inhibitors) that are present in the final solution. This is because materials that have similar electrophoretic mobilities to DNA will be extracted along with the DNA during the GEITP process. To circumvent this issue, we have performed multiple extractions to the same delivery tube. Shown in Figure 66 are PCR data that was collected after multiple extractions from the same sample into a delivery tube. On the X axis is the number of extractions that were performed and on the Y axis is the average mass of DNA, as determined by PCR, in a given series of extractions. As the number of extractions increases, the amount of DNA determined in the delivery tube increases.

Figure 66: Amount of DNA extracted with respect to number of extractions; delivery for each group of extractions was performed into one delivery tube and analyzed using PCR. The extraction solution was 50 pg/μL human DNA standard solution. The data indicates that the DNA extraction efficiency increases with number of extractions.

These data indicate that increasing the number of extractions increases the amount of DNA delivered. Table 4, below, shows the efficiency of DRAGEN extraction of DNA calculated using the mass of DNA extracted (as determined by qPCR analysis) and the mass of DNA in a standard DNA solution of a known concentration (50 pg/μL). The extraction efficiency increases with increased extraction number. Thus, future projects will benefit from a multibore capillary instrument.

Table 4: Efficiency of GEITP extraction method with respect to number of extractions performed and delivered into the same delivery tube.

Number of extractions	Apparent average mass of DNA extracted (ng)	Efficiency (%)
1	0.06976	1.38
3	0.11478	2.30
5	0.15198	3.04
10	0.34429	6.89

During the course of this project, the ARA-NIST team has been very fortunate to have had the opportunity to interface both with DFRC scientists as well as scientists from the US Armed Forces DNA Identification Laboratory (AFDIL). During one of those conversations, it was brought to our attention that the method in which we calculate efficiency, while correct, places the GEITP technique at a disadvantage. Efficiencies are calculated by dividing the average mass of DNA extracted by the total mass of DNA that is present in the vessel from which the DNA is extracted. However, during a GEITP extraction, a very small fraction of the overall sample is in an appropriate proximity of the capillary entrance to be extracted. Determining the volume of overall sample that can be applicable for

extraction is difficult and highly dependent on the electric field as well as the motion of the DNA molecules in solution.

Thus, a better method for determining efficiency of extraction is to determine the smallest volume of liquid required for the extraction. We have determined that 5 μL of liquid is required for a DNA extraction using the current DRAGEN instrument. We compared the amount of DNA extracted from 5 μL of NIST human DNA standard solution (500 pg/μL) to the amount of DNA extracted from 100 μL of NIST human DNA standard solution (500 pg/μL), Table 5. The amounts of DNA extracted from both volumes are quite similar; in fact, there is slightly more DNA extracted (on average) from the 5 μL solution. Because our efficiency calculation is indirectly dependent on volume, the calculated efficiency increases significantly after decreasing the volume of solution from which we extract.

Table 5: Extraction efficiencies of the DRAGEN instrument as calculated using the total mass of DNA in the extraction vessel.

Volume of 500 pg/μL NIST Standard DNA Solution (μL)	Apparent average mass of DNA extracted (ng) from 500 pg/μL NIST Human Standard DNA Solution (average of 10 extractions)	Standard Deviation of DNA extracted (ng) from 500 pg/μL NIST Human Standard DNA Solution	Efficiency (%)
5	0.409	0.097	16.4
100	0.384	0.093	0.77

These data indicate that the DRAGEN technique can extract DNA from solutions with volume as little as 5 μL. Further, previous results indicate that the efficiency can be increased further by increasing the extraction time, in the case of clean solutions, and/or performing multiple extractions on the sample.

3.9. Develop DRAGEN-based Method for Extracting Human DNA from Human Bone

The work completed and showcased in previous sections was concerned with the development of the DRAGEN instrument as well as determining the utility of the instrument for extracting human DNA from dirty buccal swabs. However, because human bones are often used for identification purposes, the utility of the DRAGEN device with respect to DNA extraction from bone samples was evaluated.

Because internal review board (IRB) approval was required to work with human remains, experiments were performed with pig bones prior to performing the experiments with human bones. Literature research as well as helpful conversations with the staff at DFRC and AFDIL indicated that there are several methods to prepare bone samples prior to analysis.[8-9] The method currently in use by AFDIL is the total demineralization method, whereby essentially all of the bone is dissolved in a concentrated ethylenediamine tetraacetic acid (EDTA) solution; Proteinase K is added to the solution for cell lysis. A slightly different method in use by other organizations is the casework protocol, which utilizes a less concentrated solution of EDTA in addition to NaCl. The solution that was used in this work is the casework protocol without NaCl. The components of the three extraction solutions are summarized in Table 6.

Table 6: Components of bone extraction solutions researched for this study.

	Casework protocol, total concentration	Casework protocol with NaCl, total concentration	Demineralization protocol, total concentration
Tris HEPES	25 mM	25 mM	
EDTA	50 mM	50 mM	500 mM
Triton X	0.1 %	0.1 %	1%
NaCl		100 mM	

Pig bone samples exposed to the solution required for the total demineralization protocol were very conductive and were not used for these experiments. Further, the solution prepared as the casework protocol with NaCl was also quite conductive and not used for this work. As noted in Table 6, the concentration of EDTA is 10-fold less in the solution utilized in the casework protocol than in the solution used in the demineralization protocol; as a result, the conductivity of this solution is less than that of the solution required for the total demineralization protocol. A requirement of the GEITP procedure is for the conductivity of the leading electrolyte to be higher than that of the trailing electrolyte; in the case of the 50 mM Tris 25 mM Cl^- leading electrolyte and the 25 mM Tris HEPES trailing electrolytes, the conductivities of these two solutions are 227 mS/m and 74.5 mS/m, respectively. Further, in addition to the requirement of a conductivity difference between the leading and trailing electrolytes, the conductivity of the leading electrolyte must not be so high as to promote electrolysis during the GEITP extraction. The current upon application of voltage must remain low; the current measured in the capillary at the onset of the GEITP extraction using the 50 mM Tris 25 mM Cl- leading electrolyte and the 25 mM Tris HEPES trailing electrolyte is approximately 26 μA. Solutions that are more highly conductive promote electrolysis as well as Joule heating.

Initial bone extraction experiments focused on fresh boiled pig bone. Fresh boiled pig bone from a pork chop was blended in a Waring professional blender. Approximately 0.5 g of bone powder was exposed to 1 mL of the casework protocol bone extract solution. After this slurry was prepared, 100 μL Proteinase K was added to the bone extract solution to a final concentration of 1.8 mg/mL. The solutions were incubated overnight at 56 °C in a shaker bath. The slurry was diluted 1:10 in the trailing electrolyte buffer prior to extraction. The DRAGEN instrument was equipped with 50 mM Tris 25 mM Cl^- as the leading electrolyte and 25 mM Tris 25 mM HEPES as the trailing electrolyte (including 0.1% Triton X as a surfactant for cell lysis) for the extraction experiments. The surface of the capillary was coated with 1.8 ng/μL salmon sperm DNA; the first extraction after coating was that of a control (not containing DNA) buffer.

Displayed in Figures 67-68 are representative data collected from the LIF and conductivity detectors on the DRAGEN device during a DNA extraction from pig bone; Figure 67 depicts data from a 1-min extraction and Figure 68 depicts data from a 5-min extraction. Samples were extracted from pig bone and then analyzed using qPCR utilizing primers purchased specifically for pig DNA.

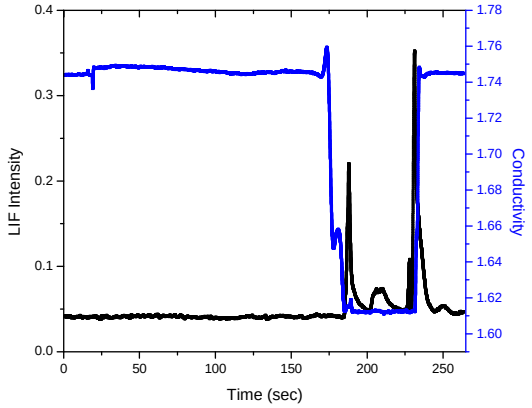


Figure 67: Representative GEITP extraction electropherograms (LIF and conductivity detector data) of DNA extracted from fresh boiled pig bone from a pork chop. Extraction time was 1 min.

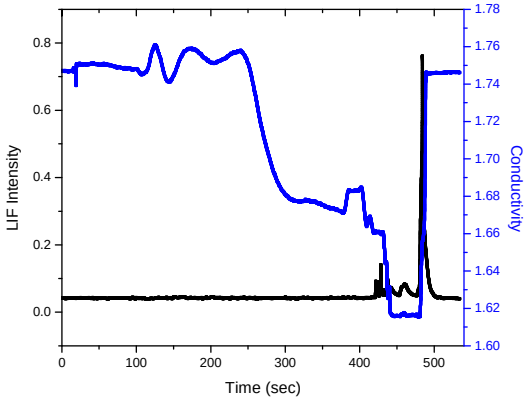


Figure 68: Representative GEITP extraction electropherograms (LIF and conductivity detector data) of DNA extracted from fresh boiled pig bone from a pork chop. Extraction time was 5 min.

Shown below in Table 7 are quantities of DNA extracted from pig bone as determined by qPCR. The quantities of DNA extracted increase with increasing extraction time; the quantity of DNA extracted also increases with number of extractions, as expected from previous experiments using standard DNA dissolved in trailing electrolyte buffer.

Table 7: Amount of DNA extracted from various extractions of fresh boiled pig bone from a pork chop

Pig DNA Extraction from Boiled Fresh Pig Bone from a Pork Chop	Apparent amount of DNA extracted (ng)
1-min extraction	12.49 ± 1.35
5-min extraction	18.92 ± 5.23
3 1-min extractions	28.23

Similar experiments performed with bone from a disjointed pig foot obtained from a skeleton supply company yielded no extracted DNA. The bone from the disjointed pig foot was prepared in the exact same manner as the bone from the fresh boiled pork chop. Shown below in Figures 69-70 are representative data from the LIF and conductivity detectors on the DRAGEN device during a DNA extraction from disjointed pig bone; Figure 69 depicts data from a 1-min extraction and Figure 70 depicts data from a 5-min extraction.

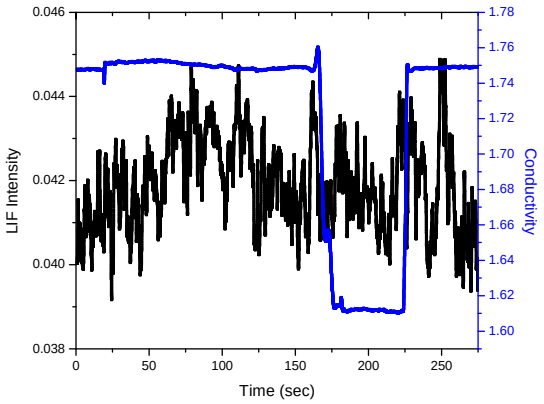


Figure 69: Representative GEITP extraction electropherograms (LIF and conductivity detector data) of DNA extracted from pig bone from a disjointed pig foot. Extraction time was 1 min.

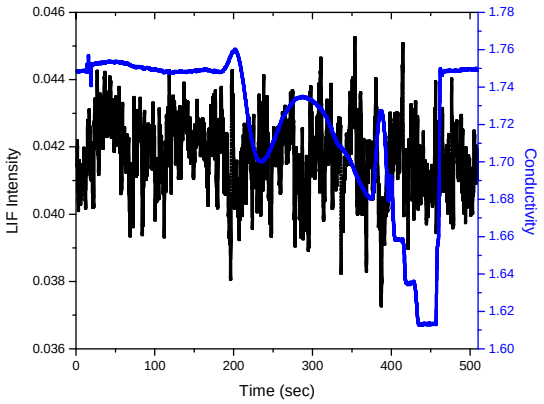
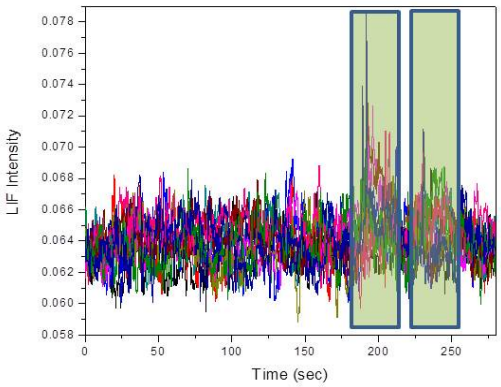


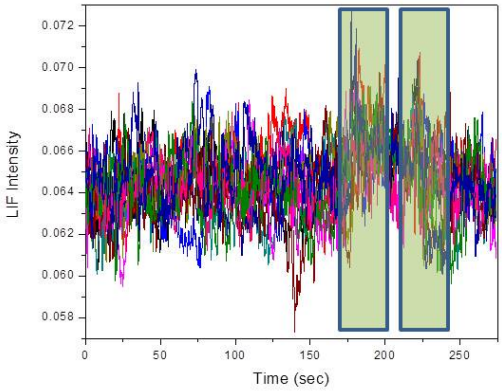
Figure 70: Representative GEITP extraction electropherograms (LIF and conductivity detector data) of DNA extracted from pig bone from a disjointed pig foot. Extraction time was 5 min.

After performing the above experiments on the fresh and processed pig bone samples, ARA personnel used the same methods for human bone samples supplied by AFDIL. The human bone samples were approximately 60 years old and contained only trace amounts of DNA. We performed extractions on four human bone samples with a forensic scientist from AFDIL. We provided the lysis/bone digestion solution, consisting of 25 mM Tris HEPES, 50 mM EDTA, and 0.1% Triton X, to AFDIL. The approximate mass of the bone that was used was 0.5 g in all cases; 1 mL of lysis digestion solution was added to the bone powder. To this solution was added 100 μL Proteinase K (20 mg/mL) to a final concentration of 1.8 mg/mL. The slurry was incubated overnight at 56 °C in a shaker bath. The slurry was diluted 1:10 in the trailing electrolyte (TE) buffer prior to extraction. The buffer system utilized in this work consisted of 50 mM Tris 25 mM Cl^- as the leading electrolyte and 25 mM Tris 25 mM HEPES as the trailing electrolyte (including 0.1% Triton X as a surfactant for cell lysis). We continued to use DNA isolated from salmon sperm as a negatively-charged surface coating.

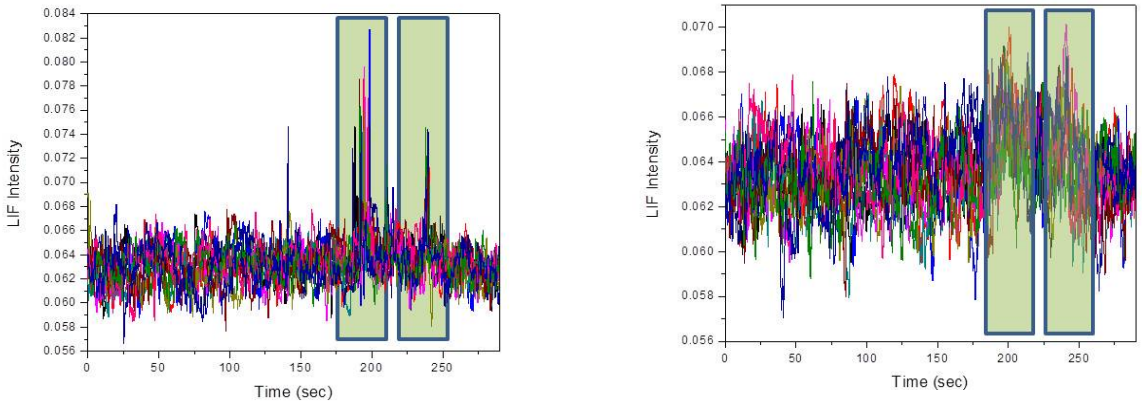
For each bone sample, we performed one extraction on a single aliquot of diluted slurry as well as ten extractions on another single, separate aliquot of diluted slurry. Shown in Figures 71A-D are representative GEITP electropherograms of each of the four bone samples. Note that the detection method highlighted in these figures is laser-induced fluorescence (LIF).



A.



B.



C. D.

Figure 71A-D: GEITP electropherograms of A. human bone 02A-5, B. human bone 05A-5, C. human bone 10A-5, and D. human bone 13A-5. The shaded rectangles are indicative of the extraction and delivery peaks in the GEITP data. Ten electropherograms are shown in each graph.

Results from AFDIL's laboratory indicate that, in the case of samples that were extracted multiple times, qPCR inhibitors are present. As indicated by the LIF data displayed in Figure 71A-D, DNA is being extracted from the bone samples.

In an effort to discourage the focusing and subsequent extraction of qPCR inhibitors that may be present in real-world samples that contain trace amounts of DNA, a study was performed to determine alternate leading electrolytes that are slightly slower than Tris Cl^-. The buffer system that we currently employ, 50 mM Tris 25 mM Cl^- as the leading electrolyte and 25 mM Tris 25 mM HEPES as the trailing electrolyte, was chosen because the electrophoretic mobility of the DNA is not quite as fast as the leading electrolyte but not as slow as the trailing electrolyte. As a result, the DNA focuses at the interface between the leading and trailing electrolytes. Any qPCR inhibitors present in samples likely possess an electrophoretic mobility between that of the leading and trailing electrolytes as well. Thus, we focused our experiments on narrowing the gap in electrophoretic mobilities between the leading and trailing electrolytes. Our first experiments concerned the leading electrolyte—because Cl^- is a very fast ion, we began our experiments by systematically decreasing the electrophoretic mobility of the leading electrolyte. We prepared a series of solutions utilizing counterions of slower mobilities than Cl^-. After the solutions were prepared, we diluted the solutions such that the conductivity of each solution matched that of the leading electrolyte that we have used throughout the course of this work, 50 mM Tris 25 mM Cl^-. We then performed extractions with each of these candidate leading electrolyte solutions on NIST human standard DNA (50 pg/μL) using GEITP. We analyzed the conductivity detector data and compared that data to the LIF data in each extraction. These data are shown in Figures 72-76. Shown in Figure 72 are LIF and conductivity data traces for the extraction of 50 pg/μL human DNA using the standard Tris Cl^- leading electrolyte. The LIF data (black trace) shows the focused DNA; the focused DNA peak, when overlaid with the conductivity detector data, is present at a slightly slower time than the large downward steps present in the conductivity detector data. This is because the detectors are at a slightly different distance along the capillary from each other; we expect the DNA peak in the LIF trace to be slightly slower than the downward step in the conductivity detector data that indicates the interface between leading electrolyte and trailing electrolyte. The data shown in Figures 74 and 75, corresponding to Tris phosphate and Tris formate leading electrolytes, respectively, indicate that the leading electrolytes are slightly slower than optimal for DNA extraction. The peaks in the LIF data coincide with the downward step in the conductivity data for both data traces. In the case of the Tris acetate buffer, Figure 75, the LIF peaks are significantly faster than the downward steps in the conductivity detector data, indicating that the leading electrolyte is significantly slower than the DNA

and is thus not appropriate for its extraction using the GEITP method. Data shown in Figure 76, corresponding to a Tris oxalate buffer, indicate that the DNA focuses on the interface between the leading and trailing electrolytes. Thus, of the four candidate trailing electrolytes presented here, only the Tris oxalate leading electrolyte showed results that were similar to the original Tris Cl^- buffer system.

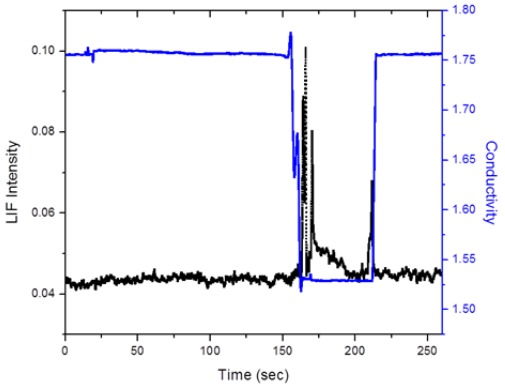


Figure 72: LIF and conductivity detector data using the standard 50 mM Tris 25 mM Cl^- leading electrolyte. The data indicates that the DNA focuses on one of the two downward steps at 155 sec and 160 sec. DNA focusing is indicated by the sharp peak in the black trace and the downward steps in the conductivity data (blue trace) indicate a decrease in the conductivity of the solution in the capillary. Because the detectors are located at slightly different places on the capillary, the sharp peak in the LIF trace does not align exactly with the interface depicted in the conductivity (blue) trace.

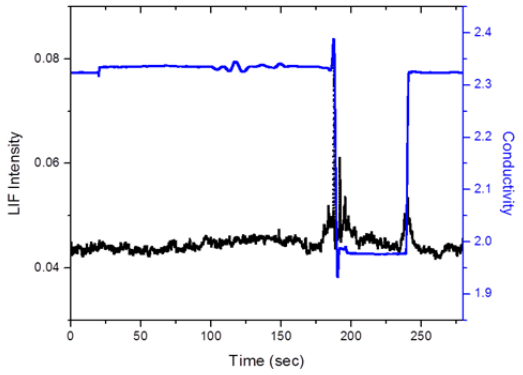


Figure 73: LIF and conductivity detector data using 100 mM Tris 37 mM phosphate leading electrolyte. The data indicates that the DNA focuses on the downward step at 180 sec. Because the detectors are located at slightly different places on the capillary, it is expected that the sharp DNA peak in the LIF data (black trace) would be slightly later than the downward step in the conductivity data (blue trace). Because the DNA peak is not slightly offset from the conductivity step, the DNA appears faster than the leading electrolyte.

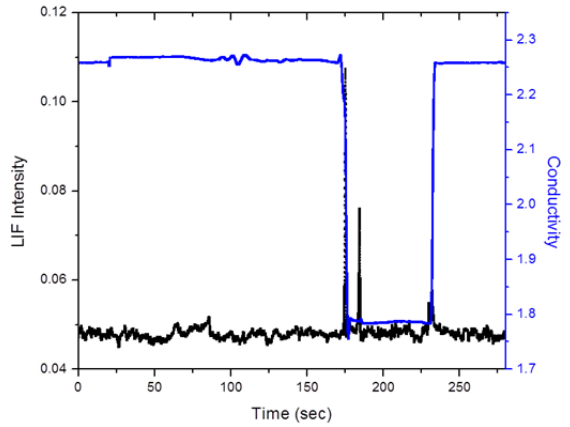


Figure 74: LIF and conductivity detector data using 60 mM Tris 35.2 mM formate leading electrolyte. The data indicates that the DNA focuses on the downward step at 175 sec. Because the detectors are located at slightly different places on the capillary, it is expected that the sharp DNA peak in the LIF data (black trace) would be slightly later than the downward step in the conductivity data (blue trace). Because the DNA peak is not slightly offset from the conductivity step, the DNA appears faster than the leading electrolyte.

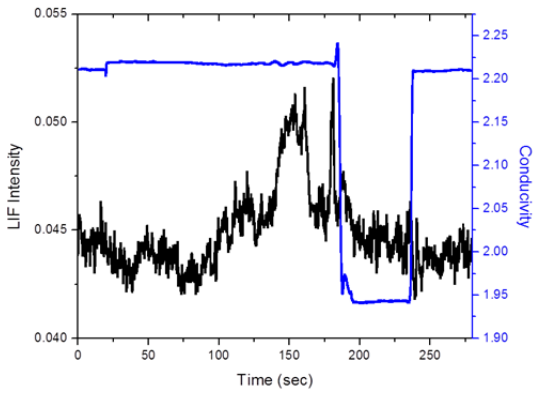


Figure 75: LIF and conductivity detector data using 100 mM Tris 50 mM acetate leading electrolyte. The data indicates that the DNA doesn't focus on the interface of the leading and trailing electrolytes.

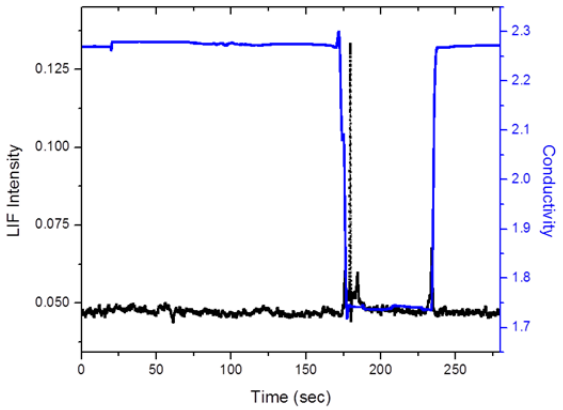


Figure 76: LIF and conductivity detector data using 63.5 mM Tris 15.9 mM oxalate leading electrolyte. The data indicates that the DNA focuses on the downward step at 175 sec.

qPCR analysis using the leading electrolytes presented above was also performed. Results indicate that the concentration of DNA extracted from all tested leading electrolytes is lower than the concentration of DNA extracted using the Tris Cl^- leading electrolyte. Further, bone extract solution was used in the extraction experiment for some of the experiments. For these experiments, bone extract solution, prepared as noted previously, was diluted in trailing electrolyte. Into this solution was added NIST human standard DNA at a concentration of 50 pg/μL. The amount of DNA extracted for each leading electrolyte is tabulated below in Table 8. Tris Cl^- is still the most appropriate leading electrolyte for the system due to the amount of DNA extracted using Tris Cl- as the leading electrolyte. Further, DNA extractions performed on mixtures that included diluted bone extract solution using alternate leading electrolytes did not yield appreciable amounts of DNA as determined by qPCR (Table 8).

Table 8: DNA collected from solutions extracted using the DRAGEN apparatus

Leading electrolyte (trailing electrolyte is Tris HEPES)	Apparent amount of DNA extracted from 50 pg/μL human DNA solution (ng)	Apparent amount of DNA extracted from 50 pg/μL human DNA solution (ng) prepared in diluted bone extract solution
Tris Cl^-	0.070	0.000
Tris phosphate	0.007	0.003
Tris formate	0.018	0.002
Tris acetate	0.027	0.000
Tris oxalate	0.021	0.003

In addition to performing a study on the leading electrolyte, we also similarly changed the trailing electrolyte. In the case of changing the trailing electrolyte, the Tris HEPES trailing electrolyte employed for the majority of this work is electrophoretically very slow. The electrolytes that were used in the leading electrolyte study presented above are very similar in electrophoretic mobility to DNA; thus, we utilized three of these materials, Tris oxalate, Tris formate, and Tris acetate, in addition to Tris lactate, in studies designed to determine if these materials will be appropriate as trailing electrolytes. Shown in Figures 77-80 are GEITP electropherograms of human DNA focused with 250 mM Tris 125 mM Cl^- as the leading electrolyte and 12.5 mM of either Tris oxalate, Tris formate, Tris acetate, and Tris lactate as the

trailing electrolyte. Note that the leading electrolyte in these experiments is significantly more concentrated than in previous experiments. This is because, at the time when these experiments were performed, we had determined that the conductivities of various real-world samples were very high and, as a result, it was necessary to increase the conductivity (and thus the concentration) of the leading electrolyte such that a conductivity difference between the leading and trailing electrolytes was maintained. As a result, experiments were performed and it was determined that concentrations of 250 mM Tris 125 mM Cl^- could be used as the leading electrolyte with little effect of hydrolysis or Joule heating due to increased measured current. Of the four trailing electrolyte buffers selected for further analysis as a potential trailing electrolyte, the 12.5 mM Tris oxalate buffer appeared to focus but not on a specific interface (Figure 77) and the DNA extracted using 12.5 mM Tris formate appeared not to focus (Figure 78). Data from both the 12.5 mM Tris acetate and 12.5 mM Tris lactate trailing electrolytes appear to contain focused DNA (Figure 79-80).

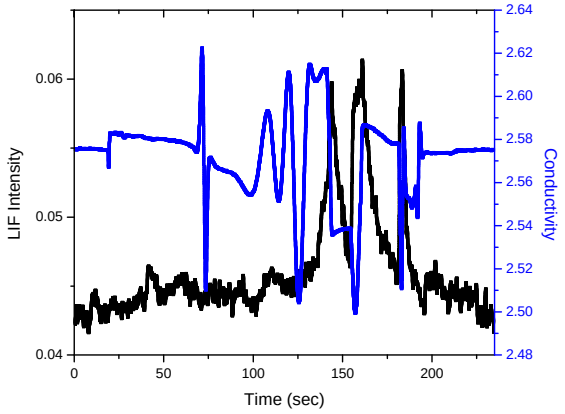


Figure 77: LIF and conductivity detector data using 12.5 mM Tris oxalate trailing electrolyte. The data indicates that the DNA focuses but it is difficult to pinpoint an interface where it focuses.

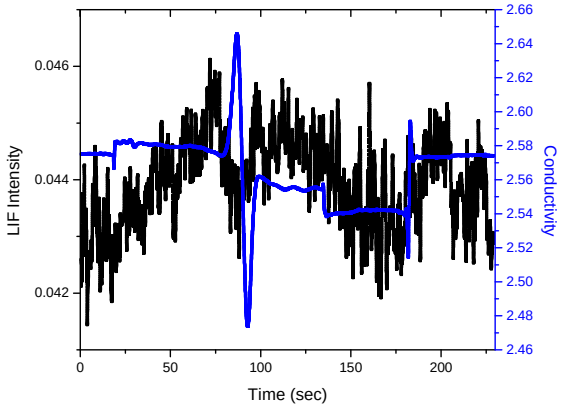


Figure 78: LIF and conductivity detector data using 12.5 mM Tris formate trailing electrolyte. The data indicates that the DNA does not focus well with this buffer system.

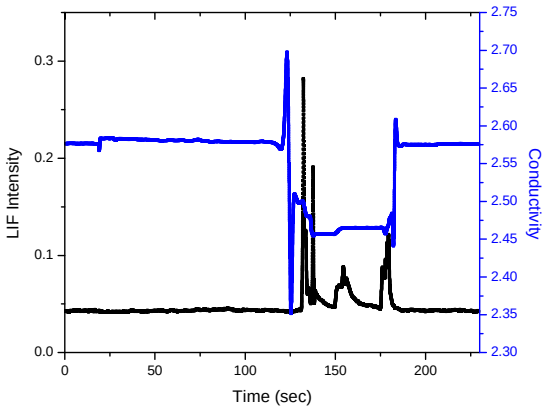


Figure 79: LIF and conductivity detector data using 12.5 mM Tris acetate trailing electrolyte. The data indicates that the DNA likely focuses on the downward step at 125 sec.

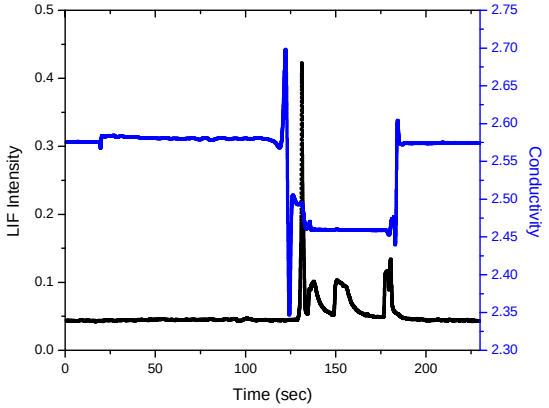


Figure 80: LIF and conductivity detector data using 12.5 mM Tris lactate trailing electrolyte. The data indicates that the DNA likely focuses on the downward step at 125 sec.

Data collected using qPCR to assess the mass of DNA extracted are shown below in Table 9.

Table 9: Masses of DNA extracted using various trailing electrolytes (*--this data was collected on a separate day from all other data shown in this table and may not reflect the increase in efficiency we were able to achieve).

Trailing electrolyte	Apparent mass of DNA extracted from 500 pg/μL DNA in buffer	Apparent mass of DNA extracted from 500 pg/μL DNA spiked in bone extract solution
12.5 mM Tris oxalate	0.527 ± 0.17	0.217 ± 0.05
12.5 mM Tris formate	0.031 ± 0.02	Not performed
12.5 mM Tris acetate	0.903 ± 0.66	0.282 ± 0.12
12.5 mM Tris lactate	0.796 ± 0.12	0.036 ± 0.001*

The 12.5 mM Tris lactate trailing electrolyte buffer showed a slightly higher mass of DNA extracted than data collected using the 12.5 mM Tris oxalate buffer. Further, data collected with the 12.5 mM Tris lactate buffer was less variable than data collected with 12.5 mM Tris acetate buffer. The 12.5 mM Tris formate buffer trailing electrolyte yielded very little DNA. However, qPCR data collected from DNA extracts from 500 pg/μL DNA spiked in bone extract solution indicates that DNA has been extracted when 12.5 mM Tris oxalate and 12.5 mM Tris acetate buffers are used as trailing electrolytes. Data collected from qPCR runs of the internal positive control IPC in the presence of DNA isolated from bone dissolution solution indicated that the IPC was not greatly inhibited. However, because the extract was only in the presence of the IPC for a short period of time, it is possible, depending on the mode of inhibition, that the extracted DNA and not the IPC is inhibited.

Note that because extractions of standard NIST DNA in pristine 12.5 mM Tris formate leading electrolyte solution yielded very little DNA, experiments extracting DNA from bone extract solution were not performed. A clear follow-on to this project is to utilize 12.5 mM Tris oxalate and 12.5 mM Tris acetate buffers as well as buffers with similar electrophoretic mobilities in DNA extraction experiments featuring bone matrices.

3.10. Develop DRAGEN-based Method for Extracting Human DNA from Chewing Gum

Shown in Figure 81 is a photograph of a gum sample that was used in this work. Fresh (unchewed) gum sticks were provided to anonymous volunteers. The gum provided, and used almost exclusively in this work, was Wrigley's Doublemint chewing gum. Volunteers were asked to chew the gum and place the used piece of gum into the gum's wrapper. The samples were placed in an envelope and dropped in a drop box in the lab for analysis.

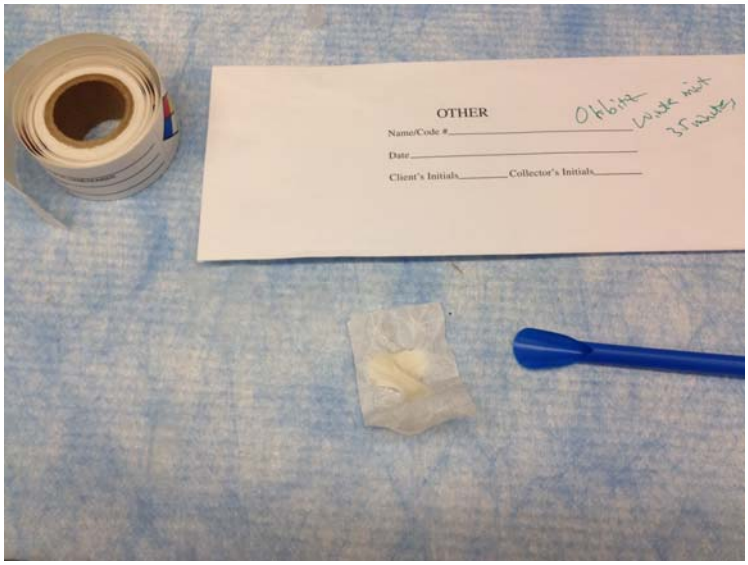


Figure 81: Photograph of chewed gum sample prior to exposure to lysis media.

The ingredients in Wrigley's Doublemint gum (the test gum for this study) include sugar, gum base, dextrose, corn syrup, natural and artificial flavors; as well as less than 2% of glycerol, aspartame, gum Arabic, soy lecithin, acesulfame K, color (TiO_2), and butylated hydroxytoluene (BHT). Of these ingredients, the charged species, or species that can be electrophoretically mobile in the GEITP experiments, include (at minimum) aspartame (may or may not be hydrolyzed), acesulfame K, and carvone (and other "natural and artificial flavors," the exact names and structures of which are proprietary). In addition, gum base (different from gum Arabic), in many cases, is a synthetic proprietary material dependent on the specific type of gum and consists of acetate-based polymers; thus, acetate is likely present as a contaminant. Because of the presence of these materials, experiments were undertaken at low pH values to potentially exclude inhibitors that are charged (and thus susceptible to GEITP extraction) at pH 7-8.

Gum samples weighing approximately 0.5 g were exposed to 500 μL lysis solution (15 mM gallic acid, 0.1% Triton X total concentration; 0.54 mg/mL Proteinase K) and incubated at 56 °C for 30 min. After incubation, the liquid was removed from the sticky, elastomeric mass and placed in a clean tube. This gum lysate was diluted by a factor of 10X and extracted in 100 μL aliquots. Lysis protocols were determined from our experience with buccal swabs.

Displayed in Figure 82 is representative DRAGEN LIF intensity data shown together with representative conductivity detector data from the same extraction. The extraction conditions are as follows: 10 mM sodium citrate leading electrolyte, 15 mM gallic acid with 0.1% Triton X trailing electrolyte, pH 5.5.

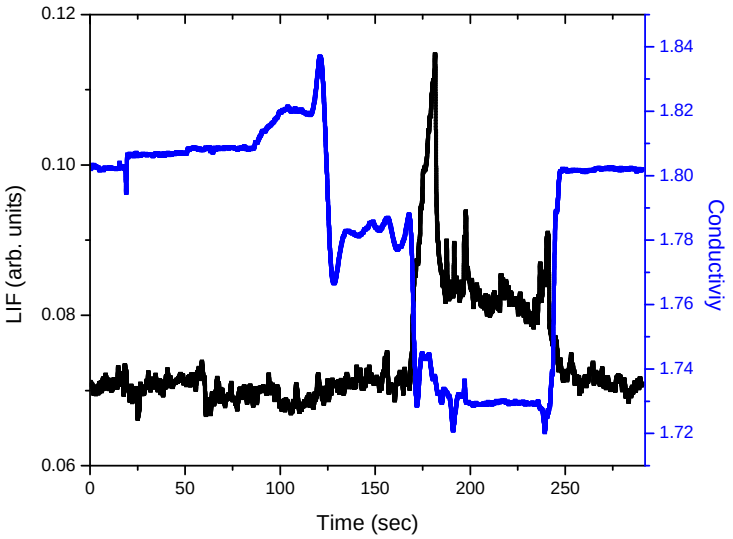


Figure 82: DRAGEN extraction of human DNA from chewing gum. The peak at 175 sec (black trace) corresponds to the DNA extraction and the peak at 230 sec corresponds to the DNA delivery peak. The conductivity detector trace (blue trace) shows that the DNA focuses at one of two steps in the focused region. The step at 125 sec in the conductivity detector trace potentially indicates that contaminants are present in the sample.

The data in Figure 82, particularly the presence of two steps in the conductivity detector data, indicate that sample ions (including ingredients from the gum and DNA) are focusing at two different steps. The analytes with a high electrophoretic mobility at pH 5.5 are focusing at the step centered on 125 sec and the analytes with a lower electrophoretic mobility, DNA included, are focusing at the step at 175 sec. The conductivity detector trace is not uniform and includes features that are not typically present in clean samples. Data from this experiment indicates that, even at a lower pH, matrix analytes are present that may interfere with the extraction. In addition, the LIF detector data indicates that the DNA moves slowly at this pH—after extraction, the baseline does not decreases to an intensity similar to the run before the extraction peak. This indicates that the DNA plug is moving very slowly past the LIF detector and hesitates at the LIF detector while the device is removed from the extraction vessel to the delivery vessel.

We have performed DNA extractions at even lower pH values, such as pH 4. Shown in Figure 83 is DRAGEN LIF intensity extraction and delivery data together with representative conductivity detector data from the same extraction. The sample utilized in this extraction is 500 pg/μL human standard DNA. As is readily noted, the DNA is very broad and focuses at a small step prior to the large step at 215 sec that indicates the difference of the conductivities of the two buffers. Because the DNA moves so slowly at this pH and because it does not focus at the interface of the two buffers, we increased the pH of the buffer system for the gum experiments. Further, experiments with low-pH buffer systems indicated that the DNA precipitates at pH values less than pH 5.5. Thus, while decreasing the pH of the buffer system to decrease the charges present on contaminants is a reasonable course of action, decreasing the pH to a value less than pH 5.5 causes DNA precipitation. Thus, buffers at pH 5.5 or higher must be used for this technique.

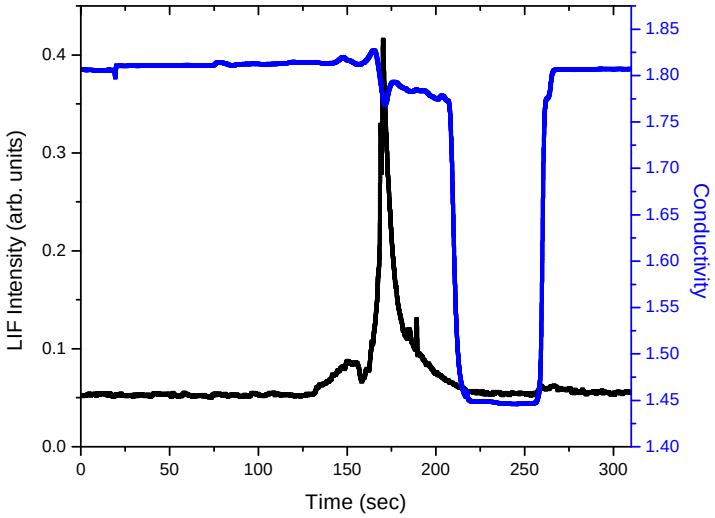


Figure 83: DRAGEN extraction of human DNA from a standard solution containing 500 pg/μL DNA. The peak at 175 sec (black trace) corresponds to the DNA extraction and the peak at 260 sec corresponds to the DNA delivery peak. The conductivity detector trace (blue trace) shows that the DNA focuses at one of two steps in the focused region. The step at 125 sec in the conductivity detector trace indicates that significant contaminants are present in the sample.

Shown in Figures 84 and 85 are representative DRAGEN DNA extraction traces from both the LIF and conductivity detectors. The extraction was performed using 50 mM Tris 25 mM Cl^- leading electrolyte, 25 mM Tris HEPES trailing electrolyte. Figure 84 are representative data from a 1-min extraction and Figure 85 are representative data from a 5-min extraction.

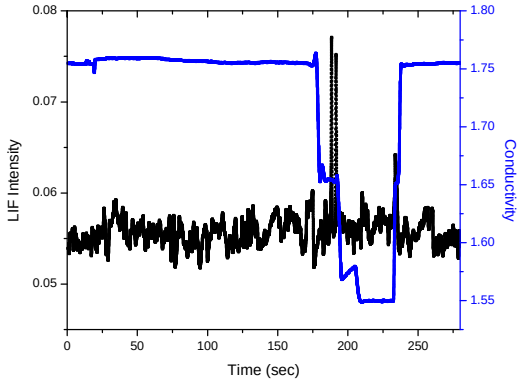


Figure 84: DRAGEN extraction of human DNA from chewing gum. The extraction time is 1 min.

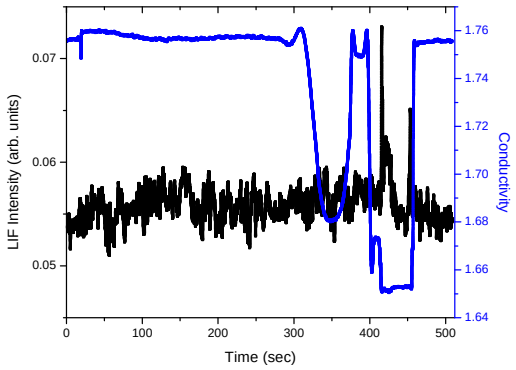


Figure 85: DRAGEN extraction of human DNA from chewing gum. The extraction time is 5 min.

The LIF data from the 1-min DNA extraction time for the gum matrix, Figure 84, shows a clear extraction peak at 195 sec and a delivery peak at 225 sec. The data from the conductivity detector suggests that there are materials present in the sample that possess a high conductivity, as evidenced by the step at 175 sec that does not correspond to DNA focusing. The data shown in Figure 85 were collected during a 5-min DNA extraction of chewing gum. Readily noticeable is a broad, downward peak at 350 sec in the conductivity detector data. We are unclear of the origins of this peak, though it is quite consistent with gum samples that are extracted for 5 min. Our hypothesis is that the additional materials that are present in the gum are changing the buffer matrix in such a way during the focusing step that the DNA is no longer focused to any appreciable extent, though the DNA that is focused is extracted from the sample. In fact, the LIF data for both the 1-min and 5-min extraction times are quite similar to each

other, though, in experiments that utilize standard DNA and a buffer matrix as the sample, the delivery peak for the 5-min extractions are significantly more intense than those collected during the 1-min extractions.

We have performed multiple extractions on one type of gum sample and delivered the extracted DNA to the same delivery vessel. Experiments focused on performing up to 10 extractions on chewing gum. Approximately 0.5 g chewed gum was exposed to a lysing solution consisting of 25 mM Tris HEPES and 0.1% Triton X. Lysing solution was mixed prior to incorporation of sample; Proteinase K was added to a final concentration of 0.9 mg/mL. The gum samples were exposed overnight to the lysis solution at a temperature of 56 °C under agitation. PCR experiments indicated that there was no DNA replication for the 10-extraction samples obtained from gum; further, qPCR experiments performed with an IPC concluded that there was little to no replication of the internal standard. However, the LIF data indicates that DNA was extracted from the samples (see, for example, Figure 86 for a representative data trace)—our conclusion is that there was DNA extracted from the gum samples but there was PCR inhibitor extracted from the samples as well.

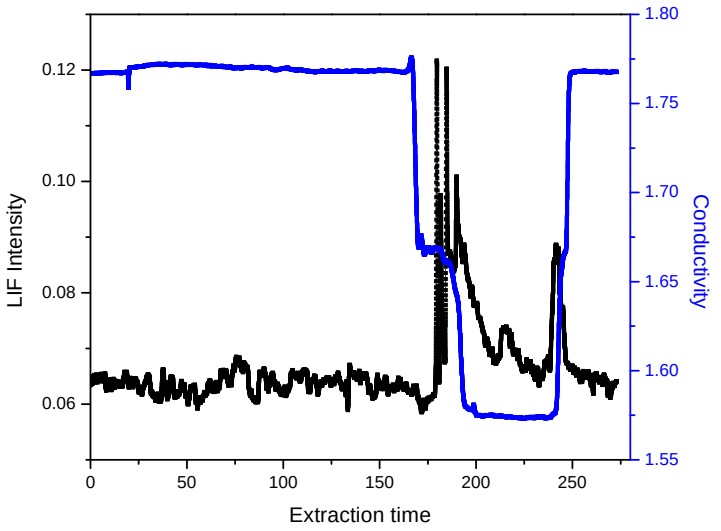


Figure 86: DRAGEN DNA extraction of human DNA from 0.5 g gum exposed to lysis solution. The peak at 175 sec (black trace) corresponds to the DNA extraction and the peak at 240 sec corresponds to the DNA delivery peak.

Our next set of experiments thus focused on performing fewer extractions for gum samples. Displayed in Table 10 are PCR results from five different gum sample aliquots; DNA extractions were performed over two days. Each gum sample aliquot was exposed to 1, 3, and 5 extractions.

Table 10: Mass of DNA extracted from gum samples extracted multiple times.

Number of extractions	Apparent average mass of DNA extracted (ng)
1 (average of 8 extracts)	0.051 ± 0.047
3 (average of 5 extracts)	0.145 ± 0.30
5 (average of 5 extracts)	0.089 ± 0.19

In the case of the 3- and 5-extraction samples, the standard deviation of the average of the 5 samples is double the average. The 5-extraction samples yielded no PCR results for either the human DNA or the IPC for three of the 5 samples, indicating that there is inhibitor in the sample. For the 3-extraction

samples, there appears to be less inhibitor, according to the IPC results. For the 1-extraction samples, there appears to be little to no inhibitor.

Because the gum extract solution is highly conductive due to the presence of charged species in the solution, we performed experiments with leading electrolytes that were higher in conductivity than 50 mM Tris 25 mM Cl^-. The concentration of leading electrolyte for these experiments was 250 mM Tris 120 mM Cl^-. The conductivity of this solution was measured at 786 mS/m. The trailing electrolyte in these experiments was 12.5 mM Tris lactate and possess a conductivity of approximately 40 mS/m. The differences in conductivity lead, in principle, to higher amounts of DNA extracted from solution. The trailing electrolyte in this instance is Tris lactate and was chosen due to its higher theoretical electrophoretic mobility that Tris HEPES. Initial experiments were performed by spiking human standard DNA into a solution containing gum extract prepared above. The final concentration of human DNA in these samples was 500 pg/μL. Extractions performed on spiked samples yielded masses of DNA that were consistent with samples that did not contain gum extract, Table 10, seeming to indicate that the alternative leading electrolyte-trailing electrolyte pair was more applicable to gum samples. However, DNA extracted from the gum samples with no human DNA spike still yielded masses of DNA that are in the 35 pg-range (Table 11). Multiple extractions from the same sample did not yield an appreciable difference in mass of DNA extracted.

Table 11: Masses of DNA extracted from human DNA solution, human DNA solution spiked into gum extract, and gum extract.

Sample	Apparent mass of DNA extracted (ng)
500 pg/μL human DNA	0.346 ± 0.14
500 pg/μL human DNA spiked in 1:10 gum extract	0.322 ± 0.09
Gum extract (1:10 dilution in trailing electrolyte)	0.035 ± 0.012

These data indicate that DNA is being extracted from the gum using the DRAGEN apparatus. Further, because the same amount of DNA is extracted from a pristine 500 pg/μL sample of DNA as a gum extract solution that has been spiked with 500 pg/μL human DNA, it is possible that the lysis methodology is simply not efficient for lysing cells embedded in a gum matrix. Further work requires the optimization of the GEITP technique for trace DNA samples.

3.11. Develop DRAGEN-based Method for Extracting Plant DNA from Plant Material

For extraction development of plant DNA from plant material, spinach was used as the trial plant material. Spinach was a reasonable choice because pure spinach genomic DNA was available commercially and we were able to find primers for qPCR analysis in the literature.[5] A plant cell wall disruption procedure was used to release spinach DNA from spinach cells.[8] Initially, the plant cell disruption procedure was performed in 50 mM Tris HEPES, 50 mM EDTA, and 0.2% Triton X. However, as the project progressed and as it became apparent that a significant conductivity difference (factor of approximately 3) between the leading and trailing electrolyte was necessary, the cell wall disruption procedure was changed such that the resulting conductivity of the extract solution was as low as possible. Thus, the plant cell disruption solution was 50 mM EDTA and 0.2% Triton X. The spinach material was then exposed to this solution for 1 h at 100 °C. For fresh spinach material, approximately 1.5 g of fresh leaves and stems from spinach purchased as a salad mix was macerated in a mortar and pestle. The 1.5 g of spinach pulp was added to 5 mL of the cell wall disruption solution. For experiments with dried spinach, approximately 0.15 g of dried spinach leaves were ground in a mortar and pestle and exposed to the cell wall disruption solution. The mass of dried spinach leaves was determined based on

the biomass of the spinach (spinach leaves without intrinsic water); fresh spinach was determined to be comprised of approximately 90% water. *Papaver somniferum* plant material (0.16 g) was also ground in a mortar and pestle and exposed to cell wall disruption solution and further DNA extraction and analysis.

Shown in Table 12 are conductivity values of the fresh spinach, dried spinach, and dried *Papaver setigerum* plant material that was used in this work. The conductivity of the plant material after exposure to the cell wall disruption solution is significant.

Table 12: Conductivities of plant solutions and leading and trailing electrolytes.

Solution	Conductivity (mS/m)
Fresh spinach in cell wall disruption solution	1127
Dried spinach in cell wall disruption solution	1726
Dried P. somniferum in cell wall disruption solution	964
12.5 mM Tris lactate (trailing electrolyte)	40
250 mM Tris 125 mM Cl- (leading electrolyte)	786

For GEITP-based DNA extraction, plant solutions were diluted 1:10 in 12.5 mM Tris lactate buffer. Displayed in Figures 87-89 are representative GEITP data for DNA extracted from fresh spinach (Figure 87), dried spinach (Figure 88), and dried *P. setigerum* (Figure 89). The GEITP data collected using both the LIF and conductivity detectors for the fresh and dried spinach samples are extremely similar. Further, data collected from the *P. setigerum* samples shown in Figure 89, show similar traces in the conductivity detector though less intensity in the LIF detector.

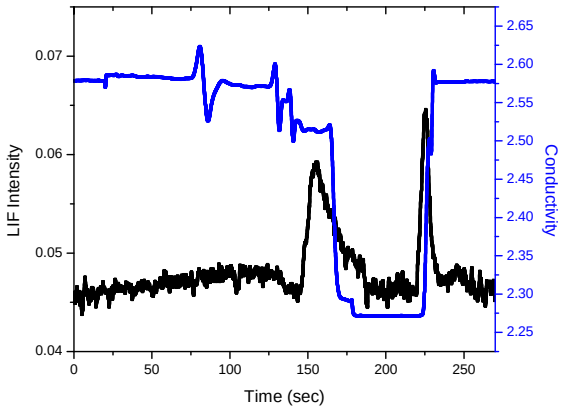


Figure 87: GEITP traces of DNA extracted from fresh spinach.

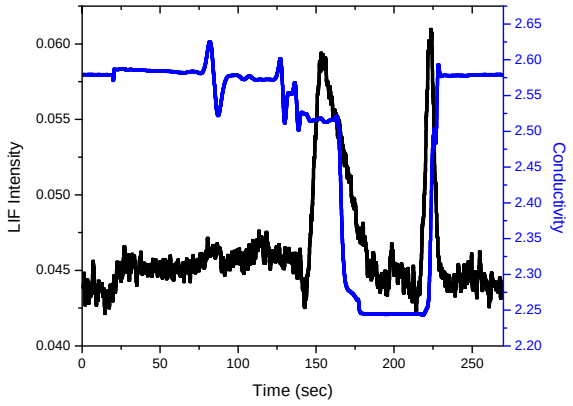


Figure 88: GEITP traces of DNA extracted from dried spinach.

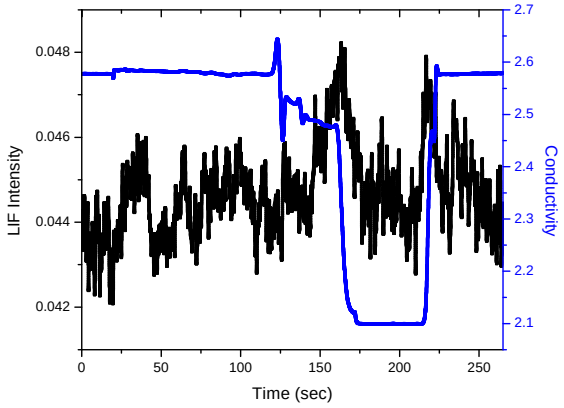


Figure 89: GEITP traces of DNA extracted from dried *P. setigerum*.

Analysis of the DNA extracted from the plant material using qPCR results presented in Table 13. Interestingly, on experiments that were performed with the low conductivity leading electrolyte-trailing electrolyte pair (50 mM Tris 25 mM Cl^- leading electrolyte, 25 mM Tris HEPES trailing electrolyte), the amount of DNA extracted was significantly lower. Ten extractions performed to one delivery tube was required to obtain 0.5 ng of spinach DNA. Conversely, when the leading electrolyte-trailing electrolyte pair was changed in both conductivity and electrophoretic mobility to 250 mM Tris 125 mM Cl- leading electrolyte and 12.5 mM Tris lactate trailing electrolyte, the amount of DNA extracted increased by over an order of magnitude. This increase in extracted DNA may be due to the conductivity difference in the leading electrolyte and trailing electrolyte solutions as well as the lesser concentration of qPCR inhibitors from the plant solutions extracted into the delivery tube due to the use of a more electrophoretically mobile trailing electrolyte.

Sample Matrix	Electrolyte system (leading:trailing)	Mass of DNA extracted (ng)
Fresh spinach	50 mM Tris 25 mM Cl^-:25 mM Tris HEPES	0.479 ± 0.056 (10 extractions were necessary)
Fresh spinach	250 mM Tris 125 mM Cl^-:12.5 mM Tris lactate	1.170 ± 0.361
Fresh spinach (different batch)	250 mM Tris 125 mM Cl^-:12.5 mM Tris lactate	0.486 ± 0.187
Dried spinach	250 mM Tris 125 mM Cl^-:12.5 mM Tris lactate	0.015 ± 0.003
Dried *P. setigerum*	250 mM Tris 125 mM Cl^-:12.5 mM Tris lactate	0.005 ± 0.004

Conductivity differences in spinach cell wall disruption solutions may explain the difference in mass of extracted DNA from fresh spinach prepared in different batches. With respect to dried spinach, the mass of DNA extracted from dried material is an order of magnitude less than that extracted from fresh material, though the GEITP traces are very similar in shape and intensity. The explanation for this may be due to DNA degradation in the dried spinach, potentially at the region on which the qPCR primers are

attached and begin replication. Further work with dried plant material is necessary to make this determination. *P. setigerum* plant material exposed to DNA extraction using the DRAGEN apparatus yielded DNA but it is impossible to state the actual concentration because the standard curve in the qPCR experiments was prepared using spinach DNA. The data are included in the table to show that DNA was extracted and replicated.

4. Conclusions

In conclusion, the ARA-NIST team has succeeded in building several variations of a stand-alone device that will extract DNA from various samples and deliver the extracted DNA to a clean vessel that can be provided for further downstream analysis. The final variation of the instrument, the DRAGEN (direct rapid analysis generating extracted nucleotides) instrument, possesses a fully automated 3-axis extraction and delivery stage, fully integrated capacitively coupled conductivity and LIF detectors for detection of the focusing interface as well as detection of the fluorescent DNA molecules, a current feedback control mechanism that allows for longer "focusing" times for DNA molecules and thus more flexibility of extraction times, and a self-aligning fluorescence excitation and detection system. The entire system is controlled by an easy-to-use software package. Work performed in support of final device integration included:

- Switching the original horizontal GEMBE design to a vertical configuration. All GEMBE instruments built in our laboratories possessed a design in which the capillary was horizontal to ground; to circumvent contamination and to enable the use of an automated stage, the configuration was changed such that the capillary is vertical. As a result, we do not have any issues with run-to-run contamination and we were able to build a device that includes an extraction and delivery stage—no human intervention during the extraction is necessary.
- Performing side-by-side experiments using a fluorescence microscope as the detection mechanism in a horizontal GEMBE configuration while also performing experiments with the device in a vertical configuration. Results from these experiments indicated that the vertical apparatus yielded data that was consistent with the horizontal apparatus.
- Extracting DNA from clean buffer solutions using vessel switching. Sample extracted in this manner were further analyzed using qPCR. This method of extracting and delivering DNA was a precursor method to the autosampling and delivery stage, and was a useful tool for determining whether carryover was an issue.
- Extracting DNA from dirty solutions and from dirty buccal swabs.
- Designing and building an LIF detector that provides a limit of detection of approximately 11-33 pg DNA.
- Integrating a contactless conductivity detector with the LIF detector. This detection system allows for the detection of the fluorescent DNA plug as well as the detection of the interface between which the DNA focuses.
- Total integration of all hardware and software components into a seamless instrument that extracts DNA from crude matrices and delivers it into a fresh vessel for further downstream analysis.

In addition to designing and building the instrument to the specifications outlined in this document, we have also performed additional experiments with other crude matrices; namely, bone samples, gum samples, and plant material. The work performed in support of developing procedures for extracting DNA from these matrices include:

- Determining methods for increasing the extraction efficiency. Extraction efficiency was ultimately increased by adding a negatively-charged surface coating to the capillary, increasing

the extraction time, and increasing the number of extractions into the same delivery tube. In addition, we have shown that we can extract DNA from volumes of solution that are as small as 5 μL.

- Developing a method for the extraction of DNA from bone. DNA was extracted from fresh pig bone and was quantified using qPCR. LIF results indicate that DNA was extracted from human bone from remains that are decades old, though PCR results indicate that the DNA is inhibited. Experiments performed using various buffer systems indicate that an alternate trailing electrolyte, 12.5 mM Tris acetate, or 12.5 mM Tris oxalate, should be used in future experiments. Further, experiments indicate that more DNA is potentially extracted from samples when the leading electrolyte and trailing electrolytes are different in conductivity. The concentration of the leading electrolyte should therefore be increased to 250 mM Tris 125 mM Cl^-.
- Developing a method for extracting DNA from chewing gum. DNA was extracted from chewing gum and was quantified using qPCR. Experiments performed using various buffer systems indicate that 12.5 mM Tris lactate should be used as the trailing electrolyte.
- Developing a method for extracting plant DNA from plant material. Spinach DNA was extracted from both fresh and dried spinach stems and leaves and quantified using qPCR. Results indicate that 12.5 mM Tris lactate is an appropriate trailing electrolyte and that the amount of DNA extracted varies from batch to batch. Further, experimental results indicate that DNA is extracted from dried *Papaver setigerum* stems; however, the results were not quantified with qPCR because genomic DNA from *P. setigerum* is unavailable.

Future work will include a systematic downselect of additional buffers for use as leading and trailing electrolytes. In addition, chemical modifiers will be explored to potentially decrease the electrophoretic mobility of DNA to possibly further reduce inhibitors from the extracted sample.

5. Recommendations

5.1. Extraction of DNA from a Wide Range of Crude Sample Matrices

The work completed by the ARA-NIST team utilized a dirty buccal swab (approximately 50-100 mg soil adhered to a freshly-collected buccal swab) as its notional sample. The soil that was used for all experiments was collected from under a small grove of oak trees on the NIST campus. This soil is fairly high in organic material content and low in salt content versus soils found in, for example, more acrid environments. In addition, the soil used in our experiments contained bacteria that are likely to not be present in, for example, sandy soils. Because of the chemical differences between soils found in different parts of the world as well as the differences bacteria content in various soils, an obvious follow-on project utilizing the DRAGEN instrumentation is the extraction of human DNA from cells in various soils. Experiments should focus on the extraction of human DNA from silty, loamy, and sandy soils. Further, bacterial content and identification should be a known variable in these extractions because bacterial cells could potentially lyse in the lysing solution and contribute to the overall mass of DNA extracted from the sample. Results from these experiments will allow the ARA-NIST developers to better tune the extraction parameters based on the chemical and biological constituents of the sample.

Determining the extraction parameters for human DNA in other crude samples, such as sludge, plant matter, and food materials is a relevant topic for follow-on experimentation. Extraction of DNA from these materials may require slight modifications to the extraction program. For example, there are very likely molecules in these matrices that will focus similarly to DNA, thus potentially interfering with the extraction or subsequent PCR analysis. Further research using these and other matrices is required to determine if the DRAGEN method will be favorable for a wide variety of crude matrices.

5.2. Extraction of Trace Quantities of DNA from Crude Environmental Matrices

Buccal swabs typically contain approximately 5 μg DNA from epithelial cells; these cells are then lysed and the DNA is extracted from the lysate. Currently, we routinely collect upwards of 5 ng of human DNA per extraction when the sample is a dirty human buccal swab, and we collect that DNA in approximately 5 min using the DRAGEN procedure. A clear follow-on project to our current work is to increase the extraction efficiency to collect a larger fraction of the DNA in a sample. This would extend the applicability of the technique to the analysis of samples containing only trace quantities of DNA.

Extracting more DNA with the current instrumentation may be possible by simply extracting multiple times at different positions within the sample fluid. Because the extraction efficiency of the DRAGEN system per unit time is competitive with other techniques, it is potentially possible to modify the extraction program to extract for longer times and extract the full volume of the capillary over several extractions in the same sample. This will increase the time required for extraction but will allow for the extraction of more DNA per sample as well as the extraction of very small amounts of DNA within a sample. In addition to repositioning the capillary between extractions, further work in this direction would be likely to include a detailed study regarding the positioning of the electrode with respect to the capillary end as well as the shape of the electrode used to best spread the electric field lines throughout the full volume of the sample tube to extract as much DNA as possible.

An additional approach to collecting larger amounts of DNA from a given sample is to utilize a multi-bore capillary rather than the single-bore capillaries that are used in the present work. Multi-bore capillaries are available from several commercial sources. The hypothesis behind using a multi-bore capillary is that, because there are several, rather than one, capillaries into which the DNA may focus, the amount of DNA extracted will thus multiply with the number of bores. This will increase the likelihood of extracting trace quantities of DNA and will increase the overall amount of DNA per extraction without increasing the time for extraction. Thus, the extraction efficiency would potentially increase without increasing the time required for extraction.

Extracting trace quantities of DNA from crude samples will require a more sensitive detector than what is currently utilized in order to better detect small quantities of DNA. A more sensitive detector may include a higher-power laser or a better focused laser. In addition, it may be possible to better optimize the detector electronics to eliminate baseline noise.

5.3. Extraction of DNA from Human Samples (Blood, Semen, Hair, etc.)

While the current project is focused on extracting DNA from a dirty buccal swab containing healthy epithelial cells, the ability to extract human DNA from other human samples in dirty matrices for, in some cases, post-mortem identification purposes would be a worthwhile and challenging follow-on project. A project that aims to extract DNA from blood, semen, hair, teeth, and tissues would require first improving the extraction efficiency of the DRAGEN technique. The methods for improving the DNA extraction efficiency were discussed above; namely, repositioning the capillary within the sample extraction-to-extraction, optimizing the placement of the electrode, and using multibore capillaries during the extraction process. These methods would be assessed in the laboratory and further developed for trace DNA extraction.

In addition to the necessity of increasing the efficiency of the extraction process, it will also be necessary to develop digestion protocols specific to the samples requiring analysis; these digestion protocols must be applicable to the reagents chosen for the extraction. It may be necessary to modify the extraction protocol to accept buffers that are more aggressive for digestion purposes or modify known digestion protocols such that the digestion product can be readily incorporated into the DRAGEN system. Both

instances will require a thorough investigation of reagents required for various digestion protocols and reagents suitable for the GEITP focusing method.

5.4. Field-Portable DNA Extraction Apparatus

For a future effort, decreasing the footprint of the tabletop device to one that is amenable to a hand-held size is proposed. The fluid reservoirs and capillary can be decreased in size fairly easily; the pressure controller, high voltage power supply, and detector will require significant modifications to their design to afford a size decrease. With respect to the pressure controller and the high voltage power supply, there are several manufacturers of pressure controllers and power supplies that are available off-the-shelf—they will need to be integrated into the device. The detector that is utilized in the tabletop system is based on fluorescence; it has been theorized, however, that a commercial conductivity detector can potentially be used to detect the appropriate amounts of DNA in slightly smaller diameter capillaries. Modifying the electronics of the detector built for the current project such that its limits of detection are feasible for 1-5 ng DNA will be necessary. If conductivity detection in a hand-held design is not feasible, detection of the DNA in a handheld device can be achieved using fluorescence if necessary. The detector must be decreased in size; it will be necessary to utilize a light emitting diode (LED) as the interrogation light and a photodiode as the detector.

With the current table-top system, the DNA is collected and dispensed through the same end of the extraction capillary. This is convenient for delivery of purified DNA to small PCR tubes for further analysis, but requires moving parts (a three-axis motion stage) that would be undesirable in a hand-held device. Alternate methods for DNA delivery for a hand-held system would be explored including the use of a microfluidic chip (with separate extraction and delivery channels) in place of the simple fused silica capillary.

5.5. Extraction of DNA from Biothreats in Complex Matrices

A logical follow-on project to the current work is the extraction of bacterial DNA from complex matrices such as environmental samples and food products. The tasks involved with this project first require improving the extraction efficiency of the DRAGEN technique. The methods for improving the DNA extraction efficiency include repositioning the capillary within the sample extraction-to-extraction, optimizing the placement of the electrode, and using multibore capillaries during the extraction process. In addition to improving the extraction efficiency, it will be necessary to improve the digestion/lysis protocols utilized in the current work due to the necessity of identifying the presence of spores in the sample matrix. Further experimentation will also involve the extraction of DNA from a wide variety of matrices, including different types of soils and foodstuffs.

An additional approach for this follow-on project is the potential of incorporating a sequence-specific detection mechanism to the DNA extraction protocol. For sequence-specific detection, the use of molecular beacons will be necessary. A molecular beacon is a hybridization probe with a sequence complimentary to a specific target. The probe contains two molecules at either end—a fluorophore and a quencher. When the probe is in free solution, the quencher molecule is in close enough proximity to the fluorophore so as to quench its fluorescence. However, when the probe molecule hybridizes with a specific nucleic acid strand, the quencher molecule is no longer in close proximity to the fluorophore and the fluorophore will fluoresce. Thus, an increase in fluorescence indicates that DNA specific to a given entity is present in the sample. Molecular beacon technology coupled with DNA extraction using the DRAGEN technique could be a powerful detection tool for BWA.

5.6. Integration of DNA Extraction Apparatus with a Complete Forensic DNA Analysis System

The DRAGEN system, in its current configuration, was made to extract DNA from a crude sample and then deliver the purified DNA into a clean sample tube for further analysis and/or STR typing. A logical follow-on project to this work is to utilize the DRAGEN technology as the front end for a complete rapid DNA analysis system for forensic use.

6. References

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