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Study of microtip-based extraction and purification of DNA from human samples for portable devices

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Abstract

Study of microtip-based extraction and purification of DNA from human samples for portable devices

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DNA sample preparation is essential for genetic analysis. However, rapid and easy-to-use methods are a major challenge to obtaining genetic information. Furthermore, DNA sample preparation technology must follow the growing need for point-of-care (POC) diagnostics. The current use of centrifuges, large robots, and laboratory-intensive protocols has to be minimized to meet the global challenge of limited access healthcare by bringing the lab to patients through POC devices.

To address these challenges, a novel extraction method of genomic DNA from human samples is presented by using heat-cured polyethyleneimine-coated microtips generating a high electric field. The microtip extraction method is based on recent work using an electric field and capillary action integrated into an automated device. The main challenges to the method are: (1) to obtain a stable microtip surface for the controlled capture and release of DNA and (2) to improve the recovery of DNA from samples with a high concentration of inhibitors, such as human samples.

The present study addresses these challenges by investigating the heat curing of polyethyleneimine (PEI) coated on the surface of the microtip. Heat-cured PEI-coated microtips are shown to control the capture and release of DNA. Protocols are developed for the extraction and purification of DNA from human samples. Heat-cured PEI-coated microtip methods of DNA sample preparation are used to extract genomic DNA from human samples.

It is discovered through experiment that heat curing of a PEI layer on a gold-coated surface below 150°C could inhibit the signal of polymerase chain reaction (PCR). Below 150°C, the PEI layer is not completely cured and dissolved off the gold-coated surface. Dissolved PEI binds with DNA to inhibit PCR. Heat curing of a PEI layer above 150°C on a gold-coated surface prevents inhibition to PCR and gel electrophoresis. In comparison to gold-coated microtips, the 225°C-cured PEI-coated microtips improve the recovery of DNA to 45% efficiency. Furthermore, the 225°C-cured PEI-coated microtips recover more DNA than gold-coated microtips when the surface is washed.

Heat-cured (225°C) PEI-coated microtips are used for the recovery of human genomic DNA from whole blood. A washing protocol is developed to remove inhibiting particles bound to the PEI-coated microtip surface after DNA extraction. From 1.25 μL of whole blood, an average of 1.83 ng of human genomic DNA is captured, purified, and released using a 225°C-cured PEI-coated microtip in less than 30 minutes. The extracted DNA is profiled by short tandem repeat analysis (STR).

For forensic and medical applications, genomic DNA is extracted from dried samples using heat-cured PEI-coated microtips that are integrated into an automated device. DNA extraction from dried samples is critical for forensics. The use of dried samples in the medical field is increasing because dried samples are convenient for storage, biosafety, and

contamination. The main challenge is the time required to properly extract DNA in a purified form. Typically, a 1 hour incubation period is required to complete this process. Overnight incubation is sometimes necessary.

To address this challenge, a pre-extraction washing step is investigated to remove inhibiting particles from dried blood spots (DBS) before DNA is released from dried form into solution for microtip extraction. The developed protocol is expanded to extract DNA from a variety of dried samples including nasal swabs, buccal swabs, and other forensic samples. In comparison to a commercial kit, the microtip-based extraction reduced the processing time from 1.5 hours to 30 minutes or less with an equivalent concentration of extracted DNA from dried blood spots. The developed assay will benefit genetic studies on newborn screening, forensic investigation, and POC diagnostics.

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DEDICATION

This dissertation would not have been possible without the support of my family and friends. I have a family whose love has provided a foundation for me to be successful. My friends have provided invaluable motivation to continue my other passions in life, while being supportive of my pursuit of a doctoral degree. And Alexandra Cali, who inspires me everyday to be a better person and provides immeasurable joy to my life.

Chapter 1. DNA SAMPLE PREPARATION

1.1 INTRODUCTION

DNA sample preparation is essential for genetic analysis. However, sensitivity, ease-of-use, and cost are major challenges moving forward[1]. Currently, methods for DNA extraction are cumbersome and require centrifugation or large, expensive robots that require routine maintenance. There are significant needs for simpler and higher-throughput methods to extract and purify DNA for medical, forensic, environmental, or military purposes[2]. Typical samples for DNA extraction are blood[3] (fresh and dried blood spots), saliva[4,5], and buccal swab[6,7]. The technology can be expanded to include dried samples, tissue samples[8], urine[9], animal samples[10], and plant matter[11].

The focus of this work is to study the use of a heat-cured polyethyleneimine (PEI) layer with an emerging method of DNA extraction using an electric field to concentrate DNA with microtips. The motivation for investigating DNA sample preparation and current methods will be discussed, along with the challenges that need to be addressed for the future of DNA sample preparation. Emergence of electric field-based DNA extraction is discussed with an emphasis on its use with microtips to separate DNA from solution.

The main objective of this work is to evaluate the use of heat cured PEI coated onto a microtip surface to improve the recovery of DNA from complex biological samples. The conclusion of this work provides an understanding of the effect of heat cured PEI for DNA sample preparation, the role of PEI in microtip-based DNA extraction, and demonstrates the purification of DNA from human samples for newborn screening, forensic analysis, and POC diagnostics.

1.2 MOTIVATION

DNA is everywhere and our understanding of genetic information has improved immensely over the last twenty years. Genetic information has shown notable impact in disease diagnostics, prenatal care, and crime scene analysis. Consequently, the utility of genetic information has catalyzed significant growth in these fields.

1.2.1 *Motivation in medicine*

In 2012, the Center for Disease Control (CDC) reported that cardiovascular diseases (782,985 deaths), cancer (582,623 deaths), and diabetes (73,932 deaths) account for 50% of deaths in the United States[12]. Moreover, heart conditions and cancer have been the leading causes of death for decades in the US (Fig. 1) and thus the leading focus of disease detection, evolution, treatment, and cure. Detection methods for cardiovascular disease and cancer include symptom-based diagnosis, cell culture diagnosis, biopsy, and imaging. The treatment for cardiovascular disease involves medication, surgery and device therapies, while oncological therapies involve surgery, chemotherapy, and radiation therapy. These methods can be highly invasive, and inaccurate due to their mainly qualitative nature and ineffective due to the nonspecific design. The ideal scenario for diseases such as cancer is (1) early detection through non-invasive molecular diagnostics from easily attainable samples or in vivo molecular imaging methods, (2) a complete, accurate biological understanding of the disease and host, and (3) specific and selective methods for efficient personalized prevention and treatment[13].

Advances in microscale and nanoscale technology have been a catalyst for understanding at a molecular level. The human genome project (HGP) is the world's largest

collaborative effort to study a single molecule. The HGP has spurred the identification, characterization, and quantification of nucleic acid sequences. Utility of genetic information toward many chronic conditions, including cardiovascular diseases, cancer, diabetes, and psychiatric disorders has propelled the world market for molecular diagnostics to be an estimated $5 billion in 2012 and projected to grow to $7.6 billion by 2017[14]. Molecular diagnostics has found primary clinical markets in oncology, histological analysis, women's health, infectious diseases, and organ transplant testing[15]. Genetics provides a foundation for molecular diagnostics to meet the challenges of current methods for detection, evolution, treatment, and cure[3]. Consequently, this market will require simple, rapid methods for the extraction and purification of DNA to meet the challenges molecular diagnostics are striving to solve.

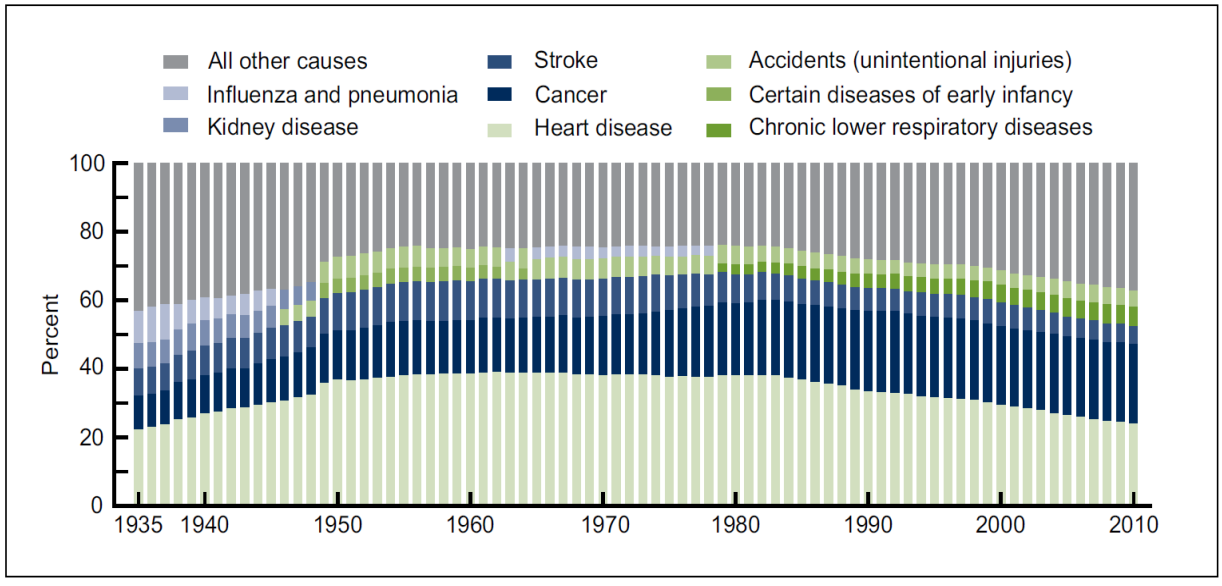

Figure 1. Percentage of deaths in the United States from 1935-2010 (CDC/NCHS, National Vital Statistics System, Mortality)[16].

1.2.2 *Motivation beyond medicine*

The molecular diagnostics market goes beyond medical applications to include use in the criminal justice system and veterinary medicine[7]. Furthermore, the use of genetic information has become increasingly important to the Food and Drug Administration's (FDA) regulation of food mislabeling[17,18], along with the agricultural industry[19].

DNA diagnostics has become an increasingly important tool in the criminal justice system; however, efficiency of processing samples is a major challenge as highlighted by the National DNA database. Saliva is the most processed sample by the National DNA database, but only 37% of samples provided suitable profiles[20]. The extraction and purification of DNA is crucial to providing suitable DNA profiles needed for criminal identification. To improve DNA diagnostics in criminal justice, DNA sample preparation methods need to eliminate inhibitors that hinder analytical tools and obtain sufficient quantities of DNA[21]. Furthermore, addressing the bottleneck of forensic casework through a reduction in the time and cost of DNA sample preparation is critical to the forensic community[22].

Food mislabeling has become a growing concern over the past few years. In 2007, the mislabeling of toxic puffer fish as a monkfish hospitalized a woman for over 3 weeks after ingestion[23]. Additional concerns include multiple reports of DNA barcoding that show high percentages of mislabeled fish in markets and restaurants[24-26]. As a result, the FDA is increasing their funds to ensuring seafood is "safe, wholesome, and properly labeled."[27] Starting with identification libraries (Regulatory Fish Encyclopedia, Barcode of Life, and the Fish Barcode of Life campaign)[28], the FDA is collaborating with the University of Guelph and the Smithsonian National Museum of Natural History to provide a standardized method for DNA barcoding[29]. Considering the efforts of the FDA and the Safety and Fraud

Enforcement for Seafood Act (H.R. 1012) being referred to Committee, improved DNA sample preparation methods will be necessary to support these efforts.

1.2.3 *Motivation for point-of-care diagnostics*

The cost of healthcare is a major concern in the United States. The equipment, facilities, experienced technicians, and time of testing are all major contributors to the high costs attributed to healthcare. Furthermore, one of the grand challenges of healthcare is to remove the barrier of providing appropriate services to underdeveloped countries[30]. Diseases such as tuberculosis are most prevalent in countries that do not have the equipment, facilities, or experienced personnel needed for diagnosis, treatment, and cure[31,32].

Point-of-care (POC) diagnostics meet the challenges of healthcare by bringing the "lab" to patients. Loosely defined, POC tests can be described as bed side, near patient, physician's office, extra-laboratory, decentralized, off site, satellite, kiosk, ancillary, alternative site, or self-test[33]. With the use of POC devices, the healthcare industry will be able to reduce, or eliminate, traditional facilities, equipment, and personnel. The challenge of costly healthcare can be addressed through developing simple, portable devices for nucleic acid POC diagnostics[34], which currently are not available. DNA sample preparation will be held to the same criteria in aiding the growth of POC diagnostics.

1.3 METHODS FOR DNA EXTRACTION

The complex matrices that DNA is typically found require methods of extraction for successful use in downstream applications. Aqueous-based biological samples typically include a complex of cells, small molecules, ions, proteins, lipids, carbohydrates, and unwanted nucleic acids[35]. Solid (or dry) samples, such as blood stains, tissues, and plant

matter can contain an inexhaustible variety of dirt, fibers, biological materials, and environmental substances that further complex the target sample. The first method to extract DNA from the many components of a complex sample was discovered by Friedrich Miescher in 1869[36].

Miescher's initial method extracted purified DNA from patient surgical bandages. First, samples are disrupted by eluting the pus off the bandages using Glauber's salt. Then, the cells are allowed to sediment and the supernatant is removed containing a majority of the unwanted, inhibiting particles. Then, isolated cells are put through a hydrochloric acid wash to remove the cytoplasm and vigorously shook in ether to lyse the cell lipids. Acetic or hydrochloric acid is used to precipitate DNA for isolation[37]. Miescher's method paved the way for the future of DNA sample preparation by addressing the four key challenges to the extraction of DNA: (1) disruption and lysis, (2) denaturing of proteins, (3) inactivation of unwanted nucleases, and (4) isolation of DNA from all contaminates[38].

A generalized description of these processes, common techniques, and most used chemicals for the extraction of DNA are described in Section 1.3.1. An in-depth look at conventional and solid-phase extraction methods, along with the challenges that still need to be addressed can be found in Section 1.3.2 and 1.3.3. These challenges motivated the use of electric field-based extraction methods (Section 1.3.4) to improve DNA sample preparation. Tip-based extraction is a recent technique that extracts DNA using electric fields generated through high-aspect ratio tips. Tip-based DNA extraction is used in this work for simple, timely preparation of purified DNA.

1.3.1 *DNA sample preparation process flow*

In general, the extraction of DNA from collected samples for use in downstream applications follows a simple process flow: (1) preparation, (2) separation, (3) purification, and (4) isolation.

(1) *Preparation*

The key to DNA sample preparation is the variety of separation techniques from ethanol precipitation to centrifugation to solid-phase binding. However, as described in Section 1.3.2, 1.3.3, and 1.3.4, DNA separation techniques require highly controlled solution properties such as density, conductivity, salt concentration, and pH. Therefore, the preparation of collected samples is critical to proper separation of DNA and includes a variety of mechanical, thermal, and chemical processes. The aim of the preparation step is to (1) release DNA into solution form through disruption and lysis mechanisms, (2) denature proteins, (3) deactivate nucleases, and (4) tune the solution properties for optimal DNA separation conditions.

The first step in preparing collected samples is to freely disperse DNA into solution through disruption and lysis of collected complex matrices. To date there are no methods of extracting DNA from a solid sample directly, so tissues, plant matter, and dried biological samples must be eluted into solution form. Miescher's work provided the foundation for the solubility of DNA by dissolving DNA precipitate using an alkaline solution, sodium carbonate[37]. Currently, Tris-EDTA (TE) Buffer is the standard elution solution for dry samples[39]. TE Buffer is made up of tris(hydroxymethyl)aminomethane [$(HOCH_2)_3CNH_2$] - hydrochloric acid (Tris-HCl) and Ethylenediaminetetraacetic acid [$C_{10}H_{16}N_2O_8$] (EDTA). Tris-HCl has a buffering range

from 7-9 pH allowing for the formulation of a pH stable weak base for the dissolution of DNA. EDTA is a chelating agent[40] and is used to suppress the reactivity of metal-dependent enzymes, such as DNase[41], that could cause damage to DNA.

In most samples, DNA is contained within cells and lysis is performed to release DNA freely into the solution. Sodium dodecyl sulfate (SDS) is a common detergent used to lysis cell membranes[35,42-44]. SDS also provides the additional function of denaturing proteins. Proteinase K is another widely known treatment of samples. Proteinase K digests proteins which are bound to cell membranes causing lysis, along with digesting inhibitory proteins[43]. Other chemical lysing methods include salts such as guanidine that cause osmotic shock to cells and denature proteins aiding in the cell lysis process. Mechanical and thermal methods are used to lyse cells through heating, freezing, bead milling, and ultrasonication[35].

Once DNA is dispersed freely within the solution, contaminants and DNA must be separated to purify the DNA. Fortunately, lysing agents such as SDS, Proteinase K, and guanidine salts also denature proteins and deactivate nucleases. The addition of Proteinase K improves DNA extraction[8,43] by preventing hydrolysis of DNA by cellular nucleases[35]. Agricultural samples can be rich in polysaccharides which inhibit polymerase activity[45]. Polymerase is an important function to polymerase chain reaction (PCR) analysis, which is used to amplify and detect specific DNA sequences. Cetyltrimethylammonium bromide (CTAB) is a cationic surfactant that forms a complex with polysaccharides to precipitate out of solution, aiding in the preparation process of DNA[46]. Due to the multifunctional activity of TE buffer, surfactants, salts, and

proteinase treatments, the process of lysing and denaturing of inhibitors is typically done simultaneously in the preparation step.

Finally, the sample is treated to provide specific solution properties for separation. For DNA precipitation, a sensitive range of alcohol and salt concentration is needed (See *Separation* segment below). For gradient extraction, the density of the medium is critical to separate DNA from contaminants (See Section 1.3.2). For silica extraction, chaotropic salts are added to the sample to induce DNA-silica binding (See Section 1.3.3). The preparation step is complete when DNA is properly released into solution form through disruption and lysis mechanisms, proteins and nucleases have been treated, and the solution properties are tuned for optimal DNA separation conditions.

(2) *Separation*

The separation step is the physical separation of DNA from a complex target matrix and the added reagents from the preparation step. The first method for separating DNA from the rest of the sample by Miescher required a long sedimentation process. DNA separation has been accelerated through centrifugation techniques demonstrated in early hot trichloroacetic acid DNA extraction[47]. A variety of solution treatments allows DNA to separate from contaminants into different layers due to differences in density after centrifugation. The use of centrifugation has become the primary method for DNA separation and isolation, highlighted by the work of Meselson and Stahl on density gradient centrifugation to isolate DNA in solution[36]. The initial methods of DNA extraction highlighted here by Miescher, Meselson, and Stahl's work are the foundation for what is now defined as solution-based (Conventional) methods for DNA separation.

Recently, Solid-Phase Extraction (SPE) methods[48] have been developed to improve the speed, quality, and quantity of extracted DNA. SPE methods bind DNA to a solid surface and separate the DNA by removing the solid from the solution. Innovation has focused on microporous filters imbedded in spin columns and magnetic beads to separate DNA from samples. The advantages of spin column and magnetic bead extraction methods have led to commercially available kits for the separation of DNA from complex samples.

(3) *Purification*

In the separation step, the goal is to isolate DNA from any potential inhibitors, providing a pure solution for downstream applications. In reality, no separation technique is specific to DNA only. Inevitably, some inhibiting particles will remain with the separated DNA. Hence, the purification step is critical to further remove inhibitors. Conventional methods separate DNA in liquid form and should be pipetted out to physically separate from the sample. Repeating the centrifugation step, or combining multiple treatment methods will enhance the purity of the DNA. Protein denaturation, deactivation of nucleases, and further treatment for inhibitors can improve the purity of the separated DNA.

One of the major advantages of SPE methods is the purity of the final DNA solution. The DNA-bound surface can be aggressively washed (e.g. centrifugation to push washing solutions across the surface) to remove inhibitors, but still retain a usable amount of DNA. The ease and quickness of washing procedures have aided in the success of SPE methods.

(4) *Isolation*

The isolation (or elution) of DNA is done to provide a usable form for downstream analysis. TE buffer is the most common medium for the suspension of pure DNA. For conventional methods, DNA is typically isolated using a combination of DNA precipitation and centrifugation. Precipitation of DNA is most commonly done using ethanol and isopropyl alcohol[35,49]. In the presence of salt (Sodium Chloride or Sodium Acetate), ethanol and isopropyl alcohol increase the electrostatic attraction between the salt ions and the phosphate backbone of the DNA, neutralizing the DNA. Due to its negatively charged phosphate backbone, DNA is hydrophilic and water soluble. Ethanol and isopropyl alcohol disrupt the electrostatic interaction between the polar water molecule and negatively charged DNA molecules. This allows the positively charged ions (salts) to form bonds with the DNA phosphates. The neutralization due to the salt ions causes the DNA to become hydrophobic and precipitate. In practice, ethanol is added to the separated and purified volume of DNA causing the DNA to precipitate. The DNA is pelleted at the bottom of the vial through centrifugation and the ethanol is removed. The pelleted DNA is then rehydrated in TE buffer. The centrifugation technique differs from the separation step. The separation step concentrates DNA in the upper regions of the solution, while the contaminants are pulled to the lower region of the solution. This allows for the upper region of separated DNA to be removed from the rest of the sample for further processing. However, the addition of ethanol causes DNA to aggregate and the larger precipitates of DNA are pulled to the lower region forming a pellet of DNA.

For SPE methods, DNA needs to be unbound from the solid surface used for extraction. DNA is eluted (unbound from the solid surface) into solution through the use of pH control, surface charge manipulation, heat, and fluid flow.

1.3.2 *Conventional methods*

Conventional or Liquid-Liquid DNA extraction methods extract, purify, and isolate DNA within liquid form. As described below this is accomplished with centrifugation. Cesium Chloride gradient centrifugation, Phenol-Cholorform, Salting-out, Alkaline, and Chelex are the most common conventional DNA extraction methods and are described below.

(1) *Cesium Chloride (CsCl) Gradient Centrifugation*

Gradient centrifugation extraction methods separate DNA by molecular weight in an equilibrium gradient solution[50]. An equilibrium density gradient solution is created due to centrifugal pressure effects on a compressible fluid from ultracentrifugation. The density gradient of Cesium Chloride (CsCl) due to ultracentrifugation fits most macromolecules effective density in solution. This is because Cs^+ atoms have a high molecular weight and are highly soluble in water, allowing for a relatively low concentration of CsCl in solution and a clearly defined density gradient[51]. An intercalating dye, Ethidium Bromide (EtBr), is used to differentiate bands of DNA within the density gradient[52]. Separate bands for chromosomal DNA, plasmid DNA, rDNA and organellar DNA can form and be isolated by CsCl gradient centrifugation (Fig. 2). Therefore, CsCl gradient centrifugation became the standard for the isolation of highly pure DNA since its development in the 1950s[53].

The preparation of the target DNA solution for CsCl gradient centrifugation involves the disruption and lysis of cells to release DNA freely into solution. Mechanical grinding[54] and surfactants[55] are typically used. Surfactants, such as SDS, also act to denature proteins and EDTA is typically added to deactivate nucleases[52]. Finally, CsCl and EtBr are added to the target solution for separation.

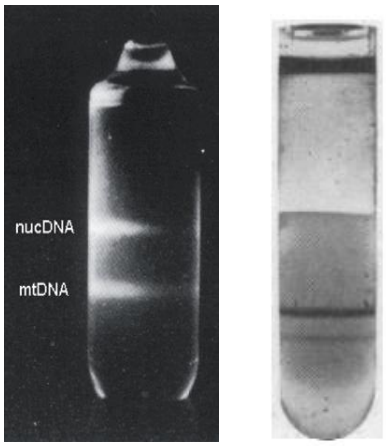

Figure 2. Image of the separation of DNA using CsCl gradient centrifugation techniques[52,53].

The separation of DNA happens through ultracentrifugation. Ultracentrifugation with CsCl will create a density gradient causing the DNA to migrate dependent on molecular weight. The centrifugation commonly takes between 24-48 hours to reach the isopycnic point[53]. After centrifugation, DNA is isolated in bands close to the center of the gradient, isolated from proteins which typically float to the top and RNA which migrates to the bottom[55]. DNA is extracted from the centrifuge tube using a drop collector to separate DNA from the original solution[52] or a needle and syringe can pierce the tube used for centrifugation to extract the desired band containing DNA[35].

Purification of the extracted sample is required to remove EtBr and dilute the concentration of CsCl. EtBr is extracted through n-butanol and the sample is diluted in

TE buffer to reduce the CsCl concentration[56]. Then the DNA is precipitated in 95% ethanol and the pellet can be further washed. The purified DNA pellet is dissolved and isolated in a buffer solution for downstream applications.

The advantages of CsCl gradient centrifugation are providing a high yield of DNA with high purity. However, the assay cost is high because CsCl and EtBr are expensive materials. Based on a standard protocol for CsCl extraction[35] and the current prices (2015) of research grade materials, the assay cost can be $4-$6. The use of EtBr in the process can increase the assay by $2-$4. Additionally, the overhead cost of an ultracentrifuge can be a significant disadvantage to using this process. Furthermore, the 24-48 hour processing time and use of EtBr, which is a mutagen, are further drawbacks to this sample preparation method.

(2) *Phenol-Chloroform Extraction*

Phenol-Chloroform develops a phase separation in solution to isolate nucleic acids when centrifuged. The solution separates into an organic (lower) phase and an aqueous (upper) phase. The lower organic phase develops due to the higher density of phenol (1.07 g/cm^3) than water. Chloroform is miscible with phenol and creates a sharper phase transition with water due to its high density (1.47 g/cm^3)[57]. The polar nature of nucleic acids forces them to the upper aqueous phase, dissolving readily in the presence of polar water molecules. Other cell lysates interact with phenol, moving to the lower organic phase. At a 5:1 phenol-chloroform ratio, the acidic nature of the solution will cause DNA's charge to be neutralized and DNA will also move to the organic phase[58]. Therefore, Phenol-Chloroform extraction methods are most commonly used to isolate RNA[58-61]. However, at a 1:1 ratio, DNA will be isolated with RNA[57], making phenol-chloroform a viable method for DNA extraction[62].

The preparation of the target DNA solution for Phenol-chloroform phase extraction involves the disruption and lysis of cells to release DNA freely into solution through heat incubation with reagents such as Proteinase K, SDS, EDTA, and sodium acetate[63]. The solution is treated for separation with phenol and chloroform. The mixture of phenol with chloroform is also effective in denaturing proteins along with inducing a density gradient.

The separation of DNA is done through centrifugation. Typically, centrifugation is done in two steps. First, only phenol is mixed in the preparation step at equal volumes with the lysate and centrifuged. The aqueous phase is extracted and mixed with equal volumes of phenol and chloroform for a second centrifugation[64]. If any interphase exists, purification is accomplished by repeating the centrifugation process to ensure proper separation[35]. The aqueous phase is extracted and DNA is isolated by precipitation with ethanol[49].

Phenol-chloroform is the preferred method of DNA extraction in the forensics community due to its effectiveness with small amounts of DNA[65]. The significant reduction in centrifugation time reduces the process time to as little as 20 minutes[66]. However, long lysing procedures required for certain samples can significantly increase the processing time to over a day[67]. For forensic analysis, an overnight incubation is recommend for dried blood samples[68]. Phenol-chloroform extraction is a labor intensive process, which significantly reduces its cost effectiveness of using low cost reagents. Phenol is highly corrosive and requires the use of a fume hood, deterring its use.

(3) *Salting-out Methods*

The salting-out method is a common technique to purify DNA samples through the precipitation of proteins and other contaminants. Sodium chloride, potassium acetate,

lithium chloride, and ammonium acetate are common salts used to precipitate contaminants[49]. Preparation of the target DNA solution for salting-out involves denaturation and lysis. A high concentration of salt solution is then added to precipitate contaminants. The high salt concentration, typically 6 M NaCl[69], causes proteins to precipitate and form a pellet after centrifugation[70]. The separation of DNA is done through centrifugation and the inhibitors that are precipitated in the preparation step are removed to improve the purification of the sample. The isolation of DNA is done by precipitation with ethanol. The salting-out procedure has been tested on a variety of plant and animal tissues showing high purity (>1.77 A260/A280 ratio) of the DNA samples[71], as well as blood samples[70].

(4) *Alkaline extraction*

Alkaline solutions are known to denature DNA[72]. However, between a solution pH of 12~12.5 plasmid DNA will remain intact meaning a carefully adjusted alkaline solution can be used as a method for the isolation of plasmid DNA[44,73]. Plasmid DNA is resistant to alkaline denaturation due to its supercoiled circular shape[35]. Therefore, alkaline solutions are commonly used for the isolation of plasmid DNA in combination with the salting-out method and centrifugation. Genomic DNA will renature with neutralization of its surrounding alkaline medium[73]. Therefore, alkaline extraction is viable for more than plasmid DNA[74,75].

The preparation of the target DNA solution for alkaline extraction involves the denaturation and lysis with a SDS/NaOH solution. Using the alkaline solution for plasmid DNA extraction, pH needs to be accurately monitored to ensure NaOH does not also denature the plasmid DNA. Sodium acetate is added to precipitate contaminates. The sodium acetate is vital to the removal of contaminants because it causes the

chromosomal DNA to aggregate into an insoluble mass along with precipitating protein-SDS complexes and RNA[73]. For chromosomal DNA extraction, the solution must be neutralized. The separation of DNA is done through centrifugation. Purification is aided by the use of SDS, NaOH, and sodium acetate in the preparation step. The inhibitors that are denatured in the preparation step will be precipitated and removed after centrifugation to improve the purity of the sample. The isolation of DNA is done by precipitation with ethanol[49].

Currently, alkaline extraction is used for newborn screening by extraction of human genomic DNA from dried blood spots[76]. The novel CASM method uses a pre-extraction washing step to remove red blood cells and other inhibitors from the dried blood spots before the DNA is released off the swab. NaOH and heating are used to release DNA from the spots and denature residual inhibitors. The CASM method is shown to not cause significant damage to genomic DNA with use of 100mM NaOH in combination with Tween-20 and neutralization in a TE solution[77]. However, the eluted DNA solution containing NaOH needs to be significantly diluted to prevent inhibition of NaOH and Tween downstream. An ethanol precipitation is not performed in this method. Currently, this procedure is rapid (45~60 minutes) and easily adaptable because it follows the typical centrifugation-based procedure. Furthermore, the use of common and inexpensive reagents ($0.1/per sample) makes alkaline extraction an advantageous DNA extraction process.

(5) *Chelex 100*

Chelex 100 resin is formed through the copolymerization of styrene (vinylbenzene) and divinylbenzene combined with paired iminodiacetate ions. The iminodiacetate groups act as a chelating agent to bind polyvalent metal ions[78]. For DNA

extraction, Chelex 100 binds to metal ions and other cellular components that cause the degradation of DNA[79].

The process for DNA extraction using the Chelex 100 extraction method[80,81] starts with the preparation of the target solution by mixing with Chelex (typically 5%)[79]. The mixture is boiled to lysis the sample. At a high temperature (100°C), DNA denatures and can breakdown. The role of Chelex is to prevent the DNA from breaking down by chelating metal ions that may act as a catalyst for the breakdown of DNA[67]. Separation of the DNA is done by centrifuging the solution. The purification of DNA is done through repition of extracting the supernatant and centrifugation. The final supernatant contains isolated DNA that can be used for downstream applications.

The process can be completed in under 30 minutes for some samples, but requires over 2 hours for difficult samples such as dried blood spots[82]. The Chelex method has been shown to yield comparable DNA to organic methods[67] and Qiagen[79]. However, the denaturation of DNA is inhibitive for some downstream applications and Chelex needs to be thoroughly removed to not inhibit PCR.

1.3.3 *Solid-phase extraction methods*

SPE methods extract DNA in liquid form onto a solid surface. The DNA can be purified by washing the solid surface. DNA can then be released off the solid surface into liquid form for downstream analysis. As described below this is typically done on a silica surface. Microfiltration and magnetic beads are the most common SPE DNA extraction methods and are described below, along with two developing methods: anion exchange membranes and electric-field concentration techniques.

(1) *Silica microfiltration*

Silica has been shown to have a high affinity to nucleic acids,[83] however this interaction is highly dependent on the environmental condition of the solution[84]. DNA binds to silica in the presence of a high concentration of a chaotropic salt[48,85,86]. Therefore, the extraction of DNA through silica-DNA binding has been the foundation for many commercial protocols: Qiagen QIAamp®, Clontech NucleoSpin™, Mo Bio Laboratories Ultraclean™ BloodSpin™, Promega Wizard™, Akonni TruTip®, and Sigma Aldrich GenElute™.

Typically, the silica is manufactured in the form of a microporous filter. The filter is inserted into a spin column so the target solution can be centrifuged, forcing the solution to flow through the microporous filter (Fig. 3). This allows the DNA to flow past the silica surface so that binding can occur. The solution passes through the filter and is discarded, separating the DNA. Washing solutions are used to remove any inhibitors that can collect on the silica surface through the same centrifugation process.

Preparation of the target DNA solution for silica-DNA binding first involves denaturation and lysis through the use of Proteinase K, surfactants, and heat. A chaotrope and ethanol are added to the solution to allow for efficient binding of DNA to the silica surface. Chaotropic agents, such as guanidine thyocynate[48], sodium iodine[87], and sodium perchlorate[87], are critical to the binding of DNA to silica because the negative charge of silica is electrostatically unfavorable with negatively charged DNA. In the presence of water, silica holds a negative charge due to the dissociation of silanol groups[88]. More specifically, the surface charge of silica is negatively charged in the higher pH range and has been shown to be neutral at low pH (1.5-3.6)[89]. However,

different conformations and pronation states can increase the pH range of charge neutrality in silica. The addition of ions, such as salts, can shield the negative charge on the silica surface, reducing electrostatic repulsion[84]. Chaotropic salt has a strong affinity to water molecules reducing the hydration of silica and DNA, and promoting binding through electrostatic repulsion shielding[90]. The complex mechanism of adsorption of DNA onto the silica surface is not completely understood. Some research suggests alternative binding mechanisms through the hydrophobic effect, hydrogen bonding, and amino acids[91,92].

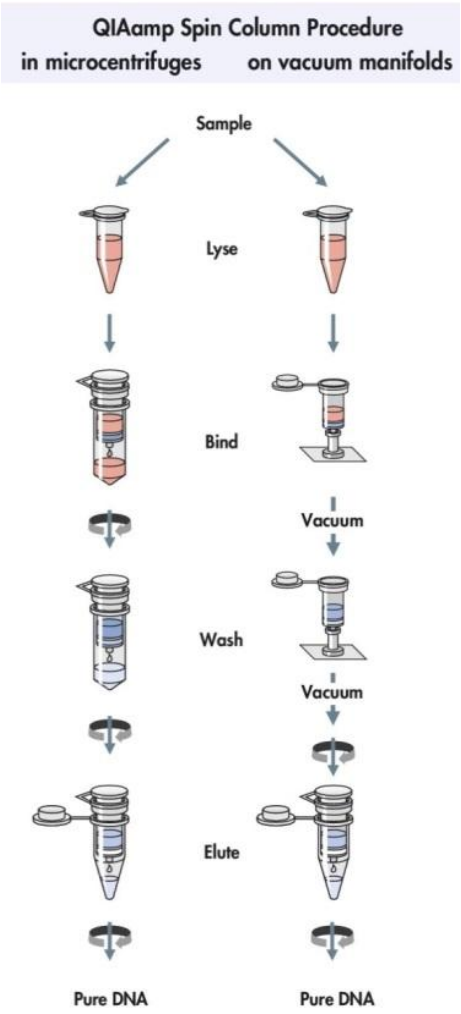

Figure 3. Silica microporous filtration method using Qiagen QIAamp® kit[93].

Microporous silica filters are the most common separation technique. The separation of DNA is done by allowing the solution to contact the silica. This is accomplished by centrifuging the prepared solution through the filter contained within a spin column. Some contaminants may non-specifically bind to the silica surface during this process. Also, the salts that induce binding of DNA to silica need to be washed away[38]. Washing procedures follow the separation step to remove these inhibitors from silica surface.

DNA is isolated by eluting from the silica using a low salt buffer. The isolated DNA is obtained quickly (< 1 hour), in a purified form with good yield. DNA can be degraded if alkaline solutions are used[72,94,95] and flow-induced shear of DNA[53] during centrifugation. The silica filter has a size-selectivity issue limiting recovery of small and large fragment DNA. A previous study[96] comparing microtip-based DNA extraction with a silica-filter based commercial kit showed that the commercial kit causes damage to DNA fragments, which can limit recovery (Fig. 4). Also, small fragments may be irreversibly bound to the silica filter, limiting DNA recovery. These results are acknowledged by the commercial vendor (Qiagen®) in their protocol description. Qiagen® suggests the use of the kit for the extraction of DNA up to 50 kbp, but states that 20-30 kbp is the predominate range of extracted DNA. Additionally, the stringent complex required within the solution to induce DNA binding to silica limits the application of silica-based filtration methods of DNA sample preparation.

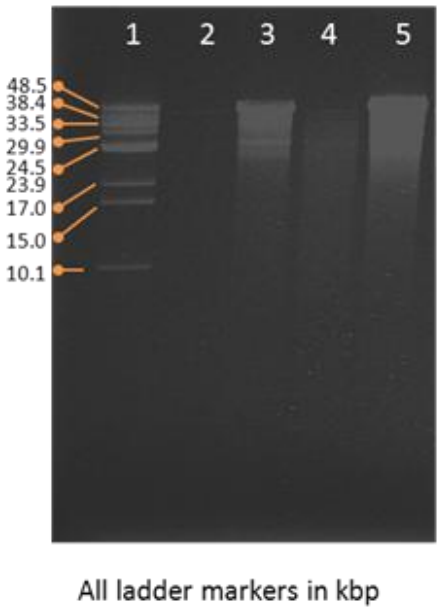

Figure 4. Gel electrophoresis of λ DNA recovered by a microtip device without an electric field (lane 2), with an electric field (lane 3), DNA by the commercial kit (lane 4) and 1/10th of original λ DNA sample (lane 5). Mono-cut λ DNA is used as the ladder (lane 1)[96].

(2) *Magnetic particle*

Magnetic particles that consist of a supermagnetic core, a protective coating, and a functionalized surface layer can be used for DNA extraction[90]. Extraction of DNA with the use of charged ligands and silica surfaces can be enhanced with magnetic particles, providing a large surface area for DNA binding[36]. When DNA is bound to the functionalized magnetic particle surface, it can be removed easily from the target solution and re-suspended in a pure form.

Preparation for magnetic-bead extraction requires that the target solution is lysed to free DNA into solution, along with denaturing proteins and nucleases that could affect the DNA. The magnetic beads are added to the lysate, and mixed to induce DNA binding. A magnet is applied to aggregate the particles, and the remaining solution is removed to separate the DNA. Washing buffers can be added as need to purify the DNA

on the magnetic beads. The DNA is isolated by adding a buffer to elute DNA off magnetic particles.

Extensive use of silica in filtration-based DNA extraction prompted its early adoption with magnetic particle extraction. The process for magnetic particle extraction eliminates the need for centrifugation[90] and has been scaled up to a fully automated 96-well platform. Magnetic bead extraction can accommodate a large range of sample volumes and has a high yield. The assay cost for magnetic beads is competitive; however the automated system requires a large initial investment and regular maintenance.

(3) *Anion-exchange*

The stringent conditions required to bind DNA to silica has motivated the use of positively charged ligands. A positively charged surface binds with the negatively charged phosphate groups that form the DNA backbone[97]. Consequently, contaminants and unwanted particles within the target solution are washed, isolating the DNA. The DNA is eluted from the positively charged surface in a high-salt solution. The salt neutralizes the charge of the phosphate groups, reducing the binding force to the positively charged surface and releases the DNA into solution. Typically, the positively charged resin is diethylaminoethyl cellulose (DEAE)[36].

In an effort to improve the capture and release of DNA from magnetic particles, different positively charged functionalization layers are being explored. Polyethyleneimine (PEI) is a positively charged polymer that is commonly used as a functionalization layer and has recently shown significant utility with drug delivery because of its affinity to DNA[98-100]. Polyethyleneimine-modified magnetic beads have been shown to be effective in the extraction and purification of DNA[101-104]. However,

surface layers such as PEI are difficult to coat on surfaces, and unstable, degrading over time or when placed in varied environments. The stability and repetition of anion exchange membrane performance needs further characterization for commercial viability.

(4) *Electric field based DNA extraction*

The manipulation of DNA in solution using an electric field[105] offers an alternative method to the extraction and purification of DNA from solution[106,107]. An electric field applied between two electrodes can induce flow due to electrophoretic, dielectrophoretic, and electroosmotic forces generated in a solution. The generation of flow and an attractive electric field force within a solution can concentrate particles such as DNA on the surface of an electrode, separating the particles from the rest of the solution. Dielectrophoresis (DEP) has received considerable attention because of the ability to tune the electric field to concentrate specific particles based on the polarization properties of the particle and medium[108].

The complex matrices of blood and saliva, which are of significant interest for DNA extraction, pose a great challenge to utilizing DEP for the specific concentration of DNA. Blood and saliva have a variety of inhibiting particles that can also be attracted. The difficulty of characterizing the exact electrical properties of the complex of particles and medium prevent a clear understanding of the optimal electric field needed for efficient DNA extraction. Continued experimental discovery and validation is needed to precisely control electric fields for optimal DNA extraction.

Preparation for electric field-based extraction requires that the target solution is lysed to free DNA into solution, along with denaturing proteins and nucleases that could affect the DNA. The solution must also be treated to ensure proper medium conductivity

and permittivity for application of the electric field. The solution is suspended between electrodes and the electric field is applied to concentrate and separate DNA onto the electrode surface. The electrode is removed from the solution and washing buffers are rinsed over the surface of the electrode to purify the DNA from inhibitors that may have been concentrated on the electrode surface. Then, the DNA is isolated by adding a buffer to elute DNA off the electrode.

In the preparation step, the lysis reagents may also be used to tune the conductivity of the solution to optimize the use of DEP. Dilution is a common method to reduce the medium conductivity[109], however, this also dilutes the concentration of DNA for volume restrictive inputs. The high conductivity of biological mediums has limited progress of DNA extraction using DEP.

Biological Dynamics has developed a microfluidic device, the ACE system[110], that has been used by the Heller Group[109,111] to concentrate DNA (Fig. 5). The ACE system uses platinum microelectrodes coated with a thin hydrogel in an array to apply an electric field through the target solution that is fed to the array through a fluidic channel fabricated on top of the microelectrode chip[109]. The microelectrode array can concentrate and purify high molecular weight DNA using an AC electric field (10 kHz, 20 V_{pp}). The AC electric field induces a positive DEP force on the DNA, while inducing negative DEP on blood cells. The separation, purification, and isolation process takes 10~20 minutes, plus the time needed to prepare the sample (cell lysis and denaturation). The limit of detection from blood is 280 pg/μL.

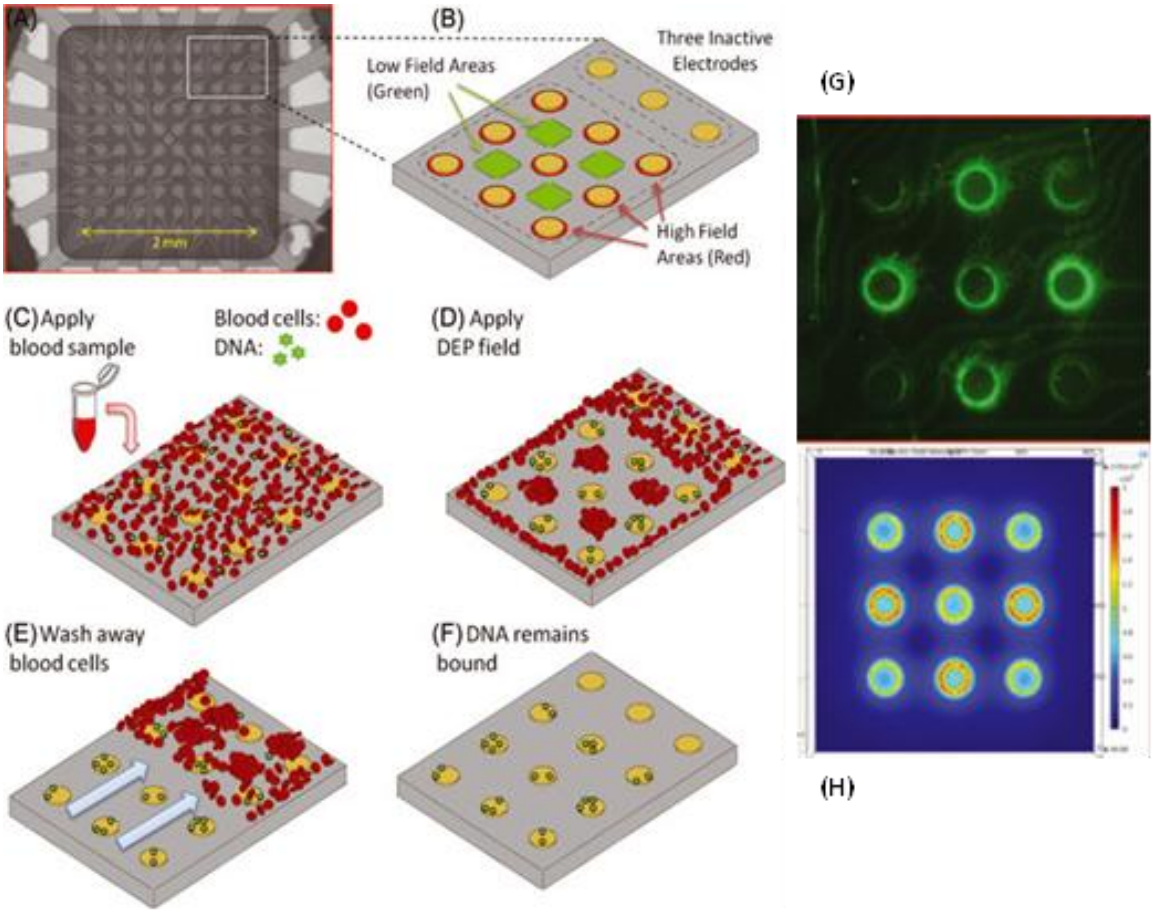

Figure 5. (A-F) Schematic and process flow for the separation of DNA from blood cells using the ACE system. (G) Concentration of SYBR Green I Dye stained DNA in rat blood to microarray electrodes. (H) Checkerboard application of high electric field to microarray[109].

1.3.4 *Tip-based DNA extraction*

High-aspect ratio tips have been shown to extract DNA with an applied electric field (Fig. 6). The concentration of particles to the surface of a tip is controlled through an applied electric field between an electrically conductive coil that holds the target solution and a tip. The applied electric field generates a combination of electrophoretic, dielectrophoretic, and electroosmotic-drag forces that manipulate particles in solution. Electrophoresis is determined by the electrostatic forces between particles and electrodes. DEP is generated due to the relationship of the electrical properties between the particles and the medium

(solution) under a non-uniform AC electric field. Electroosmosis results from the interaction between ions and charged electrodes.

Once concentrated to the tip surface, the force of the electric field combined with capillary and viscous forces due to the withdrawal of the tip from solution bind DNA to the tip surface. The mechanisms of attraction and binding of DNA to a tip surface are described in detail below.

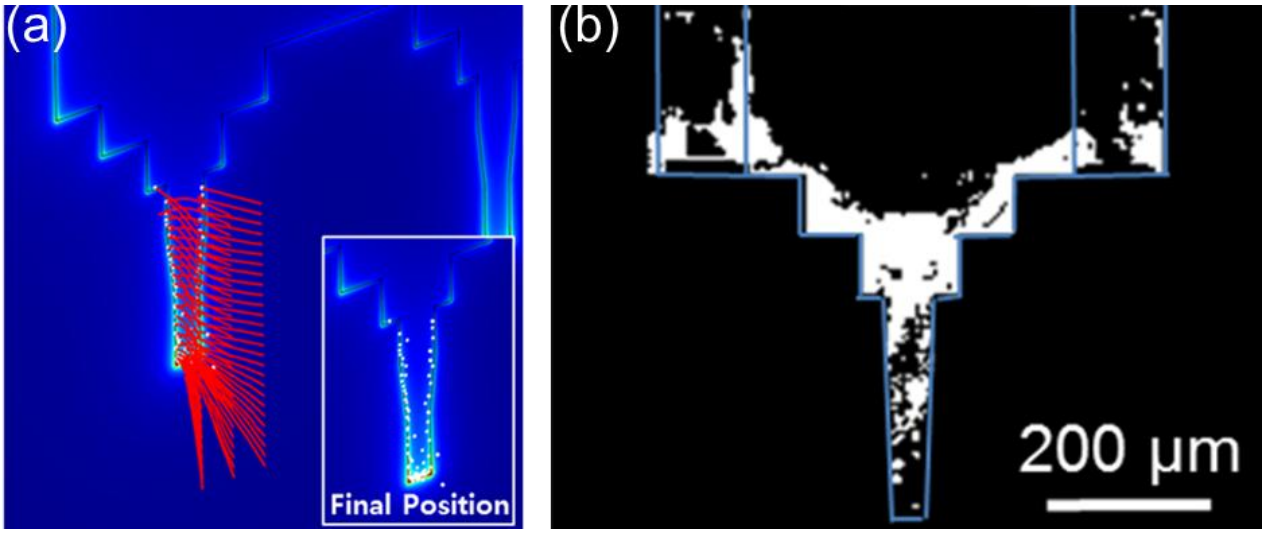

Figure 6. (a) Simulation for DNA extraction using microtips. (b) Fluorescence signal of λ DNA intercalated with picogreen captured on the microtip surface[112].

(1) Electrophoresis

The force on a particle can be derived from Coulomb's law[113]

$$\vec{F}_{12} = \frac{Q_1 Q_2}{4\pi\varepsilon_0 r^2}\vec{r}_{12} \tag{1.1}$$

where $\vec{F}_{12}$ is the force of a charged particle (Q_1) acting on another charged particle (Q_2), ε_0 is the permittivity of vacuum, r is the distance between the particles, and $\vec{r}_{12}$ is the

unit vector from Q_1 to Q_2. The electric field ($\vec{E}$) created by a point charge (Q) at the distance r is given by

$$\vec{E} = \frac{Q}{4\pi\varepsilon_0 r^2}\vec{r} \tag{1.2}$$

By combining Equation 1.1 and Equation 1.2, the electrophoretic force ($\vec{F}_{EP}$) acting on a particle that has net charge Q_{net} is

$$\vec{F}_{EP} = Q_{net}\vec{E} \tag{1.3}$$

In Equation 1.3, the electrophoretic force is the net charge of a particle under an electric field. Previously, the effect of electrophoresis on the capture of DNA on a microtip surface has been studied[112]. A microtip is immersed into a solution of λ DNA (spiked with an intercalating dye, PicoGreen®) held in an electrically conductive well. The microtip is withdrawn after 1 min and the fluorescence intensity can be measured on the surface of the microtip. When a DC potential (3 V) is added to an AC field, the fluorescence intensity on the surface of the microtip doubled from 2299 to 4500. The background signal for PicoGreen® without λ DNA is below 1000. The capturing mechanism without an electric field is due to the thin fluidic film that forms with withdrawal of the microtip as described later in this section. The 0.5 μL solution that remains on the microtip surface after withdrawal contains DNA that is bound to the microtip surface after the thin film is evaporated. The applied DC field generates an electrophoretic force, increasing the force applied to DNA molecules; therefore, increasing the concentration of DNA to the microtip surface. An increase in fluorescence indicates that more DNA is concentrated and the attracted DNA is bound on the surface when the microtip is withdrawn from the solution.

The capturing efficiency is improved due to electrophoresis. However, the study has been done under ideal conditions of λ DNA in a pure solution (TE buffer). Blood and saliva contain a variety of ions, proteins, and other particles that can be influenced by an applied electrophoretic force. Microtip extraction should separate DNA from the variety of particles found in solution, thus electrophoresis cannot translate to real samples effectively.

(2) Dielectrophoresis

Dielectrophoresis (DEP) is the movement of particles under a non-uniform electric field due to an induced effective dipole moment. The high-aspect ratio microtips create a high electric field compared with the counter electrode ring, developing a non-uniform electric field across the sample. For a homogeneous sphere, the time averaged dielectrophoretic force on a particle is[113]

$$F_{DEP} = 2\pi r^3 \varepsilon_m \mathrm{Re}[K(\omega)] \nabla |E_{rms}|^2 \quad (1.4)$$

where r is the radius of the spherical particle, ε_m is the permittivity of the medium, $K(\omega)$ is the Clausius-Mossotti factor, and E_{rms} is the root mean-squared electric field. The effective polarizability of the sphere is defined by the Clausius-Mossotti (CM) factor that relates the permittivity and conductivity of the particle and medium. The CM factor $K(\omega)$ is:

$$K(\omega) = \frac{\varepsilon_p^* - \varepsilon_m^*}{\varepsilon_p^* + 2\varepsilon_m^*} \quad (1.5)$$

$$\varepsilon^* = \varepsilon + \frac{\sigma}{i\omega} \quad (1.6)$$

where ε_m^* is the complex permittivity of the medium, ε_p^* is the complex permittivity of the particle, σ is the conductivity, and ω is the frequency of the electric field. When the frequency of an alternating current is high, the effect of conductivity is minimized

because charge cannot respond to the oscillating electric field. As a result, the permittivity of the particle and medium are dominate at high frequencies (Eq. 1.6). Therefore, a positive force (positive DEP) is developed when the particle has a higher permittivity than the medium $\mathrm{Re}[K(\omega)] > 0$ and the particle will travel to the region of high electric field strength. Alternatively, if the medium has a higher permittivity than the particle, $\mathrm{Re}[K(\omega)] < 0$, the particle will experience negative DEP force and travel to the region of low electric field strength. As the frequency of the applied electric field is reduced, the conductivity of the particle and the medium will influence the polarizability, and consequently determine if the particle experiences a positive or negative DEP force.

DEP is a critical parameter for tip-based DNA extraction. An AC electric field applied between the tips and the conductive coil creates a non-uniform electric field through the solution containing DNA. The selective concentration of DNA to the microtip surface is determined by the applied electric field, conductivity and permittivity of the complex of particles in the target solution, and the conductivity and permittivity of the medium containing the particles. The complexity of biological solutions, and the properties of the suspended particles, poses a major challenge to predicting and controlling the DEP force applied to DNA in solution.

Improving the simulative power of equation 1.4 for complex problems requires models that define the complex geometry of most molecules found in biological samples (DNA, cells, and proteins). Larger DNA fragments have been characterized as globular shapes[114], ellipsoids[115], and rods[116]. The globular shape approximation is attributed to the condensation of DNA[117]. In this case, DNA is modeled according to Equations 1.4-1.6. In solution, the negative charge of DNA attracts a counter-ion cloud[118]. This can be modeled

as a sphere with an encapsulated shell. Huang *et al*[119] developed a single-shell model to combine the effects of a cells cytoplasm and membrane, which has been used to model red blood cells[120]. This single-shell cell model can be applied to DNA with a counter-ion cloud. Assuming homogeneous properties of the counter-ion cloud (shell) and the core DNA (core), the approximate effective complex permittivity of DNA is given by

$$\varepsilon_p{}^* = \varepsilon_s{}^* \left[\frac{\left(\frac{R}{R-d}\right)^3 + 2\left(\frac{\varepsilon_c{}^* - \varepsilon_s{}^*}{\varepsilon_c{}^* + 2\varepsilon_s{}^*}\right)}{\left(\frac{R}{R-d}\right)^3 - \left(\frac{\varepsilon_c{}^* - \varepsilon_s{}^*}{\varepsilon_c{}^* + 2\varepsilon_s{}^*}\right)} \right], \tag{1.7}$$

where Equation 1.6 is transformed for the effective complex permittivity of the particle $\varepsilon_p{}^*$ to include the complex permittivity of cytoplasm or DNA core ($\varepsilon_c{}^*$), the cell membrane or counter-ion cloud shell ($\varepsilon_s{}^*$), outer radius (R), and counter-ion cloud thickness (d). The effective complex cell permittivity from Equation 1.7 can be substituted into the spherical model (Equation 1.5) to obtain the CM factor and DEP force (Equation 1.4).

Alternatively, modeling of DNA has been attempted using an ellipsoid structure[121]. The CM factor for an ellipsoid particle is adapted to account for the non-uniform geometry[122]

$$K_i(\omega) = \frac{1}{3}\left(\frac{\varepsilon_p{}^* - \varepsilon_m{}^*}{\varepsilon_m{}^* + A_i(\varepsilon_p{}^* - \varepsilon_m{}^*)}\right), \qquad i = x, y, z \tag{1.8}$$

where A_i is the depolarization factor along each axis[123]. The depolarization factor for the major axis is given by

$$A_x = \frac{1-e^2}{2e^3}\left[\log\left(\frac{1+e}{1-e}\right) - 2e\right], \qquad e = \sqrt{1-\left(\frac{b}{a}\right)^2} \tag{1.9}$$

where e is the eccentricity and a and b are the radii of the major and minor axis of the ellipsoid with prolate dimensions ($a > b$). The depolarization factor for the minor axis is given as

$$A_y = A_z = \frac{1 - A_x}{2} \tag{1.10}$$

Short DNA fragments (<1 kbp) are suggested to act similar to a rigid rod[116]. In this case, DNA can be modeled similarly to nanotubes and nanowires[124]. This model assumes homogeneous electrical properties, and the CM factor is modified to account for the rod-like shape

$$K(\omega) = \frac{\varepsilon_p^* - \varepsilon_m^*}{3\varepsilon_m^*} \tag{1.11}$$

Uncertainty of the shape of DNA under an applied electric field is a major challenge to DEP modeling. The challenge of characterizing the shape of DNA due to its complexity is also a challenge for characterizing the electrical properties of DNA and the biological medium. Although, the conductivity of λ DNA (σ_{DNA}) has been measured to be 2.4 S/m[125] and dielectric constant (ϵ_{DNA}) of 4.0[126], recent findings suggest that the dielectric constant could be underestimated[118]. Conductivities of λ DNA have been reported between 10^{-5} to 10^{4} S/m[127-129]. Blood has a conductivity of 0.1~1 S/m[109,130] and a dielectric constant that varies significantly with frequency[131]. Furthermore, DNA extraction typically requires a preparation (see Section 1.3.1) step that introduces chemicals, lysed cells, and denatured proteins, adding to the complexity of particle sizes, shapes, and electrical properties. Ultimately, more studies are required to fully understand the shape and electrical properties of DNA to better characterize its response to an AC electric field.

(3) Electroosmosis

In the presence of a solution, a charged electrode forms a layer of counter-ions on the surface due to electrostatic interaction. This is called an electric double layer. When an electric field is applied to the electrode, more ions are concentrated above an electrical double layer (diffuse layer), but are not firmly anchored to the surface due to the electrical screening effect of the first layer. The electric field decays exponentially from the edge to the electrode surface, which induces ionic motion. The electroosmotic flow for a DC electric field can be calculated by[113]

$$v = \frac{E_x \sigma_q}{\kappa \eta} \tag{1.12}$$

where E_x is the electric field, σ_q is the surface charge density in the diffuse double layer, κ is the reciprocal Debye length, and η the fluid viscosity. The movement of charge (σ_q) is a result of the electric field (E_x) tangential to the electrode surface, denoted E_t. The accumulation of charge $\Delta\sigma_q$ is given to be

$$\Delta\sigma_q = \varepsilon \kappa V_d \tag{1.13}$$

where ε as the permittivity of the medium and V_d is the induced potential across the electrodes double layer.

For an AC electric field, the fluid velocity of AC electroosomotic flow is given by[132,133]

$$v = \Lambda \frac{1}{8} \frac{\varepsilon V_o^2 \Omega^2}{\eta z (1+\Omega^2)^2}, \tag{1.14}$$

where ε is the permittivity of the medium, V_o is the amplitude of the voltage, Λ is the ratio between the Stern layer and diffuse double layer capacitance, z is the distance from the

surface of the electrode, and Ω is the nondimensional frequency. The ratio of the Stern layer and the diffuse double layer capacitance is given by

$$\Lambda = \frac{C_s}{C_s + C_d}, \tag{1.15}$$

where C_s is the stern layer capacitance and C_d is the diffuse layer capacitance. The non-dimensional frequency is given by

$$\Omega = \omega \frac{\varepsilon}{\sigma} \frac{\pi}{2} z\kappa, \tag{1.16}$$

The frequency dependence of AC electroosmotic flow is related to the response of ions to the oscillating charge on the electrodes surface. For low frequencies, ions can respond fast enough to form a complete double layer over the electrode surface. The counter ion charge of the double layer shields the potential from the electrode. The electric field in the diffuse layer decays to zero at increasingly low frequencies, decaying the force for flow generation to zero. At high frequencies, the ions do not have time to respond to the oscillating charge on the electrode, eliminating the formation of a double layer. This reduces the potential across the double layer to zero, eliminating any force for flow. For an AC electric field applied from a microtip through TE buffer, AC electroosmosis is observed from 1 kHz to 5 MHz[112].

(4) Tip-based binding

After a combination of DEP and electroosmosis concentrates DNA at the tip surface, the DNA is bound to the tip surface through a combination of DEP, capillary, and viscous forces. The viscous and capillary forces occur when the tip is withdrawn from the target solution. A thin fluidic film forms on the surface of tips during withdrawal. Film thickness is related to the tip size, withdrawal speed, contact angle, and

fluid viscosity. Thickness of the fluid film is estimated by Landau, Levich, and Derjaguin[134] to be

$$h = 1.34\, d\, C^{\frac{2}{3}} \tag{1.17}$$

where d is the microtip diameter and C is the capillary number ($\mu U/\gamma$). For the capillary number, μ is the fluid viscosity, U is the tip withdrawal speed, and γ is the surface tension. Using a 50 μm-diameter tip to concentrate polystyrene microspheres, it has been shown that microspheres with a radius smaller than the film thickness are retained on the tip surface[135].

Particles with a radius greater than the thin film thickness are dominated by capillary forces induced due to the action of the tip withdrawing from the solution. Using the 50 μm-diameter tip, polystyrene microspheres could still be captured at the end of the tip due to the dominance of the capillary capturing force (Fig. 7a). The polystyrene microspheres are released from the tip when the capillary releasing force is greater than the capturing forces due to DEP, the viscous thin film, and the capillary capturing force. The capillary releasing force on a particle is[136]

$$F_{cap} = \pi d\gamma cos(\beta) cos\,(\theta_2 + \beta) \tag{1.18}$$

where d is the microtip diameter, β is the angle between the split point and the horizontal of the particle, and θ_2 is the contact angle on the particle.

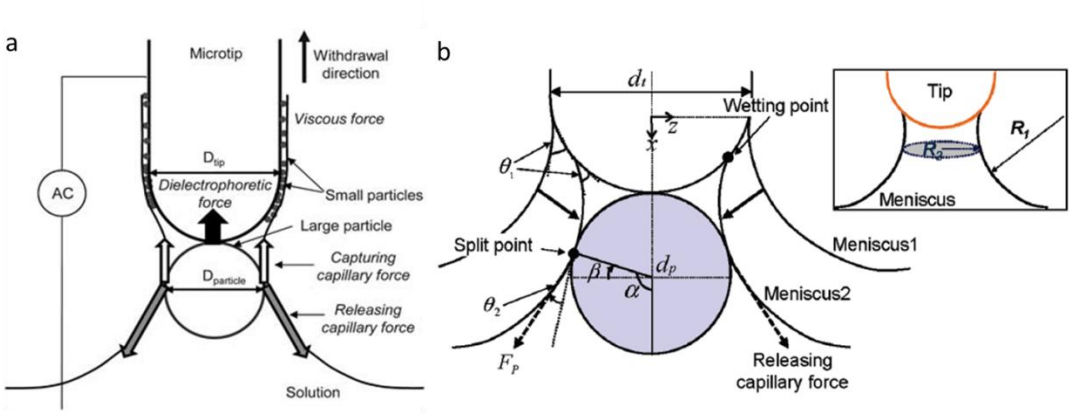

Figure 7. (a) Forces acting on a particle as a tip is withdrawn from a solution. (b) Receding meniscus on particle due to capillary action. Image courtesy from Yeo et al.[135,136]

Using this model, λ DNA is retained on a 50 μm-diameter tip surface due to the viscous thin film[136]. Larger particles could be retained on the tip surface due to dominate capillary releasing forces. Size-selective capturing can aid in the purification of DNA by automatically removing large inhibiting particles.

(5) Summary

Electric field-induced DNA extraction on a tip surface is a novel SPE method. The novelty of the separation step allows for innovations in the other steps (preparation, purification, and isolation) of the DNA sample preparation process flow that are currently constrained by current SPE methods. Also, the separation method allows for a larger variety of targets to be extracted using a singular technology.

The preparation for electric field-based tip extraction requires that the target solution is lysed to free DNA into solution, along with denaturing proteins and nucleases that could affect the DNA. Potentially, tip-based extraction can use a more extensive variety of reagents for the preparation of samples. Silica-based separation techniques require stringent pH conditions, which limits the range of reagents that can be used. The

separation of DNA with tips can be done in the presence of same preparation reagents used by silica-based methods (SDS, Proteinase K, and Guanidine HCl), along with other lysis and denaturation reagents such as Tween 20, CTAB, and NaOH. The separation of DNA is done by suspending solution in an electrically conductive coil, immersing the tip into the solution, and applying an AC electric field to concentrate and separate DNA onto the electrode surface. The tip is removed from the solution and washing buffers are rinsed over the surface of the electrode to purify the DNA from inhibitors that may also have been concentrated on the electrode surface. The DNA is isolated by adding a buffer to elute DNA off the electrode.

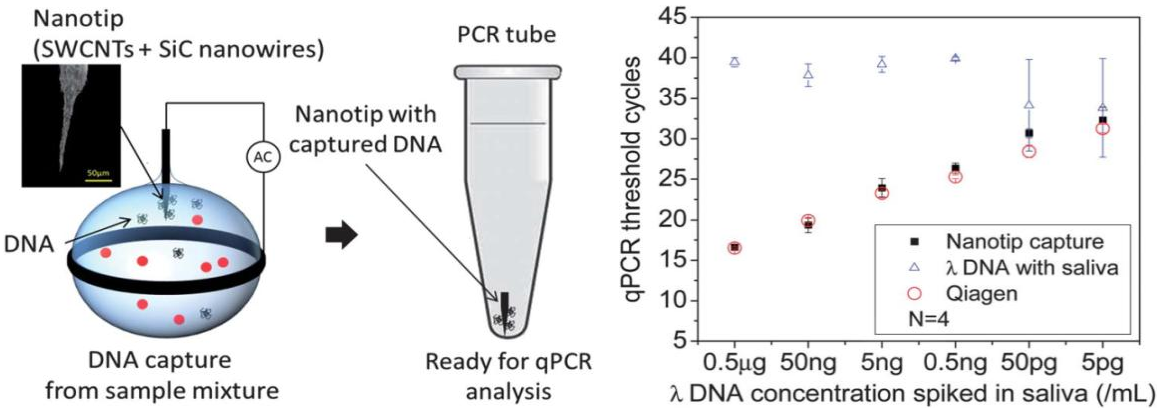

Figure 8. Extraction of λ DNA spiked in saliva using nanostructure tips. Nanostructure tip is composed of aligned SWCNTs and SiC nanowires. The nanotip with extracted DNA can go directly into PCR for amplification and shows comparable yield with Qiagen[137].

Tip-shaped electrode DNA extraction using an electric field has shown the ability to extract DNA from human samples. Microtips and nanotips[138] (Fig. 8) can concentrate DNA from a target solution suspended in an electrically conductive coil with an applied AC electric field. The method has been optimized to take less than 30 minutes to extract PCR-ready DNA from 1-5 μL of sample[96,137]. This method has also proven to

be capable of scaling up to 100 μL[112]. Nanotips have demonstrated the capability of extracting DNA from whole blood[137], however the fabrication process for nanotips is still a cumbersome, serial process. Thus, nanotips are not yet suited for mass production. Microtips are fabricated using microfabrication techniques and have demonstrated the capability of extracting DNA from saliva, and buccal swab samples[96]. The main challenges of microtip-based extraction are: (1) obtaining a stable microtip surface for the controlled capture and release of DNA and (2) improving the recovery of DNA in the presence of a high concentration of inhibitors found in whole blood and dried samples.

1.3.5 *Comparison*

Table 1. Comparison of DNA sample preparation method

Method	Cost/test	Time/test[1]	Equipment	Pros	Cons
CsCl	$4-$10	24-48 hrs	Ultracentrifuge Fume hood	➢ High purity ➢ Vairable length extraction ➢ Large volume	➢ Expensive ➢ Time consuming ➢ EtBr is a mutagen ➢ High limit of detection
Phenol-chloroform	$1-$4	Typically Overnight[68]	Centrifuge Fume hood	➢ Yield from little material ➢ Large volume	➢ Toxic reagents ➢ Time consuming/labor intensive ➢ Difficult removing phenol ➢ Contaminantion
Salting out	<$1	Typically Overnight	Centrifuge	➢ Inexpensive ➢ Simple ➢ Protein purification	➢ Typically long lysis time ➢ Inefficient ➢ Inconsistent process
Alkaline	<$1	~1 hour[77]	Centrifuge	➢ Inexpensive ➢ One-tube extraction ➢ Plasmid DNA extraction	➢ DNA degradation ➢ Alkaline PCR inhibitor
Chelex	<$1	~2.5 hrs[82]	Centrifuge	➢ Inexpensive ➢ One-tube extraction	➢ Low purity ➢ DNA can denature
Silica filter	<$5	1.5 hrs	Centrifuge	➢ High yield ➢ Reproducible ➢ Adaptable to robot	➢ Long process flow ➢ DNA shearing ➢ Low yield (large/small lengths)
Magnetic bead	$4-$8	40 min (EZ1 BioRobot)	Magnet	➢ High yield ➢ No centrifugation ➢ Adaptable to robot	➢ High investment cost for robot ➢ Large equipment ➢ Maintenance ➢ Labor intensive if not automated
Electric-field	N/A	No protocol	Small device	➢ Simple/Rapid ➢ No centrifugation ➢ Small volume ➢ Potential for high-throughput	➢ Unproven technology ➢ No efficiency validation ➢ Limited real sample testing

[1]Processing time varies considerably depending on the sample. Dried blood spots processing time is indicated here.

1.4 COMMERCIAL DNA EXTRACTION METHODS

SPE methods have been developed into a variety of commercial kits. Commercial kits described below use microfiltration, magnetic bead, and anion exchange membranes for DNA extraction. Both automated and manual methods are detailed below.

1.4.1 *Qiagen QIAamp® extraction*

Qiagen's silica-based spin column extraction has become the gold standard for nucleic acid sample preparation. A silica membrane is used in a specially designed centrifuge column to act as a filter for DNA, allowing the majority of the lysate to pass through the membrane and be removed. The lysate contains common lysing and denaturing agents such as Proteinase K to free DNA into the target solution. Proprietary reagents include an unknown concentration of guanidine and ethanol to enhance the binding of DNA to the silica membrane when the solution is centrifuged through the silica membrane spin column. Washing solutions are centrifuged through the silica membrane following the binding of DNA to remove potential inhibitors that have also bound to the silica membrane. Finally, a high pH buffer is used to elute the DNA off the silica membrane.

Qiagen's silica membrane-based extraction method provides many advantages over conventional methods. One advantage is the time of extraction has been reduced from days to hours and in some cases as quick as 30 minutes. Although, it is less labor intensive and uses less materials than phenol-chloroform[63], the steps required for spin column extraction are still cumbersome and require centrifugation. Also, centrifugation creates a high fluid shear force causing damage to DNA[53].

Qiagen provides both manual and automated methods for using its silica membrane

technology. For whole blood, the processing time for the manual QIAamp® protocol is approximately 35 minutes. For dried blood, the processing time is almost 2 hours. The protocol can be automated with the QIAcube to reduce handling; however, the processing time is similar. Qiagen also offers a magnetic bead based method of dried blood extraction using the BioRobot® EZ1 that can reduce the processing time to 40 minutes.

1.4.2 *Akonni TruTip®*

Akonni's TruTip® uses a porous rigid silica filter within a pipette tip for DNA extraction[139,140]. The TruTip® technology uses the standard process flow for extracting DNA of (1) preparation, (2) separation by binding DNA to the silica filter, (3) washing the filter, and (4) eluting the DNA into a buffer. However, the larger porosity of the silica filter used in the TruTip® requires less force for solution flow, eliminating the need for centrifugation. Instead, the pressure created within the pipette tip due to the action of pipetting, is enough to flow solution through the filter in a bidirectional manner. Though this process is repeated many times throughout the protocol, the quick action of each pipetting step can reduce the process time for TruTip® to less than 10 minutes. The total processing time is >20 minutes, accounting for preparation of the sample that includes a 10 minute heating step. The yield has shown to be similar to other commercial methods[141]. No protocol is provided for the extraction of DNA from dried blood spots using TruTip® technology.

1.4.3 *Life Technologies Dynabeads®*

Dynabeads® are formed from a polystyrene shell that encapsulates iron oxides that exhibit superparamagnetic properties[142,143]. The function of the beads to magnetize in the presence of a magnet (superparamagnetic) is critical to the separation of particles because the

beads can be uniformly dispersed and mixed within a solution when the magnet is removed. The size of the bead can be precisely controlled to produce uniform bead sizes on the micron scale. A polystyrene surface shell can be functionalized with a variety of ligands (Fig. 9) for target specific binding of cells, proteins, and nucleic acids.

Dynabeads® technology uses the standard process flow for extracting DNA of (1) preparation, (2) separation of DNA from the target solution by binding to Dynabeads®, (3) washing the Dynabeads®, and (4) eluting the DNA into a buffer. For silica-based binding (Dynabeads® MyOne™ Silane), the lysis solution includes Proteinase K and a chaotropic salt to influence binding to silica as discussed in Section 1.3.3. The actual contents of the lysis/binding buffer are proprietary. Isopropanol or ethanol is added before binding to dehydrate the DNA to optimize binding to the beads. The separation of DNA begins by uniformly mixing Dynabeads® thoroughly in the target solution. Then, the magnet is applied to aggregate the DNA-bound Dynabeads® on the side of the vial. The supernatant is removed to separate the DNA. Washing buffers are added and the process of mixing and magnet aggregation is repeated. Finally, the DNA is eluted in a buffer.

Dynabeads®-based extraction eliminates the use of centrifugation making it advantageous for high-throughput automated extraction using robots. Whether manual or automated, magnetic bead extraction shows high yield and consistent extraction. The Dynabeads® provide protocol flexibility because the bead surface can be functionalized with different materials including strong binding agents such as streptavidin and oglionucliotides. When evaluated for the extraction of viral DNA from dried blood spots, Dynabeads® performed poorly versus other commercial methods such as the Qiagen QIAamp® kit[144]. The protocol required 1 hour of processing time.

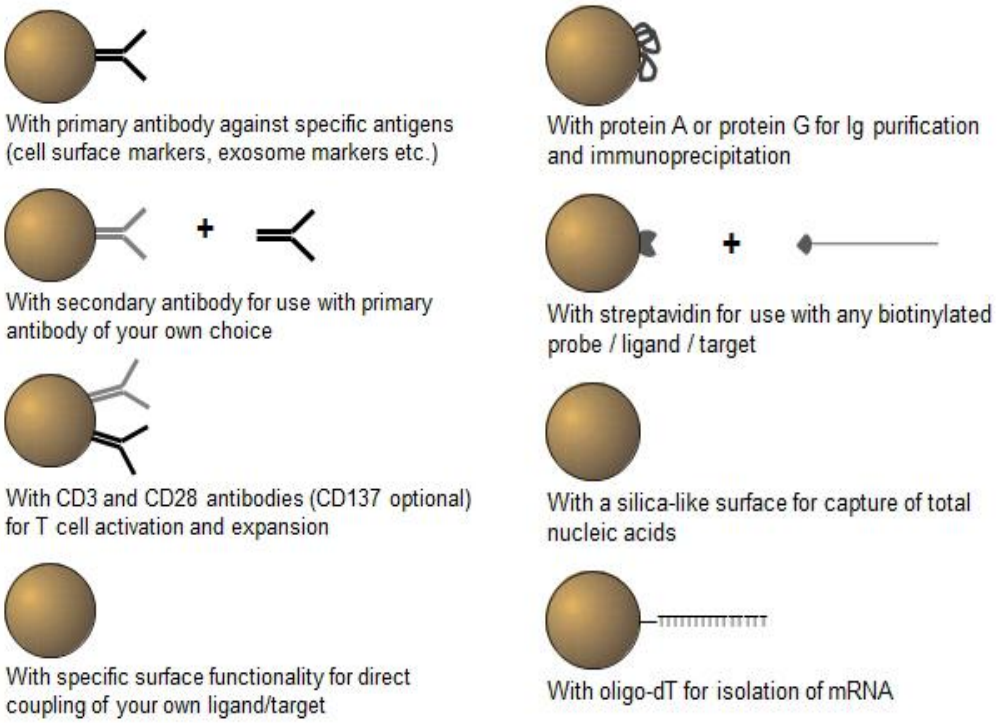

Figure 9. Surface functionalization of Dynabeads®. Image from Life technologies™.

1.4.4 *Roche MagNA Pure*

The Roche MagNA Pure is a fully automated nucleic acid extraction instrument utilizing magnetic glass particle technology. The automated robot can process up to 96 assays at one time in approximately 1 hour. The cost for the instrument is high at ~$85,000 with an assay cost of ~$8[145]. Binding of DNA to glass particles utilizes the same high salt concentration techniques as with silica particles[146]. Automated within the instrument, the MagNA Pure extraction method follows the standard process flow for extracting DNA of (1) preparation, (2) separation of DNA from the target solution by binding to the magnet glass particles, (3) washing, and (4) eluting the DNA into a buffer. For DNA extraction from dried blood spots, an overnight incubation step is recommend for the preparation of the dried blood sample[144]. However, 1 hour incubation is more common.

1.4.5 *Invitrogen ChargeSwitch®*

The Invitrogen ChargeSwitch® technology utilizes proprietary ion-exchange materials for the extraction and purification of DNA. As described in the patent[147], the ion-exchange polymer layer can be polyhistidine, Bis-Tris, Tricine, or Tris-HCl polymer. The important mechanism patented is the ability to control the charge of the surface layer through the surrounding solution pH. Since DNA is negatively charged, the pH of the binding solution is adjusted below 6.5 to create a positively charged ChargeSwitch® layer. Once contaminants are washed, the DNA is eluted by adding a solution at 8.5 pH. This causes the ChargeSwitch® layer to become negatively charged, releasing DNA into the solution. The ChargeSwitch® technology method follows the standard process flow for extracting DNA of (1) preparation, (2) separation of DNA from the target solution by binding to the ChargeSwitch® layer, (3) washing, and (4) eluting the DNA into a buffer. ChargeSwitch® technology is used with common SPE techniques of microfiltration and magnetic bead extraction, however recent developments (Direct 96) can extract DNA using one tube that is coated with the ChargeSwitch® technology (Fig. 10). This process is extremely simple, cost-effective, and uses limited consumables.

For dried blood spots, ChargeSwitch® technology on magnetic beads requires a 30 minute lysis step before extraction. From 10 μL of blood dried on an Omni swab, 3 ng/μL of DNA can be extracted[148].

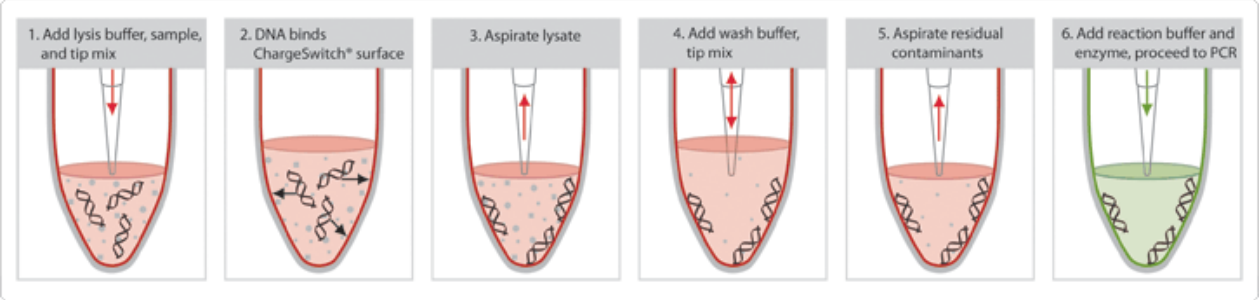

Figure 10. Surface fictionalized ChargeSwitch® well DNA extraction process. Image from Life technologies™.

1.4.6 *FTA® paper*

FTA® paper is designed specifically as a storage medium for nucleic acids in samples. Drying a target sample on FTA® paper is a simple, easy, and cost-effective method of DNA storage. Dried samples on FTA® paper are small and easy to store without concern for temperature control. The paper is functionalized with a combination of chelating agents, anionic surfactants, detergents, and uric acid to lysis cells, denature proteins and nucleases. Nucleic acids can be stored on FTA® for years.

Extraction of DNA is commonly performed using commercially available methods such as Qiagen; however, a FTA® extraction kit can also be purchased. The kit follows the standard process flow for extracting DNA. The (1) lysing of cells and (2) binding of DNA occur from drying the target sample on the FTA paper. The extraction kit includes solutions to (3) wash the paper of inhibitors, and (4) elute the DNA off the paper. The process of extracting DNA using the FTA® extraction kit is simple to perform, requiring a few pipetting steps. The FTA® needs to be dried completely before it can be used for PCR. This takes at least 1 hour.

1.4.7 *Comparison*

Table 2. Comparison of commercial kits

Method	Cost/test	Time/test[1]	Equipment	Pros	Cons
QIAamp®	$3.30	~1.5 hr	Centrifuge Heat block	➢ Variety of samples ➢ High-throughput if automated ➢ Standard	➢ 20-30 kbp optimal extraction fragment ➢ 100 µL sample volume
TruTip®	$5.00	No method (10-30 min for whole blood)	epMotion system (if automated) Rainin® pipette (manual)	➢ Rapid ➢ No centrifugation	➢ Large sample volume needed ➢ 30-50 kbp optimal extraction fragments
Dynabead®	$6.00	~1 hr	Magnet (~$700) Robot (if automated)	➢ High yield ➢ High throughput	➢ Low yield DBS If automated, ➢ High intial investment ➢ Large benchtop requirement
MagNA Pure	$7.68	~1 hr	Automated system	➢ High yield ➢ High throughput	➢ High intial investment ➢ Large benchtop requirement
ChargeSwitch® Direct 96	$2.60	>30 min lysis	No equipment	➢ No bead, filters, centrifuge ➢ One-tube extraction	➢ Variable yield with sample type
FTA®	$3.84	2 hours	No equipment	➢ Rapid elution ➢ DNA storage	➢ Slow sample drying ➢ Limited sample volume

[1]Processing time varies considerably depending on the sample. Dried blood spots processing time is indicated here.

1.5 SUMMARY

The emergence of Solid-Phase Extraction methods has been a critical stepping stone for nucleic acid analysis. SPE methods provided a platform for developing commercially viable products that reduce the processing time from days to less than 1 hour in some cases. SPE methods have provided easy access to genetic information and have been a catalyst for genetic testing. As can be seen in Table 1 and Table 2, complicated procedures still remain or have been circumvented with large, expensive equipment that require regular maintenance. Centrifugation is still the most common method of DNA separation. The influx of parties interested in genetic information and inexhaustible outcomes to study has applied more pressure by users to cut costs. Protocols for some samples such as dried blood spots still require long processing times. The required time should be reduced to <30 minutes, and the cost per assay should be $1~2. As demonstrated in Fig. 4, intact, quality DNA is still a challenge for extraction methods along with eliminating costly and toxic chemicals.

These challenges have motivated the investigation into developing new methods for DNA extraction. Electric field-guided DNA extraction using microtips has shown promise in addressing the current challenges of DNA sample preparation by providing a simple, rapid method of extracting DNA from smaller volumes. Positively charged surface coatings such as PEI have been shown to provide a favorable surface for improving the capture and release of DNA. However, there is limited characterization of PEI for DNA sample preparation.

The goal of this study is to address the challenges of microtip-based DNA extraction and expand upon the limited work on using PEI for DNA extraction. Use of electric field-guided DNA extraction onto a heat-cured PEI-coated microtip surface is the focus of this work. Contributions of this work will detail the effect of heat curing PEI for DNA sample

preparation, the role of PEI in microtip-based DNA extraction, and demonstrate the rapid purification of DNA of human genomic DNA from forensic samples.

1.6 OBJECTIVES

The main objective of this work is to address the challenges of gold-coated microtips through use of a heat-cured PEI layer. Previous work has shown PEI improves the recovery yield of DNA in magnetic particle-based extraction. The basic function of PEI toward DNA extraction is understood. However, the effect of heat curing of PEI on the capture and release of DNA, as well as the use of DNA in downstream applications, has not been studied.

The novelty of this work is the discovery of a critical curing temperature of PEI to prevent PEI from dissolving off the gold-coated microtip surface. Heat curing of PEI is investigated further to improve the recovery of DNA using microtips for applications such as PCR and gel electrophoresis. The conclusion of this work provides an understanding of the curing parameters of the PEI layer for the recovery of DNA using microtip-based extraction. Heat-cured PEI-coated microtips are used to recover purified genomic DNA from whole blood, dried blood spots, and forensic samples. A summary of this study shows:

1. The characterization of the inhibition of PCR and gel electrophoresis by high molecular weight PEI when dissolved in solution with DNA. [Chapter 3]
2. Heat curing (below 150°C) of a PEI layer on a gold-coated surface can inhibit PCR and gel electrophoresis by dissolving off the gold-coated surface and binding with DNA. [Chapter 3]
3. Heat curing (above 150°C) of a PEI layer on a gold-coated surface prevents the inhibition of PCR due to PEI dissolving off the surface. [Chapter 3]

4. PEI-coated microtips cured at 225°C improve the recovery of DNA compared with non-coated microtips. [Chapter 3]
5. A washing protocol can be used with the heat-cured PEI-coated microtips to effectively purify extracted DNA from whole blood. [Chapter 4]
6. Heat-cured PEI-coated microtips can be integrated into an automated device for rapid extraction of DNA from dried blood spots with a pre-extraction washing protocol. [Chapter 5]

Chapter 2. MATERIALS AND TOOLS

2.1 MATERIALS

Branched PEI, 50% weight per volume in water, with a mean molecular weight of M_w=750 kDa was purchased from Sigma-Aldrich.

A hand wrapped coil (1.6 mm inside diameter) was used for holding the target sample and acting as the counter-electrode for electric-field induced extraction using microtips. The coil was made of 30 AWG silver-coated copper wire (OK Industries).

Proteinase K was purchased from Qiagen for cell lysis and protein denaturation. 20% sodium dodecyl sulfate (SDS) was purchased from Fluka for cell lysis. Guanidine-HCl was purchased from Sigma for use with dried samples. N-Acetyl-L-(+)-cysteine (NALC) crystalline powder was purchased from Fisher Scientific to wash the microtips. 100% ethanol was purchased from Decon Laboratories. Pure water was purchased from Invitrogen. The Red Blood Cell Lysis (RBC) buffer contains 10mM Tris-HCl (original 1M Tris-HCl 8.0 pH purchased from Invitrogen), 320 mM Sucrose (Sigma), 5mM $MgCl_2$ (Fisher Scientific), and 1% Triton x100 (original 10% purchased from Fluka). 1x TE buffer (8.5 pH) was made in house. The concentration of Tris was 10mM and the concentration of EDTA was 1mM. The QIAamp® Blood Mini kit was purchased from Qiagen for extraction of DNA from whole blood and dried blood spot samples. The recommend protocols defined in the QIAamp® Blood Mini Handbook were used.

For PCR, SYBR® GreenER™ qPCR SuperMix Universal (SYBR) was purchased from Invitrogen with use of Lambda primers (Invitrogen) for λ DNA qPCR analysis and beta-actin primers (Invitrogen) for human genomic DNA qPCR analysis. For quantification

of genomic DNA from blood, the Plexor® HY System was purchased from Promega. The protocols for PCR analysis are described in Section 2.4 below.

For gel electrophoresis, E-Gel® 96 High Range DNA Marker was purchased from Invitrogen for use with E-Gel® EX 1% Agarose purchased from Invitrogen.

2.2 MICROFABRICATION OF MICROTIPS

Gold-coated microchips with a 3-microtip array were manufactured through microfabrication[96]. The microfabrication laboratory (Washington Technology Center) at the University of Washington was used to fabricate the microchips with the 3-tip array. A 100-mm silicon wafer was used as the substrate for fabrication. First, low pressure chemical vapor deposition (LPCVD) was used to grow a 1 μm thick layer of silicon nitride (Si_3N_4) on the silicon wafer. Conventional UV lithography was then used to pattern rectangular patterns on the back side of the Si_3N_4 layer. The pattern was etched using reactive ion etching (RIE) followed by KOH etching. The tips were then patterned on the Si_3N_4 layer and etched by the RIE process to create the needle-like microtips. Lastly, the microtips were coated with 10/30 nm-thick Cr/Au layers by electron-beam evaporation.

The design of the microchip was separated into two parts: (1) capturing area and (2) handling area. The capturing area was a 3 mm x 2 mm area that included the needle-like tips and a gold-coated chip area. The 2 mm x 2 mm rectangular area separated from the capturing area by a groove was for electrical connection to an electric field generator and convenient handling (Fig. 11b). Based on this design, 392 chips could be fabricated on one 100 mm silicon wafer. In two days, one engineer could produce 25 wafers in a single batch. The estimated manufacturing throughput of the microtips is 1.2 M ea/year/person.

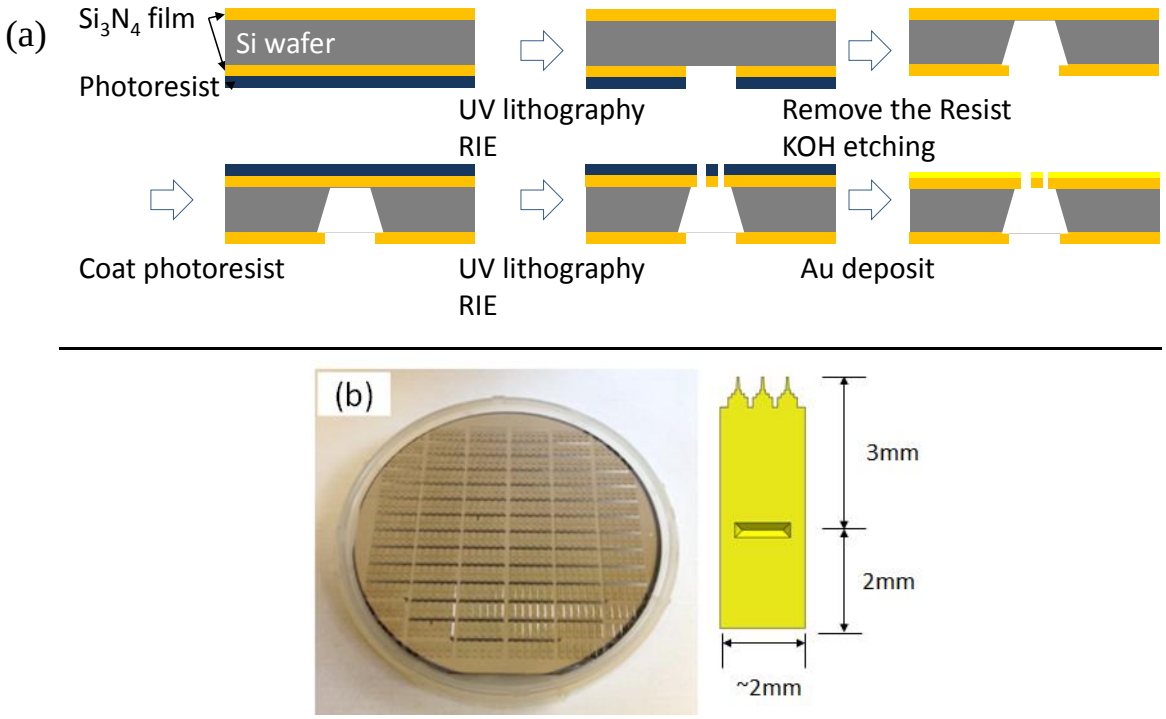

Figure 11. (a) Process flow for the fabrication of the microtips. (b) Silicon wafer with fabricated microtips and schematic of micochip. Image courtesy of Dr. Jong-Hoon Kim.

2.3 AUTOMATED DNA EXTRACTION DEVICE

In chapter 5 the microtip assays were run by using the NF DRS 420 (NanoFacture, Inc.). The role of the device is to automate the DNA extraction and washing procedures for microtip-based extraction (Fig. 12). NanoFacture Inc. provides details on the NF DRS on their website (www.nano-facture.com).

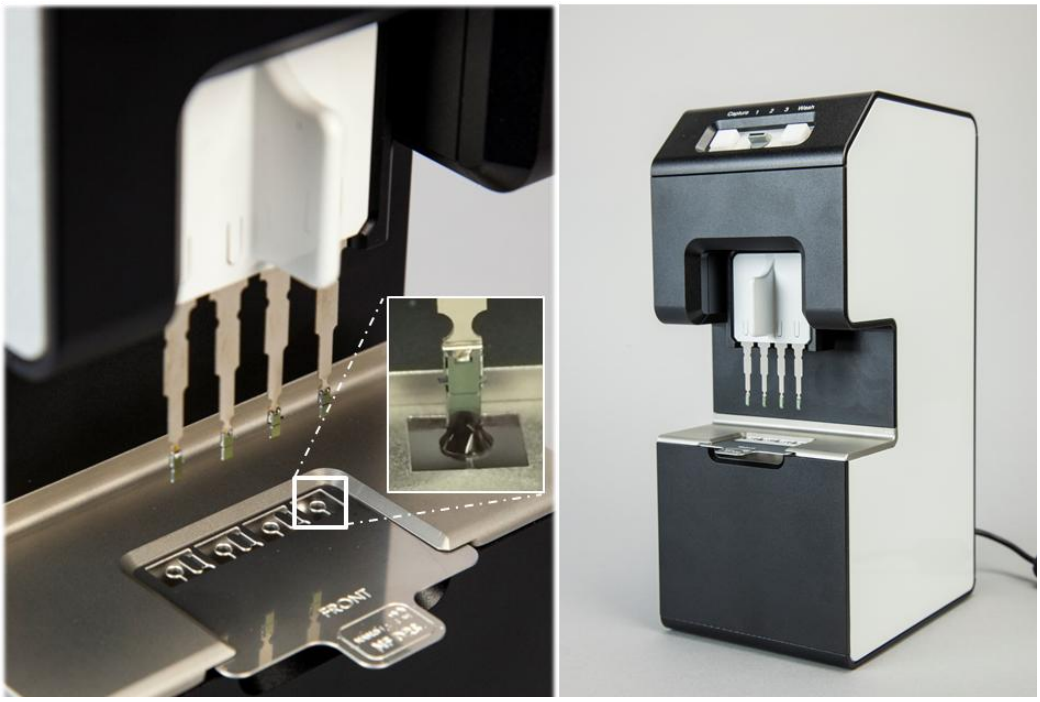

Figure 12. Automated microtip DNA extraction system

2.4 SAMPLES

(1) λ DNA

Bacteriophage lambda DNA (λ DNA) was purchased from New England Biolabs with an original concentration of 500 μg/mL (15.85 nM). The λ DNA was diluted in 8.5 pH 1x Tris–EDTA buffer (TE buffer).

(2) *Whole Blood*

Six de-identified whole blood samples with K2 EDTA were purchased from Bioreclamation and stored in K2 EDTA collection tubes (Fisher Scientific) at 4°C before use.

(3) *Dried Blood Spot*

De-identified 3-mm dried blood spot punches were received from the Mayo Clinic (Rochester, MN) for testing.

(4) *Dried Blood Swab*

A de-identified whole blood sample with K2 EDTA was purchased from Bioreclamation and stored in K2 EDTA collection tubes (Fisher Scientific) at 4°C before use. Generic cotton swabs had 10 μL of whole blood pipetted onto the tip. The samples were dried for at least 24 hours and stored at room temperature before use.

(5) *Dried Blood Denim*

A de-identified whole blood sample with K2 EDTA was purchased from Bioreclamation and stored in K2 EDTA collection tubes (Fisher Scientific) before use. A piece of retail jean material was cut into squares and 10 μL of whole blood was pipetted onto each square of denim. The samples were dried for at least 24 hours and stored at room temperature before use.

(6) *Buccal Swab*

Whatman™ OmniSwab™ was purchased from Fisher Scientific. Buccal swab samples were collected according to the recommend protocol from de-identified volunteers.

(7) *Nasal Swab*

BBL™ CultureSwab™ was purchased from BD Worldwide. Nasal swab samples were collected according to the recommended protocol from de-identified volunteer's nares. For bacterial DNA extraction, 10 μL of *Escherichia coli* (*E. coli*) at concentrations of 10^6, 10^5, 10^4, 10^3, and 10^2 cfu/mL was dried on the nasal swab.

(8) *Dried Semen Cloth*

De-identified semen sample was purchased from Bioreclamation. A piece of a cotton shirt was cut into squares and 10 μL of semen was pipetted onto each square of cotton cloth. The samples were dried for at least 24 hours and stored at room temperature before use.

(9) *Drink Swab*

Generic cotton swabs were hydrated with 100 μL of pure water and aggressively whipped over edge of a coffee mug. The samples were dried for at least 24 hours and stored at room temperature before use.

(10) *Cigarette Butt*

Fully smoked cigarette butts were collected from de-identified individuals. Only the paper surrounding the last 1 cm of the end of the filter was used for DNA extraction.

(11) *Hair root*

Hair was plucked from a de-identified volunteer.

2.5 ANALYTICAL TOOLS

2.5.1 *PCR protocol for λ DNA amplification*

The BIORAD C1000 thermal cycler with CFX96 real time system was used for all PCR experiments. Amplification of λ DNA was performed using SYBR® GreenER™ qPCR Supermix Universal (SYBR). For the amplification reaction, 14 μL of SYBR was mixed with 3 μL of the forward primer (2 μM) and 3 μL of the backward primer (2 μM) to replicate 5 μL of the target sample. The forward primer used for PCR amplification of λ DNA was[137]: 5'-GATGAGTTCGTGTCCGTACAACTGG-3'. The backward primer used for PCR amplification of λ DNA was[137]: 5'-GGTTATCGAAATCAGCCA CAGCGCC-3'. For the thermal cycling protocol, the samples were initially heated to 50°C for 2 minutes, and then 95°C for 10 minutes. The samples were then cycled 40 times at 95°C for 15 seconds followed by 60°C for 1 minute. Each sample was quantified by the cycle where the relative fluorescence unit (RFU) signal reached 10 units, which was referred to in this study as the threshold cycle (C_T). After amplification was completed, melt curve analysis was performed

by increasing the temperature from 65°C to 95°C at 0.5°C increments every 5 seconds. Each PCR products melting temperature was determined by the change in RFU at each temperature. For quantification and recovery efficiency, the threshold cycle for the target samples was fit to a standard curve. The standard curve was generated from the threshold cycles for pure λ DNA amplified directly by qPCR at concentrations of 10 pM, 1 pM, 100 fM, 10 fM, and 1 fM.

2.5.2 *PCR protocol for E. coli DNA amplification*

The BIORAD C1000 thermal cycler with CFX96 real time system was used for all PCR experiments. Amplification of *E. coli* DNA was performed using the SYBR® system. For the amplification reaction, 14 μL of SYBR was mixed with 3 μL of the forward primer (2 μM) and 3 μL of the backward primer (2 μM) to replicate 5 μL of the target sample. The forward (MotorolaF) for *E. coli* DNA was[149]: 5'-GGCGTTATCCCCAGTTTTTAGTGA-3'. The backward (MotorolaR) primer was[149]: 5'-AACGGCCATCAACATCGAATACAT-3'. For the thermal cycling protocol, the samples were initially heated to 50°C for 2 minutes, and then 95°C for 10 minutes. The samples were then cycled 40 times at 95°C for 15 seconds followed by 60°C for 1 minute. Each sample was quantified by the threshold cycle (C_T). After amplification was completed, melt curve analysis was performed by increasing the temperature from 65°C to 95°C at 0.5°C increments every 5 seconds. Each PCR products melting temperature was determined by the change in RFU at each temperature.

2.5.3 *PCR protocols for human genomic DNA*

The BIORAD C1000 thermal cycler with CFX96 real time system was used for all PCR experiments. Amplification of human genomic DNA was performed using the SYBR®

system. For the amplification reaction, 14 μL of SYBR was mixed with 3 μL of the forward primer (2 μM) and 3 μL of the backward primers (2 μM) to replicate 5 μL of the target sample. The forward and backward primers for the β-actin gene[150] of human DNA were: 5'-ACCCACACTGTGCCCATCTAC -3' and 5'- TCGGTGAGGATCTTCATGAGGTA -3'. For the thermal cycling protocol, the samples were initially heated to 50°C for 2 minutes, and then 95°C for 10 minutes. The samples were then cycled 40 times at 95°C for 15 seconds followed by 60°C for 1 minute. Each sample was analyzed through the threshold cycle (C_T) and total PCR product amplification (RFU at 40 cycles). After amplification was completed, melt curve analysis was performed by increasing the temperature from 65°C to 95°C at 0.5°C increments every 5 seconds. Each PCR products melting temperature was determined by the change in RFU at each temperature. For recovery efficiency, the threshold cycle for the target samples was fit to a standard curve. The standard curve was generated from the threshold cycles for Plexor® HY Genomic DNA amplified directly by qPCR at concentrations of 10 ng/μL, 1 ng/μL, 100 pg/μL, 10 pg/μL, and 1 pg/μL.

For quantification using the Plexor® HY System[151,152], 10 μL of Plexor® HY 2x master mix was mixed with 7 μL amplification grade water (provided by Promega), and 1 μL 20x Primer/IPC mix to replicate 2 μL of the target sample. For the thermal cycling protocol, the samples were initially heated to 95°C for 2 minutes, and cycled 38 times at 95°C for 5 seconds followed by 60°C for 35 seconds. After amplification was completed, a melt curve analysis was performed by increasing the temperature from 65°C to 95°C at 0.5°C increments for 5 seconds. Each PCR run included duplicates of Plexor® HY Genomic DNA at concentrations of 50 ng/μL, 10 ng/μL, 2 ng/μL, 0.4 ng/μL, 0.08 ng/μL, 0.016 ng/μL, and 0.0032 ng/μL diluted in TE Buffer. DNA concentration quantification was analyzed by the

Plexor® software to correlate the amplified DNA from the sample amplification with that of the control DNA. Purity of the samples was analyzed through the Internal PCR Control (IPC) included in the primer mix. The IPC primers amplified a biomarker of 150 bp sequence in every reaction. The amplification of this sequence was designed by Promega to lag in the presence of inhibiting particles. The Plexor® analysis software compared the DNA standards amplification of the IPC, which was considered inhibitor-free (pure), to the unknown sample amplification of the IPC. A significant lag of the IPC was an indicator of reaction inhibition.

2.5.4 *STR profiling for human genomic DNA*

STR multiplex amplification was performed with the Applied Biosystems® Identifiler Plus PCR amplification kit using the standard protocol. Amplified samples were separated via capillary electrophoresis on an Applied Biosystems® 3130xL Genetic Analyzer. The profiles were analyzed via GeneMapper. STR testing was performed by Karen Olsen at the Defense Forensics Science Center, Atlanta, GA.

2.5.5 *Gel electrophoresis*

For gel electrophoresis, the DNA ladders (E-Gel® 96 High Range DNA Marker) were used exclusively for this study. The DNA ladders were input into E-Gel® EX 1% Agarose gel for analysis. Electrophoresis was induced on the DNA ladders by installing the gel cartridge into the E-Gel® iBase™ Power System. The run time was 10 minutes. After electrophoresis was completed, the gel cartridge was removed and placed in the UVP Visi-Blue™ Transilluminator for visualization of the DNA ladders.

Chapter 3. STUDY OF POLYETHYLENEIMINE FOR DNA EXTRACTION

3.1 INTRODUCTION

The primary aim of this work is to investigate a heat cured PEI coating on a microtip surface for the recovery of DNA. Currently, microtip-based extraction separates DNA on the surface of a gold-coated microchip with a microfabricated array of needle-like cantilevers (tips) at one end of a microchip (See Section 2.2 Fig. 11). For the purposes of this dissertation, the chip and tips will be referred to as one unit, microtips. The binding of DNA relies on weak, nonspecific binding (the van der Waals force) on the gold-coated microtips. Combined with the gold surfaces susceptibility to contamination[153], the gold-coated microtips are not suitable for DNA sample preparation of complex samples.

Controlled binding and release of DNA with use of a polymer could be advantageous in the advancement of microtip-based DNA extraction. The binding of PEI to DNA has gained recent attention in the field of drug delivery. The affinity of PEI to DNA has been shown to be beneficial in transfection techniques through controlled release of DNA from PEI[154-156]. Positively charged PEI has been shown to bind to nanoparticles for use as an anion exchange bead-based DNA extraction method[102-104].

The foundation of electric field induced DNA extraction with the use of tips was described in detail in Section 1.3.4. For this study, the parameters for the electric field concentration of DNA to the tip surface will be fixed based on previous work. An explanation of these parameters is described below (Section 3.1.1). A background of PEI and

relevant work is described in detail below (Section 3.1.2).

3.1.1 *Microtip AC electric field*

Tip-based DNA extraction first requires the concentration of particles to the surface of a tip through an applied electric field. The microtip design is chosen for this study over the nanotip design because it has a consistent, scalable manufacturing process. An AC electric field is exclusively used for this study to minimize the attraction of unwanted particles due to electrophoresis. For the AC electric field used in this study, frequencies below 50 kHz are not suitable for use with biological samples (Fig. 13) because of electrolysis on the microtip surface. At higher frequencies, 20 V_{pp} is determined to be the maximum potential without electrolysis[112]. A high potential (20 V_{pp}) is desirable to induce a high DEP force. At 5 MHz, a flow velocity due to electroosmosis is ~35 μm/s[112]. At this speed, a particle can circulate across the 1.6 mm diameter of the 5 μL solution in 1 minute. An AC electric field of 5 MHz and 20 V_{pp} limits the attraction of inhibiting particles[102,103] due to dielectrophoresis and is used exclusively for this study.

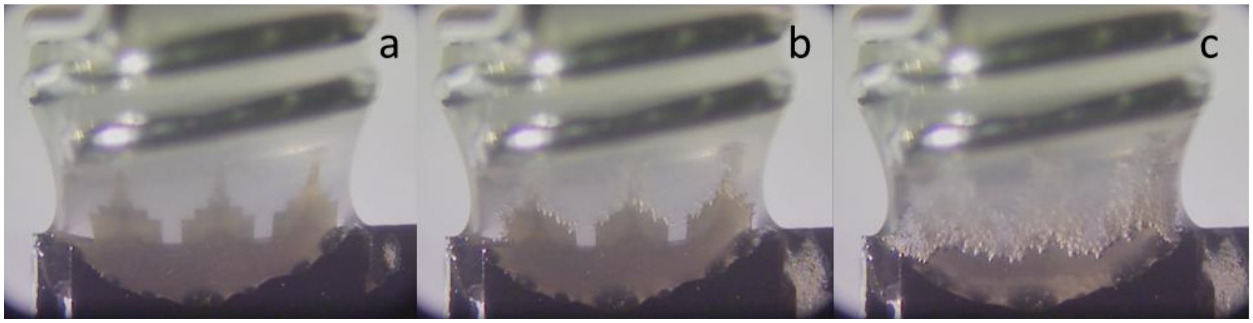

Figure 13. Applied electric field through saliva. (a) 50 kHz, (b) 5 kHz, and (c) 0.5 kHz. The formation of bubbles is an indication of electrolysis of the solution.

3.1.2 *The PEI-DNA complex*

Once DNA is concentrated to the microtip surface, DNA binds to the microtip surface due to the electric field force combined with capillary and viscous forces generated when the microtip is withdrawn from the solution. The DNA is bound through van Der Waals interaction with the gold-coated surface. Gold surfaces are susceptible to contamination, causing the performance of the microtip to be unstable. Furthermore, washing the microtip surface to remove inhibitors that could be collected from complex samples also removes the captured DNA due to the weak binding force.

A positively charged surface coating can provide a stable surface with a stronger capturing and binding force[112]. Polyethyleneimine (PEI) is a positively charged polymer due to the highly protonable nitrogen atoms within the PEI chain[157]. The polymer structure of polyethyleneimine [also cited as poly(ethyleneimine), poly(ethylene imine), polyethylenemine, polyethylenimine, or polyaziridine] is composed of chains of two ethylene ($–CH_2$-) monomers and an amine group. PEI is found in linear and branched form. Branched PEI is typically formed through ring-opening polymerization of ethyleneimine. The ring shaped monomer ethyleneimine (or aziridine) is composed of two methylene bridges ($-CH_2$-) and a secondary amine group (=NH). The ring-opening process allows for primary, secondary, and tertiary amino groups to form, creating the branched structure. Linear polyethyleneimine, $[C_2H_5N]_n$, contains only secondary amino groups[158]. The amine groups hold the positive charge of PEI and potential binding site for negatively charged DNA molecules.

Electrostatic interaction between PEI and DNA is due to the negatively charged phosphate backbone of DNA that protonates the nitrogen atoms in PEI (backbone

binding)[159]. Furthermore, it has been shown that the PEI-DNA complex has two distinct binding modes[160]. PEI can also dehydrate the DNA helix and bind through hydrogen bonds to the base pairs along the nucleic acids major groove (groove binding mode). The binding modes can change the shape of DNA and the electric charge with a change in enthalpy[161]. PEI is widely used for gene delivery because of the affinity of PEI to DNA and the ability to release DNA within a cell[162].

Recent work using PEI for drug delivery shows that the molecular weight of PEI is related to its binding efficiency with DNA. The molecular weight of PEI can range from 500 Da[163] to 800 kDa[164]. High-molecular weight PEI has a stronger binding affinity to DNA than low-molecular weight PEI[165]. However, cell toxicity of high-molecular weight PEI limits the use of high-molecular weight PEI for drug delivery[166]. Currently, 25 kDa branched PEI and 22 kDa linear PEI are most effective for drug delivery[159].

For DNA sample preparation, PEI coated on magnetic particles can extract DNA from bacterial cells[101,102], liver tissue[103], and whole blood[104]. The extraction of DNA from bacterial cells is performed by coating superparamagnetic nanoparticles (Fe_3O_4) with PEI[101]. PEI is first dispersed in 50% methanol and heated at 80°C. Then, nanoparticles are stirred in the PEI solution for 1 hour. The PEI-coated nanoparticles are rinsed with acetone, ethanol and DI water before storage and use. In this study a 1.25 M NaCl solution (9.0 pH) is optimal for the elution of DNA for UV spectrophotometry and gel electrophoresis. The optimal release of DNA from Fe_3O_4/Au nanoparticles coated with PEI is at 50°C for 20 minutes in Tris-HCl buffer (8.2 pH) with a salt concentration of 1.5 M NaCl[103]. The Fe_3O_4/Au nanoparticles are coated with PEI by mixing the nanoparticles in a PEI solution for 6 hours at room temperature. Optimal capturing efficiency of DNA on the PEI-coated Fe_3O_4/Au

nanoparticles is in PBS (5.0 pH). 25-kDa branched PEI can be coated on $FePO_4$ nanoparticles[104]. The $FePO_4$ nanoparticles are ultrasonicated for 2 hours in a PEI solution. PEI-coated $FePO_4$ nanoparticles are rinsed with DI water and heated at 50°C for 6 hours. Magnetic bead-based extraction using the PEI-coated $FePO_4$ nanoparticles can extract similar quantities of DNA from whole blood compared with a commercial kit. PEI-coated Fe_3O_4, Fe_3O_4/Au, and $FePO_4$ nanoparticles demonstrate that PEI is a viable surface coating for the capture and release of DNA.

Functionalization of PEI through physical adsorption results in an unstable layer that can dissolve in solution due to the lack of strong chemical binding forces[167]. For PEI layers used for DNA extraction, weakly bound PEI can transfer with DNA into downstream analytical tools. With analytical techniques, such as polymerase chain reaction (PCR), PEI can work as an inhibitor of the enzyme reaction and interfere with the PCR signal by binding with DNA. PEI is coated on Fe_3O_4, Fe_3O_4/Au, and $FePO_4$ nanoparticles by electrostatic interaction or covalent bonding due to the negatively charged nanoparticles surface[103,104,168]. However, PEI-coated nanoparticles are aggresively washed to remove excess PEI that is bound through physical adsorption above the electrostatically bound PEI layer[104,169,170]. DNA extracted using PEI-coated $FePO_4$ nanoparticles can be amplified through PCR, but there is no evaluation of the inhibition due to PEI. Purity analysis demostrated the elimination of contaminating proteins through UV spectrophotometry. No analysis has demonstrated that PEI does not dissolve into the final elution sample, or the effect of PEI on PCR. The concern for physically adsorbed PEI prevents the use of high-molecular weight PEI because of its high viscosity and cumbersome washing steps to remove excess PEI. Simple, reproducible processing of high-molecular weight PEI coatings has not been

demonstrated for DNA sample preparation. Understanding the effect of the PEI layer on PCR is critical to its use in DNA sample preparation. The inhibition of PEI in qPCR analysis and coating of high-molecular weight PEI are studied in this work.

3.1.3 *Summary*

PEI is a water-soluble polymer with a strong affinity to DNA. The mechanism of releasing DNA from PEI can be controlled, which is beneficial in transfection techniques[154-156]. For DNA sample preparation, PEI coated on magnetic particles has shown to be viable for the extraction of DNA from bacterial cells[101,102], liver tissue[103], and whole blood[104]. Magnetic particles are immersed and mixed in PEI solution to coat the surface through electrostatic interaction. The coating and drying process takes 6-8 hours and post-process washing is required to remove weakly bound PEI layers. The coating process has also been limited to PEI at molecular weights of 25 kDa. The processing of the PEI layer is a challenge for wide-spread use with DNA sample preparation methods.

Though PEI-coated $FePO_4$ nanoparticles can provide an amplifiable solution containing extracted DNA, no analysis demonstrates the PCR signal is not inhibited due to PCR. No analysis has demonstrated that PEI does not dissolve into the final elution sample or the effect of PEI on PCR. Also, the aforementioned methods show optimal release of DNA from PEI using NaCl, which is also an inhibitor to PCR. Use of PEI for DNA sample preparation needs further investigation to prevent inhibition of samples for downstream applications.

Utilization of a heat-cured PEI layer on the microtip surface was investigated for the extraction of DNA. A high-molecular weight (750 kDa) PEI layer will provide an additional

capturing force to complement the DEP, viscous, and capillary forces induced on DNA. The baking temperature of the PEI layer was investigated to cure the water-soluble PEI layer. Baking temperatures above the curing temperature were also investigated to control the capture and release of DNA with the PEI layer. Pure λ DNA was used for the experiments in this chapter as templates for the optimized extraction and purification of DNA utilizing heat-cured high-molecular weight PEI-coated microtips. The results in this chapter will demonstrate that:

1. High-molecular weight PEI binds with DNA to inhibit PCR, which was evaluated through PCR and gel electrophoresis.
2. PEI baked below 150°C dissolves off the gold-coated surface and inhibits PCR by binding with DNA.
3. PCR was not inhibited by PEI dissolving off the gold-coated surface when the PEI was cured at 150°C or above.
4. The baking temperature of PEI is related to the efficiency of the release of DNA.
5. PEI-coated microtips cured at 225°C improve the recovery of DNA compared with non-coated microtips.

3.2 EXPERIMENTAL METHODS

First, the inhibition of PEI was evaluated. 50% w/v PEI was serially diluted in pure water. The PEI solutions were mixed with λ DNA. The signal from PCR and gel electrophoresis was compared to control λ DNA samples to determine the inhibition of PEI.

Second, the effect of heat curing of PEI was evaluated to determine if PEI will dissolve in solution. PEI is coated on gold-coated microtips and baked at temperatures from

100°C to 250°C. The baked PEI-coated microtips were immersed in solution and heated at 60°C to determine if PEI dissolved off the microtip surface. The condition of 60°C was chosen because a heating step will be required to release DNA from the microtip surface potentially releasing PEI as well. The heated solution was removed and mixed with λ DNA. The mixed solution was evaluated with PCR and gel electrophoresis.

Third, PEI was coated on gold-coated microtips and baked at 100°C and 225°C. PEI-coated microtips left to dry at room temperature were also used. The PEI-coated microtips were immersed in solution for 10 seconds to determine if PEI will dissolve off the microtip surface. The 10 second immersion condition was chosen to evaluate if the PEI layer will be stable if washing steps were used. Microscopy was used to observe the change in the topography of the PEI layer after washing.

Fourth, the release of DNA off stable PEI was studied using PEI-coated microtips baked at temperatures from 150°C to 250°C. DNA was dried directly on the PEI layer and heated at 60°C to elute DNA into solution. The heated solution was removed for analysis with PCR and gel electrophoresis.

Fifth, the capture and release of DNA was investigated at temperatures from 100°C to 250°C. DNA was captured on heat-cured PEI-coated microtips through an applied AC electric field. Then, the microtips were heated in TE buffer to release the captured DNA into solution. The heated solution was removed for analysis with PCR and gel electrophoresis.

Finally, the recovery of DNA from washed PEI-coated microtips baked at 225°C was studied and compared to non-coated microtips.

3.2.1 *PEI inhibition*

To analyze the effect of PEI on qPCR, various PEI concentrations were evaluated with quantitative PCR (qPCR) and gel electrophoresis. PEI concentrations of 0, 6, 31, 62, 125, and 188 ng/mL were spiked into 167 fM λ DNA and mixed. The DNA-PEI solution was evaluated through qPCR. For gel electrophoresis, PEI was mixed with the E-Gel® 96 High Range DNA Marker and evaluated through gel electrophoresis using E-Gel® EX 1% Agarose for 10 minutes. PEI concentration of 150 ng/mL, 300 ng/mL, 750 ng/mL, 1500 ng/mL, 3000 ng/mL was spiked with the DNA markers. The effect of 95°C heat on the DNA markers with 150 ng/mL of PEI and without PEI was analyzed through gel electrophoresis

3.2.2 *Heat-cured PEI layer*

PEI was diluted to 0.1% w/v in deionized (DI) water to coat the microchip surface. The microchips were baked on a hotplate for 10 minutes at 300°C. Then, the microchips were immersed in 15 μL of PEI solution for 1 minute to coat the surface. The PEI-coated microchips were baked for one hour in a thermal chamber (Cascade Tek). PEI-coated microchips were investigated at baking temperatures from 100°C to 250°C.

The stability of the PEI-coated microchips was investigated by immersing the cured PEI-coated microchips in 30 μL of TE buffer and heating at 60°C for 5 minutes. After heating, 2.5 μL of the heated solution was mixed with 2.5 μL of 1 pM λ DNA and put directly into PCR for amplification. Also, 10 μL of the heated solution was mixed with 10 μL E-Gel® 96 High Range DNA Marker and run through E-Gel® EX 1% Agarose for 10 minutes.

The stability of the PEI layer was observed through optical and fluorescent microscopy after immersion into solution. PEI-coated microchips were immersed in TE

Buffer for 10 seconds to test the resistance of the PEI layer to dissolving. An Olympus BX41 microscope was used to image the PEI layer. Fluorescence emission was observed with a fluorescence filter (emission: 515nm, excitation: 450~480nm).

The efficiency of eluting DNA off the PEI surface was investigated for PEI-coated microchips baked at 150°C, 175°C, 200°C, 225°C and 250°C. After baking, 1 μL of 1 pM λ DNA was dried on the PEI surface at room temperature. The PEI-coated microchips with dried DNA were immersed in 30 μL of TE buffer and heated at 60°C for 5 minutes. The eluted solutions were analyzed through qPCR. For gel electrophoresis analysis, 1 μL of the E-Gel® 96 High Range DNA Marker was dried on the PEI surface at room temperature. The PEI-coated microchips with dried ladder were immersed in 30 μL of TE buffer and heated at 60°C for 5 minutes. The elution solution was run through E-Gel® EX 1% Agarose for 10 minutes. ImageJ was used to quantify the intensity of the different samples tested through gel electrophoresis. The fluorescence intensity of each lane was determined by averaging the intensity of the 5 ladder bands that correspond to the different lengths of DNA present. The intensity was normalized to the background signal from the agarose gel.

3.2.3 *Capture and release of DNA*

For electric field-induced extraction of DNA with PEI-coated microtips, baking temperatures of 100°C, 150°C, 175°C, 200°C, 225°C and 250°C were investigated. The baked PEI-coated microtips were evaluated for the capture and release of λ DNA. Non-PEI coated gold microtips were also investigated for the capture and release of λ DNA. To extract λ DNA onto the microtip surface, 5 μL of λ DNA solution was suspended in a coil made of silver-coated copper wire. The inside diameter of the coil was 1.6 mm. The microtip and coil

were connected to a function generator to apply an electric field through the solution when the microtip was immersed into the sample solution (Fig. 14). An AC voltage of 20 V_{pp} at 5 MHz was applied between the microtips and the coil for 10 minutes to concentrate λ DNA. The microtip was then withdrawn from the sample solution and placed in an elution vial. An aliquot of 30 µL-TE buffer was pipetted into the vial and placed in a heat block at 60°C for 5 minutes to elute λ DNA from the microtips surface. The extraction and release of λ DNA was analyzed through qPCR.

Elution temperature for the release of DNA was also investigated. Under the same capturing conditions as above, PEI-coated microtips baked at temperatures of 175°C, 200°C, 225°C and 250°C were evaluated for the recovery of λ DNA at different elution temperatures (60°C, 70°C, 80°C and 90°C). All elutions were done for 5 minutes. The extraction and release of λ DNA was analyzed through qPCR.

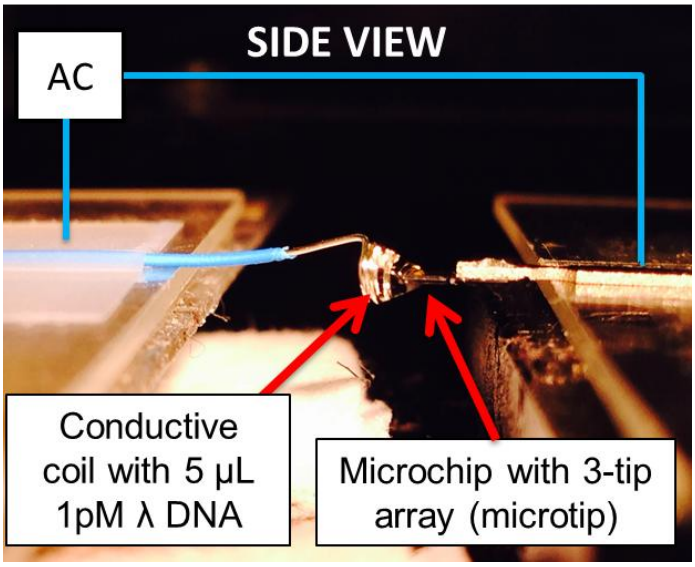

Figure 14. Experimental setup (side view) for the concentration of DNA from a 5 µL sample.

3.2.4 *Storage of PEI-coated microtips*

To evaluate the long term stability of PEI-coated microtips, microtips coated with PEI baked at 225°C were stored at room temperature for up to one month. Microtips stored for 0, 1, 7, 14, and 28 days were used for the extraction of λ DNA as outlined above to evaluate the efficacy of DNA recovery.

3.2.5 *Recovery of DNA after washing*

To evaluate the recovery of DNA from washed PEI-coated microtips, microtips coated with PEI baked at 225°C and non-PEI coated gold microtips were used for the extraction of λ DNA as outlined above. After extraction, the microtips were washed for 10 seconds in TE buffer. An aliquot of 30 μL-TE buffer was pipetted into a vial containing the washed microtip and placed in a heat block at 60°C for 5 minutes to elute λ DNA from the microtips surface. The 225°C-baked PEI-coated microtips were compared to non-coated microtips through qPCR.

3.3 EXPERIMENTAL RESULTS

3.3.1 *PEI inhibition*

The effect of PEI on PCR was analyzed. Various concentrations of PEI (0, 6, 31, 62, 125, and 188 ng/mL) were spiked with 167 fM λ DNA and reached the cycle threshold at 13.74±0.03, 14.19±0.02, 19.14±0.06, 24.64±0.49, 24.51±0.32, 27.95±1.23 cycles, respectively. The results showed that as low as 6 ng/mL of PEI could affect the amplification of DNA (Fig. 15a). The amplification product of qPCR was consistent for all tests. The melting temperature was 87.5°C (Fig. 15b).

To further study the binding of PEI to DNA, PEI-DNA mixtures were tested with gel electrophoresis (Fig. 16). When 60 ng of PEI is mixed with 100 ng of the DNA ladder (Lane 4), complete inhibition of the signal was observed. However, this was a much higher concentration (3000 ng/mL) than was observed to completely inhibit the amplification of λ DNA in PCR. This can be due to the difference in the DNA template along with the concentration of the DNA template used. The concentration of the DNA ladder used for gel electrophoresis (10 ng/µL) was much larger than the concentration of λ DNA (5.25 pg/µL), meaning more PEI will be needed to bind to all the DNA. The gel electrophoresis data confirms that PEI does bind with DNA in solution, which will cause inhibition of the amplification of DNA through PCR.

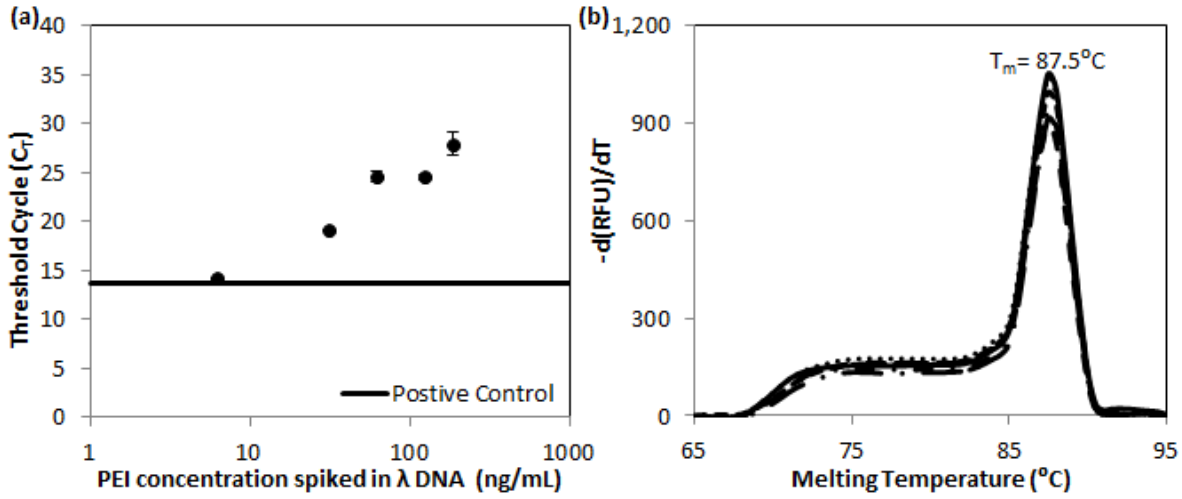

Figure 15. (a) qPCR threshold cycles for λ DNA (167 fM) spiked with PEI. Threshold cycle for positive control (0 ng/mL PEI) was 13.7 (n=3). (b) Melting curve derivative for amplified DNA after the completion of PCR.

The inhibition of PCR due to PEI could be caused by the binding of PEI to both DNA and polymerase. When PEI is in solution with DNA, the DNA will bind with PEI, as was

shown by the inhibition of DNA ladders traveling through agarose gel. When PEI is bound to DNA, it has been shown to prevent DNA from denaturing, which would inhibit PCR.

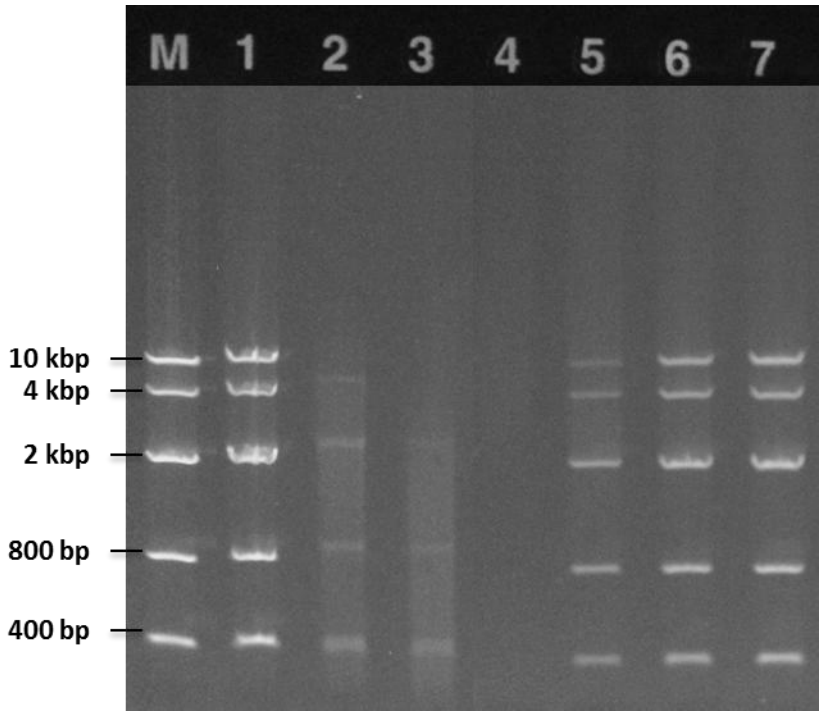

Figure 16. Gel electrophoresis for PEI mixed with the DNA ladders. Lane M: Ladder, Lane 1: Ladder + 150 ng/mL PEI, Lane 2: Ladder (heated at 95°C for 10 minutes), Lane 3: Ladder + 150 ng/mL PEI (heated at 95°C for 10 minutes), Lane 4: Ladder + 3000 ng/mL PEI, Lane 5: Ladder + 1500 ng/mL PEI, Lane 6: Ladder + 750 ng/mL PEI, Lane 7: Ladder + 300 ng/mL PEI.

3.3.2 *Heat-cured PEI layer*

The study of PEI as an inhibitor to PCR showed that as low as 6 ng/mL of PEI spiked into a PCR reaction caused inhibition. Therefore, the use of PEI for microtip-based DNA extraction must be controlled to prevent contamination of samples for downstream applications.

Gold-coated microtips coated with PEI were immersed in a buffer solution (TE Buffer) to test the PEI layers stability. The topography of the PEI layer was shown to be significantly changed. Therefore, the PEI-coated microtips were baked to cure the PEI layer.

Fig. 17 demonstrates the improvement in the resistance to dissolution when the PEI-coated microtip was baked at 225°C for 1 hour. No significant change in topography was observed after washing in TE Buffer for 10 seconds.

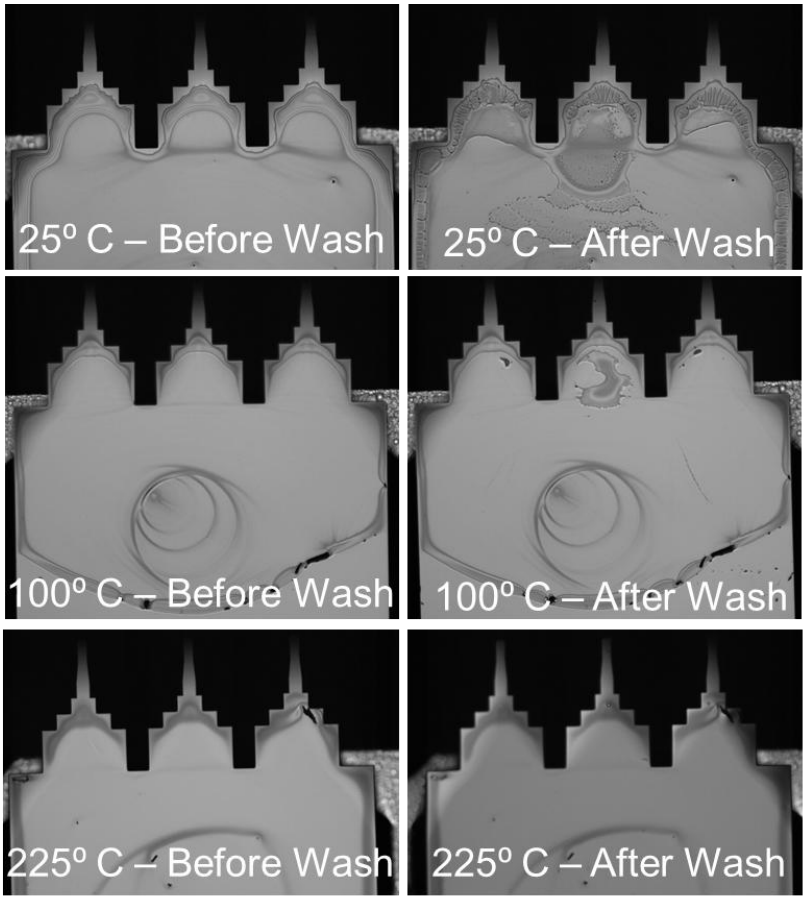

Figure 17. The resistance of the PEI layer to washing. The PEI layer in the top images was dried at room temperature (25°C) for 1 hour before testing. The PEI layer in the middle images was dried at 100°C for 1 hour before testing. The PEI layer in the bottom images was baked for 1 hour at 225°C before washing.

To further observe if PEI on microtip surface was partially dissolved in TE buffer, the fluorescence of the PEI-coated microtip surface was observed before and after washing with TE buffer (Fig. 18). When the PEI-coated microtips were baked, the cured PEI layer emits autofluorescence. The effects of the baking temperature were observed through fluorescent imaging. At 100°C, there was a significant change in fluorescence on the microtip surface

after washing in TE buffer. The increase in fluorescence was due to the coffee ring effect redistributing some of the dissolved PEI when the microtip was removed from the solution and dried[171]. However, 225°C baked microtips showed negligible change in fluorescence.

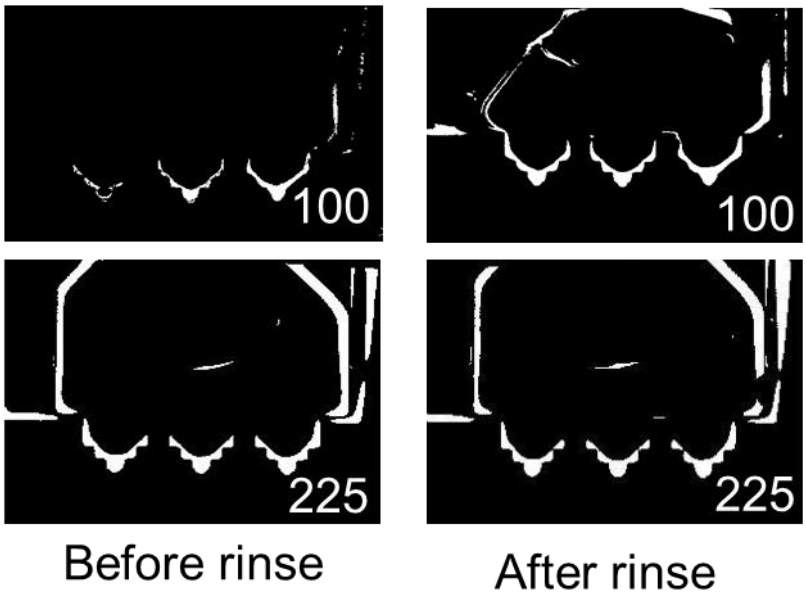

Figure 18. Autofluorescence images of PEI-coated microtips baked at 100°C and 225°C before and after washing with TE buffer. Courtesy of Andrew Cairns.

The heat-cured PEI-coated microtips were evaluated to determine if PEI could be released from the surface and inhibit qPCR. PEI-coated tips baked at 100°C, 110°C, 120°C, 130°C, 140°C, 150°C, and 160°C were immersed into TE buffer and heated at 60°C for 5 minutes. The heated TE buffer solutions were removed and mixed with λ DNA. For 100°C and 110°C baked PEI-coated tips, there was no signal from qPCR and gel electrophoresis (Fig. 19a, 19b). The inhibition indicates that PEI dissolved off the microtip surface and when that solution was mixed λ DNA, the PEI bound with λ DNA. For λ DNA mixed with TE buffer solutions heated with PEI-coated tips baked at 120°C, 130°C, 140°C, 150°C and 160°C, the threshold cycle for the amplification of λ DNA was 37.6±4.2, 23.3±2.5, 18.9±0.6,

18.5±0.3, and 18.4±0.1, respectively. The positive control sample was amplified at 18.4±0.4 cycles, indicating that, when the PEI layer is baked at 140°C or below for 1 hour, PEI can dissolve in TE buffer at 60°C. For use as a stable surface for the extraction and purification of DNA, the data shows that a baking temperature of 150°C would be required to prevent PCR inhibition.

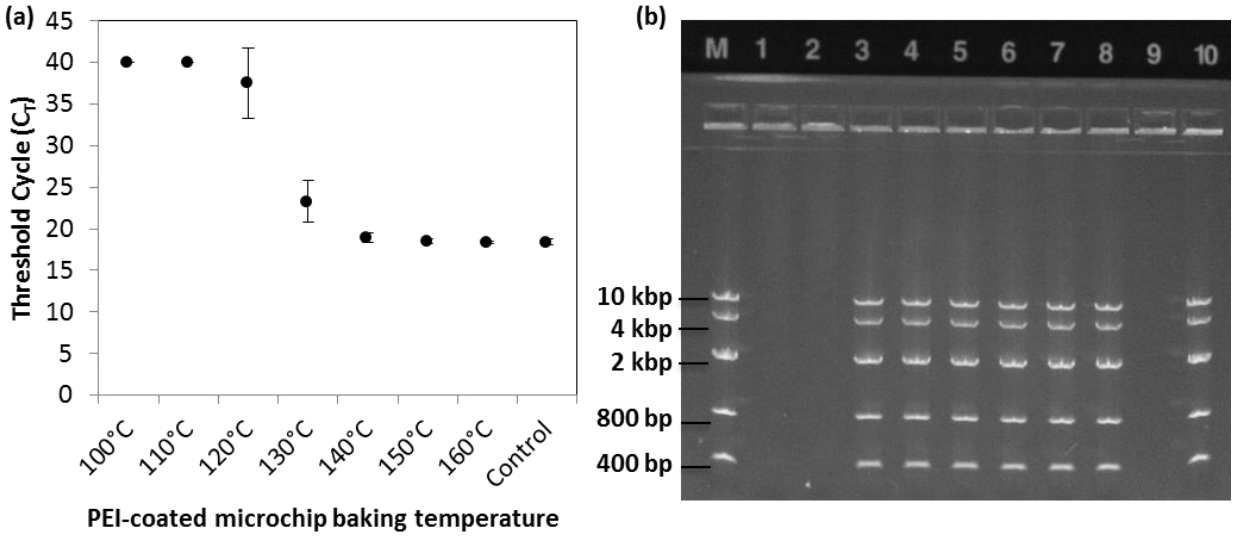

Figure 19. (a) PCR amplification of TE buffer solution heated with the PEI-coated microtips and then mixed with λ DNA. (b) Gel electrophoresis for TE buffer solution heated with the PEI-coated microtips and then mixed with the DNA ladders. Lane M: Ladder, Lane 1: 100°C cured PEI-coated tip, Lane 2: 110°C cured PEI-coated tip, Lane 3: 120°C cured PEI-coated tip, Lane 4: 130°C cured PEI-coated tip, Lane 5: 140°C cured PEI-coated tip, Lane 6: 150°C cured PEI-coated tip, Lane 7: 160°C cured PEI-coated tip, Lane 8: Ladder mixed with TE buffer, Lane 9: Blank, Lane 10: Ladder.

3.3.3 *Capture and release of DNA*

The efficiency of releasing DNA off the PEI surface was investigated. PEI-coated microchips were cured at 150°C, 175°C, 200°C, 225°C and 250°C. The threshold cycles for DNA released from the heat-cured PEI surfaces were 35.6±2.7, 24.5±1.2, 20.0±1.1, 17.7±0.6, and 17.0±0.6, respectively. For gel electrophoresis, DNA ladders were dried on cured PEI at baking temperatures of 175°C, 200°C, 225°C and 250°C (Fig. 21a). The fluorescence

intensity of the ladders showed similar intensities between the control sample and samples eluted from 200°C, 225°C and 250°C baked PEI-coated microtips. The normalized intensity for the positive control was 1.14. The normalized intensity for the released ladder DNA dried on the PEI surface baked at 150°C was 1.03. For PEI baked at 175°C, the normalized intensity was 1.09. For PEI baked at 200°C, the normalized intensity was 1.15. For PEI baked at 225°C, the normalized intensity was 1.17. For PEI baked at 250°C, the normalized intensity was 1.16. (Fig. 21b).

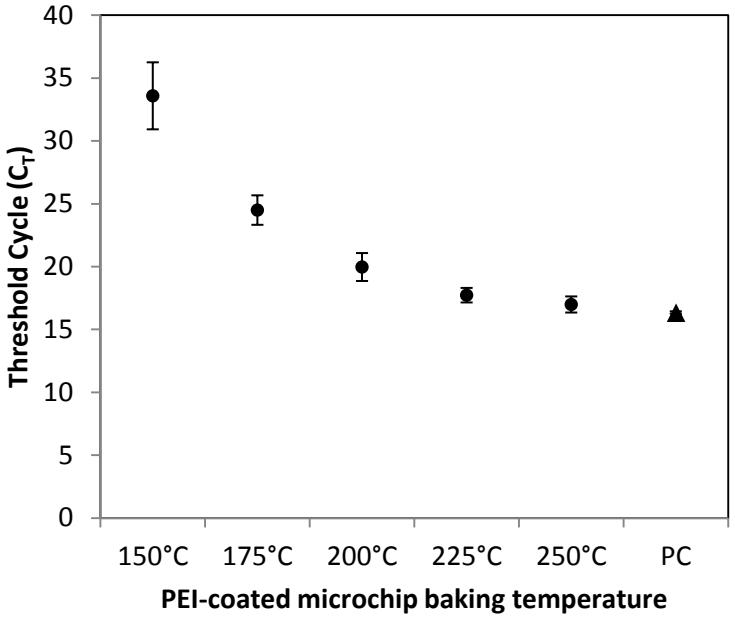

Figure 20. PCR amplification of dried λ DNA eluted at 60°C for 5 minutes off baked PEI-coated microtips.

Data for qPCR and gel electrophoresis shows that when PEI is cured at 150°C, an undetectable amount of DNA was released off the surface. The gel data showed a plateau of the fluorescence signal for ladder DNA eluted off PEI-coated tips baked at temperatures of 200°C and above. From PCR analysis, as the baking temperature was increased, the threshold cycle was significantly improved showing the best signal at 250°C.

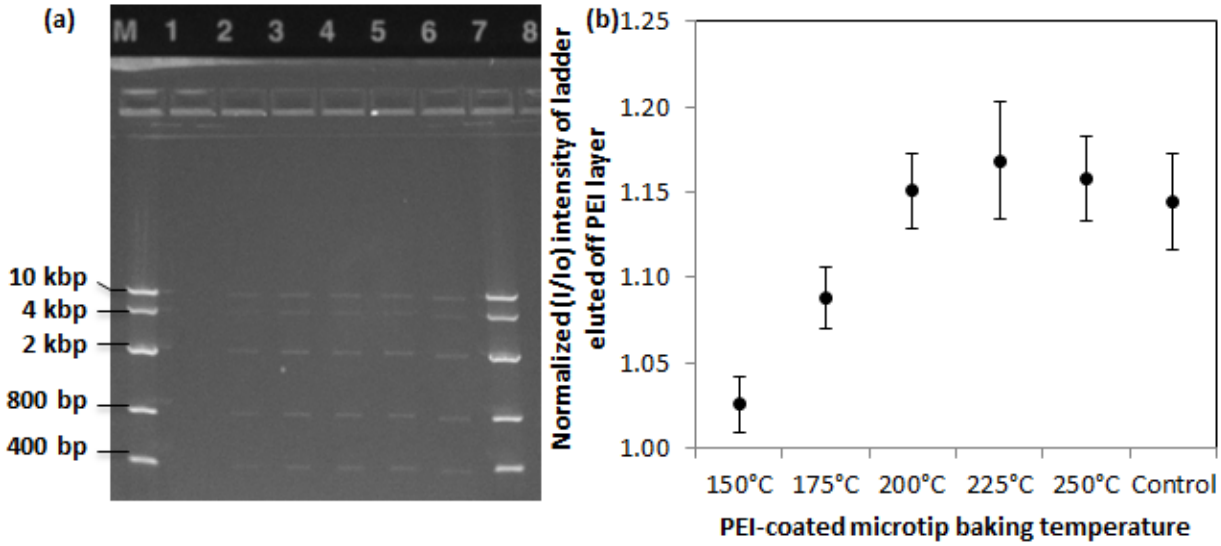

Figure 21. (a) Gel electrophoresis of dried λ DNA eluted at 60°C for 5 minutes off baked PEI-coated microtips. Lane M: Ladder, Lane 1: 150°C cured PEI-coated tip, Lane 2: 175°C cured PEI-coated tip, Lane 3: 200°C cured PEI-coated tip, Lane 4: 225°C cured PEI-coated tip, Lane 5: 250°C cured PEI-coated tip, Lane 6: Ladder diluted 1/30th, Lane 7: Ladder. (b) Normalized intensity of the fluorescence signal from DNA ladders run through gel electrophoresis shown in (a).

When the microtips were functionalized with PEI, the surface properties varied with different baking conditions. When the microtips were baked at 200°C or below, the contact angle of a 0.5 μL drop of TE Buffer was shown to be between 40°~45°. When the baking temperature was increased to 225°C, the contact angle increased to 53°. At a baking temperature of 250°C, the contact angle was 67° (Fig. 22).

The capture and release of λ DNA amplified through qPCR was significantly influenced by the baking temperature of the PEI layer (Fig. 23). For baking temperatures of 100°C, 150°C, 175°C, 200°C, 225°C and 250°C, the threshold cycles were 40.0±0.0, 27.0±9.37, 18.3±0.9, 15.8±0.2, 15.3±0.3, and 16.5±0.5, respectively.

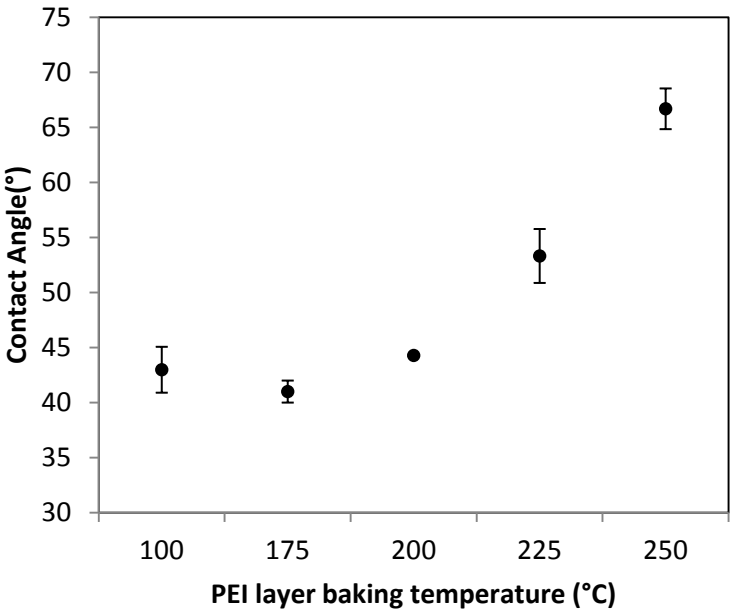

Figure 22. Contact angles for 0.5 μL of 1x TE buffer (8.5 pH) on PEI-coated microtips baked at 100°C, 175°C, 200°C, 225°C, and 250°C (n=2).

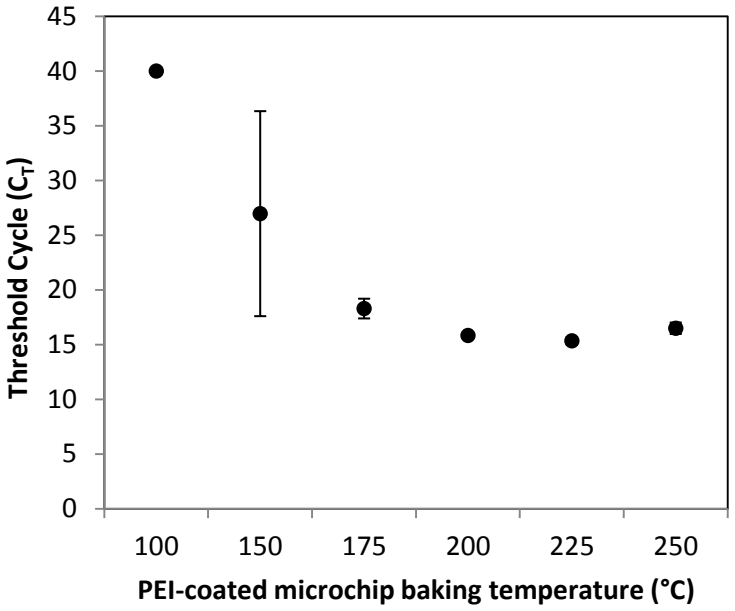

Figure 23. Recovery of λ DNA using PEI coated microtips at the baking temperatures of 100°C, 150°C, 175°C, 200°C, 225°C, and 250°C (n=3).

The capture and release of λ DNA with cured PEI-coated microtips at 175°C, 200°C, 225°C, and 250°C was further investigated with respect to elution temperatures (Fig. 24). PEI-coated microtips baked at 225°C showed the lowest threshold cycle of 15.34 at 60°C elution in TE Buffer for 5 minutes. The 175°C baked microtips showed the highest threshold cycle. This indicated that the highest yield of λ DNA was from the 225°C-baked microtips eluted at 60°C. Microtips baked at 175°C showed the lowest yield.

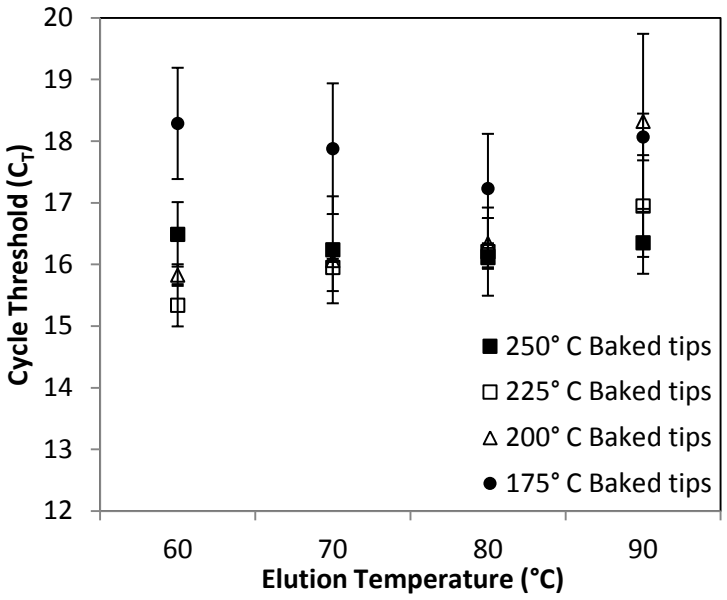

Figure 24. qPCR threshold cycles for the capture and release of λ DNA using microtips coated with PEI. Baking temperatures are 175°C, 200°C, 225°C, and 250°C. Elution temperatures are 60°C, 70°C, 80°C, and 90°C (n=3).

The capture and release of λ DNA from heat-cured PEI-coated microtips was compared with gold-coated microtips. The recovery yield of pure λ DNA in TE buffer using gold-coated microtips was 25%. The addition of the PEI layer cured at 225°C increased the recovery yield of λ DNA to 45% (Fig. 25).

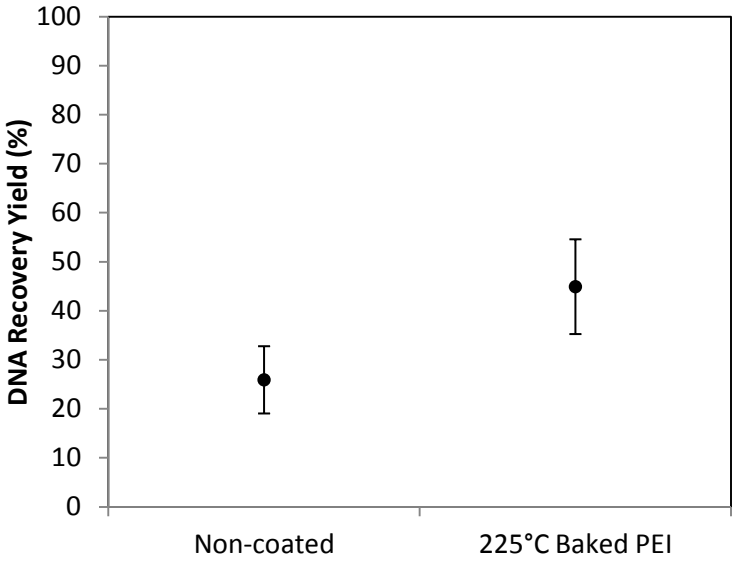

Figure 25. Comparison of PEI and non-PEI coated microtips. Recovery yield for the capture and release of λ DNA using microtips eluted at 60°C for 5 minutes (n=3).

3.3.4 *Storage of PEI-coated microtips*

The long term stability of PEI on the microtips was evaluated. Microtips coated with PEI cured at 225°C were stored at room temperature for up to one month. Microtips stored for 0, 1, 7, 14, and 28 days were used for the extraction of λ DNA to evaluate the efficacy of DNA recovery. When the PEI-coated microtips were stored for 1 month before use, the yield of DNA recovery did not degrade (Fig. 26).

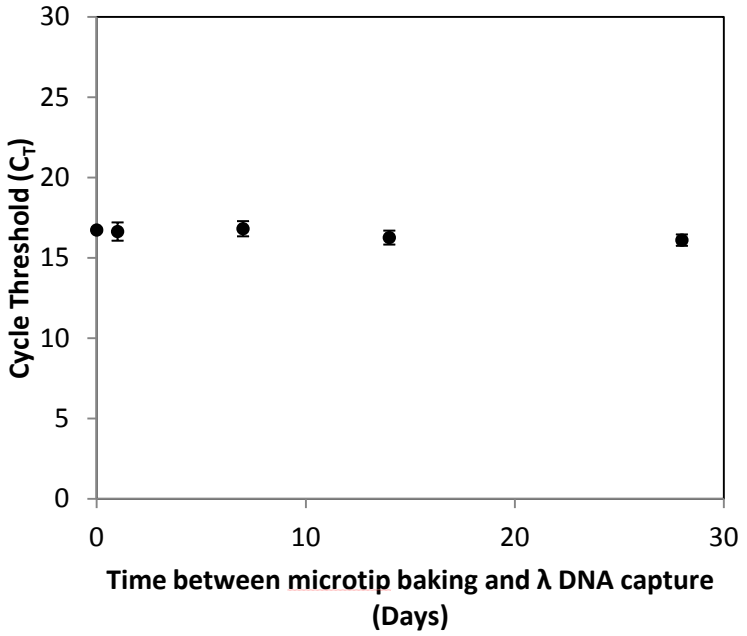

Figure 26. Capture and release of DNA using stored PEI-coated microtips.

3.3.5 *Recovery of DNA after washing*

Gold-coated microtips (Non-PEI coated) and PEI-coated microtips were washed with TE buffer after the extraction of DNA (Fig. 27). The recovery yield for the non-coated microtips and 225°C-cured PEI-coated microtips was 2.79%, and 16.90%, respectively. The results show that the 225°C-baked PEI-coated microtips perform better than non-coated microtips when a washing step was required.

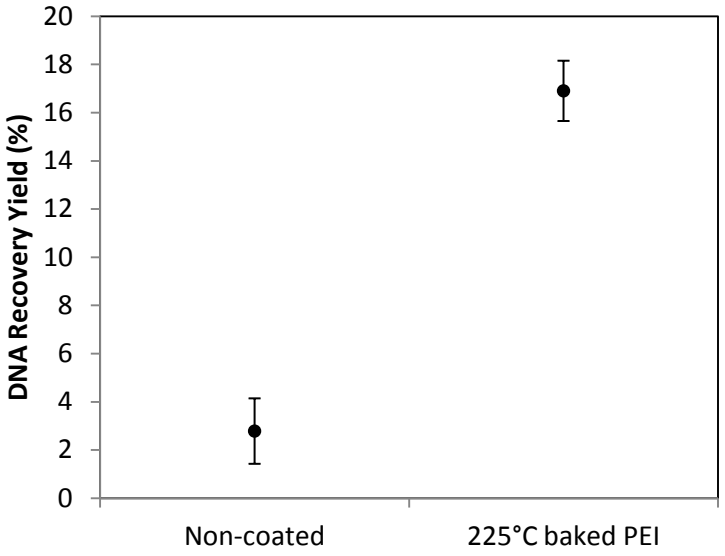

Figure 27. Washing results of the non-coated versus PEI-coated microtips for recovery of λ DNA.

3.4 DISCUSSION

The coating of PEI on gold-coated microtips was investigated. PEI was coated on the surface of the microtips to control the surface properties. During the washing and elution process of DNA extraction, PEI could dissolve off the microtip surface decreasing the recovery yield of DNA. The dissolution of PEI was observed through optical and fluorescence imaging. PEI that was dissolved off the microtip could inhibit PCR amplification by binding with DNA in solution. Gel electrophoresis confirmed that PEI does bind with DNA. PEI bound to DNA has been shown to prevent DNA from melting[100]. Melting (denaturing) of DNA is required for amplification using PCR. Our study shows as low as 6 ng/mL of PEI spiked into a PCR reaction shows an underrepresentation of DNA present in solution. PEI inhibits DNA from melting; therefore, an underrepresented PCR signal of amplified DNA was produced when PEI is in solution with DNA. Thus, the stability of the PEI layer was critical for microtip-based DNA extraction.

Heat curing PEI was crucial for a stable surface. For curing temperatures below 150°C, the PCR signal was inhibited. The gel electrophoresis validated that PEI was dissolving into solution and binding to DNA. Curing temperatures above 150°C showed negligible amounts of PEI dissolved off the surface. This was determined through observing no change in signal of PCR and gel electrophoresis in the experiments run in Figure 19. For temperatures above 150°C, the PEI layer was considered stable for microtip-based DNA extraction. However, at baking temperatures below 200°C, it was observed that DNA could not be effectively released off the PEI surface. As a result, the yield of DNA recovery was reduced (Fig. 20). The result indicated that 250°C showed the optimal release of DNA from the surface. The increase in baking temperature could increase the thermal oxidation of the PEI layer[172,173]. Oxidation of the amine groups reduces the surface charge of PEI[174], which could reduce the binding force to DNA resulting in more effective release of DNA. However, when combined with the electric-field induced concentration and capture of DNA on the microtip surface, a curing at 225°C showed the optimal recovery of DNA. Previously, Idris et al. observed the change in structure of PEI baked at 250°C[173]. The change in surface structure was shown in our study by changes in the contact angle. As the baking temperature increased, the contact angle of the PEI layer increased. The change in surface structure could decrease the capture of DNA with the PEI-coated microtips, resulting in lower yield. PEI-coated microtips baked at 225°C were stored at room temperature up to a month after the PEI coating and curing process. The recovery of λ DNA did not degrade for the stored microtips.

At this temperature, DNA could be bound on the microtip surface and eluted from the surface at 60°C with a yield of 45% with the positively charged 225°C cured PEI coating. Finally, the heat-cured PEI-coated microtips improved the recovery of DNA after undergoing

a washing step in comparison to non-PEI coated microtips. Some DNA was lost due to the washing step, indicating that PEI was not able to bind securely to all captured DNA. Future investigation into the washing/binding mechanism will be needed to improve the recovery of DNA when the microtip surface was washed.

3.5 SUMMARY

This study shows that λ DNA was captured and released from small volume samples using heat-cured PEI-coated microtips. A simple dip coating method was implemented to functionalize the surface of gold-coated microtips with high-molecular weight PEI and heat processed for 1 hour before use. It was discovered that PEI can dissolve off microtip surface when the baking temperature was below 150°C. The dissolved DNA was shown to bind to DNA and inhibit the qPCR and gel electrophoresis signals. Heat curing the PEI layer above 150°C on the gold-coated microtip surface prevented PEI from inhibiting PCR. Electric field-induced concentration and capture of DNA on PEI-coated microtips baked at 225°C were shown to recover the highest amount of λ DNA when eluted in TE buffer for 5 minutes at 60°C. PEI-coated microtips cured at 225°C addressed the challenges of gold-coated microtips by (1) improving the capture and release of DNA from 25% to 45%, and (2) improving the recovery of DNA when the surface was washed from 3% to 17%, making the heat-cured PEI-coated microtips viable for the recovery of DNA from complex samples.

The improved performance of the heat-cured PEI-coated microtips allows for the investigation of microtip-based extraction with complex samples. The following work (Chapter 4 & Chapter 5) demonstrates the use of heat-cured PEI-coated microtips for the extraction and purification of DNA from whole blood, dried blood, and forensic samples.

Chapter 4. EXTRACTION AND PURIFICATION OF DNA FROM WHOLE BLOOD

4.1 INTRODUCTION

As mentioned in Section 1.3, DNA is typically found within a complex matrix of particles, many of which can be inhibitive to DNA itself or downstream applications such as PCR. While Chapter 3 addressed the optimal capture and release DNA using PEI-coated microtips cured at 225°C, the challenge of providing purified DNA from human samples still remains.

Binding of DNA to the gold-coated microtips relies on nonspecific binding, van der Waals force, on the gold-coated microtips. After DNA is concentrated to the microtip surface due to electrohydrodynamics, binding occurs when the microtip is withdrawn from the sample. As the microtip pulls away from the solution, a thin film forms due to the contact angle, the density of the solution, the viscosity of the solution, and withdrawal speed of the microtip[134]. The thin film retains DNA concentrated in close proximity to the microtip surface by the electric field. Van der Waals interaction between DNA and gold binds DNA to the microtip surface. The weak binding force prevents the use of gold-coated microtips with complex samples where washing of the microtip surface is required to remove inhibiting particles that can non-specifically bind to the microtip surface in the same mechanism as DNA during the extraction process. In Chapter 3, the ability to use a washing step with cured PEI-coated microtips was demonstrated. PEI provides an additional binding force to improve the capture and retention of DNA. Here, a variety of washing steps were evaluated for the

effectiveness of removing inhibitors from the PEI surface that can be left behind from whole blood. The purified DNA from whole blood is evaluated through qPCR and STR profiling.

4.2 EXPERIMENTAL METHODS

Previously, human genomic DNA has been extracted from whole blood using nanotips[137]. The protocol for the preparation of the whole blood sample for extraction used with the nanotips was used for DNA extraction using heat-cured PEI-coated microtips.

The first study evaluated the use of washing steps after the extraction of DNA using the heat-cured PEI-coated microtips. The washed microtip samples were heated in TE buffer to elute DNA into solution. The heated solution was analyzed through qPCR.

Second, the extraction and purification of human genomic DNA from six whole blood donor samples was evaluated in comparison with the QIAamp® Mini Blood Kit.

4.2.1 *Purification of extracted DNA*

Prior to microtip capture, a cell lysis step was performed. 5 µL of whole blood was added to 5 µL of Proteinase K, 5 µL of TE buffer, and 5 µL of 1.12% SDS diluted in pure water. The mixture was then lysed at 60°C for 10 minutes. 5 µL of the lysate was suspended on the coil, and a 225°C-cured PEI-coated microtip was immersed for 5 minutes with an applied AC electric field (5 MHz, 20 Vpp). The microtip was then withdrawn from the sample and allowed to air dry.

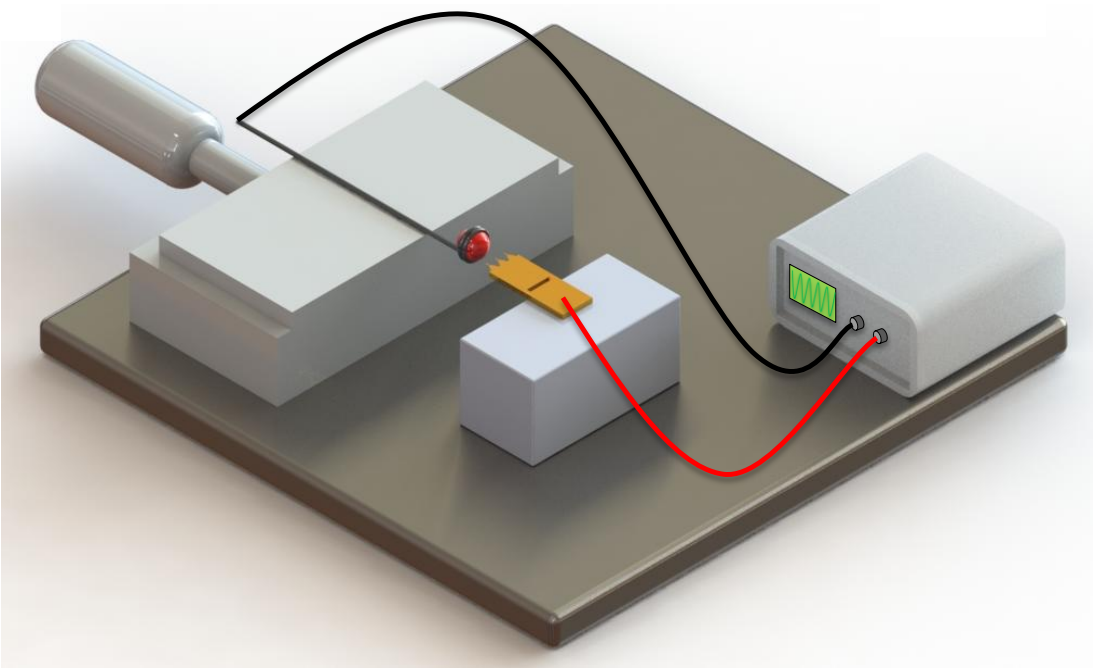

Figure 28. Experimental schematic for the concentration of DNA from a 5 µL sample. A function generator applies 5 MHz, 20 Vpp to the microtip and coil.

The effect of post-capture procedures was explored to improve the purity of the eluted sample for DNA extraction from blood. Different washing procedures were explored to improve the purity of the eluted DNA solution and compared to DNA eluted from microtips without a washing process. Three washing procedures were performed: (1) a 10 second dip and withdrawal of the microtip in TE Buffer, (2) a 10 second dip and withdrawal of the microtip in 4 mg/mL of NALC, followed by a 10 second dip and withdrawal of the microtip in TE Buffer, and (3) a 1 µL 100% ethanol drop on the microtip surface, followed by a 10 second dip and withdrawal of the microtip in 4 mg/mL of NALC and a 10 second dip and withdrawal of the microtip in TE Buffer.

After washing, the dried tips were eluted in 30 µL of TE Buffer for 5 minutes at 60°C and 5 µL was used for qPCR analysis using the SYBR system.

4.2.2 *Recovery of human genomic DNA*

The preparation and separation of human genomic DNA from whole blood using the heat-cured PEI-coated microtips was described in the previous section. For recovery yield and STR analysis, the three washing steps were implemented to remove inhibitors that could be left on the microtip surface. First, 1 µL of ethanol was pipetted onto the microtip surface. The microtip was then allowed to dry. Second, the microtip was dipped into a vial of 4 mg/mL of NALC for 10 seconds. Finally, the microtip was dipped into a vial of TE buffer for 10 seconds. The microtip was then allowed to dry. The dried microtip was placed into an elution vial where 30 µL of TE buffer was dispensed into the vial. The vial was placed in a heat block for 5 minutes at 60°C for elution. Microtip extraction from blood was performed on 6 donor samples.

After eluting DNA from the tip surface, the concentration and purity of human genomic DNA were measured using the standard protocol for the Promega Plexor® HY System. The samples were also run through qPCR using the SYBR amplification system. For comparison of the yield, a commercial kit (QIAamp® DNA Blood Mini Kit, Qiagen) was used to extract genomic DNA from the same 6 samples. A volume of 1.25 µL of whole blood was used for each experiment, which is equivalent to the volume of whole blood used for one microtip extraction. The DNA eluted from the microtips was also used for STR analysis and compared with DNA extracted from the commericial kit.

4.3 EXPERIMENTAL RESULTS

4.3.1 *Purification of extracted DNA*

Among the washing protocols, NALC combined with ethanol and TE buffer showed the best total amplification product through qPCR (Fig. 29). For the non-washed microtips, the amplification threshold was reached at 24.0 cycles and the amplification reached a RFU of 302 after 40 cycles. An RFU of 302 was very low and indicated inhibitors affecting the amplification of DNA. For the TE buffer only wash, the amplification threshold was reached at 23.61 cycles and the amplification reached 551 RFU after 40 cycles. The eluted samples for the non-washed and TE buffer only washed microtips showed quick precipitation of dark blood lysate particles (Fig. 30). For the NALC wash and TE buffer wash, the amplification threshold was reached at 24.21 cycles and the amplification reached 901 RFU after 40 cycles. For the ethanol, NALC wash, and TE buffer wash, the amplification threshold was reached at 23.56 cycles and the amplification reached 951 RFU after 40 cycles. The washing procedure using ethanol, NALC, and TE buffer showed a clean elution buffers, which demonstrated effective removal of inhibitors.

The inhibition of the PCR amplification process can be caused by many mechanisms. When inhibition of PEI was studied, a lag in threshold cycles was observed. The presence of inhibiting particles from blood was observed to affect the total amplification (RFU). Hematin in red blood cells is a known inhibitor of PCR and at low concentrations will only affect the total PCR amplification product[175]. Hematin has been shown to cause underrepresentation, which is shown by a lag in the threshold cycle. Such inhibition can also occur for the microtip assay. Although heat-cured PEI-coated microtips improved the recovery of DNA when washed, DNA was removed due to washing. However, when the microtip was washed

with TE buffer, the threshold cycle improved. A significant amount of hematin could be causing an underrepersentation of the threshold cycle for the non-washed microtips.

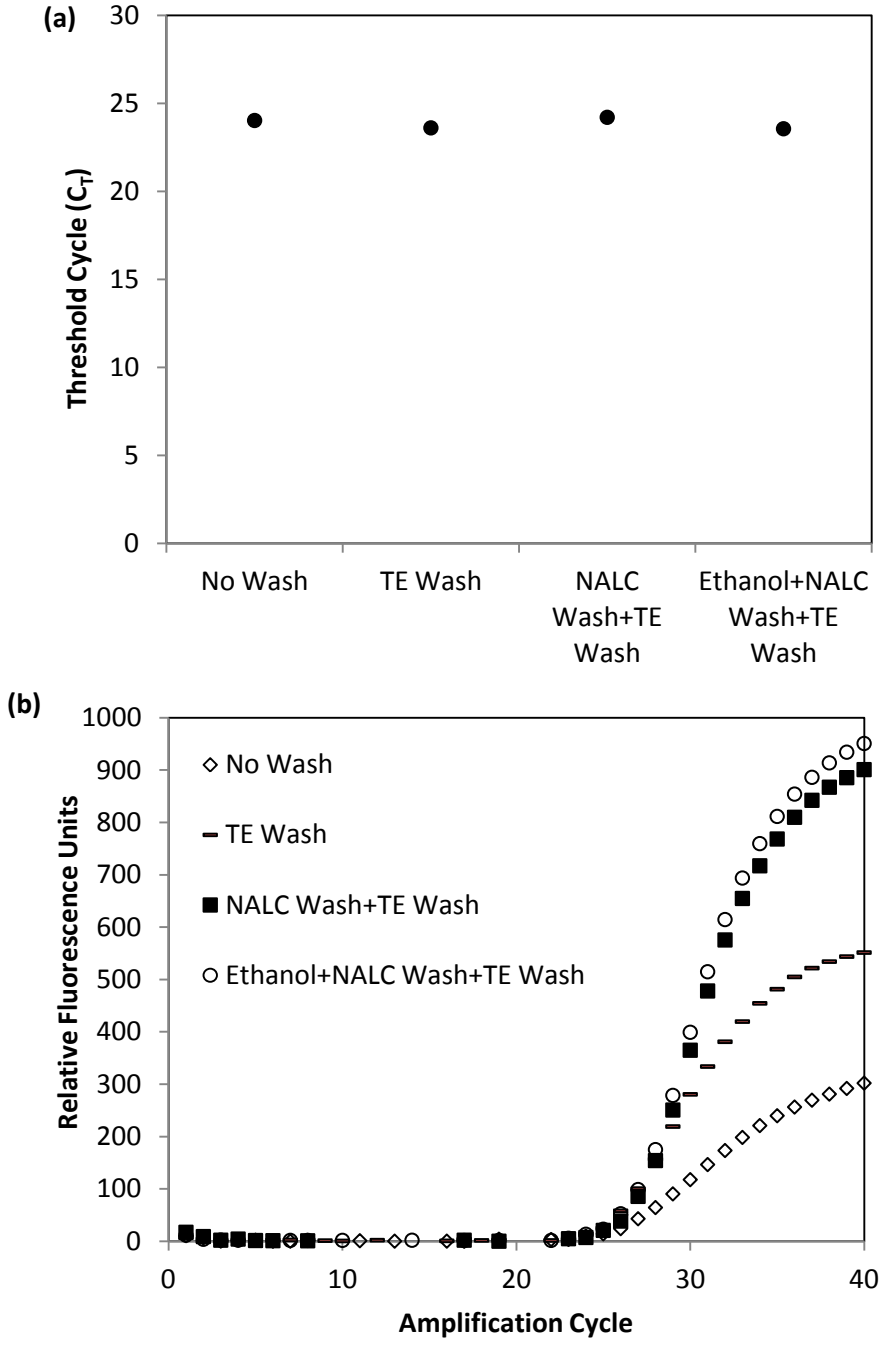

Figure 29. (a) Amplification cycle threshold reached for the capture and release of genomic DNA from 225°C baked PEI coated microtips under different washing conditions. (b) Amplification curves for microtip samples quantified by fluorescent emission in PCR.

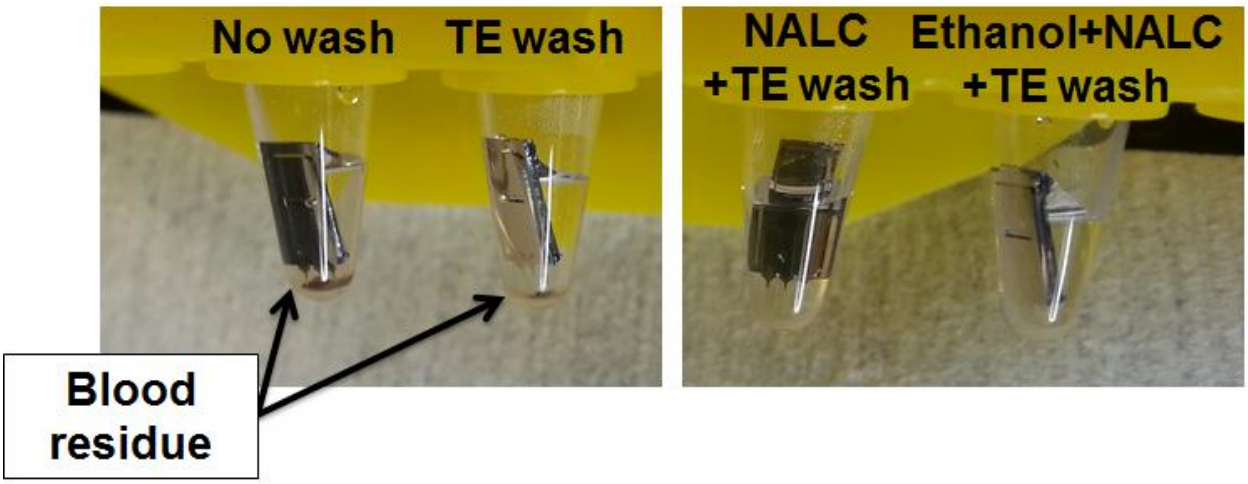

Figure 30. Microtips in 30 µL of TE Buffer after incubation at 60°C for 5 minutes according to the different washing protocols.

The washing results for the addition of ethanol to the NALC and TE buffer washing procedure (n=3). For the NALC wash and TE buffer wash, the amplification threshold was reached at 23.57±0.41 cycles. For the ethanol, NALC wash, and TE buffer wash, the amplification threshold was reached at 23.11±0.22 cycles (Fig. 31).

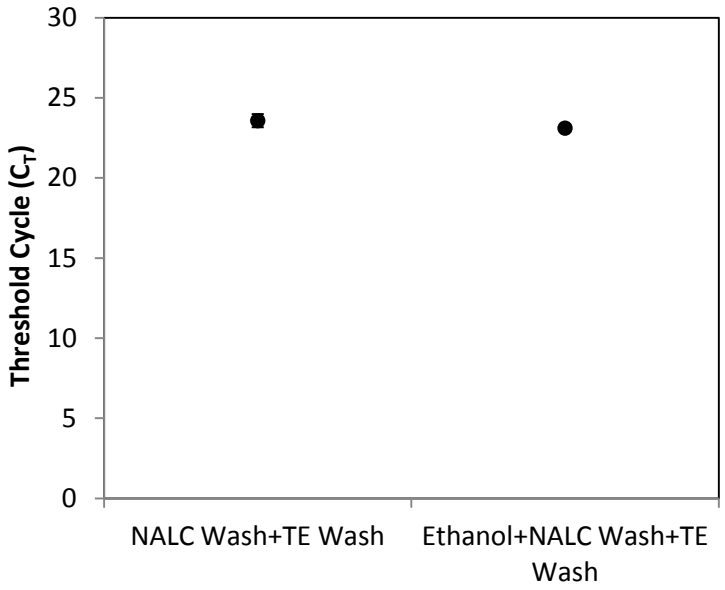

Figure 31. Amplification cycle threshold reached for the capture and release of genomic DNA from 225°C baked PEI coated microtips with and without an ethanol wash step (n=3).

4.3.2 *Recovery of human genomic DNA*

The extraction of genomic DNA from whole blood using 225°C-cured PEI-coated microtips was compared to the extraction efficiency of the commericial kit using the same blood volume (1.25 μL) for each sample (Fig. 32b). The eluted samples were analyzed using SYBR and Plexor. For SYBR, the threshold cycles for the microtip extraction and the commercial kit were 22.9±0.6 and 21.8±0.7, respectively. From Plexor analysis, the DNA concentration was 0.061±0.021 ng/μL for the microtip and 0.133±0.062 ng/μL for the commercial kit. The results for SYBR and Plexor were consistent in that the microtip assay showed half the concentration in comparison to the commercial kit.

For purity analysis, the threshold cycle of the IPC for the DNA standards was compared to those for the microtip samples. The threshold cycle for the IPC biomarker for the DNA Standards, microtips, and commericial kit samples was 23.75±0.30, 23.89±0.34, and 23.91±0.22, respectively. The results indicate that the microtip samples and Qiagen samples were pure enough for PCR amplification analysis (Fig. 32a).

For forensic application, STR multiplex amplification was conducted on DNA extracted by the microtips. Each multiplex STR amplification was separated by capillary electrophoresis and resulted in a single-source, full STR profile. Representative electropherograms are shown in Fig. 33. The profile matched with the DNA processed through a commercial kit.

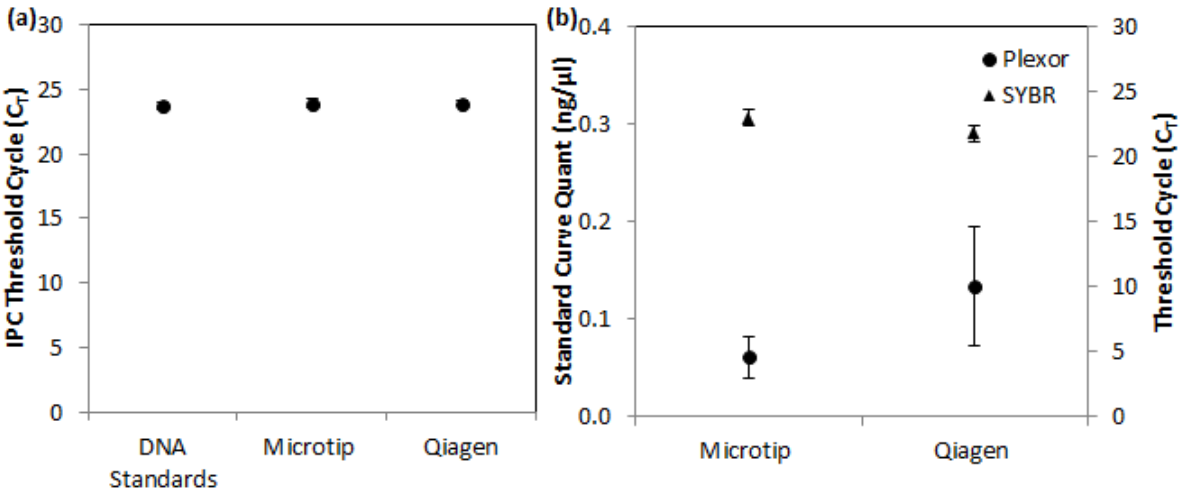

Figure 32. (a) Threshold cycles for the Internal PCR control. (b) Concentration quantification analysis (Plexor) and threshold cycle analysis (SYBR) for the capture and release of genomic DNA from 225°C baked PEI coated microtips from six different blood donors in comparison with a commercial kit.

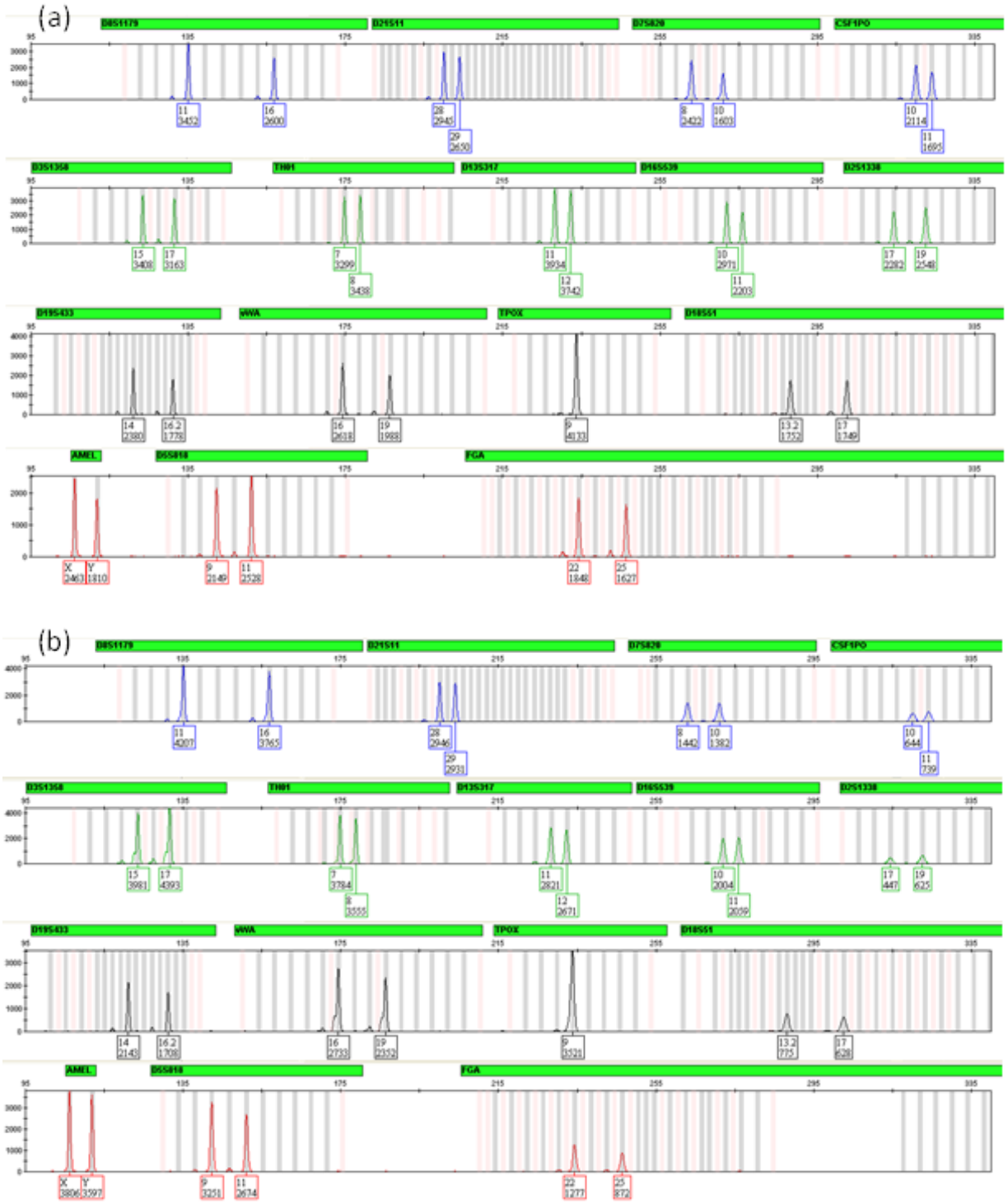

Figure 33. Electropherogram for human genomic DNA from whole blood sample. (a) STR profile generated from a commercial kit-purified DNA sample. (b) STR profile generated from microtip-purified DNA sample.

4.4 DISCUSSION

The surface area of the microtip immersed in a whole blood sample was shown to collect some unwanted inhibiting particles that need to be removed for downstream use. Hematin, IgG, hemoglobin, and lactoferrin are known PCR inhibitors found in blood. In addition, SDS, which was used for cell lysis is a known inhibitor of PCR. Using qPCR, different washing procedures were studied to purify the microtip surface (Fig. 29). The heat-cured PEI-coated surface was shown to be stable after undergoing washing with ethanol, NALC, and TE buffer (Fig. 34). Washing the microtip with NALC was found to remove dark residue that precipitated at the bottom of the elution solution for non-washed microtips (Fig. 30). Adding ethanol to the surface of the microtip before the NALC wash improved the cycle threshold with similar amplification. The washing procedure demonstrated here effectively removed inhibitors to create a purified DNA sample.

In comparison to the commercial kit, the microtip offers a simple and rapid protocol. The microtip required only 13 steps while the commercial kit required 21 steps including centrifugation. The microtip extraction protocol required 25 minutes from the raw sample to a PCR tube. The volume of reagents used was only 235 μL. On average, 1.83 ng of DNA was extracted from whole blood using microtips. For Qiagen, an average of 26.6 ng of DNA was extracted from 1.25 μL of whole blood. Performance of the microtip for blood in comparison with the commercial kit is summarized in Table 3. In consideration of the performance characteristics, the microtip assay could be useful for point-of-care diagnostics, simple genetic analysis, and forensic applications. With further development for a higher yield, the microtip assay can be useful for whole genome analysis and other disease diagnosis.

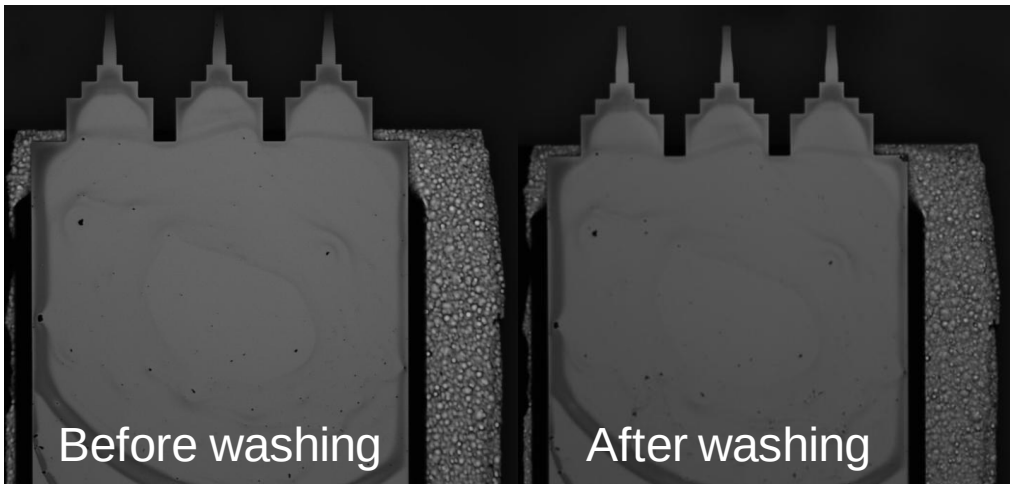

Figure 34. Heat-cured PEI-coated microtip before and after washing procedure (Ethanol, NALC, and TE buffer).

4.5 SUMMARY

The 225°C-cured PEI-coated microtips were used for the extraction and purification of genomic DNA from whole blood samples. A washing protocol was demonstrated for the purification of DNA for downstream applications. The purified genomic DNA samples showed full STR profiles, indicating that this system has utility in the field of forensics.

Table 3. Comparison between microtip extraction and a commercial kit

For a whole blood sample (1.25 µL)	Microtip extraction	Commercial kit extraction
Number of steps	13	21
Centrifugation steps	0	6
Processing time (single sample)	25 min	30 min
Threshold cycles/eluted volume	22.9/30µL	21.8/200µL
Reagent volume (µL)	235	1720

Chapter 5. AUTOMATED EXTRACTION OF DNA FROM DRIED SAMPLES

5.1 INTRODUCTION

DNA extraction has become an increasingly important tool in medical diagnostics and the criminal justice system[7]. In an effort to support the rapid advancement of genetic technology over the past decade, the National Institutes of Health (NIH) solicited a Funding Opportunity Announcement (FOA) in 2013 to catalyze the development of technology for obtaining genomic information. The FOA (PAR-13-203: *Methods Development for Obtaining Comprehensive Genomic Information from Human Specimens that are Easy to Collect and Store*) identifies dried blood spots (DBS) as a desirable sample from which DNA can be extracted, because it can be collected, handled, and stored under less stringent and costly conditions as compared with whole blood. The NIH's solicitation highlights a growing trend on the evolution of pre-analytics for genomic testing in the future[176].

5.1.1 *Medical field*

Since the 1960s, DBS have been used for newborn screening as a method for storage and transport. The use of DBS for newborn screening has been critical to the identification of diseases and early treatment. The collection of DBS is typically done on filter paper. Whatman 903® filter paper is an in-vitro class II medical device registered by the FDA and primarily used for DBS. Whatman 903® filter paper is manufactured from 100% pure cotton linters. FTA® paper is a chemically-treated form of filter paper (Section 1.4.6). The chemical additives act as part of the preparation step for DNA extraction by aiding in the lysis of cells

and denaturation of inhibiting particles.

Currently, analytics from dried samples is not preferred because DNA extraction methods are expensive, labor intensive, and difficult to automate. Typically, a limited sample is collected in dried form and needs to be rehydrated in larger volumes, reducing the concentration of DNA in final liquid form for extraction. DNA can be damaged due to drying and rehydration mechanisms. Also, DNA can become tangled or bound tightly to the fibers of the collection medium. Thus, DNA extraction methods require more steps and longer processing times to extract and purify DNA. Recovery yields are typically low. A simple, rapid DNA extraction method with higher recovery of DNA is needed to obtain usable genomic information from DBS, along with other forms of dried samples.

5.1.2 *Forensics*

The extraction and purification of DNA is critical to the criminal justice system[7]. DNA backlogs for forensic casework has been identified by the National Institute of Justice (NIJ) as a growing problem in the United States[22]. Hiring and retaining additional skilled analysts , as recommended by the NIJ, is not a viable solution to the problem, because time and cost of DNA processing are highlighted as main components of the bottleneck in casework[177]. This puts a paramount on the development of new technology that will simplify and speed up the processing of DNA casework, while reducing the cost per case. Automated methods of extracting DNA from forensic samples have been developed to improve the processing of forensic samples[178]. However, extraction from forensic samples, such as dried blood spots, is still considered labor intensive and expensive[77].

In forensics, cotton swabs are a common method of collecting samples from hard

surfaces (floors, tables, drinks, hand-held devices, etc.). Linens, clothing, and cigarette butts are other forensic samples that face the same challenges as dried blood spots. A DNA extraction method that can provide useable DNA from the variety of dried samples is desirable.

5.1.3 *Summary*

The extraction of genetic information contained in dried samples is critical to improvements in medical fields and forensics. A rapid and simple method of extraction is required to reduce the costs aliquoted on skilled personnel to retrieve genetic information from dried samples. As mentioned in Chapter 1, there is no DBS method available in 30 minutes or less. The majority of processing time is due to long preparation steps. For example, Qiagen QIAamp Mini Kit requires over 1 hour of preparation of the DBS sample before extraction. Reducing the preparation step is critical to developing a rapid extraction method for DNA from DBS samples and other dried samples.

5.1.4 *Objective*

In this chapter, heat-cured PEI-coated microtips integrated into an automated device were used for the extraction of DNA from dried samples. The goal is to demonstrate a rapid DNA extraction method using heat-cured PEI-coated microtips. For rapid and easy use, a pre-extraction washing protocol was investigated to remove inhibiting particles from DBS samples to reduce preparation time. The developed protocol was adapted for use with a variety of forensic samples. For medical applications, the extraction of bacterial DNA (*E. Coli*) from a nasal swab was demonstrated using the automated microtip assay.

5.2 EXPERIMENTAL METHODS

Heat-cured PEI-coated microtips were integrated into an automated device by NanoFacture, Inc (Section 2.3). Using the automated microtip extraction device, a pre-extraction washing protocol was optimized for use with DBS samples to provide a rapid extraction method. The pre-extraction washing protocol was modified from the CASM method described in Section 3.3.3. The rapid DNA extraction method was then adapted for use with a variety of dried samples. Experimental methods for rapid extraction of DNA from DBS, dried blood swabs, dried blood on denim, buccal swabs, nasal swabs, nasal swabs (spiked with *E. coli*), dried semen on cloth, cigarette butts, hair roots, and drink swabs are described below.

5.2.1 *Dried blood washing*

Human genomic DNA was extracted from DBS samples to evaluate the effect of pre-extraction washing of the DBS to remove inhibitors. De-identified 3-mm DBS punches from the Mayo Clinic (Rochester, MN) were used. RBC buffer (100 µL) was added to three punches placed in 1.5 mL vial. The sample was placed in a vortex for 5 minutes at 600 rpm. After vortex mixing, the washing solution was removed and placed in another vial. Then, the punches were rinsed with 100 µL pure water for 2 seconds with vortex mixing at 3000 rpm. The number of 100 µL pure water rinses was evaluated from 1 to 3 rinses. The pure water rinsing solutions were removed after mixing. The removed pure water rinse solutions were combined with removed RBC wash solution. The total volume of washing solution was evaluated for the loss of DNA due to washing. To quantify the amount of DNA removed from the punches, the washing solution was purified using the Qiagen QIAamp® Blood Mini

kit and analyzed through qPCR. The recommended protocol by Qiagen for large volume samples was used to extract DNA potentially removed from the DBS sample due to the washing procedure.

The washed punches were used for DNA extraction using the NF DRS. TS solution (15 µL of TE buffer and SDS), Guanidine HCl (15 µL), and Proteinase K (2 µL) were added to the washed punches. The mixture was vortex mixed for 10 seconds at 3000 rpm, then lysed at 95°C for 10 minutes. 10 µL of the DBS solution was placed in the sample well plate. The capture button was pressed to initiate DNA extraction with the timer set to 5 minutes. After extraction was completed, the microtips were removed from the device and broken into individual vials. An aliquot of 15 µL-TE buffer was pipetted into the vial and vortex mixed for 5 seconds at 1800 rpm to elute human genomic DNA from the heat-cured PEI-coated microtip surface. The extraction and release of human genomic DNA was analyzed through qPCR.

In comparison with a commericial kit, 2 microtips were used because >20 µL of solution was used to released DNA from the swab. The 2 microtips were eluted in 30 µL TE buffer for comparison. The Qiagen QIAamp® Blood Mini Kit reccommended protocol for dried blood spots was used for the 3-mm DBS punches from the Mayo Clinic (Rochester, MN). The elution volume was 50 µL, which resulted in 40 µL due to some solution being held within the microporous filter.

5.2.2 *Forensic samples*

(1) *Dried Blood Spot*

Human genomic DNA was extracted from DBS samples to evaluate the extraction efficiency of the automated extraction system. De-identified 3-mm DBS punches from the Mayo Clinic (Rochester, MN) were used. RBC buffer (100 µL) was added to three punches placed in 1.5 mL vial. The sample was placed in a vortex for 5 minutes at 600 rpm. After vortex mixing, the washing solution was removed and discarded. Three (100 µL) pure water rinses with 2 seconds of vortex mixing at 3000 rpm were performed on the sample. The solution from the pure water rinses were removed and discarded. TS solution (15 µL of TE buffer and SDS), Guanidine HCl (15 µL), and Proteinase K (2 µL) was added to the punches. The mixture was vortex mixed for 10 seconds at high speed then lysed at 95°C for 10 minutes. 10 µL of the DBS solution was placed in the sample well plate. The capture button was pressed to initiate DNA extraction with the timer set to 5 minutes. After extraction was completed, the microtips were removed from the device and broken into individual vials. An aliquot of 15 µL-TE buffer was pipetted into the vial and vortex mixed for 5 seconds at 1800 rpm to elute human genomic DNA from the microtip's surface. The extraction and release of human genomic DNA was analyzed through qPCR.

(2) *Dried Blood Swab*

Human genomic DNA was extracted from dried blood on cotton to evaluate the extraction efficiency of the automated extraction system. Generic cotton swabs with 10 µL of dried blood were used. RBC buffer (100 µL) was added to three punches placed in 1.5 mL vial. The sample was placed in a vortex for 5 minutes at 600 rpm. After vortex mixing, the washing solution was removed and discarded. Three (100 µL) pure water

rinses with 2 seconds of vortex mixing at 3000 rpm were performed on the sample. The solutions from the pure water rinses were removed and discarded. TS solution (15 μL of TE buffer and SDS), Guanidine HCl (15 μL), and Proteinase K (2 μL) was added to the washed swab. The mixture was vortex mixed for 10 seconds at high speed then lysed at 95°C for 10 minutes. 10 μL of the DBS solution was placed in the sample well plate. The capture button was pressed to initiate DNA extraction with the timer set to 5 minutes. After extraction was completed, the microtips were removed from the device and broken into individual vials. An aliquot of 15 μL-TE buffer was pipetted into the vial and vortex mixed for 5 seconds at 1800 rpm to elute human genomic DNA from the microtip's surface. The extraction and release of human genomic DNA was analyzed through qPCR.

(3) *Dried Blood Denim*

Human genomic DNA was extracted from dried blood on denim to evaluate the extraction efficiency of the automated extraction system. Denim pieces with 10 μL of dried blood were used. RBC buffer (100 μL) was added to the dried blood sample placed in a 1.5 mL vial. The sample was placed in a vortex for 5 minutes at 600 rpm. After vortex mixing, the washing solution was removed and discarded. Three (100 μL) pure water rinses with 2 seconds of vortex mixing at 3000 rpm were performed on the sample. The solutions from the pure water rinses were removed and discarded. TS solution (15 μL of TE buffer and SDS), Guanidine HCl (15 μL), and Proteinase K (2 μL) was added to the sample. The mixture was vortex mixed for 10 seconds at high speed then lysed at 95°C for 10 minutes. 10 μL of the DBS solution was placed in the sample well plate. The capture button was pressed to initiate DNA extraction with the timer set to 5 minutes. After extraction was completed, the microtips were removed from the

device and broken into individual vials. An aliquot of 15 μL-TE buffer was pipetted into the vial and vortex mixed for 5 seconds at 1800 rpm to elute human genomic DNA from the microtip's surface. The extraction and release of human genomic DNA was analyzed through qPCR.

(4) *Buccal Swab*

Human genomic DNA was extracted from buccal swab to evaluate the extraction efficiency of the automated extraction system. OmniSwab™ was ejected into a 1.5mL vial for use. The previously detailed washing protocol was omitted for the buccal swab protocol due to the limited inhibitors collected from buccal swab samples. TS solution (45 μL of TE buffer and SDS), Guanidine HCl (45 μL), and Proteinase K (6 μL) was added to the OmniSwab™. The mixture was vortex mixed for 10 seconds at 3000 rpm then lysed at 95°C for 10 minutes. 10 μL of the solution was placed in the sample well plate. The capture button was pressed to initiate DNA extraction with the timer set to 5 minutes. After extraction was completed, the microtips were removed from the device and broken into individual vials. Then, the microtip was broken into a vial. An aliquot of 15 μL-TE buffer was pipetted into the vial and vortex mixed for 5 seconds at 1800 rpm to elute human genomic DNA from the microtip's surface. The extraction and release of human genomic DNA was analyzed through qPCR.

(5) *Nasal Swab*

Human genomic DNA was extracted from nasal swab to evaluate the extraction efficiency of the automated extraction system. BBL™ CultureSwab™ was broken into a 200 μL vial for use. TS solution (45 μL of TE buffer and SDS), Guanidine HCl (45 μL), and Proteinase K (6 μL) was added to the nasal swab. The mixture was vortex mixed for 10 seconds at 3000 rpm then lysed at 95°C for 10 minutes. 10 μL of the solution was

placed in the sample well plate. The capture button was pressed to initiate DNA extraction with the timer set to 5 minutes. After extraction was completed, the microtips were removed from the device and broken into individual vials. An aliquot of 15 μL-TE buffer was pipetted into the vial and vortex mixed for 5 seconds at 1800 rpm to elute human genomic DNA from the microtip's surface. The extraction and release of DNA was analyzed through qPCR.

(6) *Dried Semen Cloth*

Human genomic DNA was extracted from dried semen sample to evaluate the extraction efficiency of the automated extraction system. A piece of a cotton shirt with 10 μL dried semen was added to a 1.5mL vial for use. TS solution (15 μL of TE buffer and SDS), Guanidine HCl (15 μL), and Proteinase K (2 μL) was added to the sample. The mixture was vortex mixed for 10 seconds at 3000 rpm then lysed at 95°C for 10 minutes. 10 μL of the solution was placed in the sample well plate. The capture button was pressed to initiate DNA extraction with the timer set to 5 minutes. After extraction was completed, the microtips were removed from the device and broken into individual vials. An aliquot of 15 μL-TE buffer was pipetted into the vial and vortex mixed for 5 seconds at 1800 rpm to elute human genomic DNA from the microtip's surface. The extraction and release of human genomic DNA was analyzed through qPCR.

(7) *Drink Swab*

TS solution (15 μL of TE buffer and SDS), Guanidine HCl (15 μL), and Proteinase K (2 μL) was added to the drink swab sample in a 1.5 mL vial. The mixture was vortex mixed for 10 seconds at 3000 rpm then lysed at 95°C for 10 minutes. 10 μL of the solution was placed in the sample well plate. The capture button was pressed to initiate DNA extraction with the timer set to 5 minutes. After extraction was completed,

the microtips were removed from the device and broken into individual vials. An aliquot of 15 μL-TE buffer was pipetted into the vial and vortex for 5 seconds at 1800 rpm to elute human genomic DNA from the microtip's surface. The extraction and release of human genomic DNA was analyzed through qPCR.

(8) *Cigarette Butt*

TS solution (45 μL of TE buffer and SDS), Guanidine HCl (45 μL), and Proteinase K (6 μL) was added to the cigarette butt sample in a 1.5 mL vial. The mixture was vortex mixed for 10 seconds at 3000 rpm then lysed at 95°C for 10 minutes. 10 μL of the solution was placed in the sample well plate. The capture button was pressed to initiate DNA extraction with the timer set to 5 minutes. After extraction was completed, the microtips were removed from the device and broken into individual vials. An aliquot of 15 μL-TE buffer was pipetted into the vial and vortex mixed for 5 seconds at 1800 rpm to elute human genomic DNA from the microtip's surface. The extraction and release of human genomic DNA was analyzed through qPCR.

(9) *Hair root*

TS solution (15 μL of TE buffer and SDS), Guanidine HCl (15 μL), and Proteinase K (2 μL) was added to 5 hair roots in a 1.5 mL vial. The mixture was vortex mixed for 10 seconds at 3000 rpm then lysed at 95°C for 10 minutes. 10 μL of the solution was placed in the sample well plate. The capture button was pressed to initiate DNA extraction with the timer set to 5 minutes. After extraction was completed, the microtips were removed from the device and broken into individual vials. An aliquot of 15 μL-TE buffer was pipetted into the vial and vortex for 5 seconds at 1800 rpm to elute human genomic DNA from the microtip's surface. The extraction and release of human genomic DNA was analyzed through qPCR.

5.2.3 *Extraction of DNA from E. coli cells on nasal swab*

E. coli DNA was extracted from *E. coli* cells (10^0, 10^1, 10^2, 10^3, and 10^4 CFU) spiked on a nasal swab to evaluate the dose response of the automated extraction system. BBL™ CultureSwab™ was broken into a 200 µL vial for use. TS solution (45 µL of TE buffer and SDS), Guanidine HCl (45 µL), and Proteinase K (6 µL) was added to the OmniSwab™. The mixture was vortex mixed for 10 seconds at 3000 rpm then lysed at 95°C for 10 minutes. 10 µL of the solution was placed in the sample well plate. The capture button was pressed to initiate DNA extraction with the timer set to 5 minutes. After extraction was completed, the microtips were removed from the device. Then, the microtip was broken into individual vials. An aliquot of 15 µL-TE buffer was pipetted into the vial and vortex mixed for 5 seconds at 1800 rpm to elute *E. coli* DNA from the microtip's surface. The extraction and release of *E. coli* DNA was analyzed through qPCR.

5.2.4 *Storage of DNA on heat-cured PEI-coated microtips*

DNA extracted using the DBS protocol was stored for 1 day, 1 week, 1 month, 3 months, and 1 year. The release of human genomic DNA into TE buffer was performed after storage and analyzed through qPCR.

5.3 EXPERIMENTAL RESULTS

5.3.1 *Dried blood spot washing*

Dried blood samples, provided by the Mayo Clinic, were used to evaluate the extraction of genomic DNA from DBS samples using the automated microtip extraction method. Three-3mm punches were used as the standard template for extraction. The number

of pure water washing steps was found to be critical to the recovery of purified DNA. The threshold cycle was 21.8, 20.32, and 19.2 for 1, 2, and 3 pure water washes, respectively (Fig. 35).

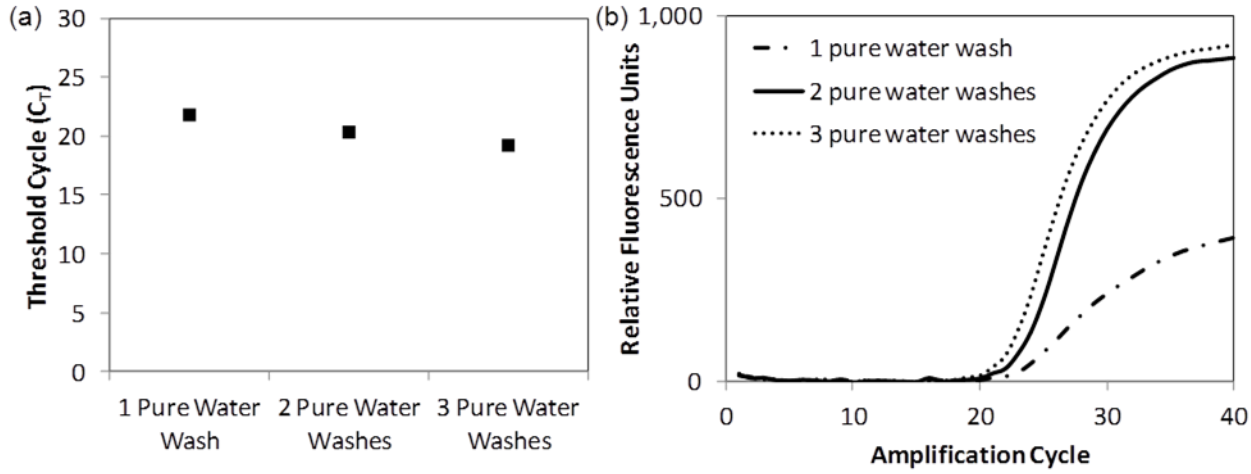

Figure 35. (a) Amplification cycle threshold reached for the capture and release of genomic DNA from PEI-coated microtips under different washing conditions of the 3 punches before capture. (b) Amplification curves for microtip samples quantified by fluorescent emission in qPCR.

The amount of DNA lost during the washing steps was quantified through Qiagen extraction and qPCR analysis. All the washing solution removed from the punches was input into Qiagen to extract and purify the DNA from the inhibitors in blood and reagents used for washing. The results showed that 0.214±0.024 ng of DNA was extracted by Qiagen from the washing solution (Fig. 36).

When the washed 3 punches were eluted and extracted with the automated microtip device, 24.74±4.09 ng of DNA was extracted into 30 μL of TE buffer from two microtips (n=3). When 5 punches were washed, eluted, and extracted with the automated microtip device, 55.32±10.97 ng of DNA was extracted into 30 μL of TE buffer from two microtips (n=3).

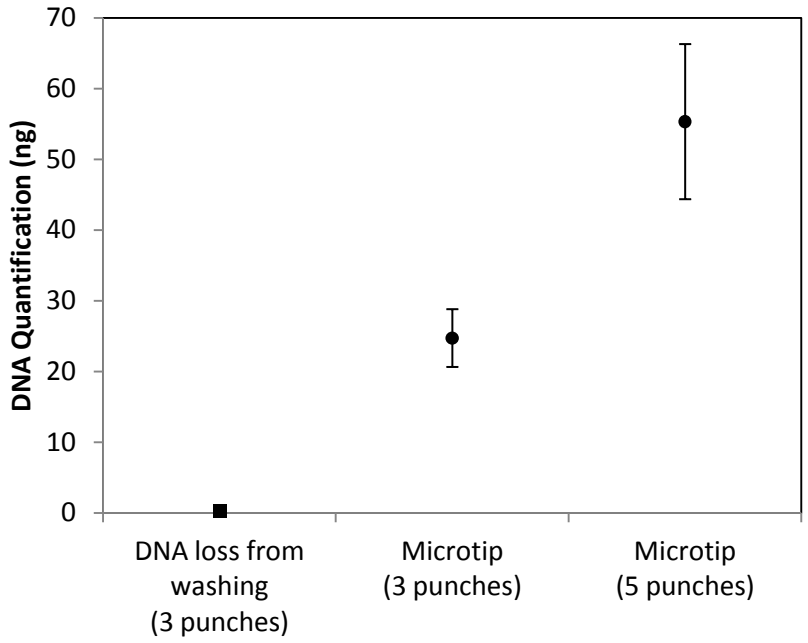

Figure 36. Quantification of DNA from 3 pure water wash dried blood spot procedure. DNA loss from washing (3 punches): The removed washing solution from the 3 punch test was run through Qiagen to extract the DNA lost in the washing solution. Microtip (3 punches): DNA eluted into 30 μL of TE buffer from two microtips. Microtip (5 punches): DNA eluted into 30 μL of TE buffer from two microtips.

The automated microtip extraction was run in parallel with the dried blood protocol using the Qiagen QIAamp Mini Kit. Automated microtip DNA extraction from the three DBS samples was accomplished in 30 minutes. The average threshold cycle was 19.17±0.22 for the three samples. Qiagen QIAamp® Mini Kit DNA extraction from the three DBS samples was accomplished in 100 minutes. The average threshold cycle was 19.22±0.20 for the three samples. A comparison of the methods can be found in Table 4.

Table 4. Comparison of microtip and QIAamp® DNA extraction from DBS

For 3 punch sample	Microtip	QIAamp®
Number of steps	22	26
Centrifugation steps	0	8
Processing time (single sample)	30 min	100 min
Threshold cycles/eluted volume	19.2/30µL	19.2/40µL
Reagent volume (µL)	460	1650

5.3.2 *Forensic samples*

Automated microtip DNA extraction was evaluated for a variety of common forensic samples. Genomic DNA was extracted from a cotton swab with 10 µL of dried blood, denim with 10 µL of dried blood, OmniSwab™ with cheek swab collection, BBL™ CultureSwab™ with nasal swab collection, cotton cloth with 10 µL of dried semen (DS cloth), cotton swab with drink swab collection, 1 cm^2 sample of cigarette butt paper sample, and five hair root samples. The results shown in Table 5 demonstrate recovery yields greater than 0.1 ng/µL for all samples except drink swabs, cigarette butts, and hair roots.

Table 5. Recovery of human genomic DNA from forensic samples

Forensic Sample	DNA extracted (ng/μL)
3 DBS punches	0.825
Dried blood swab	0.358
Dried blood denim	0.307
Buccal swab	0.192
Nasal swab	0.521
Dried semen cloth	0.214
Drink swab	0.022
Cigarette butt	0.014
5 hair roots	0.028

5.3.3 *Extraction of DNA from E. coli cells on nasal swab*

The dose response for *E. coli* cells spiked on a nasal swab was extracted on heat-cured PEI-coated microtips and evaluated by qPCR (Fig. 37). The threshold cycle for microtips eluted and amplified through PCR for 10^4, 10^3, 10^2, 10^1, and 10^0 CFU spiked on nasal swabs was 23.68±0.36, 26.99±0.52, 29.56±1.30, 30.98±0.31, and 31.93±1.03, respectively. The negative control for qPCR was 31.64±0.29.

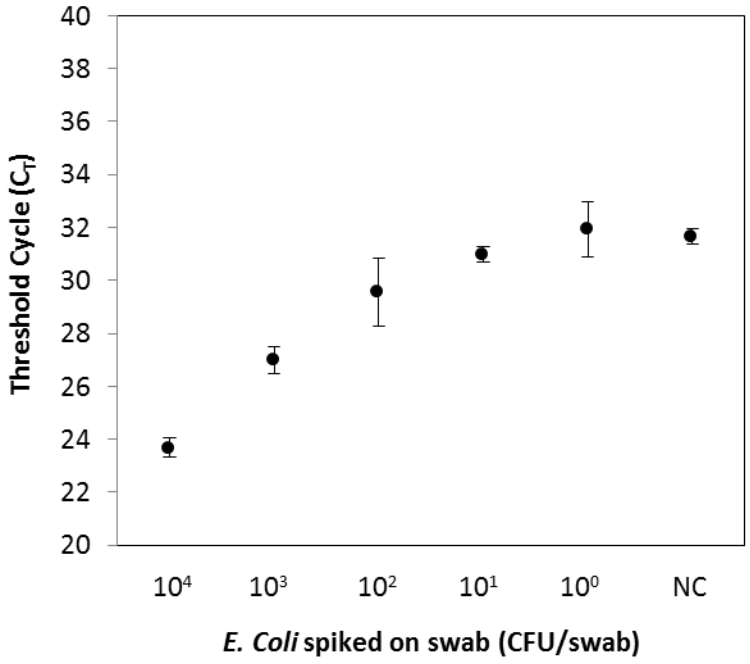

Figure 37. (a) Amplification cycle threshold reached for the capture and release of DNA from *E. coli* spiked on nasal swabs.

5.3.4 *Storage of DNA on heat-cured PEI-coated microtips*

The storage of DNA extracted on heat-cured PEI-coated microtips from a dried blood swab sample was tested (Fig. 38). The threshold cycle for microtips eluted and amplified through PCR the same day, 1 week, 1 month, 3 months, and 1 year after extraction was 21.13±0.18, 21.03±0.14, 20.88±0.31, 20.74±0.17, and 21.23±0.67, respectively.

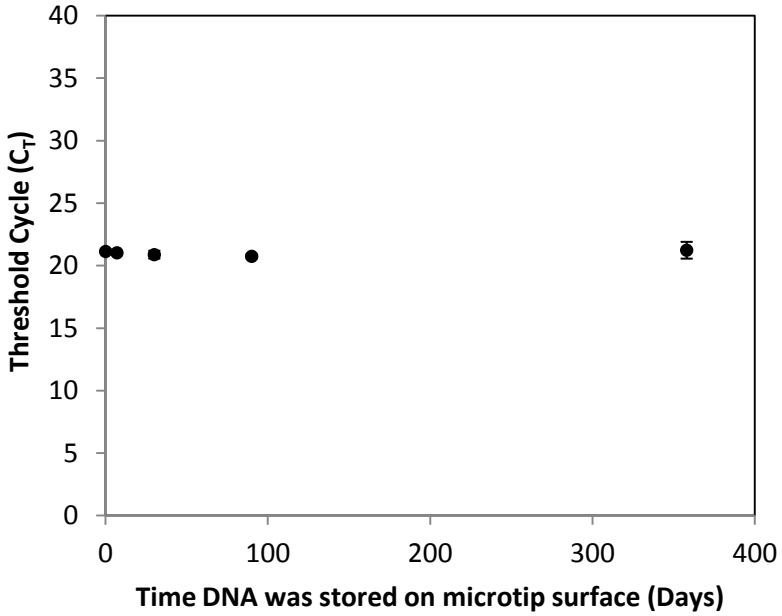

Figure 38. Genomic DNA extraction from dried blood swab samples and stored on the microtip (n=4).

5.4 DISCUSSION

The recovery of DNA from dried samples was demonstrated using the automated microtip extraction method. Instead of using a purification step after microtip capture, contaminants and inhibitors were washed before capture for the dried blood spot protocol (Fig. 39). This protocol was adapted from the CASM method used by the New York State NBS program[77]. An RBC buffer wash was used initially to release the complex of inhibitors found on a dried blood sample. The pure water rinses helped remove leftover inhibitors along with residual RBC buffer. The CASM method removed less than 10% human genomic DNA due to the washing steps. In the process, the effect of the pure water washing steps showed that 3 pure water rinses effectively removes inhibitors to improve the qPCR signal from DNA captured and released through microtip extraction. The DNA removed during the washing steps was extracted and purified using the QIAamp mini kit. An average of 0.21 ng of human genomic DNA was extracted from the washing solution from the three-3mm

punches. The adaption and modification of the RBC/pure water washing steps was shown to be critical toward removing inhibitors and retaining DNA for microtip extraction. Furthermore, the washing time was reduced from 25 minutes used for the CASM method to 10 minutes.

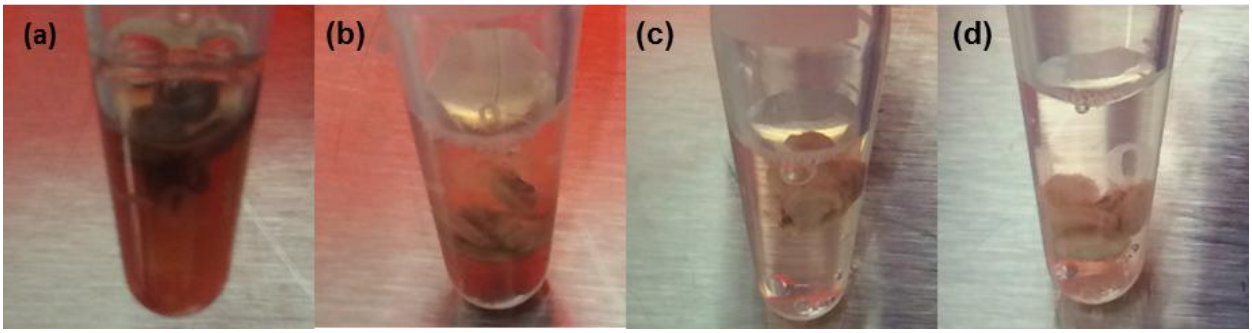

Figure 39. (a) 5 punches immersed in RBC buffer after five minute washing. (b) 5 punches immersed in pure water after 1st two minute washing. (c) 5 punches immersed in pure water after 2nd two minute washing. (d) 5 punches immersed in pure water after 3rd two minute washing.

Human genomic DNA was eluted off the punches using high heat to release DNA entangled within the cellulose fibers. Remaining inhibitors were denatured by EDTA, SDS, guanidine-HCl, and Proteinase K. The elution volume of the swab (30 µL) allowed for two microtips to be used for extraction. Combining the tips together and eluting in 30 µL of TE buffer generated the same concentration of extracted DNA as the QIAamp(R) Blood Mini Kit. Furthermore, the DBS protocol could be used for other dried blood samples such as blood dried on a cotton swab or denim.

The protocol was then modified for the extraction and purification of forensic samples. Human genomic DNA was extracted from a nasal swab, buccal swab, dried semen (cloth) sample, drink swab, cigarette butt, and hair roots. For STR profiling, 1 ng of DNA is required. Microtip extraction was able to meet this criterion for dried blood spot, dried blood swab, dried blood on denim, dried semen on cloth, buccal swab, and nasal swab samples.

However, the extraction did not meet the criteria for drink swab (0.33 ng), cigarette butt (0.21 ng), and hair roots (0.42 ng). Drink swabs, cigarette butts, and hair roots are known to have variable amounts of DNA. The efficiency of the microtip assay will need to be improved to be applicable for these samples.

For medical application, nasal swabs were spiked with *E. coli*. The limit of detection for the automated microtip assay was 10 cfu/swab. The results demonstrate that the microtip assay can be used for non-invasive disease diagnosis.

5.5 SUMMARY

Heat-cured PEI-coated microtips were adapted for use with a device (NF DRS) that automated the immersion, application of electric field, and withdrawal of the microtips from a target sample. This study evaluated the use of a pre-extraction washing step to reduce the processing time for DNA extraction from DBS. The use of a RBC buffer wash and three pure water rinses was shown to effectively remove inhibitors. The washing protocol required less than 10 minutes and allowed for the extraction of DNA using the NF DRS in 30 minutes. The PEI-coated microtips showed the capability of extracting human genomic DNA from a variety of dried samples commonly used for forensics. The quick (<30 minutes) and simple method of extraction could be advantageous to the forensic and medical communities.

Chapter 6. SUMMARY

6.1 CONCLUSIONS

Heat-cured polyethyleneimine was shown to address the main challenges of gold-coated microtip-based DNA extraction. This study demonstrated:

1. PEI was viable for DNA extraction when heat cured above 150°C on a gold-coated surface. When PEI layer was cured above 150°C, PEI did not inhibit qPCR . [Chapter 3]
2. Below 150°C baking temperature, PEI dissolved off the microtip surface and inhibited qPCR by binding with DNA.
3. PEI-coated microtips cured at 225°C could recover 45% DNA from pure samples. The recovery rate was close to twice the recovery demonstrated by non-coated microtips (25%). Heat-cured PEI-coated microtips also improved the recovery of DNA when washed. [Chapter 3]
4. PEI-coated microtips baked at 225°C were stable for the purification of DNA from whole blood. [Chapter 4]
5. PEI-coated microtips could be integrated into an automated device for rapid extraction and purification of DNA from dried samples in less than 30 minutes. [Chapter 5]

6.2 SUMMARY OF WORK

The goal of this study was to address the challenges of microtip-based DNA extraction and expand upon the limited work on using PEI for DNA extraction. This work was motivated by the goal of molecular diagnostics to provide rapid and easy tool for disease detection, monitoring, treatment, and cure. DNA sample preparation was critical to molecular diagnostics because research was limited by the genetic information available for testing.

However, challenges still remained with DNA sample preparation that inhibited the goals of personalized medicine. Chapter 1 detailed the current status of DNA sample preparation and its challenges for the future. Current methods still used complicated procedures or have been circumvented with large, expensive equipment. Centrifugation was still the predominate method of DNA separation confining the use of DNA sample preparation to a laboratory setting. Commercial kits could process samples in less than 1 hour, but the increase in demand for genetic information is putting more pressure to reduce the time of extraction to <30 minutes.

Chapter 3 demonstrated the use of heat cured polyethyleneimine to improve the recovery of DNA of microtip-based DNA extraction. High-molecular weight PEI was coated on the microtip surface using a simple 1 minuted dip coating method and heat processed for 1 hour. A stable PEI surface for the capture and release of DNA was obtained when it was baked on the microtip at 225°C. The PEI-coated microtips baked at 225°C were shown to recover the highest amount of λ DNA in TE Buffer with a 5 minute, 60°C elution. The heat-cured PEI layer retained more DNA after washing than gold-coated microtips.

Chapter 4 studied the development of a rapid protocol for the release of DNA from

cells in whole human blood samples, suppression of inhibiting particles, and efficient release of DNA from the 225°C baked PEI-coated microtips. Proteinase K, SDS, and TE buffer were mixed with whole blood and heated at 60°C for 10 minutes to lysis and prepare the sample for separation using the microtips. A purification method was developed using ethanol, NALC, and TE buffer to wash the microtip surface after capture. The recovery of genomic DNA using the whole blood protocol for PEI-coated microtips was shown to be useful for PCR and STR analysis.

Chapter 5 studied the integration of the heat-cured PEI-coated microtips into an automated device for rapid DNA extraction. A pre-extraction washing protocol was demonstrated to reduce the processing time of extraction and removal of inhibitors. The PEI-coated microtips showed the capability of extracting human genomic DNA from a variety of dried samples commonly used for forensics. The rapid (<30 minutes) and simple method of extraction could be advantageous to the forensic community and disease diagnostics.

The heat-cured PEI-coated microtips integrated into an automated device is in a portable format. The device used required an electrical outlet, but could be easily be modified for operation with batteries as the power source. The device was small in size (less than 10 pounds), allowing for portablity. Currently, heating and vortexing limit portablilty of the DNA extraction method, however a heat source and small mixer could be integrated into this device for POC applications.

6.3 FUTURE WORK

For scope of this study, the AC electric field was confined to 5 MHz, 20 V_{pp}. This work demonstrated that these parameters could capture DNA on the microtip surface.

However, analysis of the effect of the AC electric field in the presence of complex samples and reagents has not be investigated. Experimental and simulation data that investigates these parameters could improve the performance of microtip-based extraction. As mentioned in the background, there is still a poor understanding of the electrical properties of the consituents for an AC electric field applied across a complex sample. As more powerful simulative models are developed, addressing the complexity of this problem can be realized in the future. As the first step, it is suggested that the medium conductivity is measured for prepared complex samples from whole blood and the dried samples and related to the positive DEP force applied to DNA and possibly other particles.

In chapter 3, it was speculated that the contact angle of the heat-cured PEI layer influenced the capture of DNA. Specifically, that low contact angles improved the capturing efficiency. No study was done to isolate the capturing effeciency of DNA on the heat-cured PEI-coated microtip surface. This could be done through fluorescent microscopy. Using an intercalating dye, fluorescently-labelled DNA can be captured on the microtip surface and the capturing efficiency can be quantified by the fluoresecent intensity. This analysis could support the speculation that the capturing efficiency was related to the contact angle. Furthermore, it was determined in this study that baking the PEI over 150°C was necessary to prevent PCR inhibition. However, 150°C and 175°C baked PEI-coated microtips showed poor release of DNA. If the contact angle does effect the capture of DNA, further investigation is needed to determine a mechanism to release DNA effectively from 150°C and 175°C baked PEI-coated microtips. The results of this study could provide a better understanding of how to use heat-cured PEI effectively for DNA extraction and improve the performance of the microtips.

High-molecular weight PEI (750 kDa) was used for this study due to its strong affinity to DNA. However, smaller molecular weight PEI has been shown to be viable for DNA extraction. An investigation into coating the microtip surface with lower molecular PEI will provide a better understanding of the role of PEI with microtip-based extraction. As an extenstion of this work and previous worked performed with magnetic beads, investigating the heat-curing of 25 kDa PEI and its inhibition effect on PCR would provide a better understanding of optimal parameters for using PEI with DNA extraction methods.

For POC diagnostics, the development of a simple and portable DNA extraction method only addresses half the problem. Extracted DNA will require additional protocols and devices for analysis. The PEI surface of the microtips can be functionalized with target-specific probes that can be used to capture specific DNA molecules. Detection of specifically captured DNA targets can be done by binding fluorescenctly-labelled probe molecules to create a sandwich assay on the surface of the microtip. Thus, the automated DNA extraction method using heat-cured PEI coated microtips can be transformed into an automated DNA diagnostic method for Point-of-Care use.

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