Optimization Study of Polyethyleneimine Surface Coating for Microtip DNA Purification

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Abstract

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DNA purification is a process to separate and extract DNA from various samples. As such, it is an initial step in molecular diagnostics, forensics, pharmacogenomics, and many other genetic analysis based disciplines. Current commercial solutions employ microfilters, centrifugation, or magnetic beads to extract DNA. Recently, silicon microtips have shown equivalent yield to the aforementioned methods, while using fewer reagents and shorter processing time. The working principle of the microtip system relies on dielectrophoretic force and capillary action to concentrate and bind DNA to the microtip surface. There remain two challenges associated with the microtip method: (1) increase efficacy of the process in the presence of a high concentration of inhibitors, such as in blood and (2) improve stability of the microtip surface chemistry during storage. In this thesis, we investigate a polymer film of Polyethyleneimine (PEI) as a potential surface layer to address these challenges. To evaluate the performance of PEI, a coating is applied to the microtips, and their purification performance is tested in a variety of conditions. The operation parameters including curing temperature, capture time, elution time, and elution temperature are optimized. Subsequently the PEI tips are tested after

storage; results are consistent for up to 1 month. To address the need for analysis of blood samples, PEI tips are tested with a modified procedure including “washing” steps. Using PEI tips, the overall results with blood are comparable to other commercial kits. By optimizing the conditions of the PEI layer, the challenges associated with microtip based DNA purification systems have been addressed to enhance the DNA purity without compromising yield.

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Chapter 1: Introduction

Genetic analysis is a key aspect of many different commercial and medical fields. Molecular diagnostics relies on the analysis of bacterial and viral DNA found in patients to prepare a proper treatment [1-3]. Pharmacogenomics uses the analysis of a patient's genome to determine a bespoke treatment to best suit their needs [4, 5]. Forensics requires the sequencing of human or animal DNA to aid in a criminal investigation[6, 7]. Food safety testing for genetically modified animals and plants requires sequencing of livestock and crop DNA [8, 9]. For these, as well as many other applications of genetic analysis, the ability to extract nucleic acids from a sample of interest is essential.

Extraction of DNA from a specific sample is a complex problem. Most samples from which genomic material is to be extracted contain very little DNA, but a high level of inhibitors. A simple process which is capable of capturing purified DNA while avoiding contaminants is the key challenge in the field of DNA sample preparation [1].

Although there are numerous commercial solutions to this challenge, all current methods have significant drawbacks. Filter based methods require centrifugation, which can shear DNA [3]. Extraction techniques without filtration steps generally require a high sample volume to produce a useable amount of genomic material [1]. Methods which use alcohol can denature the DNA [10]. Of these various techniques, microtip based DNA capture shows high promise due to its comparably low use of reagents, lack of centrifugation and potential for high throughput [3, 11]. However, there are two main challenges which limit the use of microtips: (1) increasing efficacy of the process in the presence of a high concentration of inhibitors, such as in blood and (2) improving stability of the microtip surface chemistry for reliable capture.

Here, the use of a Polyethyleneimine (PEI) surface layer to address the two aforementioned issues will be investigated. By studying the parameters of the PEI layer applied to the microtip surface, binding affinity between DNA and the microtip surface is increased. This is shown to increase capturing efficacy with lambda DNA and blood samples. The optimized PEI layer is also found to be stable for up to 1 month of storage.

The chemical properties of PEI and the mechanism of the PEI-DNA bond are studied, as well as previous use of the polymer for DNA manipulation. Other commercially available methods of DNA purification will be briefly reviewed, as well as their advantages and disadvantages. The main objective of this thesis is to study the following parameters of a PEI layer on a microtip surface: baking temperature, capture time, elution temperature, and elution time. The optimized parameters will be used to improve DNA capture from highly impure samples and creating a more stable microtip surface chemistry.

Chapter 2: DNA Sample Preparation Review

DNA purification can be generally separated into two main sub-categories: liquid-liquid (traditional), and solid phase extraction (SPE) methods. Traditional methods such as buoyancy density gradient extraction have been in use since the 1950s [12, 13], and were the only viable means of isolating DNA prior to the advent of SPE techniques. In the late 1980s, SPE began to show great promise when it was demonstrated that binding nucleic acids to silica particles allows for rapid DNA purification [14]. This promise was quickly realized; at present the most common commercial extraction kits are silica based spin columns and filter elements [15]. Recently, new SPE methods have been developed which do not rely on silica [11, 16]. These have the potential to be significantly faster than even the most popular silica based solutions. Here, the working principle of each method, as well as its advantages and disadvantages, will be discussed.

2.1. Traditional Liquid – Liquid DNA Purification Methods

2.1.1. Cesium Chloride Density Gradient

This technique involves the use of an ultracentrifuge to force cell lysate through a density gradient, where it separates based upon molecular weight. The DNA can then be isolated based on the band formed in the spin column. More specifically, the steps are: (1) Cells are lysed to release nucleic acids (2) Cesium Chloride (CsCl) density gradient is formed in water via ultracentrifugation (3) Macromolecules from cell lysis are spun through gradient (4) DNA migrates a specific distance through the gradient, thus separating it from all material with a different molecular weight (5) The band containing the DNA is manually separated from the rest of the gradient.

Although differential centrifugation was developed in the mid-1920s, the use of a CsCl density gradient to isolate DNA was first established in the 1950s [13]. In 1967, it was shown that coiled forms of DNA could be separated from linear forms through the use of buoyant intercalating ethidium bromide (EtBr) dye [17]. Since then, it has become possible to separate the bands of chromosomal, plasmid, and organellar nucleic acid [15]. This process, despite its age, is still used today for many applications in which a large amount of highly pure DNA is required. The issues with this method are its significant processing time of up to 48 hours, the requirement of an expensive ultracentrifuge, and the use of the mutagen EtBr.

2.1.2. Phenol-Cloroform Phase Separation

Phenol was first used to create phase separation for RNA extraction in 1956 [18]. However, phenol-water process did not result in a highly pure sample and was not widely adopted. In 1986 the addition of chloroform and guanidinium thiocyanate allowed for an extremely quick extraction of highly pure RNA molecules [19]. This method was able to exceed the purity and yield of traditional CsCl gradient methods, and became the gold standard in RNA isolation.

The process works by adding equal volume phenol-chloroform to cell lysate, followed by centrifugation. This results in a biphasic mixture of aqueous and organic phases. The addition of chloroform to the mixture allows for a sharper transition between the phases [20], while gaunidinium thiocyanate is an effective inhibitor of ribonucleases [19]. The key to this method is that nucleic acids tend to remain in the upper aqueous phase due to their highly polar nature, while other cell lysate material is forced into the lower organic phase.

In order to further differentiate between various nucleic acids, the pH of the phenol can be modified to only allow RNA to stay in the aqueous phase. This can even be extended to specifically separate mRNA from other types of RNA [21]. The charges associated with RNA are less easily neutralized than those of DNA, thus as the pH is lowered, DNA is removed from the aqueous phase.

To remove the nucleic acids from the mixture, the aqueous phase is manually separated, and ethanol precipitation, followed by washing, is used for extraction. Phenol-Chloroform purification creates highly pure nucleic acids and only requires minutes of centrifugation. Due to these reasons, it is still widely used today [22]. The potential challenges with this method are its use of toxic reagents (phenol), minimum requirement of $1x10^6$ cells [19], and use of a centrifuge (although brief).

2.1.3. Chelex

The use of Chelex 100 chelating resin to extract DNA is a relatively modern method when compared to the previously described liquid-liquid extractions. It was first demonstrated to be effective in this regard in the early 1990's [23], when it was used to purify DNA from blood and semen samples for forensic work. Chelex 100 is a chelating resin made up of styrene divinylbenzene copolymers which contain paired iminodiacetate ions. The iminodiacetate ions act as chelating groups which give Chelex an extremely high affinity for heavy metal ions under alkaline conditions [24].

The procedure for using Chelex to purify DNA requires the resin to be well dispersed in water. This mixture is then added to a sample so that the final resin concentration is 5%. By boiling this sample, cells are lysed and the DNA is released and subsequently denatured.

The boiling step would normally destroy the DNA so as to render it useless for analytic applications. However, the Chelex resin prevents this from occurring by binding to the heavy metals in the solution. Heavy metal ions are required for the activation of DNA digesting enzymes which normally degrade DNA during boiling [23]. The boiled sample is centrifuged and the cellular debris is forced into a pellet while the lighter nucleic acid material stays in the supernatant. By manually removing this supernatant, the DNA can be extracted from the solution. In the process, resin should not be removed from the centrifuged sample, as it inhibits downstream processes such as Polymerase Chain Reaction (PCR).

The main disadvantage of the Chelex process is the lack of filtration or other means of removing inhibitors [25]. It also denatures DNA which can be an issue for some downstream analytic processes. The advantages of Chelex are its extreme speed and low cost. This method does not allow for the same yield or purity of DNA as organic (Phenol-Chloroform) techniques [25], but it takes significantly less time.

2.2. Silica Based Solid Phase Extraction DNA Purification Methods

There have been many silica based purification protocols developed since the initial concept of silica-DNA binding for extraction was developed in the 1980s. A short list includes: Qiagen QIAamp; Clontech NucleoSpin; Mo Bio Laboratories Ultraclean and BloodSpin; Promega Wizard; Epoch Biolabs Econospin, and Sigma Aldrich GenElute. However, all of the kits and tools created since then have the same general protocol: the lysed target sample is allowed to bind to silica in the presence of a chaotrope, all undesired

materials are washed from the silica via rinsing steps, and the purified nucleic acid is eluted with a low salt buffer.

The working principle of these techniques is the binding of the nucleic acids to the silica. DNA is highly negatively charged due to its phosphate backbone, while silica also has a negative charge at basic to near neutral pH [26]. While the exact mechanism of silica-DNA binding is not fully understood, there are two common theories. One possibility is that by adding chaotropic salts, a salt bridge can be formed between the negative DNA and negative silica OH^- groups [1]. Another possibility is that by adding chaotropic salts, both the DNA and silica are dehydrated which changes the form of the DNA and reduces the electrostatic repulsive forces between the surfaces. The hydroxyl groups on the surface of the silica are then able to form hydrogen bonds with the base pairs which make up the nucleic acid [27]. In practice, the silica-DNA bond is capable of holding the DNA in the filter element during any washing steps. The DNA is then released when a low-salt buffer is introduced and the electrostatic forces, or lack of salt bridge, separate the nucleic acid from the silica.

As there are numerous possible examples of silica based techniques, but all share a similar principle, only two will be presented in this section.

2.2.1. Qiagen QIAamp

Although there are many different silica spin column based extraction methods, the Qiagen QIAamp system is the most popular. The system is based around the use of a porous silica filter element mounted in a microcentrifuge spin column. By spinning a cell lysate and chaotropic salt mix through the filter, it is possible to trap a high percentage of the

DNA present. Rinsing steps with washing buffers are used to remove the cellular debris and inhibition molecules which are present in the target solution. The trapped DNA is then eluted from the filter using either water or TE buffer.

The QIAamp system is marketed by Qiagen for use with various sample types: Blood, urine, stool, buccal swabs, hair and dried stains [28]. The main difference between the procedures for different sample types is the lysis and washing buffers.

In practice, the QIAamp spin column method has proven to have a comparable limit of detection to traditional methods such as Phenol-Chloroform [29], and be considerably less time and labor intensive [30]. The disadvantages of this method are that it requires a centrifuge, and there are many pipetting steps which increase the likelihood of error. It has also been shown that the high fluid shear forces created in the spin columns can shear nucleic acids [15]. This is potentially problematic for downstream processes.

2.2.2. Akonni TruTip

The Akonni TruTip system utilizes a monolithic porous silica binding matrix. The matrix is situated inside of micropipette tip. The system uses the pressures created by the pipette to drive the target sample and various buffers through the matrix, which allows for bidirectional flow. The procedure starts by adding ethanol, guanidine and sodium acetate to the target, then pipetting the solution through the matrix seven times. This allows DNA, along with other contaminants, to bind with the silica. The micropipette tip is then washed via 5 cycles each of a guanidium-sodium acetate-ethanol buffer and an ethanol-acetone buffer. Subsequently the micropipette tip is air-dried by pipetting air through the matrix for 15 cycles. Finally the DNA is eluted from the silica by cycling Tris-HCL buffer through

the micropipette tip 5 times [31]. The aforementioned procedure was used for the capturing of DNA from nasopharyngeal samples; the exact number of cycles may change depending on target sample.

The TruTip method has proven to be as effective as, if not better than, the Qiagen QIAamp kit in several applications [31, 32]. It is also extremely fast, even when compared to other silica based methods, taking about 4 minutes per sample after initial set up. This is possible due to its lack of centrifugation. Another advantage of the TruTip system is its ability to handle larger sample volumes than most spin column or bead based methods. The disadvantage of the TruTip method is its required use of toxic reagents.

2.3. Non-Silica Based Solid Phase Extraction DNA Purification Methods

2.3.1. Aminosilane Silicon Channels

Amino silane DNA extraction is another method which takes advantage of electrostatic interaction between DNA molecules and a substrate surface. In this case, the substrate, silicon, is coated with 3-[2-(2-aminoethylamino)-ethylamino] - propyltrimethoxysilane (AEEA). This method was developed in 2001 [33], specifically for use in lab-on-a-chip type microfluidic devices. It is well suited to this purpose, as the AEEA layer is easily applied to silicon microchannels, and does not require centrifugation or magnetic particles [16].

The AEEA silane compound contains 1, 2 and 3 amine groups. This makes the compound highly positively charged. As DNA carries a highly negative charge, the two bind quite readily due to electrostatic forces [34]. By allowing cell lysate to incubate in a microfluidic channel coated with AEEA, DNA can be trapped by the silane compound.

Other negatively charged proteins or debris may also be trapped however, so several washing steps are used to remove impurities. After these steps, a high pH environment (10.9) is used to elute the DNA from the AEEA surface by neutralizing the surface charges [35].

Amino silane based DNA extraction has been shown to be effective at capturing up to 40% of all possible nucleic acid material from a target solution [16]. Although this is not as high as other microfluidic DNA extraction methods, such as silica microbeads, it is significantly easier to fabricate. The advantages of this process are its lack of toxic or denaturing reagents, ease of use, and ease of application to microfluidics. The disadvantage of this process is its potentially lower yield when compared with other microfluidic DNA capture methods. At this time no attempts have been made to create a macroscale adaptation of this technology.

2.3.2. ChargeSwitch

The first reference to use of magnetic beads which have a charge switching surface coating, for the use of DNA extraction, can be found in a European patent from 2005 [36]. This technology was later commercialized by Invitrogen under the name ChargeSwtich. The ChargeSwitch system works by changing the surface charge of magnetic particles by controlling the pH of the surrounding aqueous solution. This is possible due to the polyhistidine layer functionalized to the magnetic bead surface[36].

The ChargeSwitch procedure is as follows. First the magnetic beads are added to a solution of cell lysate. The pH of the solution is then lowered (below 6.5). Under these conditions the beads are positively charged and thus bind to DNA electrostatically. This

solution is allowed to incubate for 10 minutes to allow for greater DNA-bead binding [37]. The beads are then collected as a pellet using a strong magnet and the supernatant is removed manually. Subsequent washing steps help to remove any remaining impurities. Finally the DNA is released from the beads by raising the pH of their aqueous environment, thus reversing the charge of the polyhistidine surface.

This method was shown to be comparable in yield and purity to spin column based extraction, using marine samples [37]. However, it seems this is dependent upon the sample type, as ChargeSwitch was shown to have a higher limit of detection, lower yield and purity when compared to the QIAamp method when using bacterial DNA [38]. The main advantage of this system is the lack of centrifugation, which makes the process more portable and decreases chances of shearing nucleic acids. Additionally, ChargeSwitch does not use chaotropic salts or ethanol, both of which can be potential inhibitors to downstream analytic methods [37, 39]. The disadvantages of this method are its higher cost per test and longer processing time, when compared to spin column techniques [38].

2.3.3. Silicon Microtips

Recently, a silicon microtip based DNA extraction technique has been developed which relies on van der Waals forces to bind nucleic acid to a gold surface. The silicon microtips are fabricated via photolithography and consist of a rectangular silicon chip with several needle-like protrusions at one end. These protrusions, or “tips,” are only microns in width and are coated with a layer of gold [3].

The procedure for the use of these silicon microtips is as follows. First a target solution is placed in an electrically conductive well. This allows for an electrical potential to be applied between the microtip and solution. The microtip is then dipped into the target

solution and dwells there to allow for the concentration of the DNA due to dielectrophoresis and eletro-osmosis. As dielectrophoretic force is dependent upon particle size, it is possible for the electrical signal applied to the system to be tuned to create size-selective attraction [3]. The microtip is then withdrawn from the solution. During both concentration and withdrawal steps, a thin fluid layer forms near the tip surface due to capillary action. This thin fluid layer forces the DNA into extremely close proximity with the gold surface, which allows for van der Waals binding to occur, trapping the nucleic acids on the tip. The DNA is then eluted from the tip via Brownian motion induced by heating in TE buffer [11]. The combination of forces which form the working principle of this method can be seen in Figure 1.

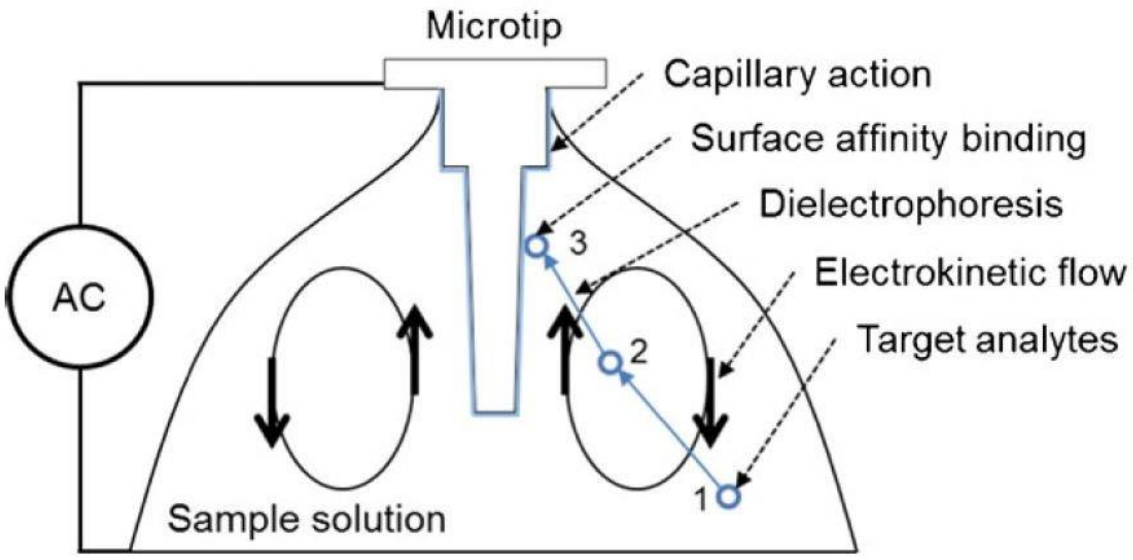

Fig. 1. The combination of these forces (electro-osmosis, dielectrophoresis, capillary action and van der Waals) allow for the concentration and capture of nucleic acids. [11]

This method has been shown to perform comparably to the Qiagen QIAamp spin column system when isolating human genomic DNA from buccal swabs. The advantages of microtip DNA extraction are its extreme speed, low use of reagents, and ease of use. The extraction process only requires 4 steps and takes about 10 minutes. However, there

are two main disadvantages to this method: the lower efficacy to extract PCR viable DNA from samples which are highly concentrated with inhibitors such as blood; and the potential instability of the microtip surface properties for reliable capture of DNA.

2.4. Summary of Different DNA Purification Techniques

Boom et all have previously described an excellent list of criteria for the optimal nucleic acid preparation technique: 1. Sensitive, reproducible, rapid and simple, requiring no specialized equipment or specialize knowledge of biochemistry 2. Extracted nucleic acid should be sufficiently pure to allow for enzymatic modifications 3. Risks for personnel with regard to pathogens should be small 4. The chance of transmission of nucleic acids from sample to sample should be small [14]. To this list we would add only one criterion: cost.

The techniques described above all have their advantages and disadvantages. These qualities are also sample-dependent, as certain methods of purification are best suited for certain target analytes. Additionally, no one method is a clear winner based on the aforementioned “ideal” criteria. For example, Akonni TruTip is fast and easy to use while producing a high yield of pure DNA; however it is quite expensive at $5 per test. The Chelex extraction method is extremely cheap, but is lacking any filtration or washing steps and is thus unable to produce usable DNA from relatively impure samples. This is well illustrated below in tables 1 and 2.

The need for a purification method which fulfills of the ideal criteria has been well established [1, 2, 4, 7, 8, 40], but at present no such method exists. It is clear that new

techniques and improvements of current protocols should be investigated to approach an ideal solution.

Purification Method	Cost/Test	Processing Time/Test	Special Equipment	Pros	Cons	References
CsCl Density Gradient	$4.06	24 - 48 Hours	Ultracentrifuge	• Compatible with high sample volume	• Extremely long process time • Expensive • High limit of detection	[15, 17]
Phenol-Chloroform	$4.00	60 - 120 Minutes	Centrifuge, Fume Hood	• Excellent for RNA specific recovery • Compatible with high sample volume • High purity	• Toxic reagents • Labor intensive • Long processing time • Expensive	[19, 29], Invitrogen
Chelex	$0.10	20 - 35 Minutes	Centrifuge	• Extremely Cheap • Simple use with very few steps • Does not use Toxic Reagents	• There is no filtration or washing steps, so highly impure samples do not yield viable DNA • Chelex resin itself inhibits PCR	[23, 24], BioRad
QIAamp	$5	35 - 40 Minutes	Centrifuge	• Cheapest silica method • High yield • Compatible with many sample types • High throughput without special equipment	• Many steps are required • Can shear nucleic acids • Some agents toxic or mutagens	[15, 41, 42], Qiagen
TruTip	$5	13 - 35 Minutes	N/A	• Fastest silica method • Requires no special equipment • Easy to use • High yield	• Low throughput without special equipment • Many steps, although simple • Expensive	[31, 32], Akonni
ChargeSwitch	$3.32	30 - 60 Minutes	Magnetic Rack	• Lack of centrifuge means more potential for field use • Does not shear DNA as readily • No toxic reagents	• Expensive • Long process time for SPE • Labor intensive relative to other SPE methods	[37-39]
Silicon Microtips	TBA	7 - 21 Minutes	Proprietary Device	• Very fast processing time • Simple use with few steps • Cheaper than silica methods • Potential for high throughput	• The process cannot remove inhibitors from highly impure samples • Tip surface properties are unstable	NanoFacture

Table 1. Comparison of different nucleic acid purification techniques. All costs and processing time based on a single extraction.

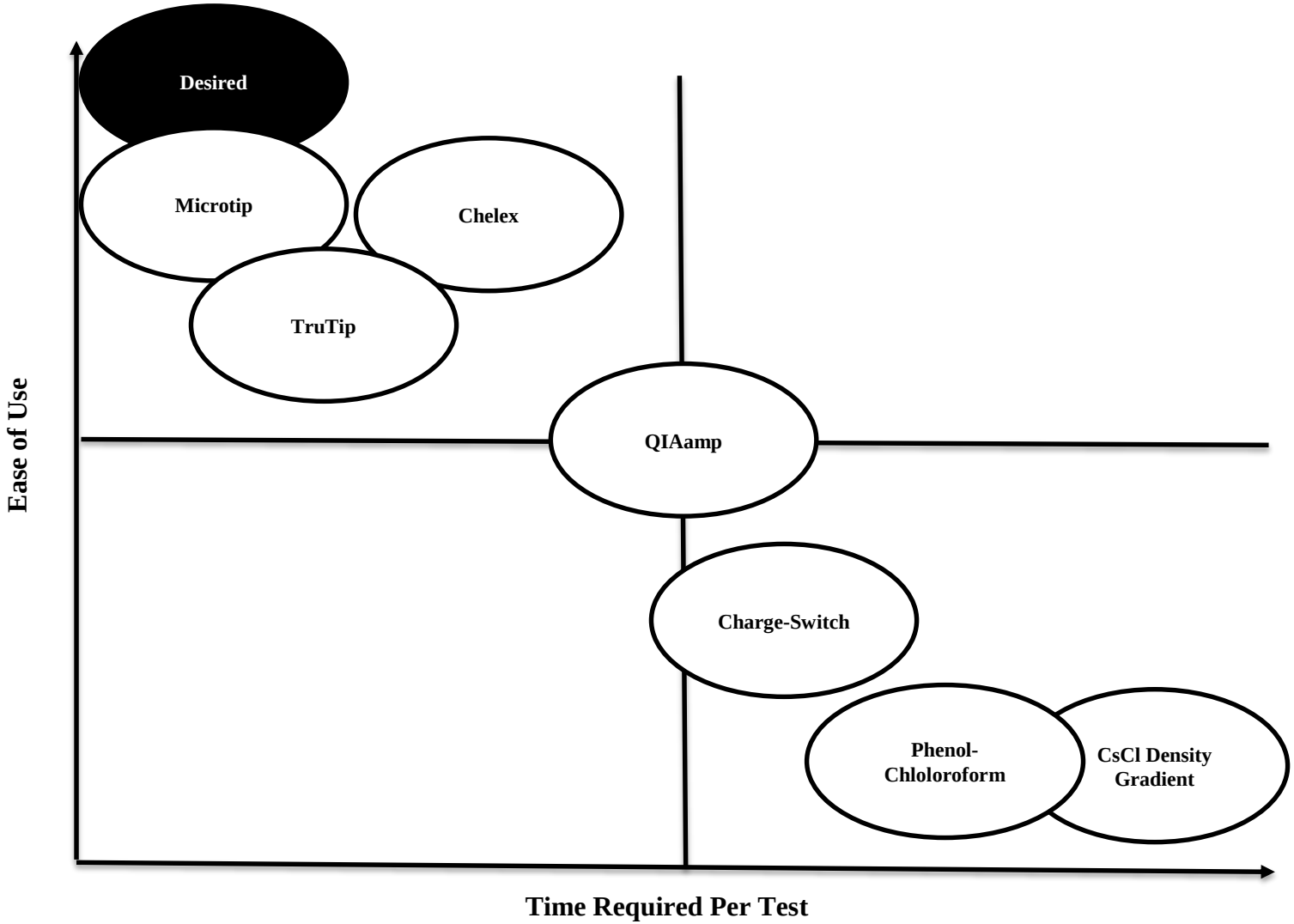

Table 2. Chart showing the relative “ease of use” of each method compared with the amount of time each method takes

Chapter 3: Polyethyleneimine Coating

Among the reviewed methods, one of the most promising is the NanoFacture Incorporated silicon microtip technique. It is cheap, extremely fast, easy to use and can be adapted for both in the field and high throughput, lab-scale use. The two disadvantages of the microtip method which keep it from nearly completely satisfying the previously outlined “ideal” criteria are: low efficacy with highly impure samples and instability of the microtip surface chemistry.

3.1. Objective of Investigation

There are then two objectives on which this investigation will focus: (1) increase efficacy of microtip DNA capture in the presence of a high concentration of inhibitors, such as in blood and (2) improve stability of the microtip surface chemistry during storage. To that end, the polymer Polyethyleneimine (PEI) has been identified as a potential solution.

3.2. PEI Background

PEI has seen widespread use for various industrial processes such as paper production, dye production, shampoo manufacturing, and water purification [43, 44]. Since the discovery of its potential as a vector for gene transfection in 1995 [45], it has also become popular in the field of gene delivery. Some of the reasons for its high efficacy as a gene transfection vector are its ability to condense DNA and readily form PEI-DNA complexes. The reason PEI is imbued with these properties is fundamentally related to its chemical makeup.

PEI is a polycationic polymer with three forms: linear (Figure 2), branched (Figure 3), and dendrimer (Figure 4). The linear form of PEI is induced from the cationic polymerization of a 2-substituted-2-oxazolline monomer, while the branched form is created through cationic polymerization of aziridine monomers[44]. Dendritic PEI has been synthesized via Gabriel amine synthesis with an ethylenediamine (EDA) core [46]. Among the three forms, the branched form is most commonly used in DNA transfection. The linear form of PEI does not perform as well in the context of gene delivery, and the dendrimer form's performance has not yet been well characterized.

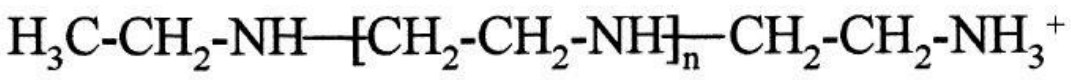

Fig. 2. Chemical Structure of Linear PEI [44]

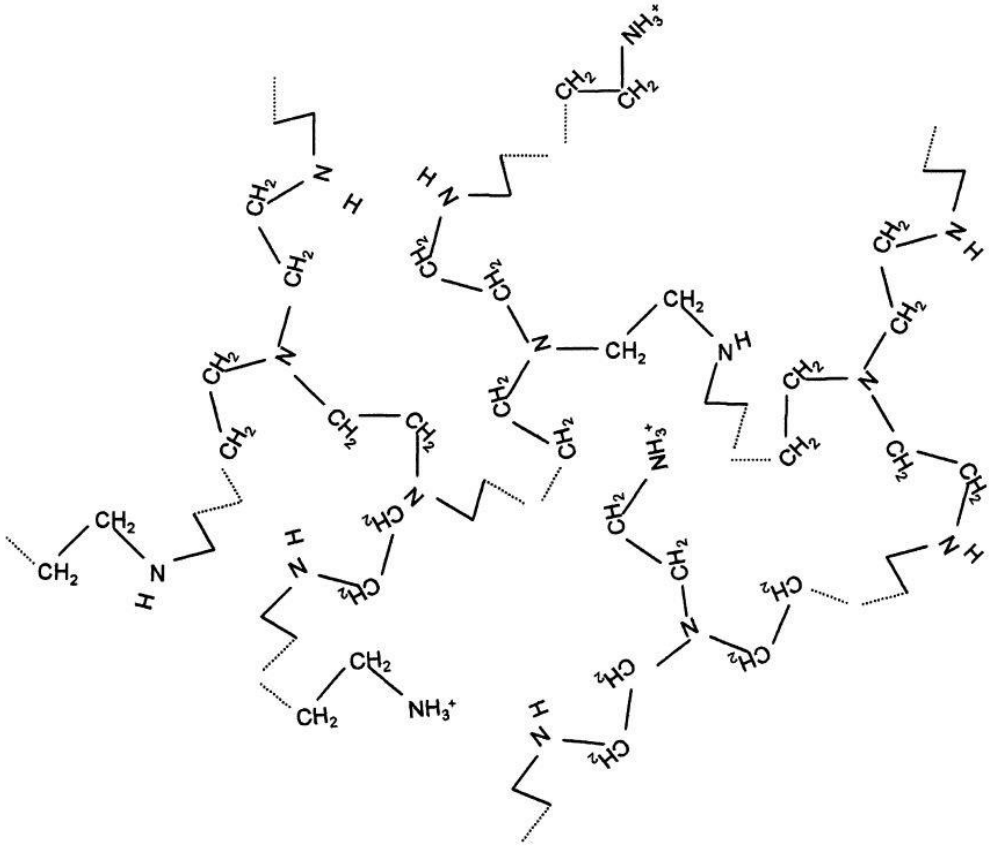

Fig. 3. Chemical Structure of Branched PEI [44]

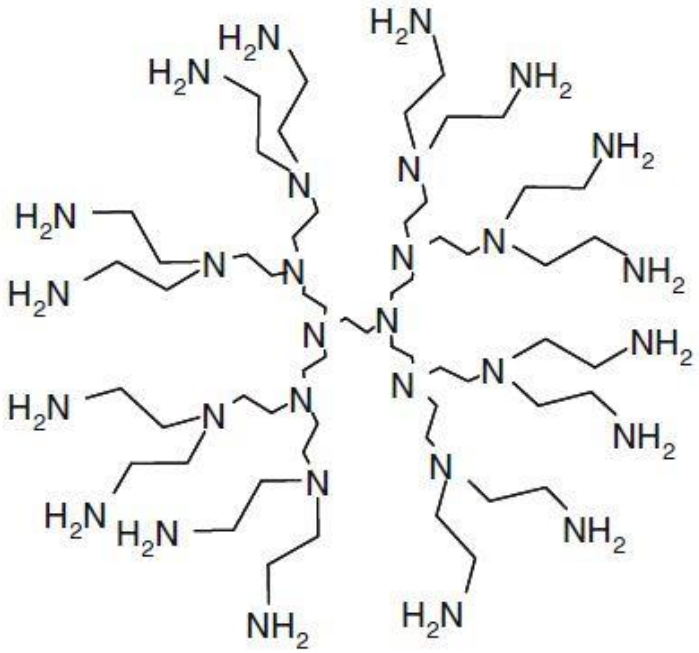

Fig. 4. Chemical Structure of Dendritic PEI [46]

There are two characteristics of branched and dendritic PEI which make it an excellent transfection agent: firstly, both forms contain 1, 2, and 3 amine groups; secondly every third atom in the PEI backbone is a nitrogen. These two traits make PEI highly protonable, and polycationic [47]. The extreme protonability of PEI makes it an effective buffer across a wide pH range, which is important for transfection in vivo [48, 49]. The other distinct attribute of PEI, its high positive charge, plays a large role in the formation of DNA-PEI complexes. In the context of this investigation, the formation of DNA-PEI complexes is extremely important, while pH buffering is not, so only the former will be discussed further.

3.3. DNA-PEI Binding

3.3.1. DNA-PEI Binding Modalities

The binding of PEI and DNA has been shown to have two distinct binding modes. The first is the formation of a hydrogen bond between the polymer and the nucleic acid. This first formation constitutes “groove binding” in which the polymer dehydrates the DNA helix and binds to the base pairs which make up the nucleic acid’s major groove. This groove binding arrangement can be seen in Figure 5.

In order to form a hydrogen bond with the DNA base pairs, the PEI must be de-protonated. This reaction has been shown to be endothermic and entropy driven [50], where the increase in entropy is attributed to the dehydration of the major groove. Water molecules are strongly bound to the major groove [51] of DNA in solution, and are displaced by the PEI-DNA binding. This is similar to many common protein-DNA binding modes, which have also been shown to be entropy driven [52].

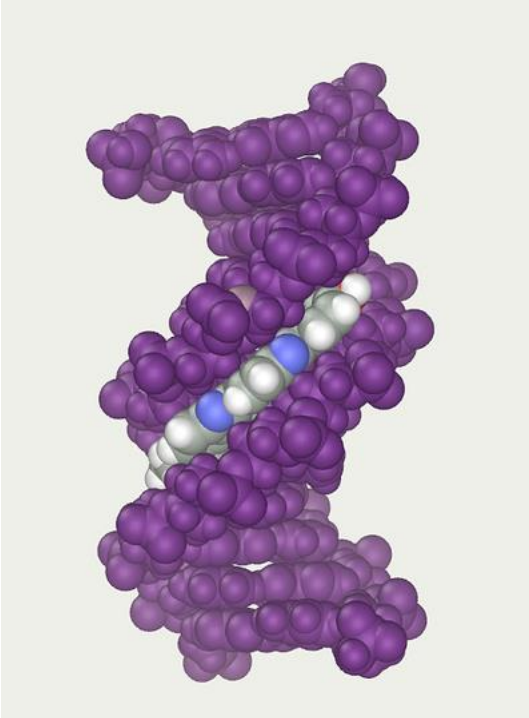

Fig. 5. Ligand binding to the DNA major groove, shown in white [53]

The second DNA-PEI binding mode is that of electrostatic interaction between the PEI's amine groups and the nucleic acid. DNA has a phosphate backbone (Figure 6) which carries a strong negative charge. This allows for electrostatic attraction and bonding to the amine groups of PEI, which are highly positively charged. The electrostatic bond requires the protonation of the PEI polymer. This reaction has been shown to be exothermic with an enthalpy change of 0.88 kJ/mol. This value is very similar to that of the positive enthalpy change associated with the first binding mode, 1.1 kJ/mol. It has been suggested that as PEI moves from one binding mode to another this enthalpy difference is caused by the de- and re-hydration of DNA [50]. This would imply PEI initiates its bond with DNA via the entropically driven first binding mode, and then moves to the second binding mode.

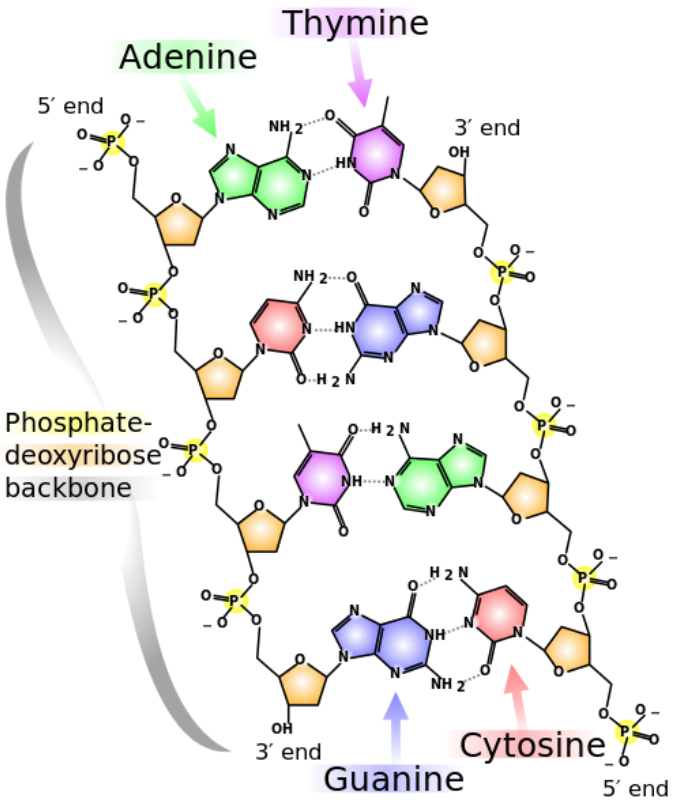

Fig. 6. DNA phosphate backbone, seen at left [54]

3.3.2. DNA Condensation

An important note on DNA-PEI bonding is the condensation of DNA. It is well established that the electrostatic repulsion between negatively charged phosphates in the DNA backbone resist the condensation of the large macromolecule [55]. However, the charge neutralization associated with the electrostatic bond between the PEI and the DNA in the second binding mode allows condensation to occur[56]. It has been shown that 90% of the charge associated with DNA's phosphate backbone must be neutralized for this process to occur [55]. It has also been shown that the ratio of nitrogen to phosphate present during the PEI condensation of PEI does not support this percentage [50]. It is suggested that the localized bending of the DNA molecule caused by the highly branched polymer

may be sufficient to initiate condensation [55, 57]. The shape of the PEI condensed DNA molecule is often toroidal, most commonly with a diameter below 50 nanometers [56].

3.4. PEI Layer for Microtip DNA Capture

As previously discussed in 2.3.3, microtip DNA purification relies on the combination of multiple forces to collect nucleic acids near the microtip surface and trap them there. Binding between the DNA and the microtip is caused by van der Waals interactions. Specifically the negatively charged DNA molecule induces a dipole force by polarizing the gold atoms which coat the silicon microtip surface. This is known as Debye force.

Although this method of adhering the DNA to the microtip has proven effective [3, 11], it requires the DNA be forced into close proximity with the microtip. This is caused by capillary forces which act upon the DNA during the concentration and withdrawal steps of the microtip purification procedure. A thin fluid layer is created on the tip surface due to capillary action, drawing aggregated DNA onto the microtip surface. The area of the thin fluid layer on the microtip surface is dependent on the contact angle between the microtip surface and the sample volume. A lower contact angle between the microtip surface and sample volume corresponds to the creation of a larger fluid film area. Young's equation for contact angle is found below:

$$\theta_c = \arccos\frac{\gamma_{SG} - \gamma_{SL}}{\gamma_{LG}}$$

Where γ_{SG} and γ_{SL} are the surface tensions for the microtip surface-air and microtip surface-sample interfaces, respectively. As γ_{SG} and γ_{SL} form the numerator

of Young's equation, they directly influence the contact angle between the microtip surface and a sample volume. However, these properties of the gold coated microtip surface are not constant or stable. Microtip surface property instability can cause the contact angle between the microitp surface and a sample volume to degrade with time.

To address this challenge, the microtip surface is modified to promote DNA extraction. From the above discussion on PEI, the polymer has excellent DNA binding affinity. For these reasons PEI will be coated as a thin layer on the microtip surface to investigate improved DNA extraction and microtip stability.

Chapter 4: Experimental Methods and Materials

The polymer PEI has been identified as a potential method to meet the aforementioned goals: (1) increase efficacy of the process in the presence of a high concentration of inhibitors, such as in blood and (2) improve stability of the microtip surface chemistry during storage. However, in order to maximize the potential benefit of the PEI coating, the following procedural parameters must be optimized: Microtip baking temperature after polymer coating, DNA capturing time, DNA elution temperature, and DNA elution time. To study how successfully the PEI coating addresses the challenges of microtip DNA extraction, the following tests must be performed: Performance of PEI coated microtips after storage, compared to that of non-coated microtips; and DNA purification from blood using PEI coated microtips. Finally, to better understand the surface chemistry of PEI coated and non-coated tips, these remaining tests must be performed: fluorescent imaging of the microtips after various PEI baking temperatures, and contact angle measurement of non-coated tips before baking, after baking, and after storage.

4.1. Experimental Materials

The Polyethyleneimine polymer used in all experiments was purchased from Sigma-Aldrich. When purchased it has a molecular weight of 750,000 kDal and is 50% water by wt/vol.

All tests performed with Lambda DNA used duplex DNA isolated from bacteriophage lambda (cl857*ind* 1 *Sam* 7), 48,502 base pairs in length, purchased from New England Biolabs. The PCR master mix and primers used for all lambda DNA tests were purchased from Invitrogen. The forward primer sequence for lambda DNA was: GATGAGTTCGTGTCCGTACAACTGG. The backward primer sequence for lambda DNA was: GGTTATCGAAATCAGCCACAGCGCC. The master mix used was Express SYBR GreenER qPCR Supermix Universal.

All blood samples were purchased from Bioreclamation. The blood used was human whole blood with K2 EDTA added as an anticoagulant. The catalog number from Bioreclamation was HMWBEDTA2. The proteinase K (PK) used for blood cell lysis was purchased from QIAGEN. The Sodium Dodecyl Sulfate solution used for blood cell lysis was purchased from Sigma-Aldrich. The primers for blood testing were purchased from Invitrogen. The forward primer sequence for blood (human genomic DNA) was: ACCCACACTGTGCCCATCTAC. The backward primer sequence for blood (human genomic DNA) was: TCGGTGAGGATCTTCATGAGGTA. All blood tests used the same master mix as described above for lambda DNA testing.

DNA elutions were performed in Tris-EDTA (TE) buffer purchased from Sigma-Aldrich followed by pH adjustment. The pH of the TE buffer was adjusted to 8.5.

The PDMS used for the wells of the functionalization device was purchased from K.R. Anderson, Inc. The PDMS used was SYLGARD 184 Silicon Elastomer base and curing agent.

4.2. Experimental Tools

4.2.1. Quantitative PCR

Quantitative polymerase chain reaction (qPCR) is a method of DNA amplification used to quantify the amount of DNA captured during an extraction procedure. This allows for evaluation of the procedure's purification performance. The process starts by adding an amino acid, primer, and polymerase mix with sample DNA. A thermocycling device then heats and cools the mixture with great precision. The temperature is increased until the DNA melts, separating the double helix into two single sided strands. The primers in the mix then bind to the single sided strands at the "start" and "end" point to be amplified. This binding occurs as the primers are designed specifically to bind at pre-designated areas of the DNA, isolating the target sequence between the start and stop primers. The polymerase enzyme is heat-activated and moves along the single sided nucleic acid strand from the start primer to the end primer, creating the matching opposite side to form a complete double sided strand. Once this replication is complete, the DNA is split again via heating and the whole process is repeated. The DNA strands are amplified exponentially with each cycle of replication. In addition to amino acids and the enzyme polymerase, there is also an intercalating dye present in the master mix. This intercalating dye is a fluorescent marker which is un-quenched when bound to the target double stranded sequence. Thus, fluorescent intensity of the bulk mixture increases exponentially. In quantitative PCR the

thermocycling device can also measure the fluorescent intensity of each vial it is heating. Once the intensity value crosses an arbitrary user defined threshold, the current cycle value is recorded. Based on the number of cycles required for the intensity to cross this threshold value, the quantity of original DNA strands can be calculated. This quantity should be a measure of the amount of DNA the extraction process captured.

Unless otherwise stated, all tests used the following qPCR thermal sequence for evaluation: 50 °C for 2 minutes, 95 °C for 10 minutes, (95 °C for 15 seconds, 60 °C for 1 minute) X 39, 65-95 °C ramp for 5 seconds. The mixture of PCR reagents for all tests was 24 µl master mix, 3 µl forward primer, 3 µl backward primer, 5 µl sample (captured DNA from elution vials). The threshold value for all qPCR tests was set at 10 relative fluorescence units (RFU). The specific thermocycler used for all qPCR tests was a BIORAD C1000 Thermal Cycler with CFX96 Real Time System.

4.2.2. PEI Functionalization Device

In order to coat a consistent layer of PEI on the microtip surface (bare surface shown in Figures 7-8), a functionalization device was fabricated. This device can insert and withdraw a microtip into and from a small PDMS well, which holds 13 µl of a highly diluted PEI solution. An aluminum “coupon” (Figure 9) was fabricated which could hold four silicon microtips to simultaneously interface with the four PDMS wells (Figure 10) present in the device. A small stepper motor bought from Oriental Motors was used to move the head of the device towards and away from the PDMS wells. All parts of the device were machined from nylon or plastic, with the exception of the “head” which held

the coupon, and the motor. The “head” was created using stereolithography (SLA), a rapid prototyping process. The whole device can be seen in Figure 11.

Fig. 7. Microtip surface capture with an optical microscope at 4X magnification

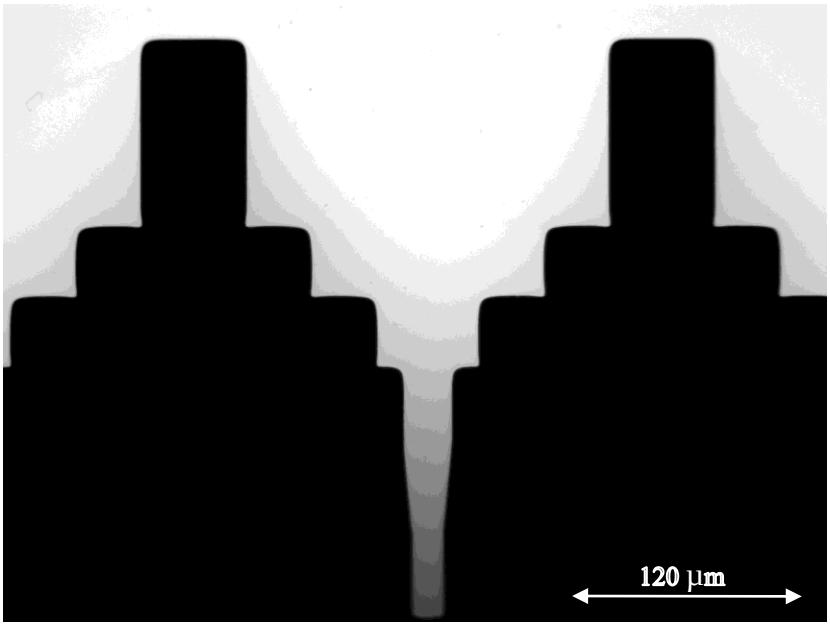

Fig. 8. Microtip surface captured with an optical microscope at 10X magnification

Fig. 9. Aluminum coupon with wire springs to hold microtips in channels at the base of the structure

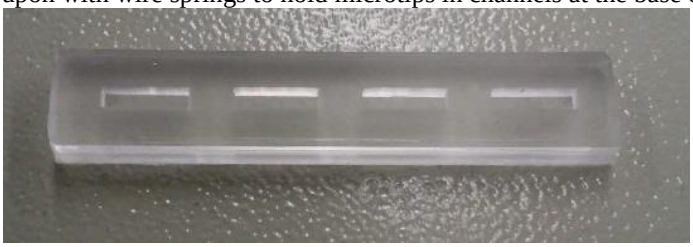

Fig. 10. PDMS wells used to hold diluted PEI solution for microtip functionalization

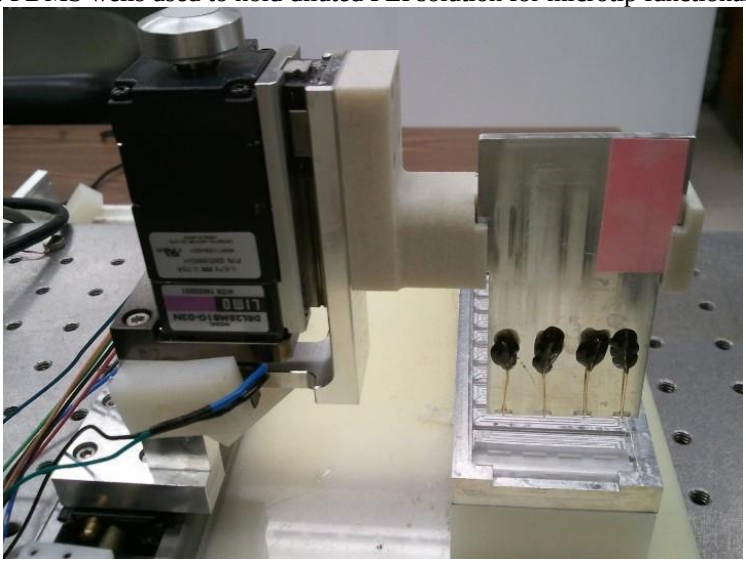

Fig. 11. Functionalization device fabricated for the purpose of coating PEI onto silicon microtips

To use the device, microtips are first cleaned of organic material by heating to 300 °C for 10 minutes using a hotplate. The microtips are then placed in the aluminum coupon and held in place by a wire spring over each channel. The PDMS well is then placed in the aluminum block below the head. Subsequently, the coupon is loaded into the head of the device and 13 µl of diluted PEI solution is added to each well. The motor is then actuated downwards manually while the PDMS well is observed with a low powered microscope. Once the microtips are immersed in the PDMS wells and roughly 25% of their surface is covered, a laptop computer is used to initiate a motor control program. The control program waits for 60 seconds before retracting the head of the device at a rate of 100 micrometers per second. This consistent withdrawal rate is the key to an even and reliable PEI coating. Finally the coupon is removed from the head of the device and the microtips are removed and baked in an oven.

4.2.3. Fluorescent Microscope

An Olympus BX41 microscope was use for all fluorescence microscopy shown in this investigation. The microscope has 4X, 10X and 50X levels of magnification. The camera used for imaging was an Olympus DP30BW. The light source used was an Olympus U-HGLGPS with the fluorescence filter (emission: 515 nm, excitation: 450~480 nm). Microtips were placed on glass slides before being imaged via the microscope camera.

4.2.4. PEI Baking Oven

All baking tests were carried out using a Cascade Tek forced air laboratory oven, model TFO-3. The oven's temperature uniformity is given as +/- 3 °C at 150 °C. The

temperature range of the oven is 15 °C to 300 °C [59]. During all tests temperatures never exceeded 250 °C.

4.2.5. Elution Heat Block

The elution device was fabricated from aluminum, a cartridge heater and a PID temperature controller. An aluminum block was machined so as to have 4 holes which are the correct size and shape to fit PCR microvials. A horizontal hole was then drilled in the side of the aluminum block, below the microvial holes, to allow for the insertion of a cartridge heater. The cartridge heater was then attached to the PID unit which controls its temperature. Finally a thermostat is attached to the aluminum block with a small bolt and wired to the PID to relay the current block temperature back to the controller.

To use the device, PCR microvials are filled with 30 µl of tris-EDTA (TE) buffer and a post capture microtip is then added. The microvial containing the microtip is then inserted into the heat block at a desired temperature. The whole device can be seen in Figures 12-13.

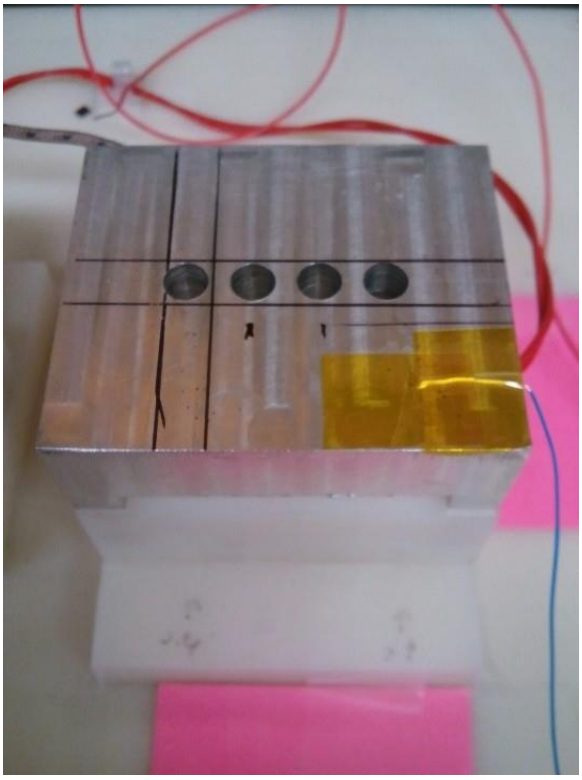

Fig. 12. Aluminum heat block with holes for microvials, termperature sensor and cartridge heater

Fig. 13. PID temperature controller

4.2.6. Wire Coil and Droplet with Electric Probe and Signal Generator

In order to capture DNA using the PEI coated microtips, a similar procedure was followed as outlined by Kalyanasundaram et all [60]. A small coil (Figure 14) was formed manually using 0.25 mm aluminum wire and a 5mm allen wrench. This coil is then placed on a glass slide using scotch tape. A microtip is placed on a glass slide using double sided

scotch tape. The microtip and coil glass slides are placed opposite each other underneath a low power microscope (2.25x), which is attached to a small television. An image from this television output can be seen in Figure 15. The coil is mounted on an XYZ stage.

A function generator (Figure 16) is used to produce a 5 MHz, 20 Vpp sine wave and this electrical potential is applied to the microtip and coil to create an electric field between them. The microtip is connected to this circuit using a small probe which is wired to the function generator and then lowered onto the tip manually for each test. The coil is connected to the circuit via an alligator clip. This whole set up can be seen in Figure 17.

5 µl of sample volume is then pipetted into the coil, and the droplet is brought into contact with the microtip. The microtip is inserted into the wire ring until all three needle like tips are directly underneath the middle of the coil (Figure 18). Once the DNA has aggregated for an amount of time decided on by the user, the microtip is withdrawn manually. The microtip can then be removed from its glass slide and allowed to dry completely in air.

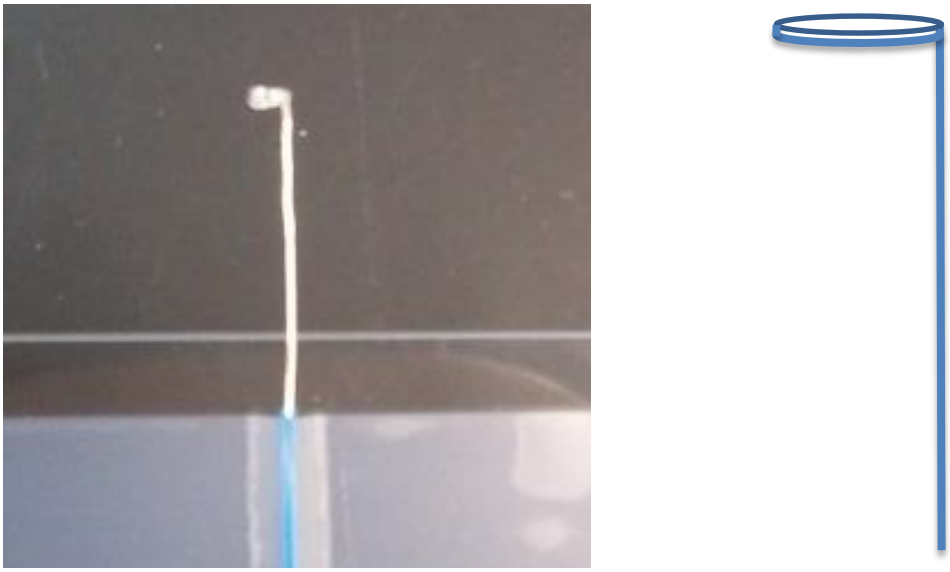

Fig. 14. Aluminum coil for 5ul solution droplet

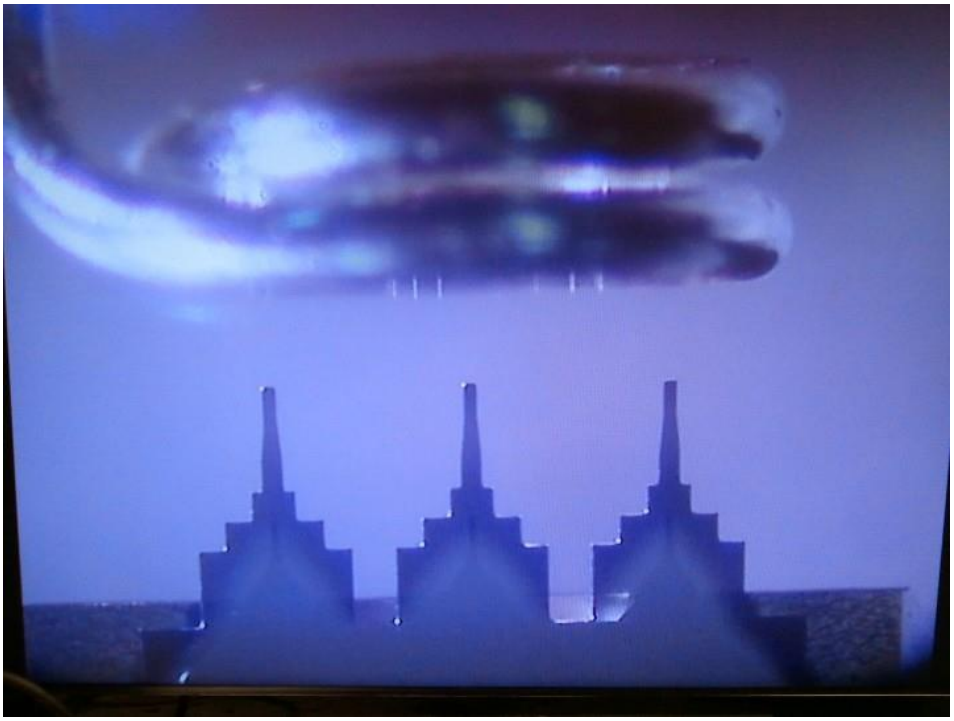

Fig. 15. Microscope output to television during capturing

Fig. 16. Function generator

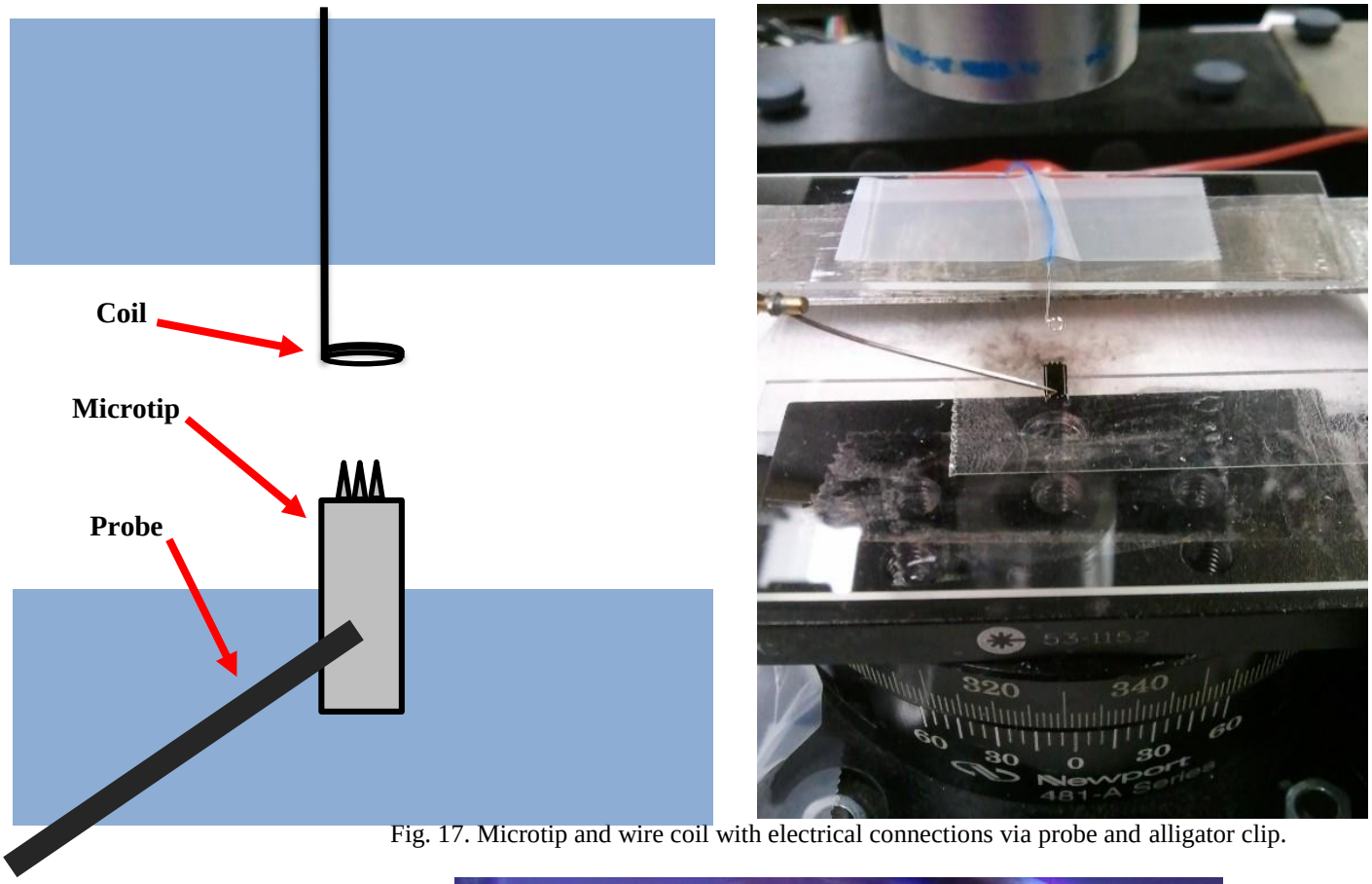

Fig. 17. Microtip and wire coil with electrical connections via probe and alligator clip.

Fig. 18. Microtip inserted into coil with solution droplet so that the end of the tips are underneath the middle of the wire coil.

4.2.7. Automated DNA Purification Device (NanoFacture Inc)

The automated DNA capture device prototype constructed by NanoFacture Incorporated was designed to incorporate all of the equipment required for the previously described coil and microtip DNA purification. The device has a small plastic coupon to hold the microtip in place (Figure 19), and a coil plate which slides into the device and holds the coil in the correct position (Figure 20). The coupon makes an electrical connection between the device and the microtip. There is also an electrical connection between the device and the coil plate. Inside of the device there is an integrated function generator which applies an electrical potential between the coil and the microtip with a 5 MHz 20 Vpp sine wave signal. The full device can be seen in Figure 21.

To use the device, a microtip is inserted in the coupon, which is then placed into the body of the device. A coil is attached to the coil plate via scotch tape and slid into the body of the device. A 5 µl droplet of sample volume is then pipetted into the wire coil. The device is then turned onto its “back” which then places the tip and coil in a horizontal configuration exactly like the previously descried method of microtip-coil capture. The device is then activated which dips the microtip into the coil for 30 seconds. The microtip can then be removed from the coupon and allowed to dry completely in air.

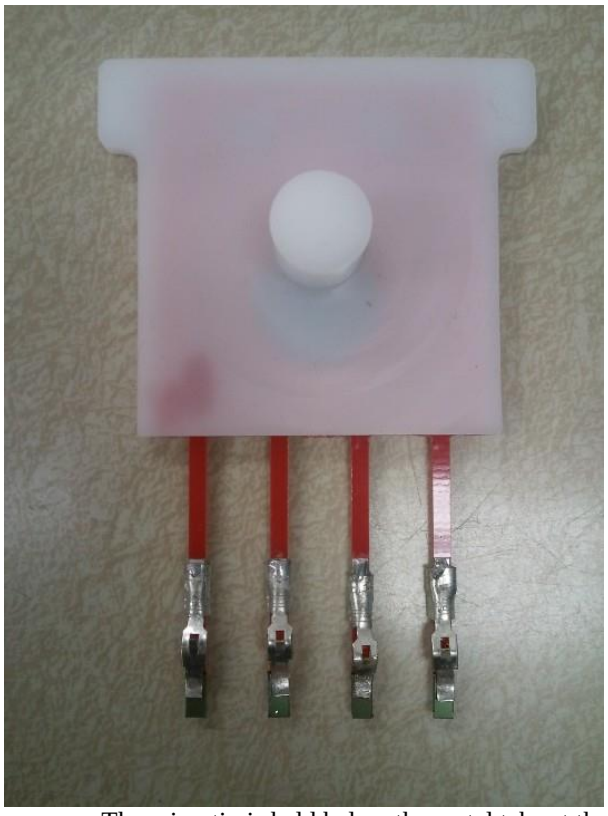

Fig. 19. Automated device coupon. The microtip is held below the metal tabs at the bottom of each arm

Fig. 20. Automated device coil plate. In practice these pre-manufactured coils were replaced with wire coils taped to the surface and stripped to make electrical contact.

Fig. 21. Nanofacture Incorporated automated DNA purification device prototype.

4.2.8. Contact Angle Goniometer

The Goniometer which was used to measure the contact angle of TE buffer on a non-coated microtip surface was manufactured by the Ramé-Hart Instrument Company. This device consisted of three components: an illuminator, a camera, and a level plate. Each microtip was placed individually on the level plate and a 0.5 µl droplet was deposited on its surface. The illuminator was used to make the droplet more visible to the optical camera. The camera was used to take an image of the droplet and DROPimage software, provided by Ramé-Hart with the purchase of the instrument, allowed for analysis of the mean contact angle. A stock image of the goniometer can be seen in Figure 22.

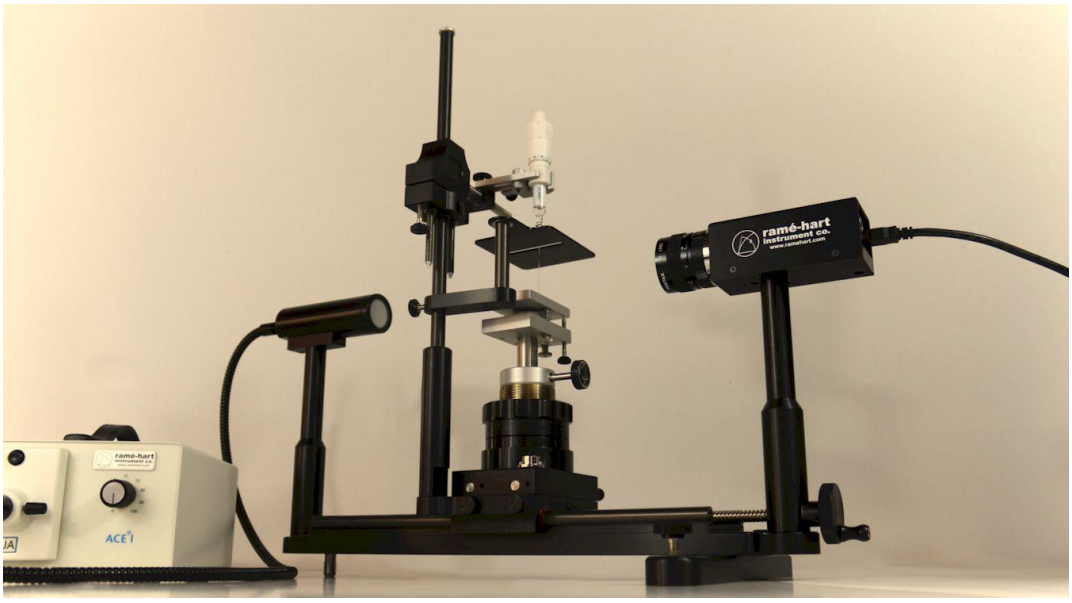

Fig. 22. Ramé-Hart Goniometer, model 500 [61]

4.3. Experimental Procedures

4.3.1. PEI Baking Temperature

In order to evaluate the DNA purification performance of PEI coated microtips with various PEI baking temperatures, the following test was conducted. Gold coated silicon microtips were first functionalized with .05% PEI solution. The tips are then baked for one hour at the following temperatures: 100, 150, 175, 200, 225, and 250 °C. After baking is complete, the tips are placed individually on a glass slide. Using the previously descried wire-coil capturing set up, each tip is left to capture from the 5 µl 1pM lambda DNA droplet for 5 minutes while an electric signal is applied. The tips are then withdrawn from the droplet manually and allowed to dry completely in air. The dried tips are eluted into 30µl of TE buffer using the previously described heating block. DNA is eluted for 3 minutes at 80 °C. All tests were performed with n = 6. Subsequently all captured DNA was quantified using qPCR as previously described.

4.3.2. DNA Capturing Time

In order to evaluate the DNA purification performance of PEI coated microtips with various capturing times, the following test was conducted. Gold-coated silicon microtips were first functionalized with .05% PEI solution. These tips were then baked at 250 °C for 1 hour. Using the previously descried wire-coil capturing set up tips were used to collect from a 5 µl 1 pM droplet of lambda DNA. The following capturing times were tested: 0.5, 1, 2, and 5 minutes. After capturing all tips were allowed to dry completely in air. DNA was eluted from the dried tips, using the heat block set up, for 3 minutes at 80 °C. All tests were performed in triplicate, n = 3. Subsequently all captured DNA was quantified using qPCR as previously described.

4.3.3. DNA Elution Temperature

In order to evaluate the DNA purification performance of PEI coated microtips with various elution temperatures, the following test was conducted. Gold coated silicon microtips were first functionalized with .05% PEI solution. These tips were then baked at 250 °C for 1 hour. Using the previously descried wire-coil capturing set up tips were used to collect from a 5 µl 1 pM droplet of lambda DNA. DNA was captured for 5 minutes, after which time the tips were removed from the droplet manually and allowed to dry fully in air. The dried tips are then placed in 30 µl of TE buffer and eluted at the following temperatures for 5 minutes: 50, 60, 70, 80, 90 °C and room temperature. All tests were performed in triplicate, n = 3. Subsequently all captured DNA was quantified using qPCR as previously described.

4.3.4. DNA Elution Time

In order to evaluate the DNA purification performance of PEI coated microtips with various elution times, the following test was conducted. Gold coated silicon microtips were first functionalized with .05% PEI solution. These tips were then baked at 250 °C for 1 hour. Using the previously descried wire-coil capturing set up tips were used to collect from a 5 µl 1 pM droplet of lambda DNA. DNA was captured for 5 minutes, after which time the tips were removed from the droplet manually and allowed to dry fully in air. The dried tips are then placed in 30 µl of TE buffer and eluted for the following times at 70 °C: 1, 2, 3, and 5 minutes. All tests were performed in triplicate, n = 3. Subsequently all captured DNA was quantified using qPCR as previously described.

4.3.5. Performance of PEI Microtips after Storage

In order to evaluate the DNA purification performance of PEI coated microtips after storage for various lengths of time, the following test was conducted. Gold coated silicon microtips were first functionalized with .1% PEI solution. These tips were then baked at 175 °C for 1 hour. The tips were then stored in air, in a sanitary environment, for the following lengths of time: 0 (control), 5, and 30 days. Following this storage, and using the previously described automated DNA purification device, tips were used to collect from a 5 µl 1pM droplet of lambda DNA. DNA was captured for 30 seconds, after which time the tips were removed from the droplet automatically and allowed to dry fully in air. DNA was eluted from the dried tips, using the heat block set up, for 3 minutes at 70 °C. All tests were performed in triplicate, n = 3. Subsequently all captured DNA was quantified using qPCR as previously described.

4.3.6. Performance of PEI Microtips with Blood

In order to evaluate the DNA purification performance of PEI coated microtips with blood samples, the following test was conducted by Gareth Fotouhi. Gold coated silicon microtips were first functionalized with .1% PEI solution. These tips were then baked at 175 °C for 1 hour. 2.5 µl of whole blood was added to 2.5 µl of PK, 2.5 µl of TE buffer, and 2.5 µl of 1.12% SDS. This solution was then heated to 60 °C for 10 minutes to fully lyse the cells, releasing their DNA into the solution. Using the previously described wire-coil capturing set up, DNA was collected from the 5 µl of lysis solution for 5 minutes. The tips are then withdrawn from the sample droplet and allowed to dry full in air. Following this drying, two rinsing steps were implemented to remove inhibitors present in the blood residue. In the first rinsing step 1 µl of ethanol is pipetted onto the tip surface. The tip is then allowed to dry. In the second rinsing step the tip is manually dipped into a microvial of TE buffer for 10 seconds. The tip is then allowed to dry completely. DNA was eluted from the dried tips, using the heat block set up, for 3 minutes at 95 °C. The Qiagen QIAamp kit was also run for sake of comparison using the same blood samples, using 1.25 µl of blood. All blood tests were performed with n = 20, all QIAamp tests were performed with n = 7. Subsequently all captured DNA was quantified using qPCR as previously described.

4.3.7. Fluorescent Imaging of PEI after Various Baking Temperatures

In order to determine the effects of baking temperature on the PEI layer, the following test was performed. Gold coated silicon microtips were first functionalized with .1% PEI solution. The tips were then baked at the following temperatures: 100, 175, 200, 225, and 250 °C. After baking, the tips were imaged with the fluorescent microscope and camera.

4x magnification was used for all images. The exposure time was adjusted between 400msec and 1sec to eliminate background noise while maximize fluorescence captured, although it was kept consistent before and after rinsing. The tips were then manually dipped into a microvial of TE buffer for 5 minutes. The tips were allowed to fully dry, before being imaged a second time with the same procedure. All tests were performed in triplicate, n = 3. Subsequently all images were analyzed using a MATLAB script for pixel counting.

4.3.8. Contact Angle Measurement

In order to determine the degradation of baked, non-coated microtip surface chemistry, the following test was performed. Non-coated gold surface microtips were baked on a hot plate for 10 minutes at 300 °C. The contact angle of TE buffer on the microtips was then measured, using a goniometer, at the following times after baking: 0, 3, and 24 hours. The contact angle of non-baked microtips was also measured. All tests were performed in triplicate, n = 3.

Chapter 5: Results

5.1.1. PEI Baking Temperature

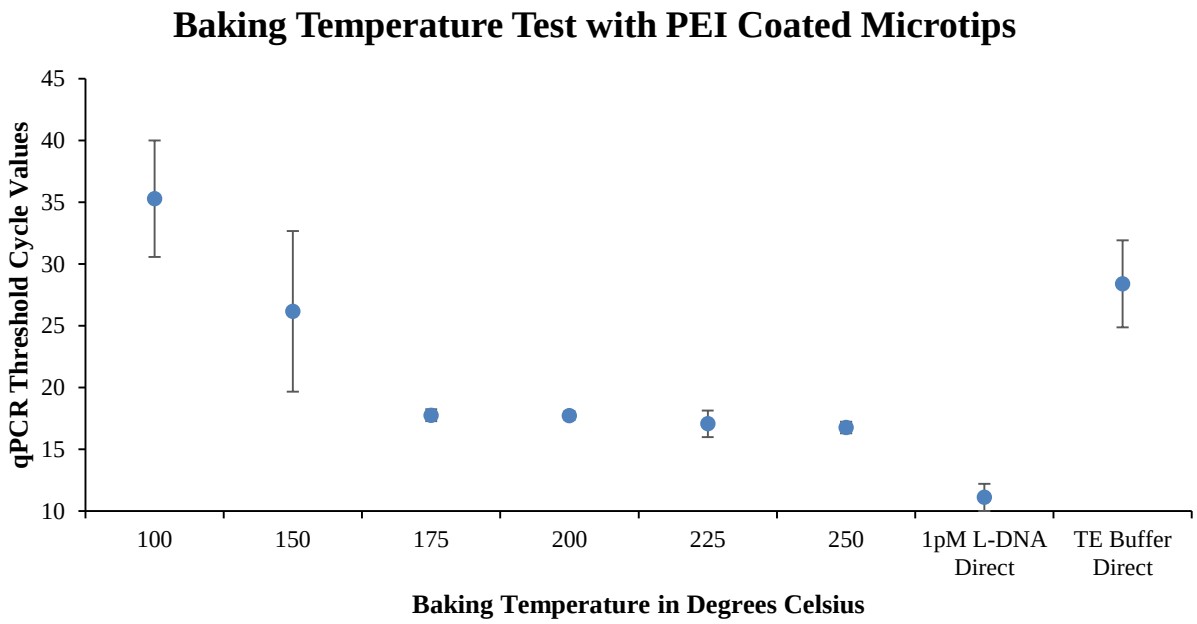

Fig. 23. Results of the Baking Temperature Test

The results of the PEI baking temperature test show a decrease in qPCR threshold cycle values as the baking temperature is increased. This corresponds to an increase in captured DNA while using the PEI coated tips baked at higher temperatures. The standard deviation of the results also decreases as the baking temperature is increased. This suggests some change is occurring in the PEI above 150 °C, as from 175 to 200 °C the results are fairly similar. Although the higher temperature (above 150 °C) results show a marked improvement over 100 and 150 °C tests, the 250 °C value is the best result. 250 °C has thus been proven to capture the most DNA, and is then the most ideal temperature to bake PEI coated tips at prior to DNA capturing. Two control results are also plotted: 1pM lambda DNA directly added to PCR (5 µl) and TE buffer added directly to PCR (5 µl).

5.1.2. DNA Capturing Time

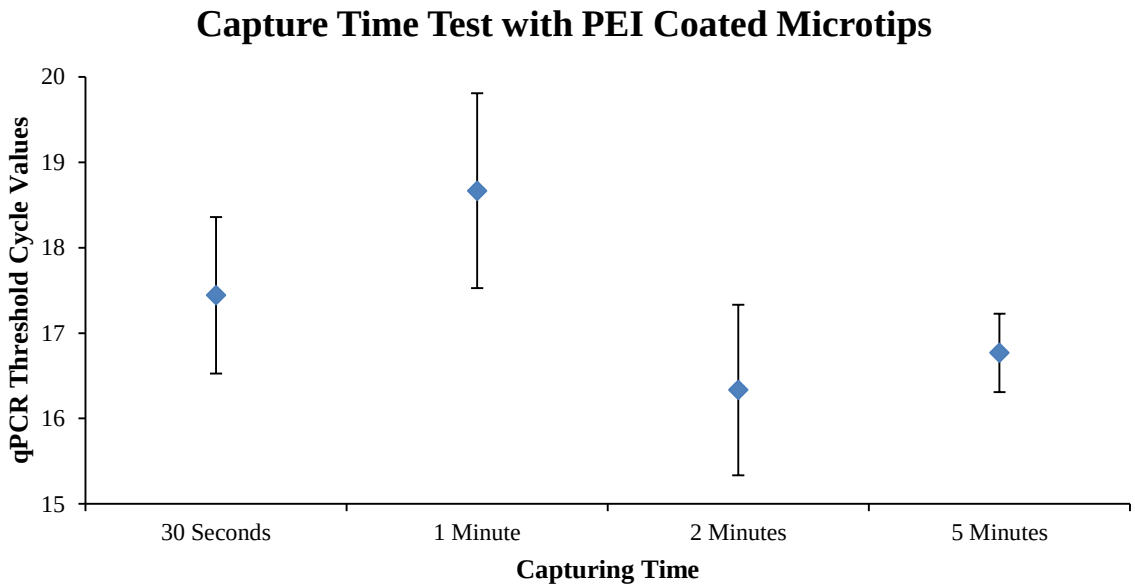

Fig. 24. Results of the Capturing Time Test

The results of the DNA capturing time test show a decrease in qPCR threshold cycle values as capturing time is increased. This corresponds to a larger amount of nucleic acid material being captured using a longer capturing time. The 5 minute average value proves to be slightly worse than the 2 minute average value. However, the 5 minute values are also much more consistent, with significantly smaller standard deviation than the 2 minute values. This is in line with observations by Kalyanasundaram et al that given more time (up to 5 minutes), more DNA particles are concentrated at the microtip surface [3], and thus put in contact with PEI for capture. The decrease in variability is most likely related to the dielectrophoretic forces becoming more dominant, in comparison to transient initial forces after microtip insertion, on a longer time scale.

5.1.3. DNA Elution Temperature

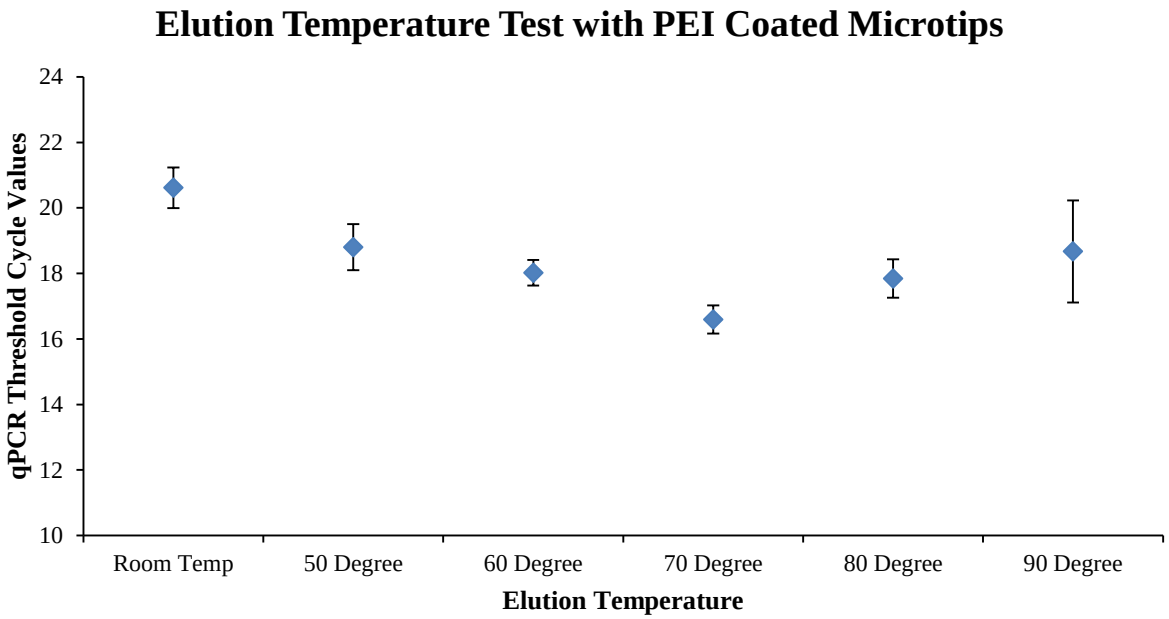

Fig. 25. Results of the Elution Temperature Test

The results of the DNA elution temperature test show a decrease in qPCR threshold cycle values with an increase in temperature, up to 70 °C. This corresponds to an increase in eluted DNA material up to 70 °C. The DNA is released from the PEI due to Brownian motion, so results are in line with the intuitive assumption that with greater heating energy, more DNA will be freed from the tip surface. However, after 70 °C the qPCR analysis shows a decrease in DNA material eluted from the tips. As it is impossible for the elution step to affect how much DNA is originally captured, this indicates that temperatures above 80 °C negatively impact the integrity of the eluted DNA molecules. It is possible that at 80 °C the DNA is denatured or otherwise damaged. It is also possible that at 80 degrees more PEI is eluted from the tip and inhibits the PCR reaction. 70 °C is then the ideal elution temperature for DNA elution in our case.

5.1.4. DNA Elution Time

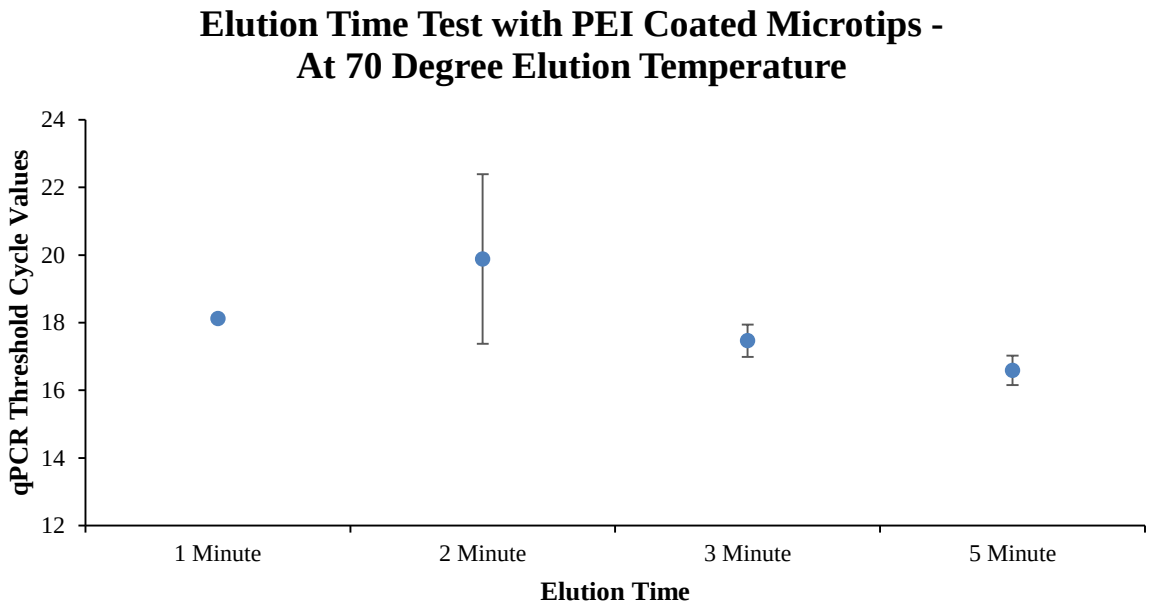

Fig. 26. Results of the Elution Time Test

The results of the DNA elution time test show a decrease in qPCR threshold cycle values with increasing time. This corresponds to an increase in eluted material from the PEI coated microtip, with increasing time. This result is again, quite intuitive, as with more time for the heating energy to effect the DNA molecules on the PEI surface, the probability they are knocked loose increases. However, it is also apparent that the difference between 3 minutes and 5 minutes is very slight. Based on this data, 5 minutes was chosen as the ideal elution time for PEI coated microtips. Longer times were not investigated due to the diminishing returns shown by the increase from 3 to 5 minute elution. Longer elution time extends the total protocol length, which is an important parameter in purification technique evaluation.

5.1.5. Performance of PEI Microtips after Storage

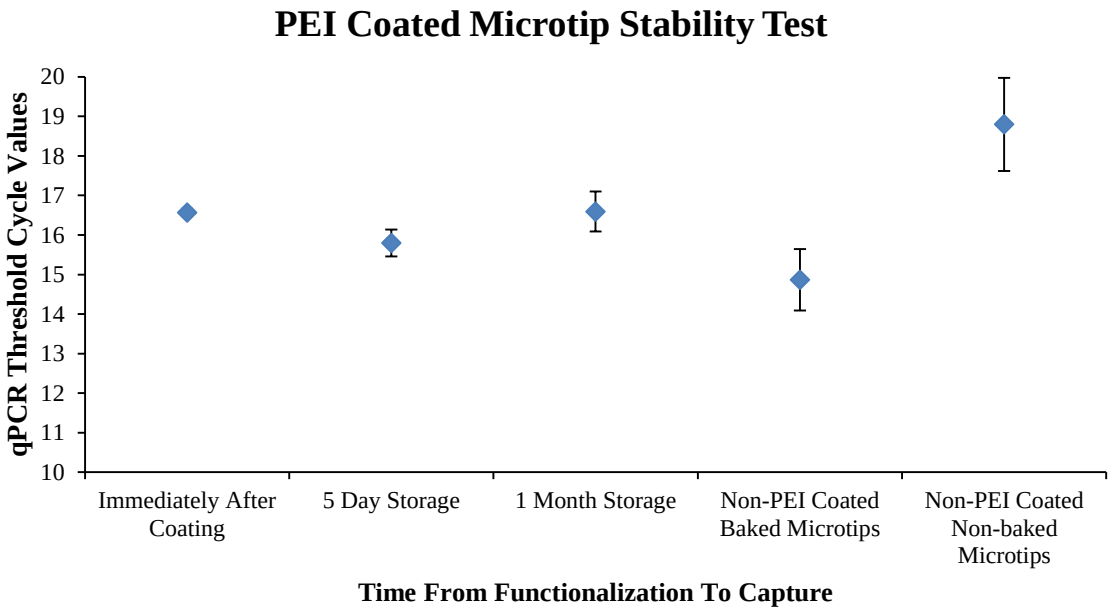

Fig. 27. Results of the PEI Microtip Storage Test

The results of the PEI coated microtip storage test shows the DNA purification performance of tips immediately, 5 days and 1 month after baking. The difference between the performance of a PEI coated microtip directly after baking and after one month of storage is not statistically significant. This is an important result as it shows the PEI surface chemistry, which allows for high DNA yield in capturing, is stable. For comparison, the performance of a non-coated microtip just after baking (300 °C for 10 minutes) and without baking is also shown. The non-coated tip performance degrades from that of the baked state back to the non-baked state after a short amount of time due to the changing contact angle. This changing contact angle is shown in the contact angle measurement result.

5.1.6. Performance of PEI Microtips with Blood

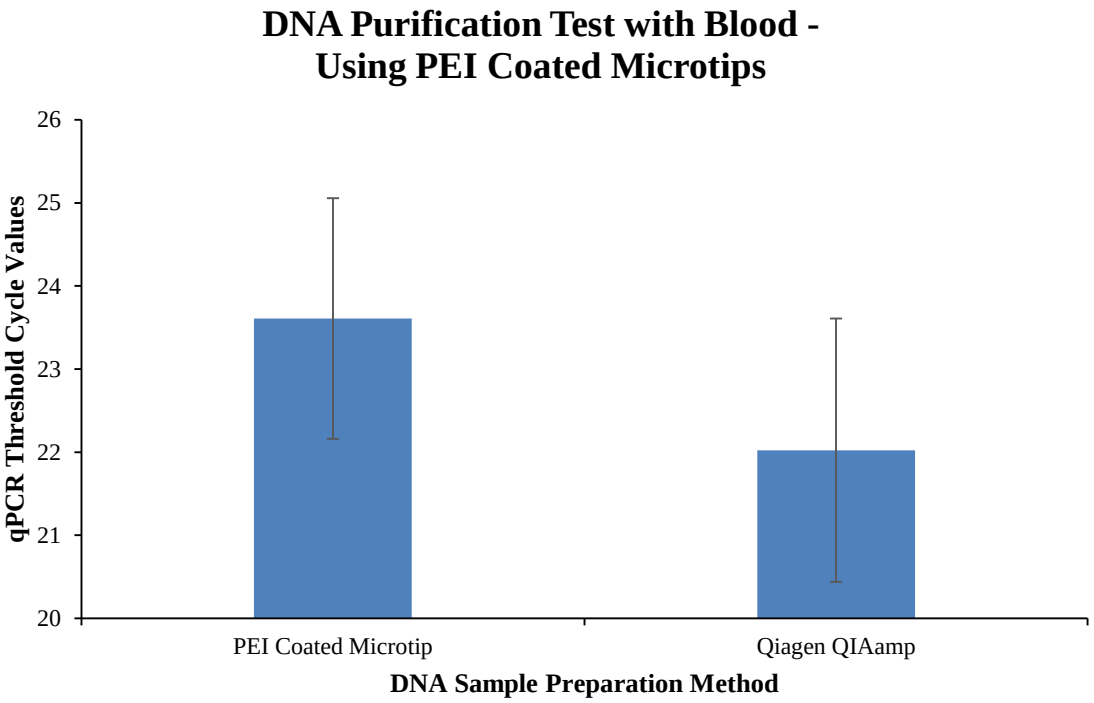

Fig. 28. Results of the DNA Purification Test from Blood Samples using PEI Coated Microtips

The results of the DNA purification test using whole blood samples, completed by Gareth Fotouhi, are shown above in Figure 28. By testing the performance of the PEI microtip extraction method with whole blood, it is possible to evaluate the performance of the technique with an extremely complex and impure sample. This type of test is often performed using the Qiagen QIAamp system because of its reliably good results. To evaluate the performance of the microtip technique relative to that of other modern DNA extraction methods, the QIAamp system was run with the same whole blood sample. It can be seen in Figure 28 that the two systems produce comparable results. The QIAamp system produces a lower average cycle value, which correlates to a higher yield of DNA. However, the error bars for both methods overlap, which suggests they are not statistically different.

This shows the microtip method's performance is similar to that of the industry standard Qiagen QIAamp system, while extracting DNA from whole blood.

5.1.7. Fluorescent Imaging of PEI after Various Baking Temperatures

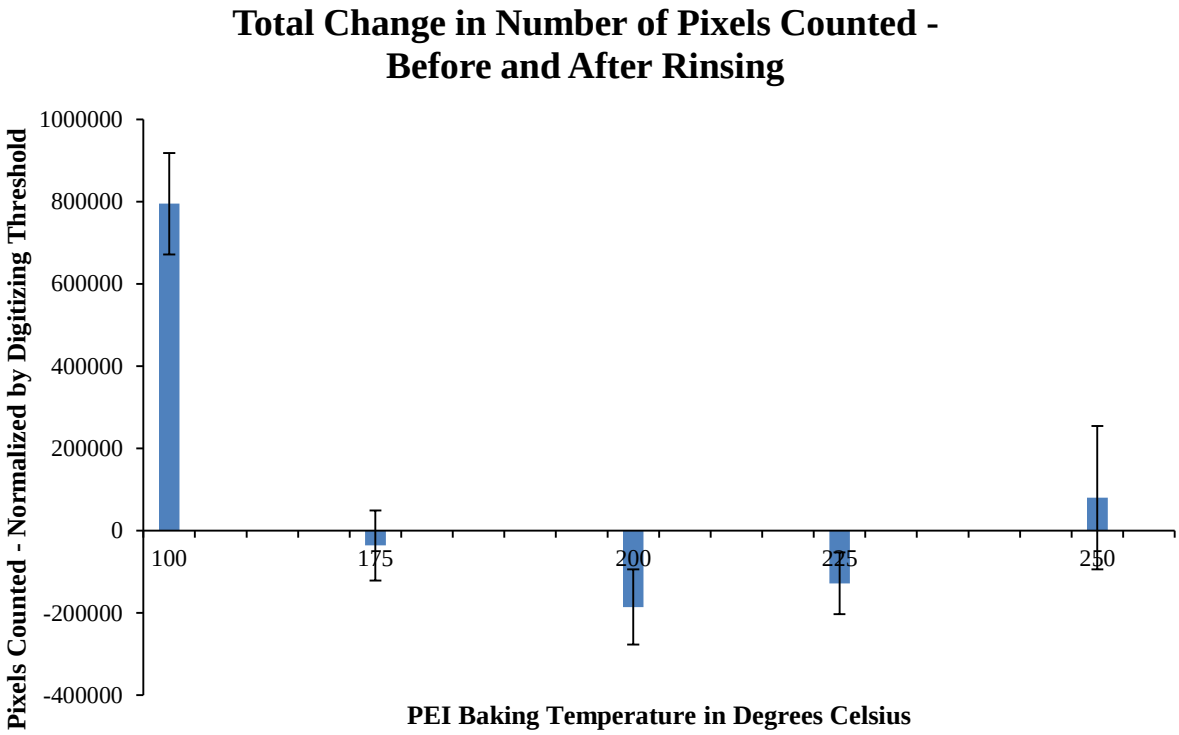

Fig. 29. Results of the MATLAB Pixel Counting Test from Fluorescent Imaging at Various PEI Baking Temperatures

The fluorescent imaging test for various baking temperatures involved the use of a MATLAB script to analyze the resulting data. The MATLAB script took each fluorescent image and changed each pixel into either a 100% bright or 100% dark spot, thereby digitizing the image. The script determined whether or not a pixel would become "bright" or "dark" by assessing the original brightness of the pixel. If the pixel was originally above some threshold value, then it became bright, if not it became dark. The script then counted all pixels which were bright and output this number.

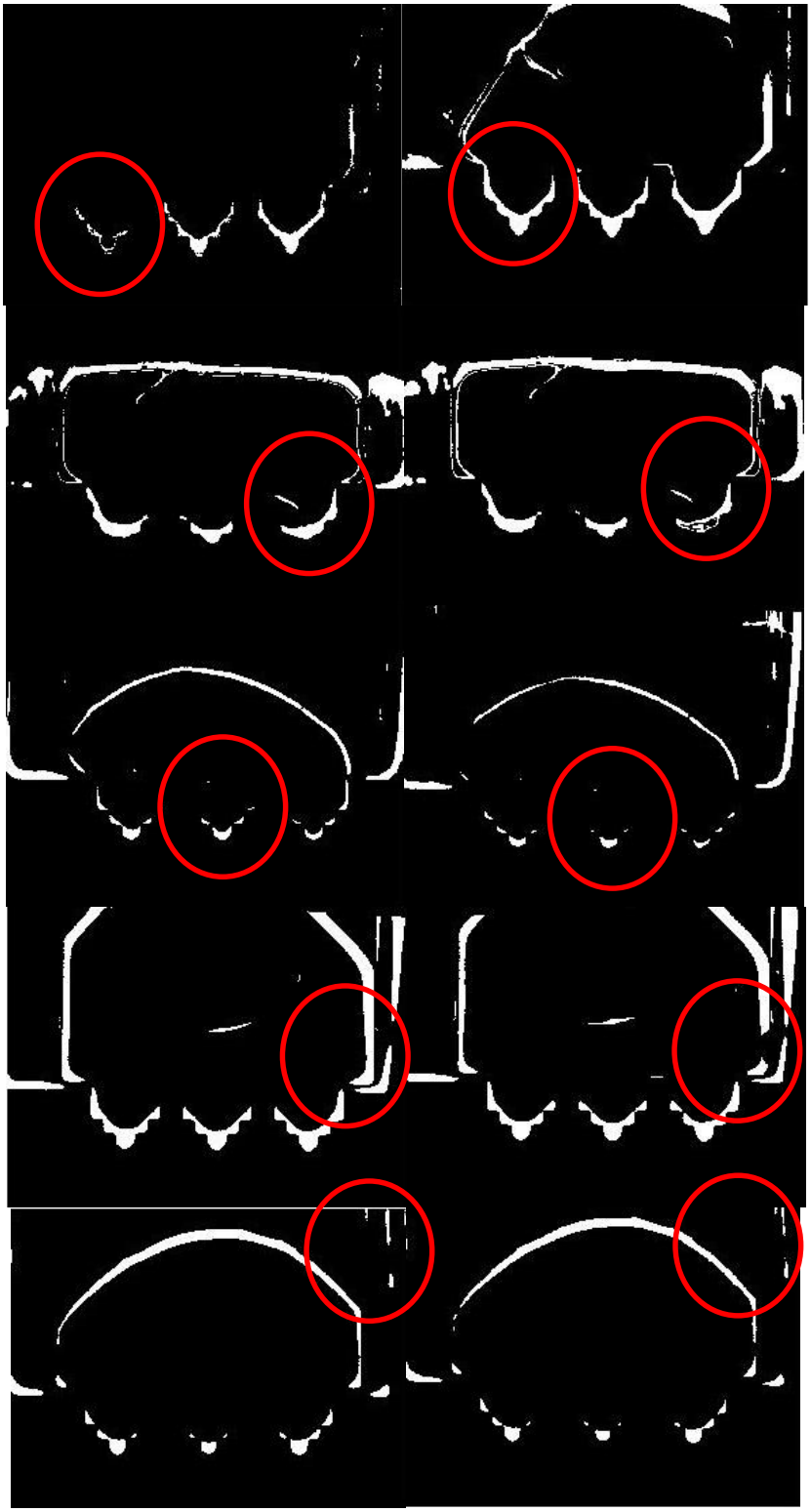

Fig. 30. *From top to bottom:* 100, 175, 200, 225, 250 °C images. Before rinsing on left, after rinsing on right

All tips were imaged before and after a rinsing step, so there are two images per one tip. The MATLAB script threshold value was adjusted for each set of two images so as to minimize the counting of background noise but maximize PEI fluorescence pixels counted. The overall change in pixels counted per tip before and after rinsing was calculated and then normalized by the threshold value used during pixel counting. These values can be seen in Figure 29.

The results show a large increase in fluorescence for the 100 °C baked tips and little to no change for all others. To better understand the difference between the PEI surfaces after various baking temperatures, the digitized fluorescent images were also be analyzed manually.

In Figure 30 we see the fluorescent images of the PEI coated tips before and after rinsing, with differences highlighted. The fluorescent images of 175 and 200 °C show obvious PEI loss due to rinsing. However, above 200 °C we see very little degradation of the PEI layer. 225 °C images show almost no change before and after rinsing, while the two 250 °C images are essentially identical. The images correlate well with the results of the PEI baking temperature test. As the temperature is increased above 175 °C, very little PEI is washed away. The least PEI washed away is after 250 °C baking. The best purification performance is also seen after 250 °C baking (Figure 23).

5.1.8. Contact Angle Measurement

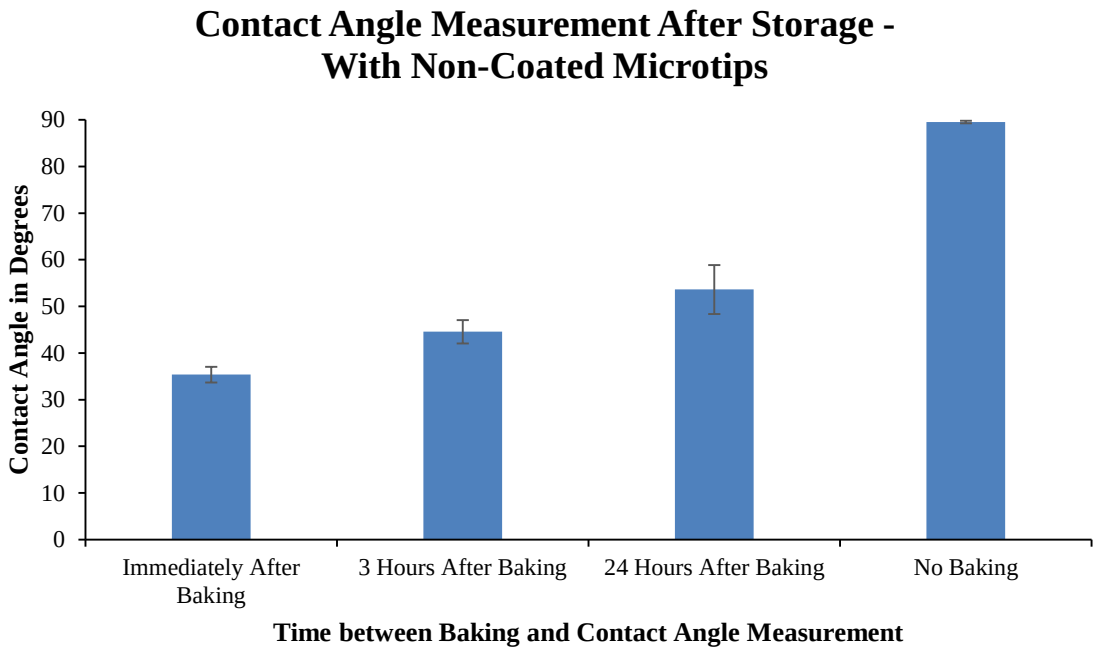

Fig. 31. Results of the Contact Angle Measurement Test

The results of the contact angle measurement test with non-coated microtips show a clear trend. Tips which were not baked at all had a contact angle of 89.5 degrees; this shows the extreme hydrophobicity of the gold surface. After baking however, the gold surface was significantly more hydrophilic, with a contact angle measuring 35.4 degrees. After three hours of storage in a gel back, the angle had degraded to 44.6 degrees, and after 24 hours the angle was 53.6 degrees. These measurements show an obvious trend of the gold surface returning to its non-baked state after storage in air. This demonstrates the need for a more stable microtip surface layer.

Chapter 6: Discussion

It is speculated the increase of the contact angle between a sample volume and microtip surface can reduce the capture yield of DNA. Capillary action creates a thin fluid layer on the microtip surface during DNA capturing. As contact angle between a sample volume and the microtip surface is increased, thin fluid area created during DNA capturing is decreased. As thin fluid area on the microtip suface is decreased, less DNA will be forced into contact with and bound to the microtip surface. The DNA yields of baked vs. non-baked gold surface microtips without PEI are compared in Figure 27. Gold surface microtips without PEI have a contact angle of 90 degrees. After the gold surface microtips, without PEI, are baked at 300 °C for 10 minutes, the microtip surface becomes more hydrophilic with a contact angle of 35.4 degrees. As time after baking increases, the contact angle between a 0.5 µl buffer droplet and the non-PEI coated gold surface microtips increases. It is speculated that the unstable surface properties of the non-PEI coated gold surface microtips will lead to the degradation of capturing performance as time after baking is increased. The coated microtip storage test shows that stable surface properties are achieved with the PEI coating. PEI coated microtip capturing performance is equivalent when tested directly following baking and one month after baking. The time-independent reproducible result shows the PEI surface does not degrade during long term storage and provides a more stable surface.

Coating microtips with PEI increases DNA capturing efficacy from highly complex samples by allowing electrostatic binding between the DNA and the microtip. PEI-coated microtips are capable of extracting DNA from blood, and achieve similar yield to that of the Qiagen QIAamp system when using the same sample. The rinsing step present in the

blood sample PEI coated microtip protocol is the key to achieving highly pure DNA. Previous tests using non-PEI coated microtips, blood samples, and no rinsing step did not produce a quantifiable DNA yield due to high levels of qPCR inhibition. Similarly, PEI coated microtips which were used with a no-rinse protocol did not produce a quantifiable DNA yield due to high levels of qPCR inhibition. The rinsing step washes away captured PCR inhibitors, but the electrostatic bond between PEI and DNA prevents DNA release prior to the elution step. In previous tests using non-PEI coated microtips, a rinsing step was implemented, but quantifiable DNA yield was equivalent to negative control. A rinsing step is not possible with non-PEI coated microtips as the van der Waals binding between the microtip and the DNA is not strong enough to prevent DNA release during rinsing.

A significant change in the molecular structure of a PEI surface coating occurs at baking temperatures above 175 °C. After baking PEI coated microtips at 100 and 150 °C, their capturing performance is significantly worse than that of PEI coated microtips baked above 175 °C. Above 175 °C, DNA yield is steadily improved as the baking temperature is increased, up to 250 °C. However, the gap between the DNA capturing performance of 175 and 250 °C baked PEI coated microtips is much less than between 100 and 175 °C baked PEI coated microtips. Pixel counting of fluorescent images, showing PEI coated microtips before and after rinsing, indicates 100 °C baked PEI is more changed from rinsing than PEI baked at or above 175 °C. The increased fluorescence of 100 °C baked PEI coated microtips is potentially due to the redistribution of melted PEI from the microtip perimeter grooves to the surface, during rinsing. Above 100 °C baking temperatures, PEI redistribution during rinsing becomes negligible, implying the PEI has more fully “cured” to the microtip surface. Instead, above 100 °C baking temperatures, the number of pixels

counted on the microtip surface area is reduced due to PEI being washed away into the rinsing solution. The fluorescent images correspond with the baking temperature test results, in that both show a distinct difference between 100 °C and 175 °C baked PEI properties. Additionally, it is clear from digitized fluorescent images that PEI is dissolved from the surface of 175, 200, and 225 °C baked PEI coated microtips after rinsing. Only 250 °C baked PEI coated microtips show no change before and after rinsing. The decrease in PEI dissolved from PEI coated microtips corresponds to the increase in DNA capturing performance of PEI coated microtips, as baking temperatures rise. It is speculated that PEI is released from the microtip surface during the elution step of the purification protocol. PEI polymer released into the elutant can bind to the target DNA. When groove binding occurs between PEI and DNA molecules, the formed PEI-DNA complexes cannot be amplified by the PCR process, as annealing or enzyme reactions are inhibited. PEI-DNA complexes may not be amplified as DNA yield by qPCR. PEI-DNA complexes, formed by PEI eluted from PEI coated microtips, explain the decrease in measured DNA yield with PEI coated microtips which are baked below 250 °C.

Chapter 7: Conclusion

In summary, we investigated (1) the efficacy of microtip DNA capture using PEI coating and (2) the stability of the microtip surface chemistry at different baking temperatures and concentration times. The ideal capturing time, elution time and elution temperature for DNA capture with PEI coated microtips were found to be 5 minutes, 5 minutes and 70 °C respectively. The performance of PEI-coated microtips was evaluated via storage and blood capture tests. PEI-coated tips produced a DNA yield comparable to that of the QIAamp system with whole blood. PEI-coated tips were also shown to provide

reproducible capturing results after 1 month of storage. From the results, PEI coating at the given experimental conditions is a successful solution for reproducible extraction of DNA from blood.

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