DNA Purification on Microfluidic Devices with a Focus on Large Volume, Forensic Biological Samples

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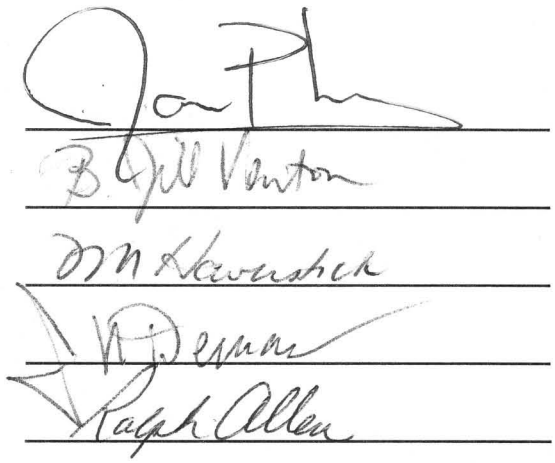

Abstract

The development and application of microfluidic sample preparation methods to a wide range of sample types, including large-volume forensic samples undergoing genetic analysis, has the potential to greatly benefit the forensic and clinical communities. The design, development and optimization of a microfluidic solid phase extraction method applicable to the purification of DNA from forensic biological samples (vrSPE) obtained in large volumes is demonstrated. Illustration of the range of samples this method can handle, the successful application to environmentally-degraded DNA samples, mitochondrial DNA from blood and blood stains, and genomic DNA from bone are also shown. Integration of vrSPE with a secondary, orthogonal, purification method is demonstrated to be advantageous for the removal of the PCR inhibitors (e.g., indigo dye), and to outperform low volume microfluidic SPE (μSPE) and vrSPE in the removal of that inhibitor. The development of a microfluidic, forensic genetic analysis system, both in a modular and integrated form, is detailed. The modular system described, used three microfluidic devices for SPE, polymerase chain reaction (PCR), and microchip electrophoresis (ME), while the integrated system used a single device for both SPE and PCR. Each method was used for the successful, forensic STR analysis of buccal swab lysate. Finally, an exploratory excursion into the development of a PMMA (Plexiglas) microfluidic device for DNA purification is shown, with a focus on device bonding and surface modification. This work provides the next step towards developing a single-use, genetic analysis microdevice.

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1. Introduction

1.1 Conventional DNA Analysis

1.1.1 Forensic DNA Analysis

The analysis of DNA is essential in both forensic and clinical fields. Forensic DNA analysis is crucial for multiple applications including, but not limited to, human identification for security purposes at check points or a criminal investigation to determine the identity of the perpetrator (samples collected at a crime scene), paternity testing, and identification of missing persons through the use of mitochondrial DNA (mtDNA).[1] Because of these widespread applications, multiple sample types are encountered such as whole blood, semen, buccal swabs, bone, and stains on various substrates exposed to a variety of conditions [i.e., sunlight exposure, heat, environmental and substrate polymerase chain reaction (PCR) inhibitors]. This variability in sample type and condition make it imperative that methods for analysis be reproducible, efficient, and applicable to a wide range of samples. Conventional DNA analysis typically involves a three step process where the DNA is first extracted from the biological sample, followed by amplification using PCR, and then separation/detection of PCR products (Figure 1).[1] In total, this process can take several hours to complete which can have a large affect on the patient, if related to clinical analysis, or identification of a perpetrator, if pertaining to forensic analysis.

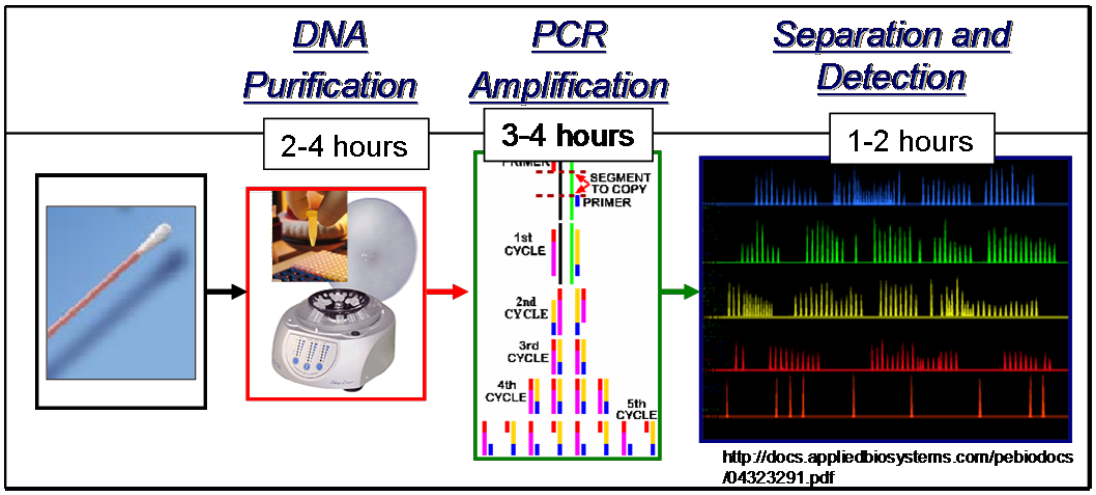

Figure 1. Schematic for analysis of DNA from a biological sample. The sample is purified, amplified, and then separated/detected.

1.1.1.1 Nuclear DNA Analysis

The use of DNA for forensic purposes was first described in 1985 by Sir Alec Jeffreys as 'DNA fingerprinting' or DNA profiling[1]. He found that there are DNA sequences within the human genome that are repeated and the number of repeats vary from person to person, providing the capability for human identification based on repeat patterns. These repeat regions became known as VNTRs (variable number of tandem repeats) and were investigated using restriction fragment length polymorphism (RFLP) (using a restriction enzyme) to cut the DNA containing VNTRs into segments. Due to the downfalls of this method including being lengthy, laborious, and requiring large quantities of DNA that is well-preserved, advancements were made towards smaller repeat units known as short tandem repeats (STRs).[1] These STRs are found within non-coding regions between or within genes at specific loci on chromosomes within the genome. The number of repeats at each locus, also termed alleles, can vary from person to person. The combination of possible alleles at a locus with analysis of multiple loci,

specifically 13 core loci set (due to their neutral mutation rates) by the national Combined DNA Index System (CODIS) launched by the FBI (Figure 2), provide the high power of discrimination that makes STRs so powerful for human identification. Also, due to the smaller size of these repeats, multiplexed amplifications are possible. Furthermore, the method works well with low quantities of starting DNA template[1] making it applicable to samples that previously could not be analyzed due to the lack of or condition (single strand breaks or sheared DNA) of starting DNA template.[1]

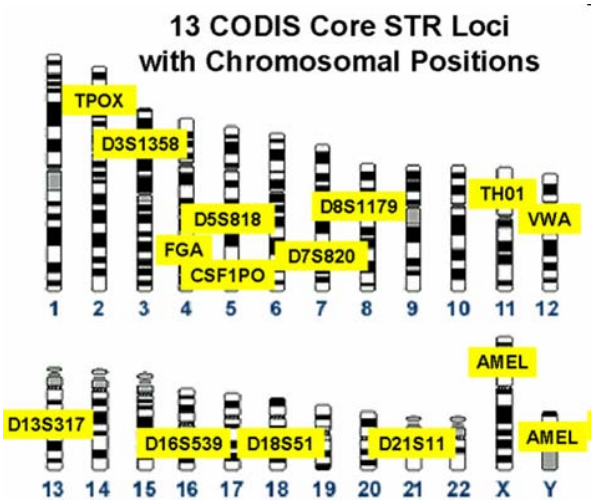

Figure 2. Chromosomal positions of each of the 13 core STR loci determined by CODIS. Adapted from http://nitro.biosci.arizona.edu/courses/EEB208-2008/Lecture08/Lecture08.html.

1.1.1.2 Mitochondrial DNA Analysis

Forensic analysis has not only taken great leaps in nuclear DNA technology but also in mitochondrial DNA (mtDNA). Mitochondrial DNA, which is solely inherited from the mother, is very different than nuclear DNA in that it is circular (Figure 3), ~ 16,569 base pairs (~ 3.2 billion for nuclear DNA), and each cell can contain > 1000 copies (only 2 copies for nuclear DNA). Due to the increased number of copies per cell and circular genome, mtDNA has a longer survival rate than genomic DNA, making it

useful in analysis of degraded or ancient samples that would contain minimal to no nuclear DNA. Comparison and analysis of mtDNA typically involves sequencing of the hypervariable I (HVI) or hypervariable II (HVII) regions within the control region of the mtDNA genome. Although termed the control region (D-loop), it has the greatest variability from person to person as it is a non-coding region, i.e., does not code for gene products, and, therefore, is more susceptible to the high mutation rate in mtDNA (also due to fewer DNA repair mechanisms in mitochondria) creating distinguishable differences in sequence between individuals. Although the information gained from mtDNA, since it is maternally inherited, may be limited in comparison to nuclear it is still used for many purposes including human identification in mass disasters where samples may be highly degraded or in missing persons investigations as it can be compared to maternal reference samples.[1] Additionally, mtDNA has become commonly used for identification of skeletal remains due to the increased survival rate of mtDNA over nuclear.

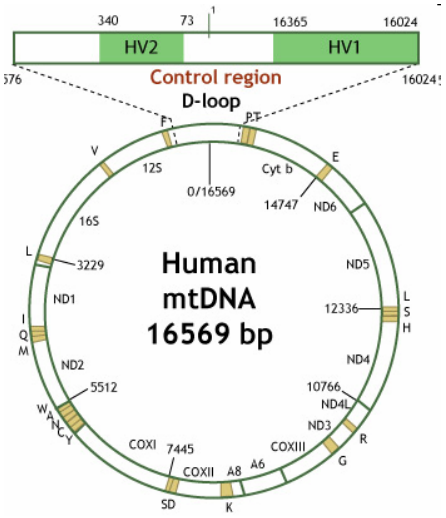

Figure 3. Mitochondrial DNA genome with the control region, (D-loop) that contains the HV1 and HV2 regions, highlighted. Both regions are typically amplified and sequenced for mtDNA analysis. Adapted from http://www.nfstc.org/pdi/Subject09/pdi_s09_m02_01_a.htm.

1.1.2 Analysis of Clinically-relevant Samples

Similar to forensic DNA analysis, clinical DNA analysis focuses on the detection of certain markers within the genome. Clinical analysis is also concerned with the detection of viruses and/or bacteria through analysis of DNA from a patient sample. The majority of conventional clinical analysis involves cell culturing which can require hours to days, a time period that is often not realistic or available for a patient. Therefore, new methods are being heavily researched that can reduce this time but still provide reproducible results and possibly allow for early detection of disease or pathogens. Along the same lines, this has also furthered research in point-of-care methods for physician offices to reduce the turnaround time for patient sample analysis.

1.1.3 DNA Extraction

The first step in genetic analysis of biological samples is DNA extraction. The DNA in the biological sample must be purified from other cellular and extracellular material, e.g. protein, heme, environmental contaminants, that may be present that can inhibit downstream processes, such as PCR.[1] Numerous methods have been developed to accomplish this including organic, chelex, FTA paper, and solid phase extractions, although some have distinct advantages over others (Figure 4).[1] Organic extractions use the addition of SDS (sodium dodecyl sulfate) and proteinase K to perform cell lysis which is then followed by the addition of a phenol/chloroform/isoamyl alcohol mixture resulting in the separation of the protein from DNA which will be in the aqueous phase after centrifugation.[1] Organic extractions have been used for a variety of samples

including bone for the purification of both genomic and mtDNA which is known to be highly susceptible to contamination.[2] Although a widely used method[3, 4], organic extractions require the use of multiple centrifugation steps, use of/exposure to hazardous chemicals, and multiple sample transfer steps that increase the points for entrance of contaminants which can be detrimental, especially when mtDNA is of interest due to its high susceptibility to contamination because of the increased copy number per cell[1].

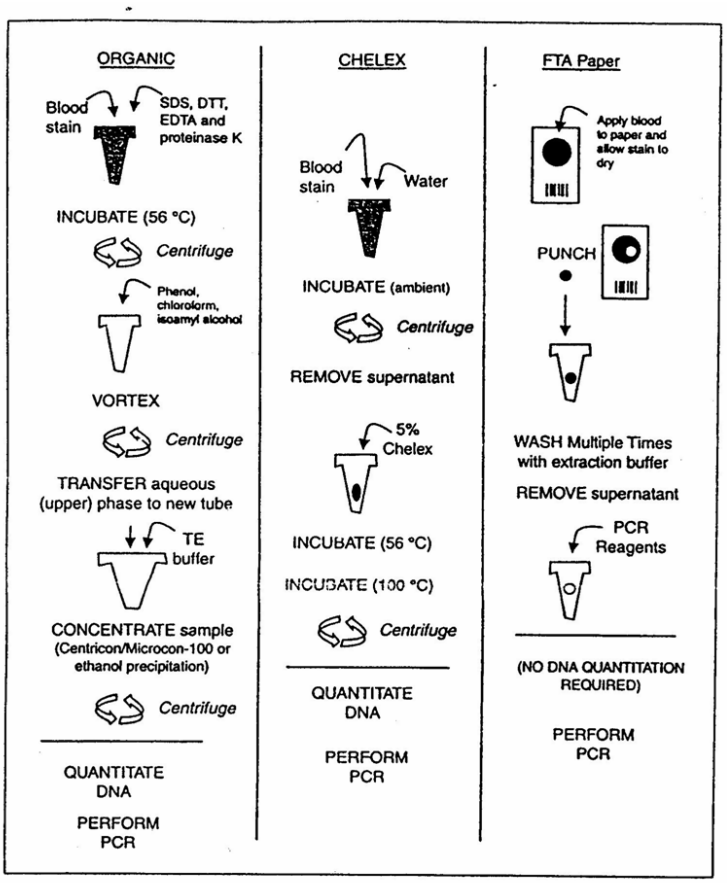

Figure 4. Schematic of the procedure for conventional DNA purification methods. Adapted from Butler, J. M. *Forensic DNA Typing: Biology, Technology, and Genetics of STR Markers*, 2nd ed.; **2005**.

In contrast, chelex extractions employ an ion-exchange resin that binds polyvalent metal ions in the sample. To perform the purification, the resin is added to the sample followed by boiling for cell lysis. The sample is then centrifuged to pull the resin to the

bottom of the tube while the supernatant, containing the purified DNA, is collected and can be added directly to the PCR amplification.[1] While resulting in amplifiable DNA, if too much template (i.e., too much blood) is added to the purification it can result in PCR inhibition.[1]

Similar to the chelex extraction which uses a solid phase, FTA™ paper also uses a solid phase, cellulose-based paper. A drop of blood is added to the paper and upon contact, the cells are lysed and DNA is immobilized. A punch from the paper is then washed to remove heme and subsequently added to a PCR reaction. Although this method has been proven reproducible it has not been used extensively as was predicted due to the static electricity of the dried punches 'jumping' from tubes which can cause cross-contamination of samples.[1]

Solid phase extraction, which is employed in such commercialized kits as QIAamp spin columns, uses a silica-based solid phase[1] that can reversibly bind nucleic acids. DNA binding to the phase was described by Melzak, et al.[5] and occurs in the presence of a chaotrope, such as guanidine, and is driven by three forces: increase in entropy from the release of water, electrostatic interactions, and hydrogen bond formation. After binding, the phase can be washed with an alcohol to remove proteins and unbound material and then the purified DNA eluted in a low ionic strength buffer. Using the same purification concept, other SPE approaches have been developed employing the use of silica-coated magnetic particles (Figure 5).[6, 7] Because of the magnetic nature of the solid phase, it is highly amenable to automation as magnets built into the instrumentation can be used to manipulate the solid phase between purification steps. Automated systems, MagneSil® Genomic Large Volume System[6] and the BioMek

2000 laboratory workstation in combination with the DNA IQ™ system[8] (Figure 6) (both of which use the silica-coated magnetic particles), have been developed but both expose the sample to possible contaminants as the sample reservoir is open between each purification step. Additionally, the MagneSil® Genomic Large Volume automated system has only been shown for large amounts of sample, i.e., 10 mL of blood, which is not available in forensic casework analysis where the sample is present on surfaces and not in a tube.

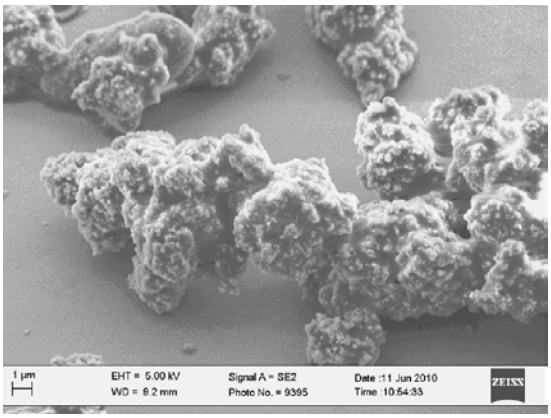

Figure 5. SEM image of 5 µm MagneSil™ particles.

Figure 6. Image of the BioMek 2000 automated laboratory workstation. This system has been shown applied to the DNA IQ™ purification system by Greenspoon et al., *Journal of Forensic Science* **2004**, 49, 29-39. Samples are placed in the plate and moved to specific locations for each portion of the extraction process. Adapted from http://www.esd.ornl.gov/facilities/genomics/equipment.html.

In addition to these silica-based SPE methods, there have also been developments made towards charge-switch SPE, including the commercially available kit developed by Invitrogen™. Other work has also been shown for pH-induced DNA binding with the use of a chitosan-coated silica phase.[9] Both purification methods utilize a simple change in pH to bind and then release DNA free of proteins and other inhibitors. Perhaps the greatest advantage of these methods is the absence of chaotropic salts and organic solvents in the purification process which are known to inhibit downstream PCR.[10, 11]

All of these SPE methods eliminate exposure to hazardous chemicals and allow for faster sample prep. The greatest advantage though, is the more simplistic translation of these SPE methods to microdevices as the solid phases mentioned (silica, silica-coated paramagnetic particles, and charge-switch beads) can be easily packed and immobilized into a microdevice, providing the closed environment that is essential in forensic DNA analysis.

1.1.4 DNA Amplification

DNA amplification through polymerase chain reaction (PCR), an enzymatic reaction, was first described by Mullins, et al.[12] Copies of a DNA template are made by cycling through three steps: denature, anneal, and extension. The first step of the amplification occurs at ~94 °C and denatures the double stranded DNA template in the reaction. This provides a single stranded DNA segment that allows for annealing of primers specific for the region of interest at ~60 °C. Extension of the primers generating new DNA fragments is then completed at ~72 °C. This three-step cycle is then repeated

anywhere from 25-35 times resulting in an exponential increase in the region of interest in the DNA template (Figure 7).[1]

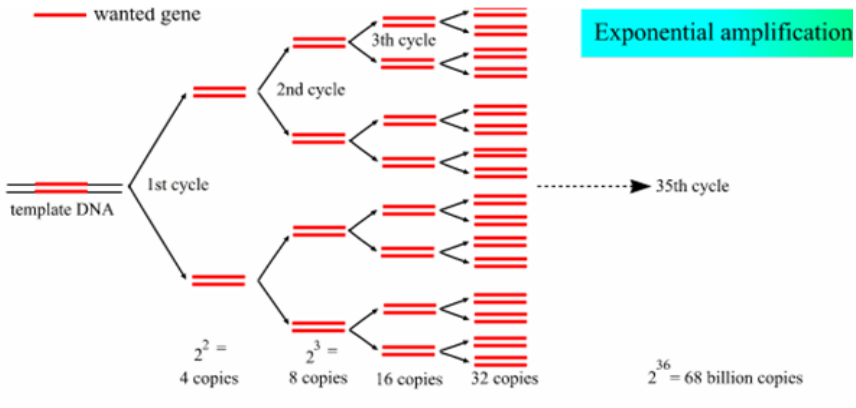

Figure 7. Schematic illustrating the exponential amplification that occurs during polymerase chain reaction. The reaction, containing the starting DNA template, is cycled through 36 cycles of a denature at ~94 °C, anneal at ~60 °C, and extension at ~72 °C resulting in, if 100% efficient, 68 billion copies of the target gene. Adapted from http://users.ugent.be/~avierstr/principles/pcr.html.

The use of PCR analysis has become routine in both clinical and forensic settings whether for disease/pathogen detection or human identification using STRs because of the sensitivity it offers as billions of copies of DNA can be made. This allows analysis of samples that contain very little starting material which is often the case in forensic casework analysis or in disease/pathogen detection. The application of PCR specifically for forensic analysis has focused on multiplex amplifications (amplifying more than one region) of STRs which requires amplification of anywhere up to 16 loci at the same time. Commercially available kits have been developed to do just that and include MiniFiler™ (discussed in Chapters 2 and 4), which differs from the previously developed STR kits [i.e., COfiler®, Identifiler™ (both discussed in Chapter 4)] because it involves amplification of miniSTRs. MiniSTRs are produced using primer sets that are designed to anneal closer to the repeat region, therefore decreasing the size of amplicons produced during PCR. The smaller-sized base pair fragments amplified make the kit applicable to

degraded samples containing damaged or sheared DNA. Although these kits have been developed and perform well, the time required for the amplification is ~ 3.5 hours creating a large bottle-neck in DNA analysis which can further increase the backlog[13] of DNA evidence to be processed. This bottle-neck could be lessened if PCR was performed on a microdevice which has reduced volumes and thermal mass allowing for faster heating and cooling, as discussed in Chapter 4 with both STRs and miniSTRs.

1.1.5 DNA Separation and Detection

Traditionally, the separation of DNA is completed using gel electrophoresis which uses agarose or polyacrylamide gels that separate DNA fragments based upon the size with smaller fragments migrating faster through pores within the gel.[1, 14] Although this has been shown to work well, preparation of the gels is time-consuming and labor-intensive. Additionally, the separation time required is lengthy because of the joule heating that occurs if the voltage is increased (decreases run time). Because of these burdensome characteristics, gel electrophoresis has been replaced with capillary electrophoresis (CE). DNA separations with CE are completed in a glass capillary filled with a sieving polymer with a voltage applied over the capillary (each end of the capillary in buffer). The sieving polymer is crucial to the successful separation of DNA fragments, as DNA has a constant charge-to-mass ratio which does not allow for separation based upon charge. With the sieving polymer in the capillary, the DNA fragments are able to migrate through the pores at various speeds depending upon their length. The DNA fragments can then be detected fluorescently, through tagging or using intercalating dyes, using laser-induced fluorescence.[1] There are numerous advantages to the use of CE for

DNA separations including automated separations and faster separation times as increased voltages can be used due to the dissipation of joule heat because of the increased surface area to volume ratio of the capillary. Although this particular step of DNA analysis will not be discussed any further in this dissertation, its use for separation and detection of PCR products will be shown in later chapters (Chapters 2 – 5).

1.2 Microfluidics

Microfluidic devices, which are fabricated using standard photolithography (Figure 8)[15], offer many advantages for both clinical and forensic analysis including the ability to integrate multiple sample processing steps[10, 16-23] which was first described by Manz as a micro total analysis system (μTAS) in 1992.[24] Numerous processes have been demonstrated on microfluidics including, but not limited, nucleic acid extraction[9, 25-32], PCR[33-36], and separation/detection of PCR and sequencing products[10, 19, 37-42]. The integration of these processes on a single device provides a closed system which eliminates the number of sample transfer steps involved and therefore decreases the points for entrance of contaminants or DNases. Integrated analysis systems have been shown for extraction with PCR[16, 17], PCR with microchip electrophoresis (ME)[19, 22], and finally, a fully integrated system[10]. These integrated microdevices have not only been shown to decrease analysis time[10] but also inherently require smaller volumes of reagents therefore decreasing the overall cost of analysis. Along the same lines, microfluidics is amenable to development into a portable, automated system which requires less time, user intervention, and a smaller laboratory footprint, all of which could greatly impact both forensic and clinical laboratories. This dissertation will focus on the development of

an integrated system for forensic analysis (Chapter 4) in addition to developments for microchip-based SPE (Chapters 2 and 3). To further the goal of developing a μTAS, microfluidics has also begun to focus on developing plastic microdevices because of the lower cost of fabrication. The development of a plastic microchip-based DNA extraction device will also be discussed in this thesis (Chapter 5), providing the first step towards a fully integrated disposable microdevice.

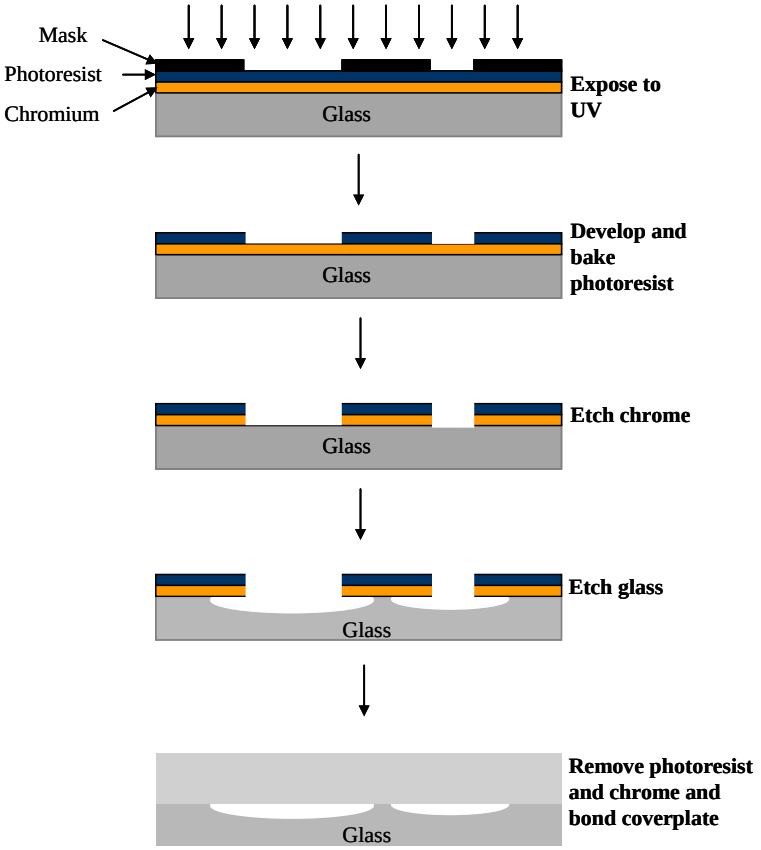

Figure 8. Schematic of standard photolithography used for fabrication of a glass microfluidic device. Briefly, a mask with a pre-determined design is stacked on top of the glass wafer that has a layer of chrome and photoresist. The stack is exposed to UV light, the photoresist developed, and the chrome removed. The exposed portion of the glass is then etched with hydrofluoric acid. All remaining photoresist and chrome are removed and a glass coverplate thermally bonded to the etched glass.

1.2.1 Microfluidic DNA Extraction

Microfluidic DNA extraction has primarily focused on the use of silica, whether through beads or sol-gel[10, 17, 26, 27, 43], as the binding phase. The very first demonstration of microchip-based SPE was shown by Christel et al.[44] using pillar silica microstructures fabricated in the device as the solid phase. Pillar microstructures, however, can be costly and difficult to fabricate. This facilitated development of other silica phases such as sol-gel, silica beads, and silica bead/sol-gel mixtures (Figure 9)[26, 27, 43]. The use of sol-gel has been shown successful for the purification of DNA from semen[26], whole blood, and cell cultures for human identification and biowarfare agent detection[27]. Although shown successful, preparation of the phase is time-consuming making it difficult for the method to become a rapid SPE technique. Because of this, work began to focus on the use of silica beads for the purification of DNA (which will discussed more in depth in Chapter 4). The successful application of ~ 30 μm silica beads for microchip DNA purification from blood for infectious agent detection[10] and semen and buccal swabs for human identification has also been shown[16, 17]. SPE with silica beads has developed into a highly reproducible method that is relevant to numerous biological samples and applications.

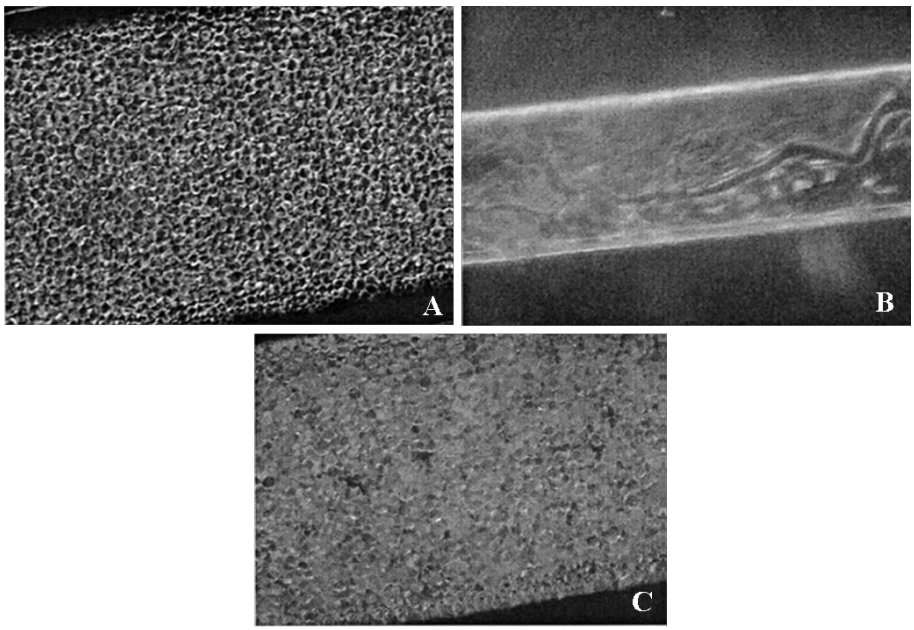

Figure 9. Images of 30 μm silica beads (A), sol-gel (B), and a silica bead/sol-gel mixture (C) within a microchannel. Adapted from Wolfe, et al., *Electrophoresis* **2002**, *23*, 727-733.

In addition to the developments made using silica beads, other silica phases have also been investigated for DNA purification. Specifically, the use of silica-coated paramagnetic particles has begun to gain popularity. This phase consists of 5 μm particles with an amorphous surface and a large surface area for increased DNA binding capacity. The particles have not only been used for robotic platforms[8] but also in microchip format where the magnetic properties of the particles are exploited to move the particles within the microchannel for DNA purification[45]. In addition, the silica-coated paramagnetic particles can also be packed in a microdevice, similar to silica beads, which will be discussed further in Chapters 2 and 3 with the application to large volume biological samples that are often encountered in forensic analysis.

In contrast to the work that has been completed with silica-based microfluidic DNA purification, chargeswitch® technology has also been developed. Chargeswitch®, developed and commercialized by Invitrogen, employs reversible binding of DNA based

upon electrostatic interaction. Using the same concept, microchip-based, pH-induced reversible binding of DNA using chitosan-coated silica has been described by Cao et al.[9] Reversible binding of the DNA is completed by a simple change in the pH from acidic (DNA binding) to basic (DNA is released). Perhaps the greatest advantage of this method is that the extraction is completely aqueous which eliminates reagents that can inhibit downstream PCR, such as those used in silica-based extractions (i.e., guanidine and isopropyl alcohol). Additionally, because of the orthogonal binding mechanism of this phase opposed to silica, contaminants that may be present and not removed by silica-based extractions could be removed using this purification method. The integration of this purification method with a volume reduction solid phase extraction method for inhibitor/contaminant removal will be discussed further in Chapter 3.

1.2.2 Microfluidic DNA Amplification

Conventional PCR is a lengthy process requiring upwards of ~ 3.5 hours which creates a bottleneck for both forensic and clinical analysis. It is typically completed in a polypropylene tube placed in a metal heating block where the entire block must be heated and cooled to do the same for the sample, creating the lengthy amplification time previously mentioned. Therefore, applying the use of microfluidics to PCR is logical because of the decreased analysis time inherent to microfluidics due to decreased volumes.

Microfluidic PCR (μPCR) has been demonstrated using various heating elements, including heating in a commercial thermocycler[16, 17, 46] and resistant heaters[20], and microchip designs using either static (one chamber is cycled through various

temperatures)[35] or flow-through (solution is flowed through regions of the device held at specific temperatures)[47]. Non-contact, where the heat source is not in direct contact with the microdevice, methods have also been developed which allow for much simpler and more inexpensive fabrication of microfluidic devices as the heating element is not incorporated into the device. A non-contact PCR method using infrared (IR)-mediated heating (IR-PCR) and forced-air through a fan for cooling was developed in our lab and allows for much sharper temperature transitions due to reduced thermal mass of the microdevice (Figure 10). Due to this increase in heating and cooling rates, faster amplifications can be accomplished using this system.[33, 35] Use of this method for both clinical and forensic applications including detection of whopping cough and B. *anthracis* has been shown.[10] To date though, IR-PCR has not been employed for forensic STR amplifications which, as previously described, are multiplexed reactions requiring amplification of anywhere from 14 – 32 segments that must exhibit inter- and intra-peak balance and complete adenylation. Application of this IR-PCR method for forensic STR analysis will be demonstrated later in Chapter 4 for 9 and 16-plex amplifications.

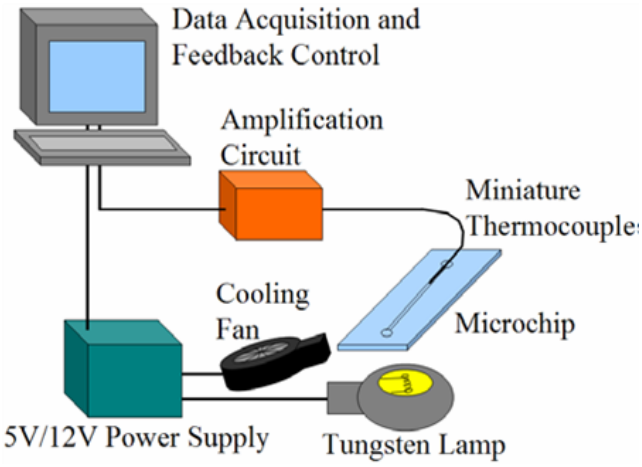

Figure 10. Schematic of the IR-PCR system developed in the Landers lab which uses an infrared source for heating and a fan for cooling the solution within the microdevice. Adapated from Easley, C., Disseration, University of Virginia, **2006**.

1.3 Microfluidic Device Substrates

Glass is the most commonly used substrate for microdevices, but more recently, focus has shifted to other substrates including poly (methylmethacrylate) (PMMA)[48], polycarbonate (PC)[49-51], and polyolefin[25, 30, 48]. Plastic microdevices, in contrast to glass devices, are more inexpensive and easier to fabricate and therefore suitable for mass production. This allows for them to be a one-time use device which not only eliminates possible carry-over between samples but also paves the way for an on-site or point-of-care disposable device for the forensic and clinical communities. Utilizing plastic microdevices allows for fabrication of high resolution features, 100-500 nm opposed to 10 μm in glass devices. In addition, the inherent advantages seen with microfluidics also apply including a closed environment to reduce points for entrance of contaminates and the ability to integrated multiple sample processing steps on one device. This would provide the quintessential fully-integrated, disposable microdevice for on-site analysis that could potentially revolutionize the forensic and clinical fields.

Various sample processing steps have been demonstrated on plastic devices including SPE[25, 30, 48], PCR[19], and microchip electrophoresis (ME)[19, 52-54]. Plastic microdevices for solid phase extraction have previously focused most on the use of a silica solid phase. For example, Bhattacharyya, et al.[25] demonstrated the use of a sol-gel containing silica beads for the purification of lambda-phage DNA. Although high extraction efficiencies were achieved, the method was only demonstrated for the purification of pre-purified DNA and no application was shown to a complex biological sample. Although silica is widely use for DNA purification, as previously discussed,

reagents that are used during purification (i.e., guanidine and isopropyl alcohol) can inhibit PCR[10] making the integration of silica-based SPE with PCR more difficult. Additionally, because of the goal of developing a single-use device, it would be beneficial for the solid phase to already be in place, requiring minimal training and user intervention. This can be achieved by fabrication of the solid phase into the device design as posts or microstructures due to the high resolution features that can be generated using fabrication techniques such as X-ray lithography. Plastic microdevices can be fabricated using various methods such as X-ray lithography, injection molding, or laser ablation.[55] Although all have been shown successful, X-ray lithography has a distinct advantage over the other techniques as high aspect ratio structures can be created which allows for incorporation of structures within the device that are not possible with the other techniques (Figure 11).[56]

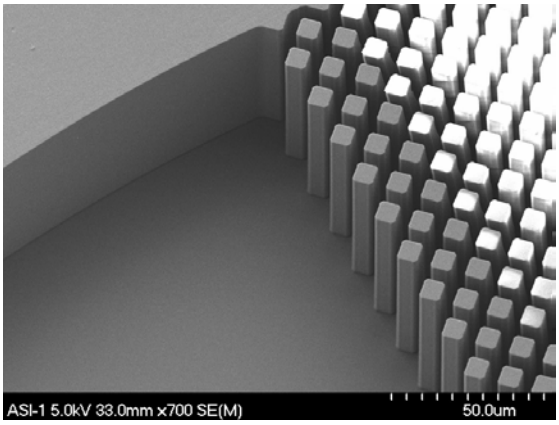

Figure 11. SEM image of PMMA microstructures fabricated with X-ray lithography in a PMMA microdevice.

This dissertation will focus on the development of a solid phase extraction technique in a PMMA microdevice fabricated using X-ray lithography (Chapter 5). The solid phase is generated within the microchannel providing a highly reproducible method for purification. Furthermore, a completely aqueous extraction method, previously

developed by Cao, et al.,[9] is used by coating the PMMA solid phase with chitosan. This eliminates PCR inhibitory reagents that are specific to silica-based purifications.

1.4 Conclusions

The fields of clinical and forensic DNA analysis continually seek techniques that can improve methods of analysis for human identification or early disease detection. The method that has become widely researched is microfluidics due to the advantages it offers for the fields of forensic or clinical sample analysis. Conventional analysis requires time consuming sample processing steps, multiple sample transfer steps exposing the sample to the environment causing possible loss or contamination of the sample, and large laboratory footprints (requiring multiple pieces of instrumentation). Microfluidics and the opportunity for integration of multiple sample processing steps on a single microfluidic device[10] address the mentioned disadvantages of conventional methodologies. This dissertation will focus on developing microfluidic methods for DNA analysis from biological samples. Chapters 2 and 3 describe the development of a volume reduction solid phase extraction method for the purification of DNA from large volume biological samples. Application of the method to mitochondrial DNA and compromised/degraded samples including bone is also emphasized. Chapter 4 focuses on the development of an integrated device for DNA purification and PCR and a modular system incorporating SPE, PCR, and ME for forensic STR analysis. Both methods provide the next step towards a portable, integrated system for forensic analysis. Finally, Chapter 5 describes the development of a plastic microdevice for DNA purification. Device development including bonding and functionalization of the microstructures in

the device are highlighted. This provides the first step towards a fully integrated, disposable genetic analysis system for both the clinical and forensic communities.

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2. Volume Reduction Solid Phase Extraction of DNA from Dilute, Degraded, and Compromised Large-Volume Biological Samples

Microdevices are often designed to process sample volumes on the order of tens of microliters and cannot typically accommodate larger volume samples without adversely affecting efficiency and greatly increasing analysis time. However, dilute, large volume biological samples are frequently encountered, especially in forensic laboratories due to dilution, solubilization from surfaces or demineralization (i.e. bone). Chapter 2 details the development of a SPE microdevice, capable of efficiently processing 0.5 – 1.0 mL samples that are commonly seen in forensic laboratories. The method utilizes silica particles and an optimized volumetric flow rate and elution buffer, resulting in a 50-fold sample volume reduction and 15-fold sample concentration enhancement. Extraction efficiencies comparable with previously reported silica-based purification methods were seen, and resulted in PCR-amplifiable DNA extracted from dilute whole blood (indicated by a proof-of-principle study of a single-plex amplification of a fragment from the gelsolin gene). Application to large volume samples that are commonly encountered including low DNA template and environmentally-degraded samples, which sometimes require mitochondrial DNA (mtDNA) analysis, is also described in this chapter. In addition, the first successful on-chip purification of mitochondrial DNA (mtDNA) from both dilute whole blood and a degraded blood stain and the first on-chip purification of DNA from bone are presented. Some of the results in this chapter are also described in Reedy et al.[1] and Reedy et al.[2]

2.1 Introduction

For the DNA analysis of clinical and forensic samples, it is necessary to isolate DNA from the cellular or extracellular components that can inhibit downstream processes, such as polymerase chain reaction (PCR)[3]. Various extraction methods can be utilized for this purpose as described in Chapter 1, including organic extractions (e.g., phenol/chloroform), which have served as the gold standard for DNA purification for forensic applications[4]. Solid phase extraction (SPE) methods provide an alternative to organic extractions, allowing for more rapid sample preparation and have been widely accepted in the clinical and forensic communities. Most SPE methods use silica as the solid phase, including kits which have been successfully commercialized with widespread distribution and use in forensic casework.[5]

One inherent disadvantage of conventional extraction methods is the sample handling required throughout the extraction, exposing the sample repeatedly to the surrounding environment and, consequently, the opportunity for contamination. Microminiaturization of the SPE method for nucleic acids [micro-SPE (μSPE)] has been offered as an alternative to macroscale methods that reduces the need for sample handling (and entry points for potential contamination).[6-18] Micro-SPE also provides the closed environment needed for working with biological samples that are especially susceptible to contamination, such as mitochondrial DNA (mtDNA), although little to no work has been shown for this sample type and other comprised or degraded samples.[4]

Although it has been shown that silica phases utilized in the microscale format can be used to extract DNA from complex biological samples, most of the sample volumes used in these purifications were on the order of tens of microliters. This poses a

fluidic limitation, especially when forensic analysis is considered, as many forensic samples are of substantially larger volumes. These often results from the volume required for the removal or solubilization of a sample from a surface (necessary for many sample types including compromised biological stains), or for adequate demineralization of bone or teeth, which are common sample types encountered in mass disasters or for determining ancestry, as they are often the only sample type available.[5, 19] In addition, these larger volumes, as large as 0.5 – 1.0 mL, are sometimes necessary for certain forensic samples where dilution of a sample minimizes the impact of contaminants that inhibit PCR which also inherently dilutes the DNA template in the sample resulting in low DNA template concentration.[4, 20] In order to effectively utilize microfluidic SPE technology for larger volume samples a *volume reduction* step, is needed to successfully interface larger volume samples with previously developed microscale technology.

The work presented in this chapter describes development of a microdevice volume reduction solid phase extraction (vrSPE) method using silica-coated-paramagnetic particles to remove impurities and concentrate biological samples to volumes more compatible for subsequent DNA processing and analysis. This research shows the development and optimization of the vrSPE method, including its capacity for various sample types. Further, successful application of the method for low DNA template, time-, heat-, and UV-degraded samples is also demonstrated. Finally, results from the use of the vrSPE device for the first on-chip mtDNA extraction and human genomic DNA (hgDNA) extraction from bone are also presented, to show potential of the system for use in forensic applications. The results presented in this chapter are also described in Reedy et al.[1] and Reedy et al.[2]

2.2 Materials and Methods

2.2.1 Reagents

Guanidine hydrochloride (GuHCl), 2-(4-morpholino)-ethane sulfonic acid (MES, enzyme grade), 2-propanol (IPA), hydrochloric acid, sodium hydroxide, acetone, dithiothreitol (DTT), 0.5 M ethylenediaminetetraacetate acid, *Taq* DNA polymerase, 10X PCR buffer, dNTPs, and $MgCl_2$ were purchased from Fisher (Fair Lawn, NJ). 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Trizma Base, 99.9%), lauryl sarcosine, and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Triton X-100 for molecular biology was purchased from Fluka (St. Lous, MO). λ–phage DNA was purchased from USB (Cleveland, OH). MagneSil™ paramagnetic particles were purchased from Promega (Madison, WI). Hyperprep silica beads (15-30 µm) were purchased from Supelco (Bellefonte, PA). Ethylenediaminetetraacetic acid (EDTA) disodium salt (reagent grade) was purchased from American Research Products (Solon, OH). Quant-iT™ PicoGreen® dsDNA reagent, an intercalating fluorescent dye, was purchased from Invitrogen™ (Carlsbad, CA). AmpF*l*STR® COfiler®, MiniFiler™, and Identifiler® PCR amplification kits were purchase from Applied Biosystems Inc. (Carlsbad, CA). Purified human genomic DNA was obtained through in-house purification from whole blood. All solutions were prepared in Nanopure water (Barnstead/Thermolyne, Dubuque, IA).

2.2.2 Microdevice Fabrication

Microdevices were prepared using borofloat glass (Telic Company, Valencia, CA) and standard photolithographic techniques[21] resulting in channel dimensions of 1 cm

to the weir, 200 µm deep, and 1 mm line width; with a weir depth of 20 µm. Reservoir holes were drilled using a 1.1 mm diameter diamond-tip drill bit (Crystalline Corp., Lewis Center, OH). A borofloat glass cover plate was thermally bonded to each device.

2.2.3 Preparation of vrSPE Apparatus

A 1 mL SGE gas-tight syringe (SGE, Austin, TX) and a 250 µL Hamilton gas-tight syringe (Hamilton, Reno, NV) were attached to the microdevice through the use of 0.75 mm and 0.25 mm i.d. PEEK™ tubing, mini-tight fittings, and nanoports (Upchurch, Oak Harbor, WA). A SP100i syringe pump (WPI, Sarasota, FL) was used to flow solutions into the device. The vrSPE channel was packed with a small frit of 30 µm silica beads by vacuum to hold in place subsequently packed 5-8 µm MagneSil™ particles (Figure 1). The channel in the device was unpacked and rinsed with 2 M HCl after each extraction to prevent any carry-over or contamination.

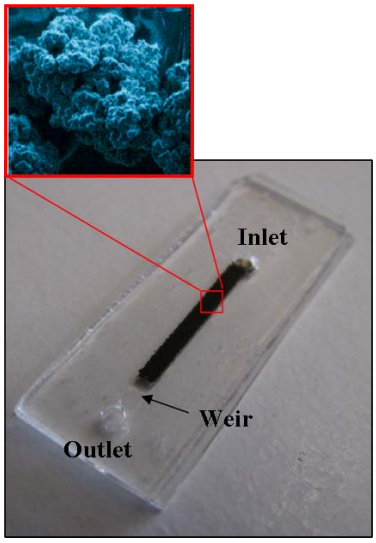


Figure 1. vrSPE device (1 cm to weir, 1 mm line width, 200 µm deep, 20 µm weir depth) packed with MagneSil™ solid phase. SEM image of a MagneSil™ particle is shown enlarged[22].

2.2.4 Sample Preparation

Solid phase, volumetric flow rate, elution buffer, and condition optimization study samples consisted of preparing and loading 500 μL of a 0.1 ng/μL solution of λ-phage or prepurified hgDNA in 6 M GuHCl, pH 6.1. To prepare dilute whole blood (for IPA wash optimization and mtDNA extraction experiments), 1.4 μL of whole blood (obtained from University of Virginia School of Medicine from fully deidentified residual clinical specimens) was lysed in 480 μL 6 M GuHCl, pH 6.1 and 20 μL proteinase K (20 mg/mL, Qiagen, Valencia, CA) by mixing prior to incubation in a 56 °C water bath for 10 min. Approximately 360 μL of this solution was then loaded onto the device. To prepare low DNA template samples 140 nL of whole blood was lysed in ~487 μL 6 M GuHCl, pH 6.1 and 12.5 μL proteinase K (20 mg/mL) and incubated in a 56 °C water bath for 10 min. All blood stains on Whatman paper were prepared at the Virginia Department of Forensic Science and then either left at room temperature for 3 years or incubated at 56 or 80 °C for 1 or 3 months. Each of the blood stains on Whatman paper were then eluted and the sample lysed in 190 μL 6 M GuHCl, pH 6.1/1% Triton X-100 and 10 μL proteinase K (20 mg/mL) and incubated in a 56 °C water bath for 30 min. Heat-degraded blood stains on cotton were prepared by pipetting 10 μL whole blood onto cotton and was allowed for dry for ~1 hr. The stain was then incubated in an oven at 56 °C for 1 month. Then ½ of the stain was cut out and immersed in 730 μL 6 M GuHCl, pH 6.1/1% Triton X-100 and 20 μL proteinase K (20 mg/mL) and incubated in a 56 ° water bath for 10 min and 500 μL of the solution loaded onto the vrSPE device. The UV exposed blood and semen stains were prepared by pipetting 10 μL of the sample onto cotton and was then allowed to dry for ~1 hr. The semen samples were obtained through a Institutional Review Board

(IRB) approved collection method. Each stain was then exposed for 56 min or 1 hr 52 min to short wave (254 nm) UV light ~1 cm away from the source (720 μW/cm^2). The blood stain exposed to 56 min of UV light was processed using the same procedure as the 56 °C 1 month stain on cotton with the exception that a 6.25 mm^2 portion of the stain was cut out for each extraction. The blood stain exposed to 1 hr 52 min of UV light was also processed the same as the 56 °C 1 month blood stain on cotton with the whole stain immersed in the lysis solution. The semen stain exposed to 56 min UV light was processed by cutting out a 6.25 mm^2 portion of the stain. This portion was immersed in 750 μL 6 M GuHCl, pH 6.1/40 mM DTT and incubated at room temperature for 15 min and 500 μL of the solution loaded onto the device. The 1 hr 52 min UV exposed semen stain was processed the same as the 56 min exposed sample with the exception that half of the stain was cut out and used per extraction. Bone samples (obtained from the Armed Forces DNA Identification Laboratory) were prepared by incubating 0.1 – 0.2 mg of ground bone in 3 mL demineralization buffer (0.5 M EDTA/0.5% w/v lauryl sarcosine, pH 8) overnight at 56 °C. The sample was then concentrated down to ~100 μL with a Centricon-30 concentrator (Amicon, Beverly, MA). The sample was then diluted to ~550 μL with 8 M GuHCl, pH 6.1 and the entire sample loaded onto the device.

2.2.5 vrSPE Procedure

A MagneSil™-packed vrSPE microdevice was conditioned with 6 M GuHCl, pH 6.1 for 10 min at a flow rate of 15 μL/min. The extraction protocol for the optimization studies consisted of two steps using pressure driven flow at 15 μL/min (or 20 μL/min for flow rate comparison study). First, the sample was loaded in 6 M GuHCl, pH 6.1 and

then eluted with 1X TE (10 mM Tris, 1 mM EDTA, pH 8) (or 10 mM Tris for elution buffer optimization studies) while 5 µL fractions (10 µL fractions for low template, 3 year old blood stain, and bone samples) were collected for subsequent PCR or fluorescence analysis. A third step, consisting of a 75 µL wash with 80% v/v (IPA/H_2O) at 15 µL/min (corresponding to 5 min), was added for the extraction of mtDNA, and phase selection optimization study, low DNA template sample, 3 year old blood stain, and all heat- and UV-degraded samples following the load of the DNA sample to ensure efficient removal of all protein and cellular debris.

2.2.6 Fluorescence Detection

Once elution fractions had been collected, a commercially developed PicoGreen® fluorescence assay[22] was utilized for analysis according to manufacturer's protocols. A standard curve was prepared with either prepurified hgDNA or λ-phage DNA for comparison and a NanoDrop 3300 Fluorospectrometer (NanoDrop, Wilmington, DE) or Fluorometer (Perkin Elmer) were used for fluorescence detection.

2.2.7 Binding Capacity Studies

Capacity studies were conducted by first conditioning the solid phase with 6 M GuHCl, pH 6.1 for 10 minutes. To determine the capacity of MagneSil™ for hgDNA, a 0.11 µg/µL prepurified hgDNA sample in 6 M GuHCl, pH 6.1 was loaded continuously while 20 µL fractions were collected from the outlet reservoir. Aliquots (1 µL) of each fraction were diluted to 100 µL with 1X TE. The samples were analyzed with a fluorescence assay as previously described. Additional capacity studies for λ-phage

DNA and whole blood involved conditioning the phase with 6 M GuHCl, pH 6.1 for 10 minutes. Increasing quantities (25-400 ng) of DNA prepared in 500 µL 6 M GuHCl, pH 6.1 (20 µL 20 mg/mL proteinase K and 10 min incubation in a 56 °C water bath was performed for whole blood lysis) were loaded. The DNA was then eluted using 10 mM Tris, pH 8 while fifteen 5 µL fractions were collected and analyzed using the previously described fluorescence assay.

2.2.8 mtDNA Sample and Reagent Preparation

All buffers prepared for mtDNA extractions were prepared with Nanopure water and filtered with 0.22 µm filters (Fisher, Fair Lawn, NJ). All solutions and PCR tubes were autoclaved. Extractions were performed in an Envirco hood (Envirco, Albuquerque, NM). Accessible areas and equipment were cleaned with 10% bleach (prepared fresh daily) and all solutions and equipment were UV irradiated for ~1 hr prior to use. PEEK™ tubing and the microfluidic device were rinsed with 2 M NaOH, 2 M HCl, 10% bleach, and filtered-nanopure-autoclaved water prior to each use. Whole blood extractions were carried out as specified in S*ample Preparation* and *vrSPE Procedure* with the exception that disposable, 1 mL plastic syringes were utilized instead of glass syringes to reduce potential contamination. To perform the degraded blood stain extraction, the sample (human blood stain that was obtained from Virginia Department of Forensic Science, Richmond, VA), which had been incubated at 56-80 °C for 1-3 months and then at room temperature for 3 years, was placed in a solution containing 190 µL 6 M GuHCl, pH 6.1, 1% Triton X-100 with 10 µL 20 mg/mL proteinase K and incubated in a 56 °C water bath for 30 min. The sample was loaded using a 250 µL Hamilton syringe

(rinsed with 2 M NaOH, 2 M HCl, 10% bleach, and filtered-nanopure-autoclaved water), washed with 75 µL 80% IPA, and DNA eluted with 1X TE while fifteen 5 µL fractions were collected in autoclaved PCR tubes. All flow rates were held constant at 15 µL/min.

2.2.9 Amplification of mtDNA

Extracted samples were sent to the Virginia Department of Forensic Science (VDFS) (forward primer: 5'-CCCCATGCTTACAAGCAAGT-3', reverse primer: 5'-GAGGATGGTGGTCAAGGGA-3') and Armed Forces DNA Identification Laboratory (AFDIL) (primer regions: MPS 2B F15971 R16410 and PSIII F15 R285) for amplification of portions of the hypervariable one (HVI) region in the mtDNA genome. Following amplification, samples were separated and detected using a DNA 1000 Series II kit and a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA).

2.2.10 Wash Optimization Studies

For wash optimization fluorescence analysis studies, each dilute whole blood sample was prepared as detailed above in *Sample Preparation*. Samples for wash optimization with respect to gelsolin gene amplification were prepared with 2.3 µL whole blood lysed in 777.7 µL 6 M GuHCl, pH 6.1 and 20 µL 20 mg/mL proteinase K, mixed well and incubated in a 56 °C water bath for 10 min. The samples, for both fluorescence and amplification studies, were loaded (500 µL) onto the device, followed by either a 0, 5 (for amplification studies), or 25 min (all for fluorescence studies) 80% IPA (v/v IPA/H_2O) wash step. DNA was eluted with 10 mM Tris, pH 8 while fifteen 5 µL

fractions were collected for fluorescence or PCR analysis. All flow rates were held at 15 µL/min.

2.2.11 Amplification of DNA Purified from Dilute Whole Blood

Elution fractions (5 µL) collected as described above in *Wash Optimization Studies* were amplified using primers for the gelsolin gene by mixing each with 1X PCR buffer, 25 mM $MgCl_2$, 0.2 mM dNTPs, 0.8 µM forward (5'-AGTTCCTCAAGGCAGGGAAG-3') and reverse (5'-CTCAGCTGCACTGTCTTCAG-3') primers (MWG BioTech, High Point, NC), and 0.5 units/µL *Taq* DNA polymerase up to 25 µL. The thermocycling protocol involved an initial denaturation step of 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s/64 °C for 30 s/72 °C for 30 s, and a final extension at 72 °C for 2 min. The samples were then separated and analyzed using a DNA 1000 Series II kit on a Bioanalyzer 2100.

2.2.12 Amplification of DNA from Low Template, Degraded, and Bone Samples

Elution fractions (5 or 10 µL) were amplified with either the AmpFℓSTR® COfiler®, MiniFiler™, or Identifiler® PCR amplification kits according to the manufacturer instructions. The elution fractions after the DNA extraction from bone were amplified using the MiniFiler™ kit with 0.5 µL extra Taq/fraction and 6 extra cycles.[19] The samples were then separated and analyzed using an ABI 310 Genetic Analyzer.

2.3 Microchip-based Volume Reduction Solid Phase Extraction

2.3.1 Silica-based DNA Binding Chemistry

The majority of commercially-available purification kits use silica-based binding chemistry for the extraction of nucleic acids. The mechanism for DNA binding to silica is an area that has not seen in-depth exploration, with the work of Melzak, et al.[23], representing the seminal work defining the physical interaction of DNA with silica. In that work, they purport that a combination of several factors are involved. DNA is predominantly anionic at most pH values, while silica surfaces are also negatively charged with a pKa of 5 – 7 creating a repulsion between the two at pH values greater than 7 – 8.[23] Although, in the presence of high ionic strength solutions the interaction is very different. The dissolution of a chaotrope such as guanidine, is an endothermic process with a positive entropy change.[23] Guanidine has been reported to bind, on average, 4.5 water molecules but also has the potential to bind upwards of 10 water molecules through additional weak interactions. This enables dehydration of the DNA and silica surface in the presence of high concentrations of guanidine.[24] With the addition of the chaotropic salt, a conformational change in the DNA from B-DNA, which binds ~20 molecules of water per nucleotide, to A- (which binds 10.5 water molecules) or C-form occurs and reduces the surface area of the DNA thereby releasing bound water molecules which add to those released by the silica surface in the presence of guanidine.[23] This release of water molecules from the DNA when transitioning from B- to A-form DNA, 20 to 10.5 water molecules bound per nucleotide, causes an increase in entropy of the solution. The high salt concentration also facilitates binding by shielding the electrostatic repulsion of the DNA and silica surface. Additionally, the pH at which

DNA-silica binding has been demonstrated in microfluidic devices[7], pH 6.1, provides increased protonation of the silanol groups on the silica surface, further reducing the electrostatic repulsion between the DNA and silica. The binding of DNA to silica can be described by the following equation[23]:

$$DNA_{hydrated} + silica_{hydrated} + counterions \leftrightarrow neutral\ DNA\text{-}silica\ complex + water$$

In addition to the increase in entropy and reduction in electrostatic repulsion driving binding, hydrogen bonding between the DNA and protonated silica surface also plays a role.[23]

2.3.2 Optimization of vrSPE Phase and Extraction Conditions

2.3.2.1 Device Design

Typical microfluidic SPE devices that use volumetric flow rates of ~4 μL/min contain channels with dimensions of ~200 μm deep and ~500 μm wide.[25] As previously shown an optimal linear flow exists for the purification of DNA in a packed microdevice[7], which can be related to the volumetric flow rate through the cross-sectional area (discussed below). Therefore, in order for large volume samples to be processed on a microdevice in a timely-manner (~30 mins), it was necessary to increase the cross-sectional area by 2.6-fold (in comparison to typical μSPE devices) resulting in channel dimensions of 200 μm deep and 1.4 mm wide.

2.3.2.2 Phase Selection

The first step in the development of this method for interfacing large sample volumes with the microfluidic environment of chips was to identify an appropriate solid phase with the requisite binding capacity for DNA[26] for the high efficiency purification

of nucleic acids from dilute samples containing interfering species. For these experiments, the efficiency of 30 μm silica and 5-8 μm silica-coated-paramagnetic particles to reversibly bind prepurified λ-phage DNA was explored to identify which phase would show optimal capacity for DNA binding. To assess the efficiency and capacity of each phase, DNA was extracted and analyzed using a commercially-available fluorescence assay. While extraction efficiencies (EE) were comparable for DNA extraction on both silica [28.8 (± 2.4)% EE (n = 3)] and silica-coated-paramagnetic particles [24.7 (± 4.4)% EE (n = 3)] (Figure 2A), extraction on the latter resulted in a sharper elution peak, and thus, a more concentrated, smaller volume eluate. Additionally, these silica-coated-paramagnetic particles have already been successfully employed for DNA extraction and concentration by Greenspoon et al. in an automated robotic platform.[27] For these reasons, silica-coated-paramagnetic particles were, therefore, chosen as the more suitable phase for applications involving large volume, dilute samples both due to this increased concentration enhancement and prior demonstrated success for use in automated platforms.

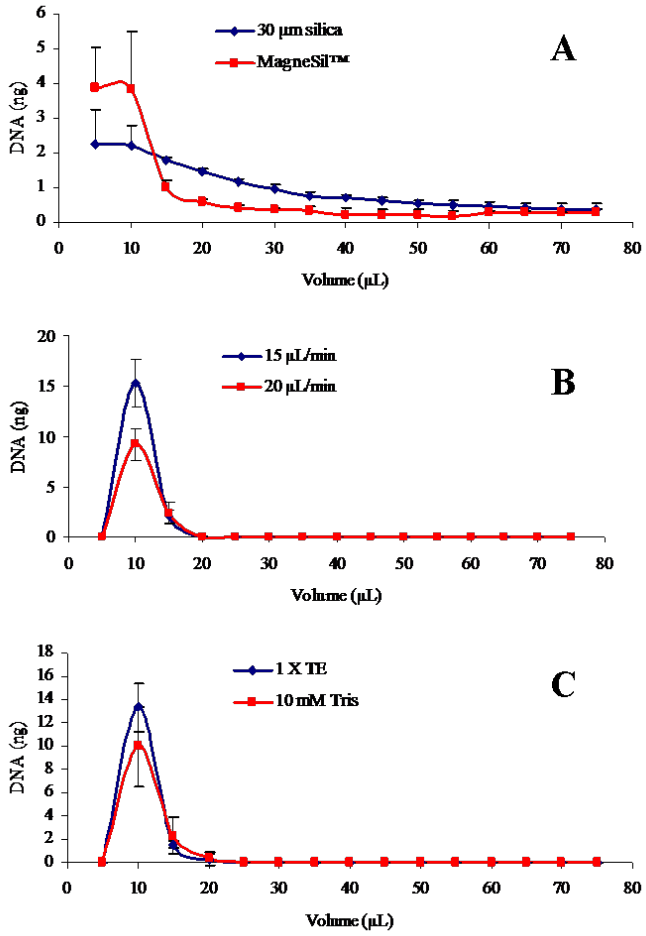


Figure 2. Elution profiles (n = 3 for each elution profile in each panel) detailing optimization of the vrSPE extraction method. (A) Extractions were performed using either 30 µm silica or MagneSil™ as the solid phase and MagneSil™ provided a more concentrated eluent. (B) Volumetric flow rates, 15 and 20 µL/min, were tested and 15 µL/min resulted in the highest extraction efficiency. (C) Two elution buffers, 1X TE or 10 mM Tris, were tested and both resulted in statistically-similar (determined by t-test with confidence level of 95%) extraction efficiencies. t_{calc} (t_{table} = 2.776) was determined to be 1.16, demonstrating that the average EE values are not significantly different.

2.3.2.3 Volumetric Flow Rate

After the selection of silica-coated-paramagnetic particles as a solid phase, the volumetric flow rate during the extraction was optimized both for efficiency and reduced purification time. It was critical that the speed of purification did not negatively impact recovery, so that the downstream analysis of DNA from forensically-relevant samples

would not be compromised. An optimal volumetric flow rate of 15 µL/min was first calculated using the relationship

$$\frac{Q}{A} = \vec{L}$$

where Q is the volumetric flow rate, A the cross-sectional area of the microchannel, and L the optimal linear velocity for silica-based microchip extractions determined by Breadmore et al.[7] A flow rate of (20 µL/min) was also tested to determine whether the increased flow rate, which would further reduce analysis time, would be detrimental to the extraction efficiency. Extraction studies were completed at both flow rates (15 and 20 µL/min), with both of the yielded elution profiles having a similar shape (Figure 2B) and the largest quantity of DNA eluting in the second fraction (10 µL), providing a 50-fold volume reduction. This degree of volume reduction is significant, especially when compared to the lower volume reduction seen when DNA is purified using conventional methods (~3-fold). Perhaps not surprising, the effect of varied flow rate was seen in the extraction efficiency (EE), with a flow rate of 15 µL/min [34.7 (± 4.0)% EE (n = 3)] providing a 15-fold concentration enhancement, in comparison to a 9-fold concentration enhancement at 20 µL/min [23.1 (± 2.2)% EE (n = 3)]. Overall, with the flow rates tested, 15 µL/min provided the greatest EE, which was comparable to traditional and µSPE silica-based purification methods.

2.3.2.4 Optimal Elution Buffer

The optimal elution buffer for the system was next determined so that the largest quantity of DNA was eluted in the smallest volume possible, allowing for a highly concentrated eluate for multiple subsequent analyses. Elution buffers 1X TE [30.3 (±

5.4)% EE (n = 3)] and 10 mM Tris, pH 8 [25.1 (± 5.6)% EE (n = 3)] (Figure 2C) were compared. Elution with both buffers resulted in similar elution profiles with statistically-equivalent (determined by t-test) EE values (Figure 2C). Based upon these results, it was determined that either elution buffer could be used for the elution of DNA from the solid phase surface. However, 10 mM Tris was selected because it was more compatible with downstream PCR analysis due to potential for EDTA inhibition of PCR[3], especially when microscale volumes are considered, and due to the 10 mM Tris concentration being routinely used in TE^{-4} for forensic samples. It should be noted, however, that for applications where stability of DNA during storage of samples may be of concern, 1X TE can be utilized with no adverse effects on recovery.

2.3.2.5 Conditioning of the Solid Phase

Prior to loading a sample onto a SPE microdevice the solid phase must first be conditioned with the buffer being used during the load process. This ensures that the phase can bind DNA at the onset of the load step, maximizing the DNA bound during the load, whereas if the phase was not conditioned, some DNA may be lost at the start of loading. Previous work using silica solid phases for DNA purification used a 10 min condition step with 6 M GuHCl in a microchip with a 2.6-fold smaller channel cross-sectional area, and, therefore, column volume, than the vrSPE device.[12] Due to this difference in area and phase, as 5 μm silica-coated paramagnetic particles were used in the vrSPE device, it was important to determine whether a longer condition step was needed. To establish the optimal condition time, a vrSPE device packed with the silica-coated paramagnetic particles was conditioned with 6 M GuHCl, pH 6.1 for 10, 30, 45, or

60 min. A sample (500 μL) containing 50 ng of hgDNA was then loaded, and the amount of DNA in each fraction eluted from the phase quantitated. Extraction efficiencies for 10, 30, 45, and 60 min condition times were calculated (Figure 3) to be 34.7 (±4.0), 23.2 (±10.7), 25.8 (±4.1), 24.4 (±13.3)%, respectively. To determine whether the EE values were statistically similar, t-tests were completed comparing the EE for each condition time. It was determined that all values were statistically-similar (at a confidence level of 95%) with the exception of the 10 vs 45 min result ($p = 0.05$). Although 10 min and 45 min were statistically different, the EE for a 10 min condition was greater than that of the 45 min condition demonstrating the superiority of a 10 min condition. Additionally, because all other values were statistically similar, a longer condition step could not be justified as valuable and the 10 min condition step would suffice while also reducing the analysis time needed for the method. As a result, a 10 min condition step was chosen for all later work.

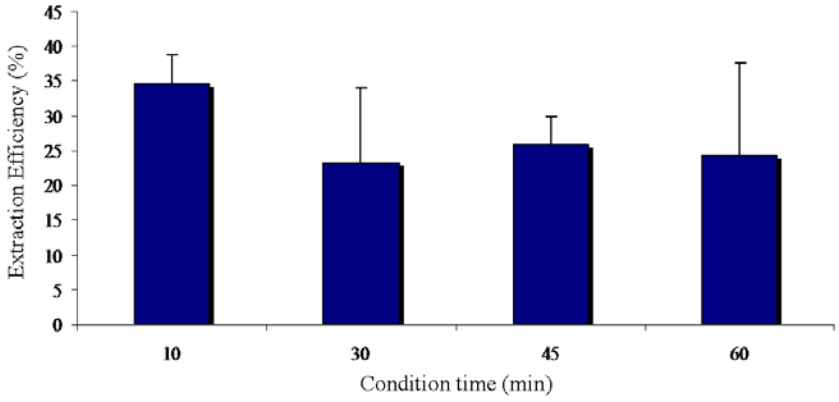
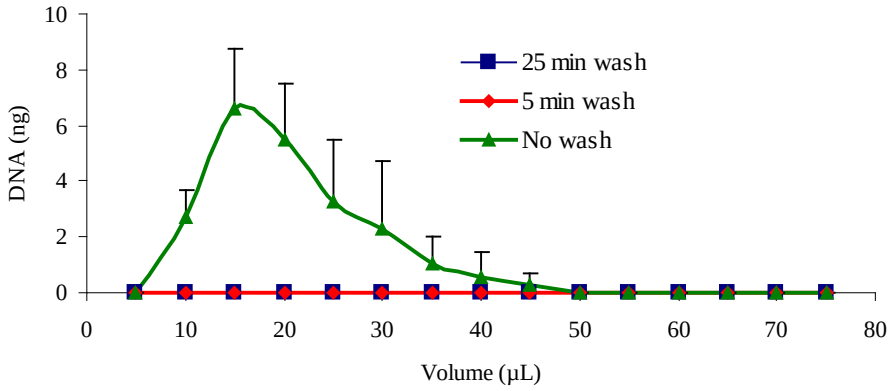


Figure 3. Extraction efficiencies determined after purification of hgDNA using the vrSPE device with varied [10 (n = 3), 30 (n = 7), 45 (n = 6), and 60 (n = 3) min] times for conditioning of the phase prior to loading the hgDNA sample. T-tests were completed and determined that all values were statistically similar with the exception of 10 min compared to 45 min. T_{calc} values were determined to be 1.75 (t_{table} = 2.306), 0.55 (t_{table} = 2.228), 0.23 (t_{table} = 2.365), 3.08 (t_{table} = 2.365), 2.67 (t_{table} = 2.776), 0.15 (t_{table} = 2.306) when comparing 10 to 30, 30 to 45, 45 to 60, 10 to 45, 10 to 60, and 30 to 60 min, respectively.

2.3.2.6 Wash Step Optimization

To further increase the quantity of amplifiable DNA extracted from a biological sample using the vrSPE device, the optimal wash time required to remove the maximum mass of protein, and other PCR-inhibitory compounds, from the solid phase was determined. A fluorescence assay was first used to investigate the optimal wash time by comparing extraction efficiencies. These experiments were conducted as described in *Wash Optimization Studies* in *Materials and Methods* with a 0, 5, or 25 min 80% IPA wash step (n = 3 for each wash time). Following comparison of extraction efficiencies, it was determined that a 0 min 80% IPA wash step resulted in the greatest quantity, and,



therefore, highest extraction efficiency, of extracted DNA (Figure 4).

Figure 4. Elution profiles representing optimization of time necessary for 80% IPA wash step during vrSPE extraction procedure for dilute whole blood samples. No 80% IPA wash step was found to be the optimal wash time providing the greatest quantity of recovered DNA.

To further test whether the wash step was needed for protein removal, extractions of dilute whole blood were performed with either no wash or a 5 min IPA wash step (25 min IPA wash step was not tested as it provided the same EE as a 5 min IPA wash step)

followed by elution with 10 mM Tris. Fractions were subjected to PCR amplification using primers for the gelsolin gene, and separated and analyzed using a Bioanalyzer 2100, which has the capability of providing a semi-quantitative measurement of amplicon concentration. When a 5 min 80% IPA wash step was performed, the gelsolin amplicon concentration was an average of 0.26 (± 0.03) ng/µL (n = 3). When no IPA wash step was used, the extraction yielded an average gelsolin amplicon concentration of 1.41 (± 0.45) ng/µL (n = 3). These results were surprising due to the residual GuHCl that was present in the packed channel which was expected to be more PCR inhibitory than residual IPA that would be present in the channel if a wash step was completed. As indicated by the ~5-fold increase in amplicon concentration (when no wash step was employed), an increased amount of template DNA was present in the elution fractions, prior to PCR, when no wash step was performed as opposed to a 5 min 80% IPA wash step (Figure 5). The superior results obtained with no IPA wash step was verified by comparison of the concentrations of the positive controls that were included for each PCR reaction where a 3.07 (± 0.58) and 3.37 (± 0.24) ng/µL concentration resulted for a 5 m wash step and no wash step, respectively. These results are not statistically different (t-test, 95% confidence interval) demonstrating that the concentration difference seen between amplicon sizes for the extracted DNA is due to the differing wash times used and not the efficiency of the PCR reaction. Additionally, the largest amplicon size was seen in fraction 3 for all extractions performed with no wash which tracks with the elution profile (Figure 4) where the greatest mass of DNA is present in fraction 3. These results demonstrate that the optimal wash is actually, no wash, resulting in the greatest concentration from amplification of DNA extracted using the vrSPE device.

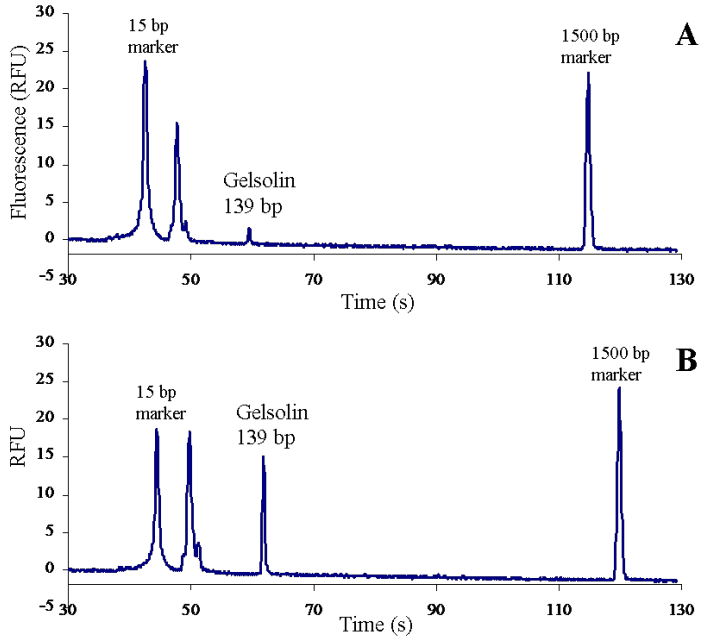


Figure 5. Electropherograms resulting from analysis of PCR products after DNA was extracted from whole blood. (A) Extractions were performed using a 5 m 80% IPA wash step and resulted in a 0.26 (± 0.03) ng/µL gelsolin amplicon concentration (139 bp peak indicative of gelsolin gene). (B) Extractions performed with no wash step resulted in a 1.41 (± 0.45) ng/ µL gelsolin amplicon concentration.

2.3.3 Binding Capacity Studies

Following optimization of conditions for the extraction process, the capacity of the selected phase using the optimized protocol was established. It was important to ascertain both binding capacity of the silica-coated-paramagnetic particles phase for DNA and also the capacity for DNA if a sample containing a high ratio of protein to DNA (such as blood) was loaded onto the phase. In such samples, proteins will begin to occupy a majority of the DNA binding sites, competing for those positions and decreasing the amount of DNA recovered during purification. Consequently, it was important to determine the binding capacity, both in the presence and absence of high protein concentrations, to ensure that the phase is suitable for a wide variety of samples

with components that may compete with DNA for binding to the solid phase. For this reason, capacity studies were completed for λ-phage DNA, prepurified hgDNA, and whole blood.

The capacity of the phase for λ-phage DNA was determined first by loading increasing quantities onto the phase. Once capacity of the phase is reached, a plateau in the relationship between DNA loaded and recovered will be seen. It is clearly seen from the linear relationship between the DNA loaded and that recovered, that even with up to 400 ng of λ-phage DNA, the capacity had not been reached [Figure 6, inset (n = 3)].

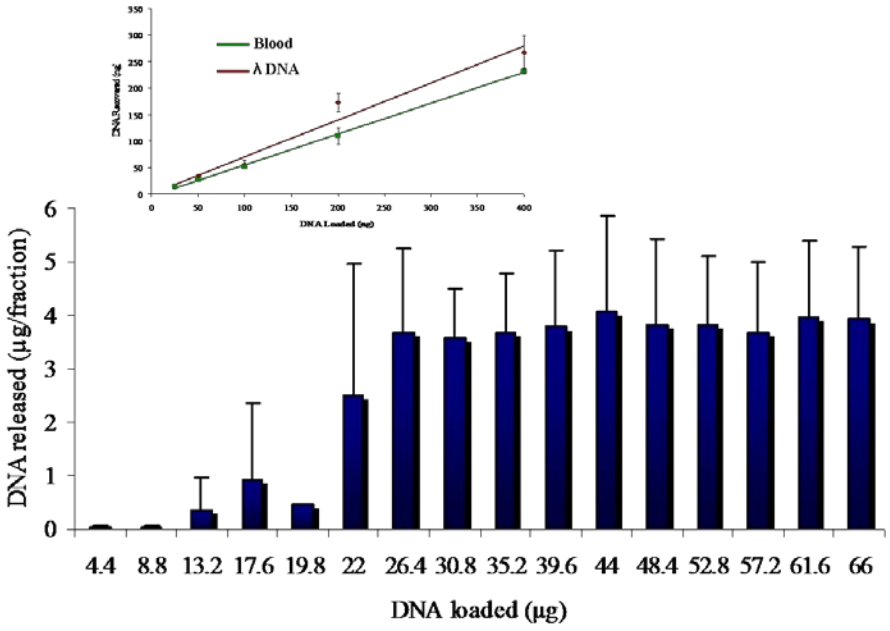


Figure 6. Breakthrough curve indicating binding capacity of the MagneSil™ phase in the vrSPE device for hgDNA, λ–phage DNA (inset), and whole blood (inset).

The capacity of the silica-coated-paramagnetic particles was then determined for hgDNA to establish whether a larger genome size would have an effect on the binding capacity by running a series of experiments to produce a 'breakthrough' curve. A breakthrough curve is generated by loading a concentrated sample, containing the analyte

(in this case DNA), onto the solid phase while the quantity of analyte is monitored over time at the outlet. For this study, a concentrated sample (0.11 µg/µL) of hgDNA in 6 M GuHCl, pH 6.1 was loaded onto the device while fractions were collected at the outlet and subjected to fluorescence analysis to generate a breakthrough curve (Figure 6). While below the capacity of the solid phase, DNA will bind efficiently to the phase, but once capacity is reached at the breakthrough point, the DNA will no longer bind and will flow through the system unretained resulting in a plateau of the DNA detected in each fraction collected. The first derivative of the data in Figure 6 has an inflection point which correlates to the volume of load solution where DNA no longer binds to the phase (breakthrough point). This value can then be used to calculate the capacity of silica-coated-paramagnetic particles by determining the quantity of DNA that had been loaded at this inflection/breakthrough point. The capacity of the phase under these conditions was found to be 50.3 (± 5.6) ng of hgDNA/µg of particles (450 µg of particles loaded into the microdevice) (n = 3).

Finally, the capacity of silica-coated-paramagnetic particles in the vrSPE device was determined for dilute whole blood representing a more complex biological sample. Increasing volumes of blood were loaded onto the phase (corresponding to 25 to 400 ng DNA) followed by determination of the mass of DNA recovered. As was detailed with λ-phage DNA, if capacity was reached, a plateau in the relationship between the mass of DNA loaded and that recovered would be seen. It is clear from the linear relationship observed (Figure 6, inset) that the capacity had not been reached even when 11.4 µL (400 ng DNA) of whole blood had been loaded (n = 3). Although most forensic samples will contain considerably less DNA than the capacity of λ-phage, hgDNA, and whole blood, it

is important to have the value defined to ensure the capacity is high enough that DNA will not be lost due to competition for binding sites on the solid phase from proteins or cellular debris when biological samples are purified.

Overall, the binding capacity was found to be >400 ng for λ-phage DNA. The capacity of the particles for hgDNA was determined to be 50.3 (± 5.6) ng hgDNA/µg particles, indicating the phase has a sufficient capacity for larger genome sizes. Lastly, the capacity for whole blood was determined to be >11.4 µL of whole blood, which is a quantity of blood that is more than sufficient for DNA extraction and subsequent analysis.

2.3.4 Extraction of Low DNA Template and Time-Degraded Samples

A large number of samples encountered by the forensic community consist of low DNA template samples. One method in which these samples are generated is through the dilution of the original sample to a larger volume to reduce the impact that contaminants within the sample will have on downstream processing. When this is completed, not only are the contaminants diluted but also the DNA template in the sample. To test the utility of the vrSPE method with this sample type, a 1:3,500-fold dilution of whole blood in lysis solution was prepared. The DNA in the sample was extracted using the vrSPE method and elution fractions amplified using the COfiler® amplification kit. COfiler® is a multiplex STR (described in Chapter 1) amplification kit that amplifies 7 loci including the sex marker amelogenin, which is used for human identification. It can be seen in Figure 7 that a full (7 of 7 loci) STR profile resulted (n = 3), demonstrating successful application of the vrSPE method to low DNA template samples. Additionally, previous

work using the silica-coated paramagnetic particles in a robotic platform showed locus drop-out at a 1:1,000-fold dilution of blood deposited on Whatman paper to create blood stains.[27] Although that work was completed with blood stains and only using a small portion of each stain (5 mm^2), Figure 7 still demonstrates the initial, successful developments of using the vrSPE method for lower DNA template samples.

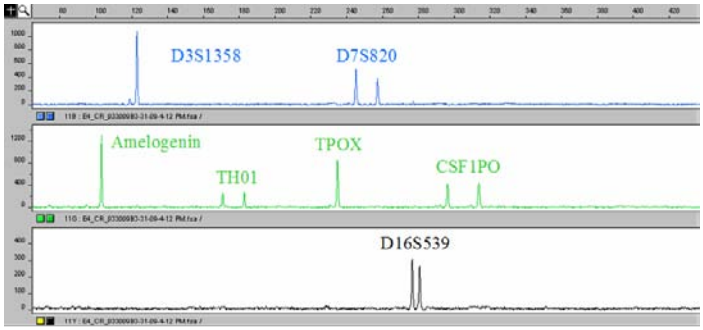


Figure 7. Full STR profile (7 of 7 loci) of DNA extracted from a large volume, low DNA template sample containing 140 nL of whole blood.

Large volume samples are also encountered in the forensic sector when a sample, degraded or not, requires solubilization or removal from a surface. So to further test the vrSPE method, DNA from a blood stain that had remained at room temperature for 3 years was extracted. The sample was then amplified using the MiniFiler™ amplification kit and resulted in a full (9 of 9 loci) STR profile (Figure 8, n = 3). These results again demonstrate the successful application of the vrSPE method to large volume samples generated during solubilization of sample from a surface.

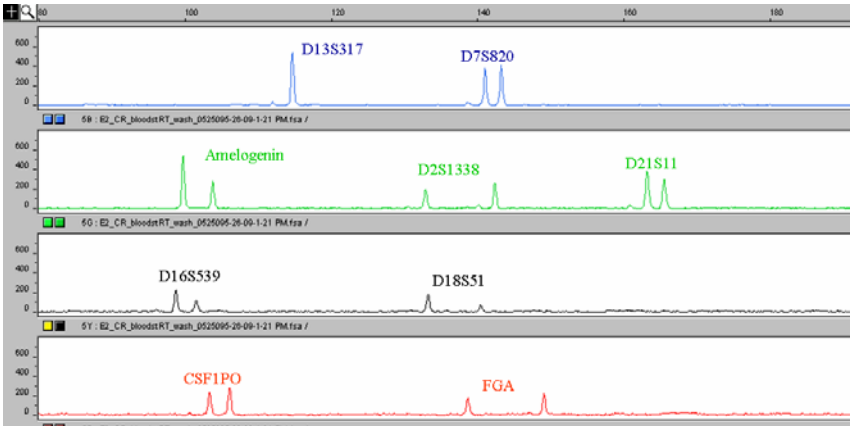


Figure 8. Full STR profile (9 of 9 loci) of DNA extracted from a 3 year old blood stain on Whatman paper that was solubilized/removed from the paper in a large volume (500 µL).

2.3.5 Extraction of DNA from Heat-Degraded Blood and Semen Stains

As previously discussed, the forensic field is focused on obtaining successful STR typing results from highly variable, degraded samples that are often obtained. One of the sample types that is encountered involves heat-degraded biological stains, which again require large volumes for removal from surfaces making the sample incompatible with processing using µSPE. Exposure of biological samples to heat can cause strand breaks within the DNA segments resulting in shorter DNA fragments. This causes the larger loci that are to be amplified with STR amplification kits to diminish in amplitude (peak height), which can decrease the discriminatory power of STR typing.[4] Blood (from different donors) stains on Whatman paper that had been incubated at 56 °C for 1 and 3 months were processed using the vrSPE method and purified DNA amplified using the MiniFiler™ amplification kit. Full STR profiles (9 of 9 loci) resulted for both 1 and 3 months (Figure 9A and B, n = 3). Spurious peaks, due to 'stutter' products or allelic drop-in and not contamination (studies were completed to eliminate it as a factor), are seen in Figure 9A which have been previously reported to occur with degraded or low

template samples or samples that were analyzed under an increased injection time during separation/detection of PCR products, both of which were the case in these samples.[4, 28] Although spurious peaks can be seen in the 56 °C/1 month blood stain sample, the true alleles are still present demonstrating the successfulness of the vrSPE method.

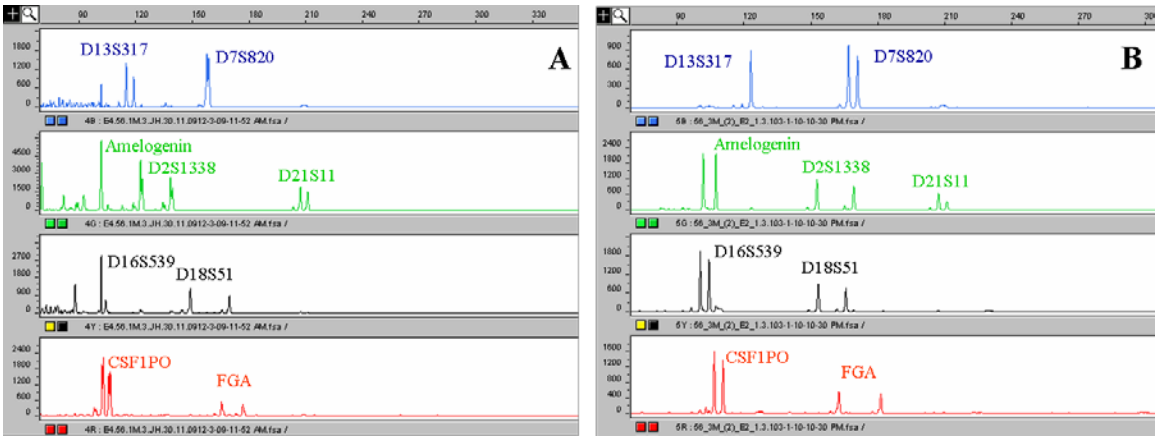


Figure 9. Full STR profiles (9 of 9 loci) of DNA extracted from blood stains on Whatman paper incubated at 56 °C for 1 (A) and 3 (B) months that was removed from the paper using a large volume lysis solution (500 µL).

To further test the vrSPE method, blood stains on Whatman paper that has been incubated at 80 °C for 1 and 3 months were also processed using the vrSPE method and the purified DNA amplified using the MiniFiler™ kit. Again, full (9 of 9 loci) STR profiles were obtained from these samples (Figure 10A and B, n = 3). It is important to note that spurious peaks (allelic drop-in) were again seen in Figures 10A and B, possibly due to the degraded nature of the sample, increased injection times during separation/detection, or a mixed sample (present during preparation). To ensure the spurious peaks were not due to contamination, blank extractions and amplifications were completed and showed no evidence of contamination. Therefore, the presence of the spurious peaks was thought to be due to the aforementioned reasons. Although these spurious peaks were seen, the success of the vrSPE method for application to degraded

samples is still demonstrated. These results provide a significant improvement over extractions reported previously using the same solid phase in a robotic platform for large volume extraction where drop-out began to occur with blood stains incubated at 56 °C for 1 month. The vrSPE method has exceeded this by demonstrating successful purification of DNA from blood stains incubated at 56 and 80 °C for 1 and 3 months.

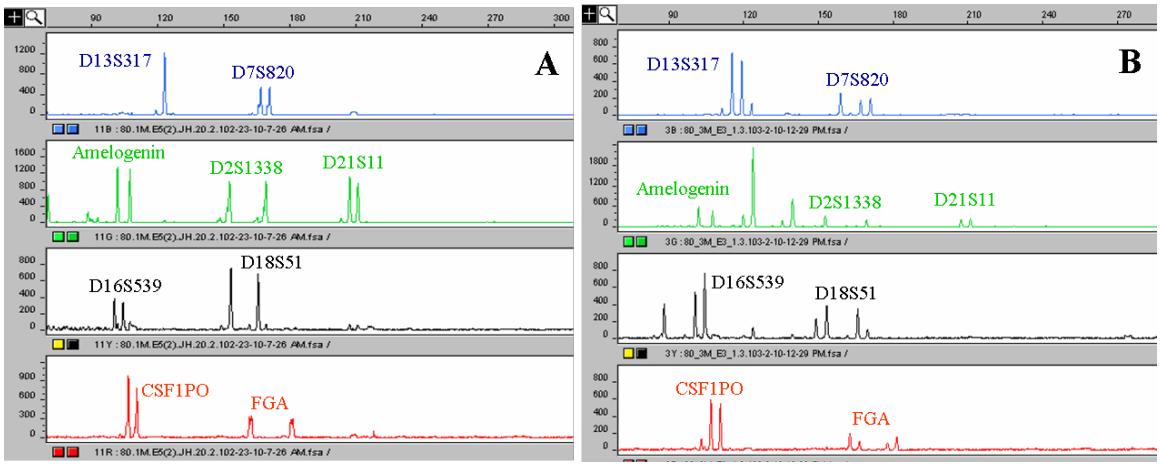


Figure 10. Full STR profiles (9 of 9 loci) of DNA extracted from blood stains on Whatman paper incubated at 80 °C for 1 (A) and 3 (B) months that was removed from the paper using a large volume lysis solution (500 µL).

All of the samples tested and described above were prepared on blood stain cards, which are often used for storage of reference DNA samples. Samples stored on these cards have been shown to yield full DNA profiles, even after 20 years at room temperature.[29] To have a closer representation to a sample that may be encountered in forensic casework, blood and semen stains were prepared on cotton cloth and incubated at 56 °C for 1 month. The blood stain was immersed in a large volume lysis buffer and processed using the vrSPE method and then amplified using the Identifiler® amplification kit. A full (16 of 16 loci) STR profile was obtained (Figure 11A, n = 3) demonstrating that the method is still successful when degraded samples are encountered. Additionally, although a slight 'ski slope' effect (decrease in RFU of the larger loci in

comparison to shorter loci) is seen with this sample (is also commonly seen in low template and degraded samples[4, 30]), the extraction from the large volume sample was still successful in providing sufficient DNA to provide a full profile after amplification. To further broaden the spectrum of sample types analyzed, DNA from a semen stain on cotton that had been heat-degraded was extracted using the vrSPE method and amplified using the Identifiler® kit. Again, a full (16 of 16 loci) STR profile was obtained (Figure 11B, n = 3). These results from both the blood and semen heat-degraded stains demonstrate that the vrSPE method can be used for application to samples that may have previously not provided a full profile when processed using other methodology.

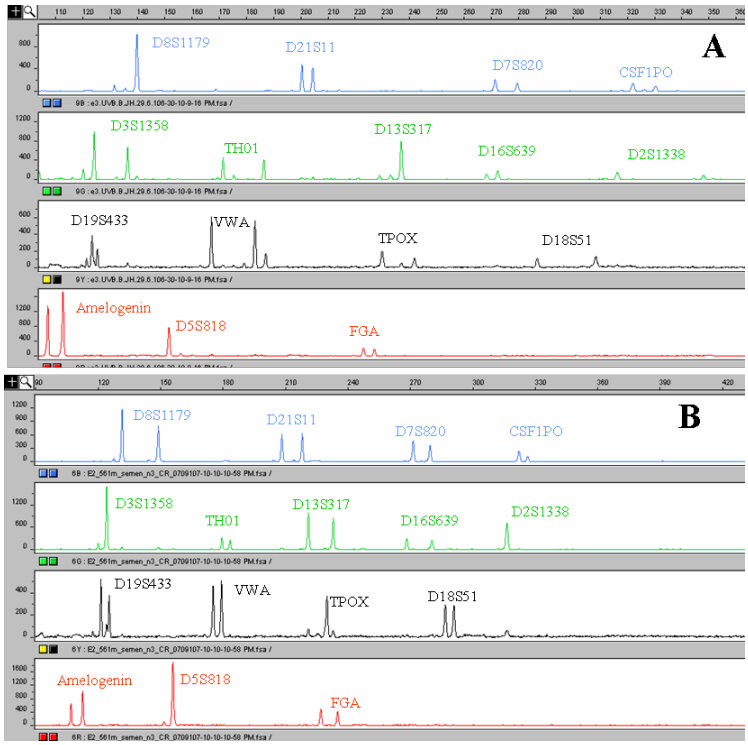


Figure 11. Full STR profiles (16 of 16 loci) of DNA extracted from blood stains on cotton incubated at 56 °C for 1 (A) and 3 (B) months that was removed from the cotton using a large volume lysis solution (500 µL).

2.3.6 Extraction of DNA from UV-Degraded Blood and Semen Stains

Large volumes are also seen with UV-degraded samples, due to a prolonged time outdoors, which still require removal from a surface resulting in a large volume, dilute biological sample. Exposure of DNA to UV light has been reported to cause cyclobutane pyrimidine dimers, oxidation products, and single strand breaks.[31] Work has been carried out by Hall and Ballantyne[31] on the degradation of blood stains and the ability to obtain a full STR profile from UV-exposed samples. Although, downfalls with this method include the use of 50 μL blood stains which is a volume of sample that is not always available in casework analysis. Additionally, a phenol/chloroform/isoamyl alcohol extraction procedure is used which, as previously discussed, is time-consuming and requires multiple sample transfer steps which can expose the sample to environmental contaminants. It was then logical to determine whether the vrSPE method, which could eliminate at least one of the downfalls, could be used. Blood and semen stains (10 μL) were applied to cotton and allowed to dry. Each stain was exposed to UV for 56 min which equates to 4 months and 8 days outside. This value is based upon a radiance of 420 J/m^2 equating to 20 hrs of natural sunlight at noon on a summer day in Los Angeles[32] and then assuming 8-9 hours of intense sunlight per day while incorporating the radiance of the UV source used in this work (720 μJ/cm^2). After removal of the blood and semen samples from the cotton in a large volume, the DNA was extracted using the vrSPE method, the purified DNA was then amplified using the Identifiler® kit. A full (16 of 16 loci) STR profile was obtained from both the UV-degraded blood (Figure 12A, n = 3) and semen (Figure 12B, n = 3) stains. This demonstrates the successful application of a microfluidic extraction method for UV-degraded samples. Additionally, the sample size

used in these studies is ~20-fold smaller than that used by Hall and Ballantyne[31] and still resulted in full STR profiles. The small sample sizes used to provide the full STRs in Figure 12 only required ~25% of the 10 µL stain prepared, indicating that multiple analyses can be completed from a single sample which can provide a more definitive STR profile for human identification.

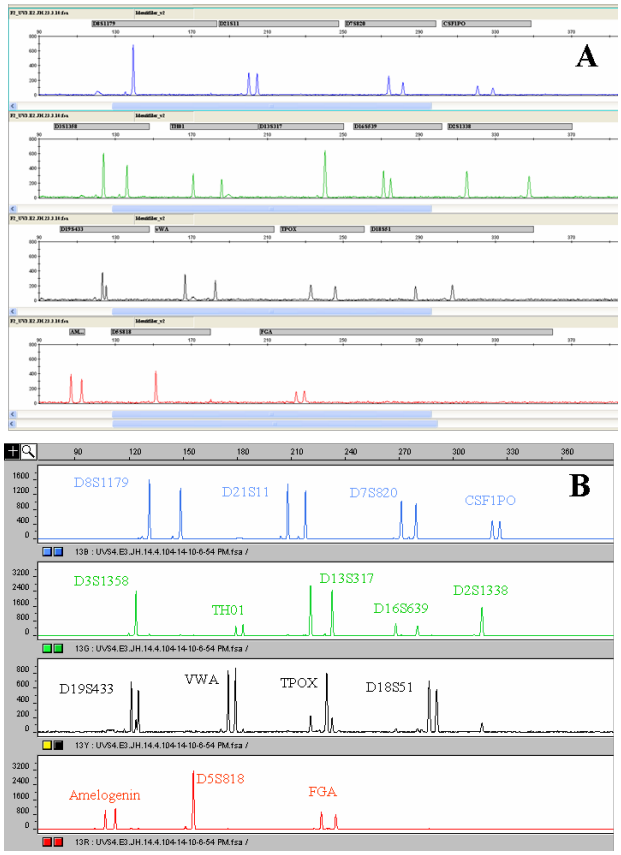


Figure 12. Full STR profiles (16 of 16 loci) of DNA extracted from blood (A) and semen (B) stains on cotton exposed to UV light for 56 min. The stains were removed from the cotton surface using a large volume lysis solution.

To further test the vrSPE method for degraded samples, blood and semen stains were exposed to 1 hr 52 min of UV light which equates to 8 months and 16 days outside. Both stains were immersed in a large volume (500 µL) lysis solution and the DNA subsequently extracted using vrSPE. Full (9 of 9 loci) STR profiles were obtained for

both the blood (Figure 13A, n = 3) and semen (Figure 13B, n = 3) stains. These results demonstrate that the vrSPE method can be used for a wide variety of large volume, degraded sample types that cannot previously be processed with μSPE. Additionally, the closed environment provided by the microdevice reduces the possible points for entrance of contaminants, which is a high priority when working with samples that already have degraded DNA templates.

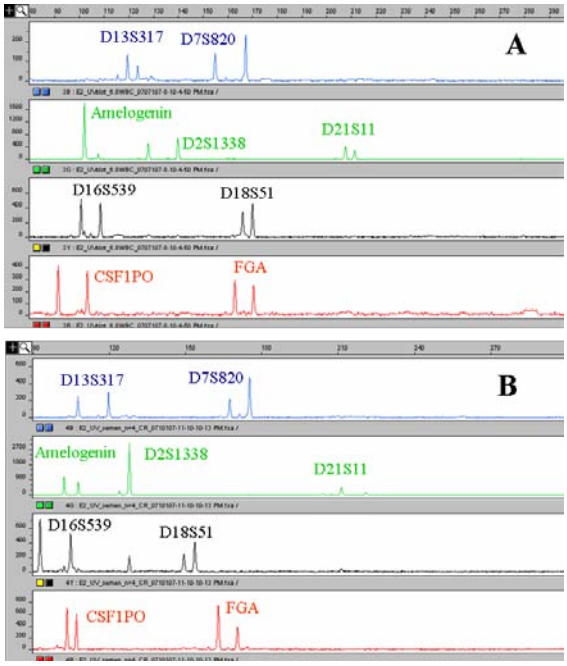


Figure 13. Full STR profiles (9 of 9 loci) of DNA extracted from blood (A) and semen (B) stains on cotton exposed to UV light for 1 hr 52 min. The stains were removed from the cotton surface using a large volume lysis solution.

2.3.7 Extraction of DNA from Bone Samples

All of the large volume samples discussed thus far have been a result of dilution of a sample or solubilization/removal from a surface. But large volume samples are also generated in analysis of bone samples due to the volumes needed for adequate demineralization, on the order of 3 mL.[19] The current protocol for DNA analysis of bone samples first requires overnight mixing and incubation of the sample (~0.2 g) in 3 mL of

demineralization buffer (0.5 M EDTA/0.5% w/v lauryl sarcosine) at 56 °C. The sample is concentrated down to ~100 μL using a concentration kit and purified using the MinElute PCR purification kit followed by amplification using an STR kit with extra *Taq* and cycles during PCR.[33]

To investigate application of vrSPE for bone analysis, the first step was to determine whether the vrSPE method could replace the MinElute PCR purification kit. To test this, bone samples [obtained from the Armed Forces DNA Identification Laboratory (AFDIL)] (0.1 g) were incubated overnight at 56 °C in demineralization buffer (0.5 M EDTA/0.5 % w/v lauryl sarcosine, pH 8) according to the AFDIL protocol[33]. The sample was concentrated down to ~100 μL with a Centricon 30 concentrator kit and then amplified with the MiniFiler™ kit (with extra *Taq* and PCR cycles). A STR profile with 15 of 16 alleles present resulted (Figure 14) which demonstrates the first development towards purification of DNA from a bone sample on a microdevice. Upon repeating this purification the 15 of 16 alleles was never obtained again, although usually 8 – 13 alleles were seen. This could potentially be due to the excess EDTA that is used in the demineralization buffer being concentrated alongside the DNA in the concentrator kit. If this concentrated EDTA is present after the purification it can result in PCR inhibition due to binding of Mg^{2+} that is crucial for the functionality of *Taq* polymerase during PCR. To determine whether improved results could be obtained using other concentration kits, filters with varying molecular weight cut-offs (MWCO) were tested below (10,000 MWCO) the previous 30,000 MWCO used with each providing similar results, less than 10 alleles. Future work will involve removal of the

EDTA from the dissolved bone sample prior to concentration to minimize the inhibitory affects it can have on PCR.

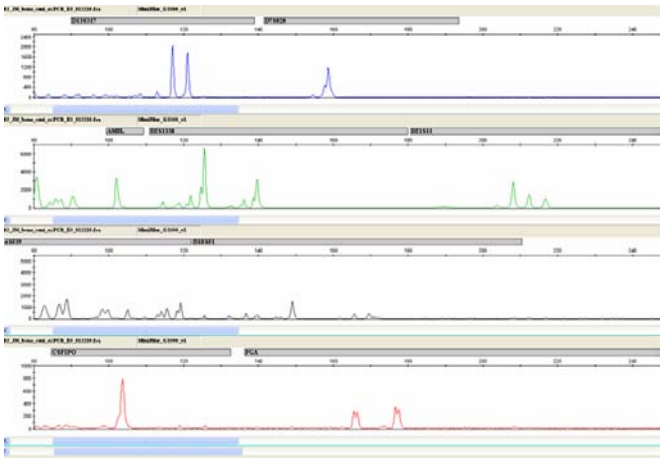


Figure 14. STR profile (15 of 16 alleles present) of DNA extracted from a bone sample that had been subjected to demineralization and concentration prior to extraction using the vrSPE device.

2.3.8 Extraction of Mitochondrial DNA from Whole Blood and Degraded Blood Stains

Not all samples submitted for DNA analysis are ideal for downstream processing using conventional short tandem repeat (STR) methods. Samples are often encountered where degradation may have reached a point where nuclear DNA typing may be challenging, and a viable alternative for genetic evaluation is mitochondrial DNA analysis. Microdevices present the ideal environment for such analysis by providing a closed environment where the number of sample handling steps is minimized therefore reducing points of contamination which is known to be a concern in mitochondrial DNA analysis.[4] Along those lines, vrSPE provides an even greater advantage for mitochondrial DNA analysis due to the large sample volumes (~500 µL) that it can accommodate in ~30 min which are comparable to volumes seen for a majority of mtDNA samples[19], making the need for application of mtDNA analysis to the vrSPE method all the more

necessary. The high volume-throughput capabilities of the vrSPE method also widens the spectrum of samples that can be extracted using the vrSPE device to such samples as blood stains, which were investigated here, which require large volumes for solubilization due to the absorbent nature of the substrate.

Having optimized the device and protocol for volume reduction and purification, the vrSPE device was next utilized to extract mtDNA from biological samples. Dilute whole blood (1 µL) was loaded onto the device and the extracted DNA eluted with 1X TE, pH 8. Elution was performed with 1X TE, opposed to 10 mM Tris, to reduce DNase activity due to the presence of EDTA in the elution buffer. While TE^{-4} (containing 0.1 mM EDTA) is routinely used for forensic samples and would not change the efficiency of the extraction if used, there was no observable PCR inhibition when using 1X TE. Elution fractions were then amplified for portions of the HVI region of mtDNA using either HVI (VDFS) or MPS2B (AFDIL) primers and separated/analyzed using a Bioanalyzer 2100. While the absence of a wash step seemed optimal, as indicated in *Optimization of vrSPE Phase and Extraction Condition* section, a wash step may still be included for samples that may be contaminated, such as the whole blood or degraded blood stain samples used in this work. This implies that, in the instance of casework samples, a wash step can still be included in the extraction process if contamination is a concern. As depicted in Figure 15A (n = 3), mtDNA was successfully extracted on-chip and amplified off-chip from dilute whole blood and is indicated by the presence of a 459-bp amplicon. These results illustrate the first successful extraction of mtDNA from whole blood using a microfluidic device. This provides a critical step in developing microdevices capable of extracting mitochondrial DNA from degraded or compromised

samples by reducing the possible points of contamination and, therefore, enhancing the likelihood of a positive result.

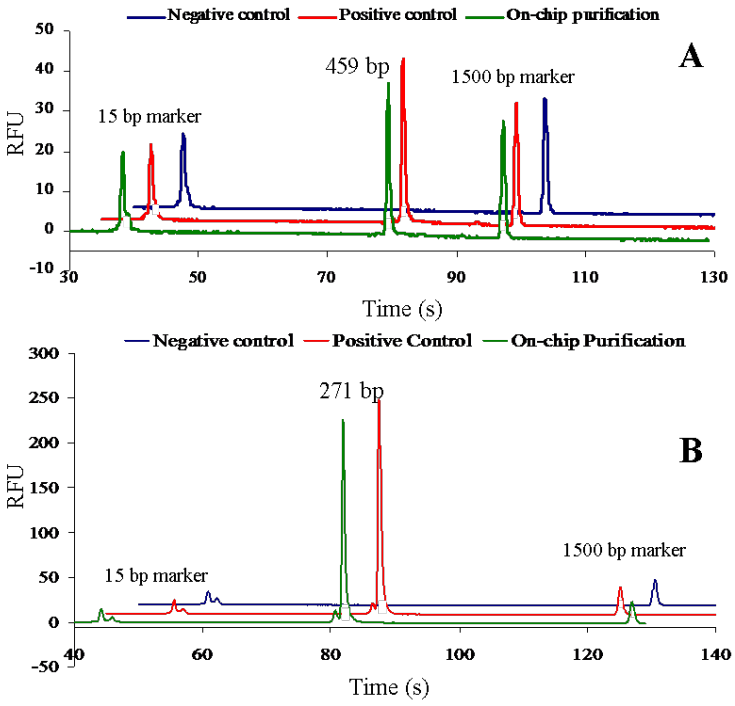


Figure 15. Electropherograms indicating successful amplification of a portion from the HV1 region of the mtDNA genome after on-chip vrSPE of mtDNA from (A) dilute whole blood (HVI primers from VDFS) and (B) a degraded blood stain (MPS 2B or PSII primers from AFDIL).

To further challenge the robustness of the vrSPE method, mtDNA was extracted from a representative forensic sample: degraded blood stains on Whatman paper incubated at 56-80 °C for 1-3 months. The blood was solubilized and removed from the surface of the paper in a large volume lysis solution (500 µL) and subsequently loaded onto the vrSPE microdevice. Following an IPA wash step to remove protein, and elution with 1X TE, pH 8, portions of the HVI region of mtDNA were, again, amplified (using HVI primers at VDFS and PSIII at AFDIL) and the resulting mtDNA product can be seen by the presence of the 271 bp amplicon [Figure 15B (n = 3)]. These results demonstrate

the first example of a successful microfluidic mtDNA purification from a degraded blood sample coupled to subsequent amplification. Although mtDNA is more susceptible to contamination than genomic DNA, the demonstrated ability to extract it from forensic samples in a microchip format allows for reduction of possible points of contamination due to the closed environment the microchip provides. In addition, the ability to load large sample volumes on this device increased the quantity of mtDNA available for purification in comparison to what is typically loaded for microchip silica-based SPE (~40 µL[13]), allowing for an increased likelihood that amplifiable product will be observed from a sample type known to be degraded or compromised. Additionally, the increased sample volume that can be loaded onto the vrSPE device reduces the possibility of inhibition of downstream analysis, such as PCR (due to contaminants in the sample such as creams and oils) by diluting the contaminants in the initial sample, which has already been shown to be beneficial in macroscale applications by Covert, et al.[20] Furthermore, the increased sample volume does not significantly change the time (~30 min) required for analysis using the vrSPE method in comparison to other µSPE methods where a 12.5-fold smaller sample volume is loaded[12, 13]. This ensures that advantages seen with microchip based systems, such as decreased analysis time in comparison to conventional analysis, is still achieved with the vrSPE method. Other advantages such as a closed environment, reduced contamination, and sample degradation, are also still achieved with loading large sample volumes using the vrSPE method in comparison to other microchip based systems.

2.4 Conclusions

This work demonstrates the use of a microfluidic device for the on-chip volume reduction and purification of hgDNA from a biological sample. Evaluations of the silica-coated-paramagnetic particles using different flow rates and elution buffers were performed to ensure the greatest quantity of DNA is eluted for downstream analysis. Using these optimized conditions, a 50-fold decrease in volume and 15-fold increase in concentration were achieved in the vrSPE channel, whereas little or no volume reduction is seen with conventional methods. The use of the vrSPE device was demonstrated effective for the extraction and subsequent off-chip amplification of hgDNA from a dilute whole blood sample, as well as for the first successful on-chip purification of mtDNA from whole blood and a degraded blood stain. The method was also shown successful for the purification of DNA from low DNA template and environmentally-degraded samples as well as the first development towards microfluidic purification of DNA from bone samples. The work in Chapter 2 provides the next step towards developing a device that integrates this vrSPE method with an orthogonal purification phase, chitosan-coated silica[9], to provide a more stringent purification and possible elimination of inhibitors that may not be removed by a silica phase (discussed further in Chapter 3).

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3. Dual-domain, Microchip-based Process for Volume Reduction Solid Phase Extraction of DNA from Dilute, Large Volume Biological Samples

As described in Chapter 2, large volume, degraded or contaminated, biological samples are often encountered in forensic DNA casework analysis. Robotic systems have been developed for processing of these sample volumes but have associated disadvantages including exposure of the sample to environmental contamination during and between processing steps. Most commercially-developed products for purification of DNA from this sample type have used only one type of binding chemistry, mainly silica. Whereas, if a second orthogonal purification method was used, a broader spectrum of samples could be processed as contaminants that may not be removed from the first phase could be using the second phase, due to the different binding mechanisms, increasing the success rate of genetic analysis for inhibited biological samples. Therefore, development of a microscale method that not only provides a 'closed environment', for reduction of sample contamination or degradation, but that also integrates orthogonal purification method is imperative.

A microfluidic device was developed to carry out integrated volume reduction and purification of DNA from dilute, large volume biological samples commonly encountered in forensic genetic analysis. The dual-phase device seamlessly integrates two orthogonal solid phase extraction processes – a silica solid phase using chaotrope-driven binding (described in detail in Chapter 2), and an ion exchange phase using totally aqueous chemistry (chitosan phase) – providing the unique capability of removing PCR inhibitors used in silica-based extractions (guanidine and isopropanol). Chapter 2 demonstrated that the silica phase (vrSPE) provides a substantial volume reduction. This

is then followed by a more stringent extraction on the chitosan phase. Key to interfacing the two steps is mixing of the eluted DNA from the first phase with loading buffer, which is facilitated by flow-mediated mixing over a herringbone mixing region in the device. The completely aqueous chemistry associated with the second purification step yields a highly-concentrated, PCR-ready eluate of DNA devoid of PCR inhibitors that are reagent-based (isopropanol) and sample-based (indigo dye), both of which are shown to be successfully removed using the dual-phase device but not by the traditional microfluidic SPE (μSPE). The utility of the device for purifying DNA was demonstrated with a variety of biological samples including a blood sample inhibited with indigo dye, with the resultant DNA from all shown to be PCR-amplifiable. The vrSPE-SPE device reliably yields a volume reduction and concentration enhancement for DNA purification on the order of 50- and 14-fold, respectively, both compatible with downstream PCR analysis. In addition, purification of all samples consumed less reagents (2.6-fold) than traditional purification methods, with the added advantage of being a 'closed system' that eliminates sample transfer steps, thereby reducing the possible entrance points for contaminants. Some of the results in this chapter are described in Reedy, et al.[1]

3.1 Introduction

As discussed in Chapter 1, the use of microfluidics for SPE has previously been demonstrated and provides a closed environment void of sample transfer steps, smaller reagent consumption, and integration with other downstream processes.[2-17] A majority of microchip-based SPE (μSPE) has focused on using a silica-based solid phase.[2, 6, 11, 18, 19] In contrast, a totally aqueous μSPE method was developed by Cao et al.[8] utilizing

chitosan to reversibly bind DNA in a pH-dependent manner (also detailed in Chapter 1). Binding of DNA to chitosan (Figure 1) occurs when the amino group on chitosan becomes protonated at an acidic pH, while a simple increase in pH releases the DNA in PCR buffer, avoiding PCR-inhibitory reagents that are often required for silica-based purification methods [guanidine hydrochloride (GuHCl) and isopropyl alcohol (IPA)].

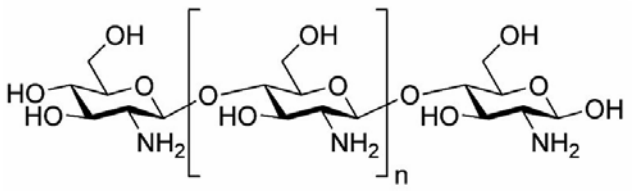

Figure 1. Chitosan structure containing amino groups with pKa ~6.3. DNA is bound to the positively charged amino group at pH 5 and then eluted by an increase in pH to 9 (with PCR buffer, 10 mM Tris/50 mM KCl, pH 9) causing the amino groups to become neutral and release the DNA. Adapted from http://en.wikipedia.org/wiki/Chitosan.

Although both chitosan and silica solid phases have been proven successful for reproducibly extracting DNA from complex biological samples using microscale approaches[6, 11, 18], input sample volumes used in these purifications were on the order of tens of microliters. This poses a challenge when encountering many forensic samples which often have volumes ranging from 0.5-1.0 mL, due to the removal or solubilization of a sample from an adsorbent surface.[20] Larger volumes may also be necessary in other forensic scenarios, as it has been shown that dilution of sample to a larger volume also dilutes the contaminants present, reducing inhibition of PCR.[21, 22] In order to effectively utilize microfluidic technology for larger volume samples, yet still harness all of the inherent analytical advantages offered by the microscale, a method, which was detailed in Chapter 2, was developed that interfaced large sample volumes with previously developed microscale technology using a high capacity silica-based solid phase (50.3 ± 5.6 ng DNA/µg particles).[23] Although this method alone provided a 50-fold reduction in

volume, a still greater reduction (and concentration enhancement) could be obtained if the sample was subjected to an orthogonal secondary purification process which would also eliminate reagent-based PCR inhibitors (GuHCl and IPA) and potentially sample-based PCR inhibitors that are not removed by the first phase. Hence, the importance of integration with a second SPE domain using a chitosan-coated phase for an orthogonal DNA capture process, which is the focus of this chapter.

While a two-stage, dual-phase microchip-based system has been previously reported for DNA extraction by Wen et al.[16], this system used one phase (C18) for protein capture followed by a monolithic silica phase for DNA extraction (Figure 2). The system described provided a substantial increase in DNA extraction efficiency over previous microfluidic systems for DNA purification (~80%) from a 110 µL load sample containing 8.8 µL blood. While advantageous for use with clinical samples, this device has not yet been tested with more variant forensic samples or larger volume samples. In addition, the time necessary for monolith preparation alone was on the order of 75 min, minimizing the gain in analytical process speed.

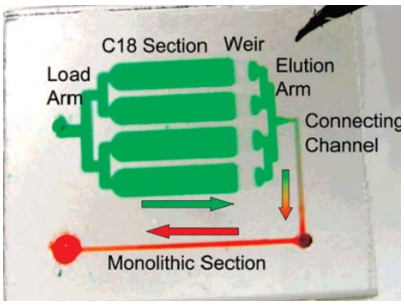

Figure 2. Dual-phase large volume purification microdevice used by Wen et al. for DNA purification. from whole blood. The C18 section is used for protein removal while the monolithic section reversibly binds DNA. Adapted from Wen, J., Guillo, C., Ferrance, J.P., Landers, J.P. *Analytical Chemistry* **2007**, *79*, 6135-6142.

Forensic samples can be challenging for DNA analysis owing to a number of undesirable attributes that include contamination with PCR inhibitors of environmental

origin (e.g., indigo dye[21], humic acid) and the fact that forensic samples are often associated with volumes that are atypical for microchip systems (i.e., >10 μL). Indigo dye (Figure 3) is a PCR inhibitor commonly-encountered with DNA extracted from biological stains on blue jeans, and has been included in studies testing the capability of different conventional STR amplification kits to amplify DNA in the presence of common PCR inhibitors.[24] Having defined microchip-based extraction systems for DNA[2, 6, 8, 11, 13, 15, 16, 18] from low volume samples[17], efforts were focused on an extraction system that could accommodate larger volume samples. Chapter 2 (Reedy et al.[23]) showed that enhancing the capacity of a standard chip-based silica phase bed could accommodate larger volume samples. Using a 1.4 mm bed instead of the standard 0.54 mm bed with the appropriately augmented flow rates, 35% of the sample DNA could be recovered in a volume that was reduced by 50-fold relative to the input sample volume. While an effective volume reduction strategy for producing concentrated DNA, downstream PCR is compromised if PCR inhibitors (e.g., indigo dye) are retained during the extraction process, and even more problematic if they are concentrated. Moreover, the very reagents used for chaotrope-driven nucleic acid extraction on silica solid phases (i.e., IPA and GuHCl) are also potent PCR inhibitors, with residual isopropanol specifically having been shown to affect chip PCR in an integrated genetic analysis system[11]. For these reasons, a two-phase, chip-based nucleic acid extraction system was developed to handle large volume samples, remove both reagent- and sample-based PCR inhibitors and provide high quality, concentrated DNA for downstream PCR. A silica phase is used to bind DNA in the presence of guanidine to concentrate it into a smaller volume, while the second phase, in a totally aqueous manner, binds the nucleic acids

from the first phase while cleansing them of PCR inhibitors (isopropanol, indigo dye). This approach is shown to be effective at eliminating the potential PCR-inhibitory effects of environmental contaminants, like indigo dye, and of silica chemistry extraction buffers (guanidine and isopropanol) from large volume samples in a PCR-ready form amenable to integration with downstream PCR.

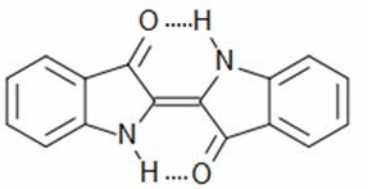

Figure 3. Structure of indigo dye which is a commonly encountered PCR inhibitor used to dye denim. Adapted from http://en.wikipedia.org/wiki/Indigo_dye.

3.2 Materials and Methods

3.2.1 Reagents

Guanidine hydrochloride (GuHCl), 2-(4-morpholino)-ethane sulfonic acid (MES, enzyme grade), 2-propanol (IPA), hydrochloric acid, sodium hydroxide, sulfuric acid, acetone, hydrogen peroxide, *Taq* DNA polymerase, 10X PCR buffer, dNTPs, and $MgCl_2$ were purchased from Fisher (Fair Lawn, NJ). 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Trizma Base, 99.9%), synthetic indigo, and bovine serum albumin were purchased from Sigma (St. Louis, MO). Potassium chloride was purchased from Mallinckrodt Chemical Company (Paris, KY). Triton X-100 for molecular biology was purchased from Fluka (St. Louis, MO). Low molecular weight chitosan (chitosan oligosaccharide lactate) and (3-glycidyloxypropyl) trimethoxysilane (GPTMS) were purchased from Aldrich (St. Louis, MO). MagneSil™ paramagnetic particles were purchased from Promega (Madison, WI). Hyperprep silica beads (15-30 µm) were

purchased from Supelco (Bellefonte, PA). Silica beads (5-15 µm) were purchased from Fuji Silysia Chemical Ltd (Aichi, JAPAN). Ethylenediaminetetraacetic acid (EDTA) disodium salt (reagent grade) was purchased from American Research Products (Solon, OH). Picogreen® dsDNA intercalating fluorescent dye was purchased from Invitrogen (Carlsbad, CA). AmpFℓSTR ® COfiler® and AmpFℓSTR® Identifiler® PCR kit were purchased from Applied Biosystems (Foster City, CA). Purified human genomic DNA was obtained through in-house purification from whole blood. All solutions were prepared in Nanopure water (Barnstead/Thermolyne, Dubuque, IA).

3.2.2 Microdevice Fabrication

Microdevices were prepared using borofloat glass (Telic Company, Valencia, CA) and standard photolithographic techniques.[25] All channels in the integrated vrSPE-SPE device were etched 200 µm deep, with a 1 cm long vrSPE channel (1.4 mm wide) and a 1.5 cm long chitosan-SPE channel (500 µm wide), and 20 µm weir depths (Figure 4A, B). Standard microchip-SPE (µSPE) and vrSPE devices were etched 200 µm deep, with a 1 cm long channel (500 µm wide for µSPE and 1.4 mm wide for vrSPE), and 20 µm weir depths. Reservoir holes were drilled using a 1.1 mm diameter diamond-tip drill bit (Crystalline Corp., Lewis Center, OH). A borofloat glass cover plate was thermally bonded to each device.

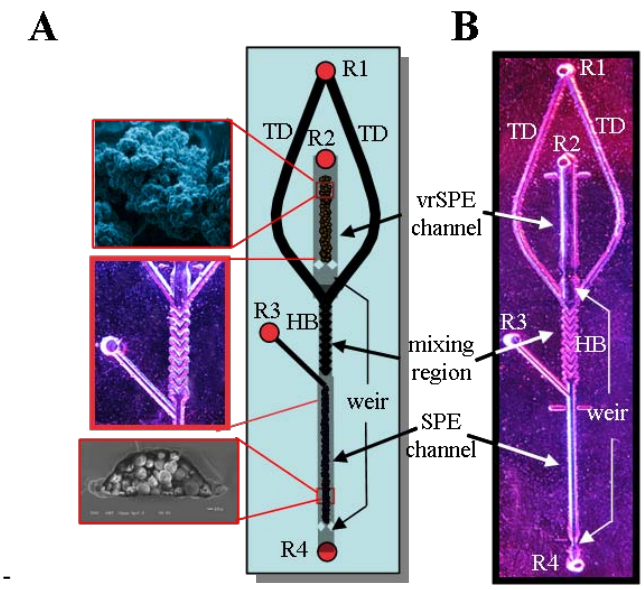

Figure 4. A) Schematic drawing of vrSPE-SPE device depicting the MagneSil™ phase in the vrSPE channel, herringbone mixing structure, and chitosan phase in the SPE channel (right). Enhanced views of MagneSil™[26] phase (top left), herringbone mixing region (middle left), and a packed SPE channel (bottom left) are enlarged. B) vrSPE-SPE device design and function. R1-4 = reservoirs 1-4 for sample and buffer loading and removal; HB = herringbone region for mixing eluted sample from vrSPE channel with load buffer for SPE channel; TD = teardrop-shaped channels for infusion of loading buffer for SPE channel; vrSPE contains silica phase and SPE contains chitosan-coated phase.

3.2.3 SPE Apparatus

SGE (1 mL) gas-tight syringes (SGE, Austin, TX) or 250 µL Hamilton gas-tight syringes (Hamilton, Reno, NV) were attached to the microdevice through 0.75 mm i.d. PEEK™ tubing, mini-tight fittings, and nanoports (Upchurch, Oak Harbor, WA). Solution flow through the device was achieved with a SP101I syringe pump (WPI, Sarasota, FL). The vrSPE channel of the device was packed with a small frit of 30 µm silica beads by vacuum which allowed packing of the rest of the channel with 5-8 µm MagneSil™ particles. The chitosan-SPE channel was then packed by vacuum with 30 µm chitosan phase.

3.2.4 Fabrication of Chitosan-coated Phase

Chitosan-coated silica beads were fabricated as described in Hagan, et al.[27] Briefly, 30 μm silica beads (0.1 g) were suspended in 1 mL NaOH, vortexed, and sonicated for 2 min. The suspension was then mixed on a LabQuake® rotator (Barnstead Thermolyne Corporation Dubuque, IA) for 30 min, centrifuged, the supernatant removed, and the beads washed with water until at a neutral pH was reached. The beads were then rinsed with ethanol and acetone and placed in a 60-70 °C oven for 5-10 min to dry. The beads were cleaned with a piranha solution (1:1 H_2SO_4:H_2O_2) for 30 min, the piranha solution removed and the beads were again washed with water, until a neutral pH was reached, then ethanol and acetone, and dried in an oven (60-70 °C) for 1-2 hrs or until dry.

A solution (500 μL in water) containing 20 mg of chitosan oligosaccharide and 2 μL of GPTMS was vortexed, sonicated for 2 min, and mixed using the LabQuake® rotator for 2 hrs. This solution was then added to the 30 μm silica beads and the suspension mixed for 18 hours using the LabQuake® rotator. The suspension was centrifuged, the supernatant removed, and the beads rinsed with 0.1 M HCl. The suspension was centrifuged and the supernatant of HCl removed and the beads dried in an oven (60-70 °C) for 2 hrs or until dry. Lastly, the beads were rinsed with water, ethanol, and acetone and placed in the oven for 30 min or until dry.

3.2.5 Sample Preparation

To prepare lysed whole blood, 4 µL of whole blood (obtained from University of Virginia School of Medicine from fully deidentified residual clinical specimens) was added to 476 µL 6 M GuHCl, pH 6.1 and 20 µL proteinase K (Qiagen, Valencia, CA), mixed well, incubated in a 56 °C water bath for 10 min, and the entire solution loaded onto the device. Semen samples for purification of hgDNA were prepared by adding 4 µL of dilute semen (1:1 mixture of semen:water) to 496 µL 6 M GuHCl, pH 6.1 with 40 mM dithiothreitol (DTT), mixed well, and subsequently loaded onto the device. The semen stain for hgDNA purification was prepared by pipetting 10 µL of semen onto a cotton cloth and allowed to dry at room temperature. The stain was cut from the cloth, immersed in 500 µL of 6 M GuHCl, pH 6.1 containing 40 mM DTT, vortexed, and subsequently loaded onto the device. The blood stain for hgDNA purification was prepared by pipetting 6 µL of blood onto a cotton cloth and allowed to dry at room temperature. The stain was cut from the cloth, immersed in 780 µL 6 M GuHCl, pH 6.1 with 20 µL proteinase K (20 mg/mL), incubated at 56 °C for 30 min and 500 µL of the sample loaded onto the device. Samples of lysed whole blood with 8 mM indigo dye were prepared by adding 918 nL of whole blood to ~459 µL 6 M GuHCl, pH 6.1 and 40 µL 100 mM indigo 0.2 % Triton X-100, mixed well, and incubated in a 56 °C water bath for 10 min, and the entire solution loaded onto the device for both vrSPE and vrSPE-SPE purifications.

3.2.6 SPE Procedure, Dual-phase vrSPE-SPE

A silica and chitosan packed vrSPE-SPE microdevice (Figure 4A) was conditioned with 6 M GuHCl, pH 6.1 for 10 min at a flow rate of 15 µL/min via reservoir 2 (R2, Figure 5A). The chitosan-SPE channel was then conditioned with 10 mM MES, pH 5 for 30 min at 5 µL/min (via R3) followed by loading of the sample of interest with a 1 mL SGE gas-tight syringe through R2 at 15 µL/min to achieve chaotrope-driven binding of DNA in the sample to silica in the vrSPE domain. DNA was eluted from the silica with 10 mM Tris, pH 8 via R2 in laminar flow with 50 mM MES, pH 3.5 infused in the teardrop-shaped channels via R1, both at 2.5 µL/min. Knowing the volumetric flow rates from R1 (chitosan SPE load buffer) and R2 (vrSPE) were both 2.5 µL/min, a load buffer of 50 mM MES, pH 3.5, was determined to be optimal for lowering the pH of the 10 mM Tris, pH 8 elution buffer (from R2) to a pH of 5 (data not shown), thus ensuring sufficient protonation of the amino groups on chitosan (pKa 6.3). In addition, the resultant flow rate of 5 µL/min is optimal for DNA binding to the chitosan-SPE phase. These solutions then mixed in the HB region (Figure 5A) and loaded onto the chitosan-SPE channel. Following washing of the chitosan phase with 10 mM MES, pH 5 buffer via R3 (5 µL/min) to remove any unbound material, the DNA was eluted with 10 mM Tris/50 mM KCl, pH 9 buffer via R3 (5 µL/min) and collected from reservoir 4 (R4) in PCR tubes for subsequent fluorospectrometric, PCR or RT-PCR analysis (Process depicted in Figure 5).

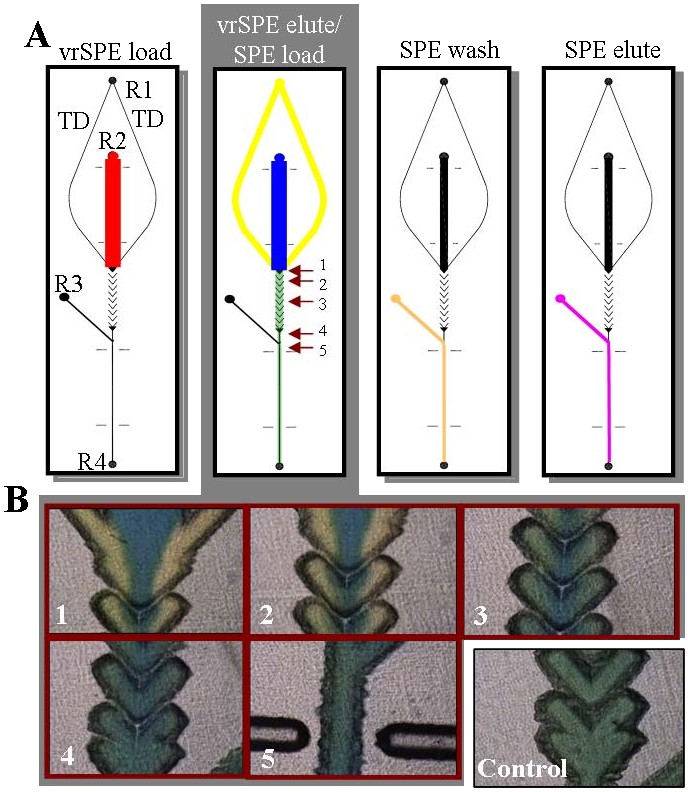

Figure 5. vrSPE-SPE device protocol and evaluation of mixing in herringbone region. A) vrSPE-SPE device protocol where sample is infused through R2 (*vrSPE load*). Nucleic acids are then eluted from vrSPE via R2 while load buffers for SPE are simultaneously infused through R1 and the teardrop (TD) structures (*vrSPE elute/SPE load*). SPE wash to remove proteins is then completed via R3 (*SPE wash*). Finally, elution buffer is infused via R3 while extracted nucleic acids are collected at R4 (*SPE elute*). B) Blue dye was flowed through the vrSPE channel while yellow food dye was flown through the TD shaped channels, both at a flow rate of 75 µL/hr. A control sample to demonstrate the resulting color change when mixing is complete was run through the herringbone mixing region, and involved mixing equal volumes of blue and yellow dye off-chip before being flowed on-chip. Arrows (in *vrSPE elute/SPE load*) and corresponding images 1-5 show fluid moving progressively further down the herringbone region as mixing occurs in each still image.

3.2.7 SPE Procedure, Single-phase vrSPE

A single-phase vrSPE microdevice[15] was packed with MagneSil™ and conditioned with 6 M GuHCl, pH 6.1 for 10 min at a flow rate of 15 µL/min. The lysed whole blood indigo sample, prepared as described in *Sample preparation*, was loaded onto the vrSPE at 15 µL/min. The DNA was then eluted from the phase with 10 mM

Tris, pH 8 at 15 µL/min while 5 µL fractions were collected and subsequently amplified using the AmpFℓSTR ® Identifiler® amplification kit. The amplified product was then separated and detected using an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

3.2.8 SPE Procedure, Microchip-SPE (µSPE)

A single-phase µSPE device was packed with 30 µm silica and conditioned with 6 M GuHCl, pH 6.1 for 10 min at a flow rate of 4.8 µL/min. The lysed whole blood, semen, semen stain, or whole blood indigo sample, prepared as described in *Sample preparation*, was loaded (40 µL) onto the µSPE device at 4.8 µL/min. A wash with 80% IPA (IPA/H_2O v/v) was completed for 5 min, to remove cellular and extracellular debri, at 4.8 µL/min. The DNA was then eluted from the phase with 10 mM Tris, pH 8 at 4.8 µL/min while 2 µL fractions were collected and subsequently amplified using the AmpFℓSTR® COfiler® (for whole blood, semen, and semen stain samples) or AmpFℓSTR® Identifiler® (whole blood indigo sample) amplification kit. The amplified product was then separated and detected using an ABI 310 Genetic Analyzer.

3.2.9 Elution Profile and Optimization of Extraction Conditions

A sample containing hgDNA (0.2 ng/µL) in 6 M GuHCl, pH 6.1 was prepared and loaded onto the vrSPE-SPE device and DNA extracted following the *SPE procedure*. During elution, ten 2 µL fractions were collected and analyzed using a fluorescence assay and a NanoDrop 3300 Fluorospectrometer (NanoDrop, Wilmington, DE). For the linear flow rate study, 500 µL of a 0.1 ng/µL hgDNA sample in 10 mM MES, pH 5 was loaded

onto the vrSPE portion of the vrSPE-SPE device while 50 µL fractions were collected at R3 (Figure 5) and analyzed using a fluorescence assay. The decreased flow rate study using the single-phase vrSPE device used the same sample type as the linear flow rate study. The purified DNA was eluted at a flow rate of 2.5 µL/min while 5 µL fractions were collected and analyzed using a fluorescence assay to quantitate the amount of DNA in each fraction.

3.2.10 On-chip Mixing Studies

Using two 1 mL SGE gas-tight syringes and a Harvard Apparatus model 22 syringe pump (Harvard Apparatus, Holliston, MA), blue food dye was flowed through the vrSPE channel while yellow food dye was flowed through the teardrop channels (TD) (Figure 5B), both at 75 µL/hr. During flow, the herringbone mixing region was visualized and recorded using a Leitz microscope with a CCD camera connected to a Trinitron screen (Sony) and DVD recorder (Panasonic). Once the mixing was recorded, the videos were analyzed using Adobe Premiere Pro software.

3.2.11 Integrated vrSPE-SPE Extraction and Conventional Off-chip Amplification of hgDNA

The device and solid phase were prepared as described above. The 500 µL (containing 100 ng hgDNA) hgDNA load sample in 6 M GuHCl, pH 6.1 was loaded onto the vrSPE bed at 15 µL/min. The load was followed by the vrSPE-SPE procedure described above in *SPE Procedure, dual-phase vrSPE-SPE*. Upon elution of DNA from the chitosan bed, ten 2 µL fractions were collected in PCR tubes.

The extracted DNA samples were mixed with PCR master mix containing $MgCl_2$, dNTPs, forward and reverse primers (5'-AGTTCCTCAAGGCAGGGAAG-3' and 5'-CTCAGCTGCACTGTCTTCAG-3', MWG BioTech, High Point, NC) for a fragment of the gelsolin gene, PCR buffer, BSA, *Taq* DNA polymerase, and autoclaved water and amplified using standard PCR protocols developed in lab and a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA) for thermal cycling. The thermal cycling protocol involved an initial denaturation step of 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s/64 °C for 30 s/72 °C for 30 s, and then a final extension of 72 °C for 2 min. Using a DNA 1000 Series II kit and a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA), the PCR products were separated and analyzed.

3.2.12 vrSPE-SPE Purification of hgDNA from Dilute Whole Blood, Blood Stain, Dilute Semen, and a Semen Stain

The vrSPE-SPE device was prepared as previously described. Dilute whole blood, blood stain, dilute semen, and a semen stain were prepared using the methods described in *Sample Preparation*. The samples were then loaded onto the device followed by the extraction procedure described above in *SPE procedure, dual-phase vrSPE-SPE*. Upon elution, a 10 µL fraction was collected and subsequently amplified using the AmpFℓSTR® COfiler® amplification kit. The amplified product was then separated and detected using an ABI 310 Genetic Analyzer.

3.2.13 vrSPE-SPE Purification of hgDNA from an Inhibited Sample

The vrSPE-SPE device was prepared as previously described. A sample of hgDNA containing 8 mM indigo dye was prepared using the methods described in *Sample Preparation*. The sample was then loaded onto the device, followed by the extraction procedure described above for vrSPE-SPE. Upon elution, a 10 µL fraction was collected and subsequently amplified using the AmpFℓSTR® Identifiler® amplification kit. The amplified product was then separated and detected using an ABI 310 Genetic Analyzer.

3.3 Dual-domain, Microchip-based Process for Volume Reduction Solid Phase Extraction

A two phase DNA extraction system has already been described by Wen et al.[16], but it is important to point out the distinctions. First, the Wen et al. system aimed to exceed the 0.2 µL volume limit (typical capacity for whole blood) that µSPE chips could accommodate, beyond which the DNA binding capacity of a silica sol-gel phase was exceeded. Second, knowing that the DNA binding capacity of the sol-gel was compromised by the overwhelming mass of protein in whole blood, it utilized a primary extraction phase of C18, which bound 70% of the protein in the sample but <1% of the DNA, allowing for efficient extraction of DNA on the sol-gel phase. The overall result was that as much as 10 µL of whole blood could be loaded for effective DNA extraction.

The current two phase system described here contains two orthogonal DNA binding phases and was designed to handle large volume samples (i.e. 500 µL, 1 mL), remove PCR inhibitors and provide high quality, concentrated DNA for downstream

PCR. The primary extraction is on a silica bead phase that binds DNA in the presence of a chaotrope (guanidine) – this is standard DNA extraction chemistry universally accepted for tube-based and automated extractions, as well as having been described on microchips.[17] The secondary extraction is achieved with an anion-exchange phase that binds DNA under aqueous conditions (i.e., without chaotropes or organic solvents, such as isopropanol). This is achieved with the chitosan-derivatized silica beads described previously by Cao et al.[8] who demonstrated the reversible binding of DNA triggered by a simple change in the buffer pH. This system is ideal for extracting DNA from large volume forensic samples that may contain inhibitors because the first DNA binding phase provides a volume reduction, as previously shown by Reedy et al.[15] (Chapter 2), and also potentially binds inhibitors, such as indigo, that would co-elute with the purified DNA because they may not be effectively removed during the wash step, while the second phase not only binds DNA but also acts as an inhibitor removal phase by not binding indigo [due to both being positively charged at pH 5 causing the two (chitosan and indigo) to repulse one another], and eliminating reagent-based PCR inhibitors due to the aqueous nature of the phase.

3.3.1 vrSPE-SPE Extraction Domain Characteristics and Phase Selection

The design for the vrSPE-SPE integrated device leverages various attributes of previous device designs for single-process vrSPE[23] and SPE[27] microchips. Chapter 2 and Reedy et al.[23] showed that a 50-fold volume reduction and 15-fold DNA concentration enhancement could be achieved using the vrSPE device packed with a silica phase for DNA purification but would inherently allow for the binding, and subsequent co-elution

of commonly encountered PCR inhibitors, such as indigo dye and humic acid, with the released DNA. Also, the chemistry involved with the silica phase utilizes two PCR inhibitors (IPA and GuHCl) that can still be present in residual yet inhibitory amounts during DNA elution. A two-phase system was, therefore, developed to allow for downstream elimination of PCR-inhibitory compounds and the silica phase was incorporated into the integrated vrSPE-SPE design as the first SPE domain for volume reduction (Figure 4A). For the second SPE domain, a smaller channel design was used with dimensions similar to previously described μSPE devices that used chitosan as a solid phase for the successful purification of DNA from biological samples.[8, 27] Extraction efficiency for DNA on a SPE device using chitosan as a solid phase is typically 47 (± 4.2)%[27] while an efficiency of 34.7 (± 4.0)% is seen using the vrSPE device for purification of DNA.[23] These studies suggested that an integrated microdevice combining these two orthogonal processes would result in an overall extraction efficiency of ~25%. Although the overall extraction efficiency is expected to be lower than the extraction efficiencies that can be obtained using a single-process individual SPE device, it was hypothesized that the use of two orthogonal SPE domains, with differing binding chemistries, on a single device would provide the benefit of increased concentration enhancement of elution fractions for downstream PCR. In addition, it was expected that the volume reduction achieved with the use of a single-process vrSPE[23] method would improve further by coupling with an additional SPE method, increasing the possibility for downstream integration with microchip-based PCR. Most importantly, it was thought that two phases with different binding chemistries would improve removal of common PCR inhibitors, particularly those involved with the silica-based extraction process. As

detailed later, the greatest advantage of the integrated vrSPE-SPE system is the out-performance of the device over a single-phase μSPE method, as well as the single-process vrSPE method for removing indigo, a forensic PCR inhibitor, from a sample prior to STR analysis.

3.3.2 Mixing and Focusing Architecture

In developing the integrated design, certain obstacles had to be overcome in order to ensure complete functionality of the device. Two structures that were necessary for the functionality of the device were a mixing region, using herringbone structures (Figure 4A expanded image), and teardrop-shaped buffer addition channels to provide a fluidic focusing effect (Figures 4A, B and Figure 5). Flow in microfluidic channels is normally characterized by low Reynolds (Re) numbers, which are related to the average flow speed and dimensions of the channel. At low Re numbers, flow within the channels is laminar where mixing only results due to diffusion, requiring a lengthy amount of time for complete mixing to occur.[28] Due to this, a mechanism must be built in or used with microfluidic devices to induce chaotic mixing or turbulent flow of solutions. Various forms of mixers have been developed for microdevices, both in passive and active forms, where the mixer is an external component or a built in moving part (active), or is simply built into the microfluidic architecture of the design (passive).[28] Although both have been shown to work, incorporation of a passive mixer would allow for more simplistic device operation.

Therefore, in this work, herringbone structures (passive mixer), developed by Stroock et al.[28], are built into the device design and used to facilitate mixing by

decreasing the length that diffusion must occur in. This decrease in length is a result of the design of the herringbone structures, which once fabricated in the device design, create ridges, which solution flows along (due to the lower resistance than flowing perpendicular to the ridge structures), and then circulates back across the top and over the cross section of the channel, forcing the fluid to the center of the channel and facilitating mixing.[28]

As previously stated, mixing is essential in this device design, as during the extraction process, buffer must effectively elute DNA from the silica bed (vrSPE domain) and this same eluent must also be loaded onto the subsequent chitosan SPE bed. To accomplish this, a loading buffer compatible with chitosan chemistry must be flowed simultaneously with elution buffer from the silica domain and be allowed to mix with the silica phase eluate, automatically adjusting the pH to allow for eluate DNA binding to the chitosan phase. In addition to the herringbone structures, the teardrop (TD)-shaped design of the buffer addition channels surrounding and intersecting the vrSPE channel (Figure 5A) was critical in the mixing process of buffer and eluate because it also helped to create a shorter diffusion distance of eluted DNA into the buffer on each side of the eluate stream compared to a design with side-by-side laminar flow streams of eluate and buffer.[28] The teardrop design also aided in mixing eluate and buffer while flowing over the herringbone (HB) structures. While the herringbone structures work to mix solutions by narrowing the flow, as described above, the angle of the structures can also push the main flow of solution out to the sides. By flowing buffer in on both sides of the eluate stream over the herringbone structures through the teardrop channels, the buffer and eluate can mix more efficiently. If a design was instead used with buffer and eluate

flowing over the herringbone region side-by-side as two laminar flow streams, some eluate would be continually forced to the sides of the herringbone structures preventing effective mixing with the buffer.

3.3.3 Mixing Evaluation Study

The fluid dynamics of the 'mixing region' is detailed in Figure 5 which shows how mixing of the vrSPE (silica) eluate with loading buffers for the chitosan phase was assured by the herringbone structures incorporated into the design during syringe-driven flow from separate inlet reservoirs[28]. The two solutions involved in this portion of the chip, eluted sample DNA in 10 mM Tris, pH 8 and 50 mM MES, pH 3.5 aqueous loading buffer, must be comprehensively mixed prior to the chitosan phase in the SPE domain. Insufficient mixing between the vrSPE eluate and the pH 3.5 MES buffer within the HB region of the device will lead to pH heterogeneity in the load solution for the chitosan, decreasing the efficiency with which DNA eluting from the silica will interact and bind to the chitosan. The herringbone design used to promote mixing was first reported by Stroock et al.[28] who showed that the design could be used to achieve passive mixing on a microdevice at a low Reynolds number. The effectiveness of this on-chip passive mixer as implemented in this device design is illustrated by the photomicrographs in Figure 5B and was tested using yellow dye infused through the teardrop-shaped channels (R1), while blue dye was infused through the vrSPE channel (R2). Videos and still images captured at various locations down the HB mixing region illustrate the mixing of the blue and yellow dyes, with the expected green color observed in the fluid reaching the end of the herringbone mixing region, indicating that effective mixing has been achieved (Figure

5B, panels 1-5). For visual calibration purposes, a control sample (Figure 5B) was created with equal quantities (1:1 vol/vol) of blue and yellow dye mixed off-chip and flowed through the chip architecture. The control sample visually matches well with the color of the fluid in panel 5, suggesting that the herringbone structure provided sufficient mixing of solutions input from the vrSPE channel and the flanking TD channels.

3.3.4 Optimization of vrSPE-SPE Extraction Conditions and Elution Profile

In addition to ensuring functionality of the device architecture and mixing, it was important to determine the flow rates through various parts of the device and the conditioning required for each phase prior to loading the DNA sample. Previous work using chitosan-coated silica beads for DNA purification in a microdevice in the Landers lab demonstrated that a linear flow rate of 0.67 mm/sec was optimal for DNA binding, opposed to a linear flow rate of 0.94 mm/sec for silica beads. This result was confirmed with preliminary experiments when hgDNA (in a 500 μL sample) was loaded, while fractions were collected at the outlet, onto 5 μm chitosan-coated silica beads packed in the vrSPE channel of the vrSPE-SPE device. The amount of DNA in each load fraction was quantitated using a fluorescence assay. Figure 6 shows load profiles for linear flow rates of 0.94 and 0.67 mm/sec. It can be seen in the load profile for 0.94 mm/sec that DNA is clearly not binding to the phase and is being lost during this load step. In comparison, little to no DNA is lost during the load when a linear flow rate of 0.67 mm/sec is used, confirming that a slower linear flow rate is necessary when using chitosan-coated silica beads. For this reason, a linear flow rate of 0.67 mm/sec was used for all DNA purification on chitosan-coated silica beads.

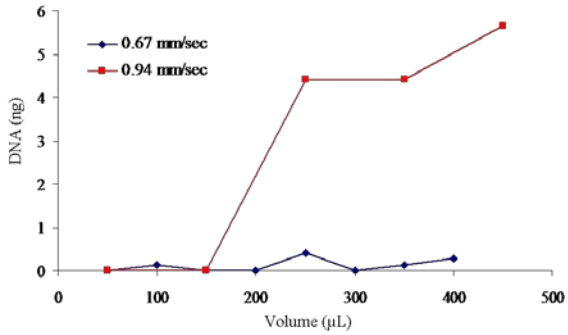

Figure 6. Preliminary load profiles generated when a sample containing hgDNA in 10 mM MES, pH 5 was loaded onto 5 µm chitosan-coated silica beads within the vrSPE channel of the vrSPE-SPE device, while fractions were collected at R3 (Figure 4). The increase in DNA seen for 0.94 mm/sec indicates insufficient DNA binding, while little to no DNA is lost with a linear flow rate of 0.67 mm/sec.

Having determined that a linear flow rate of 0.67 mm/sec (corresponding to a volumetric flow rate of 5 µL/min based upon the cross-sectional area of the SPE portion of the vrSPE-SPE device) was optimal for DNA binding to chitosan, it was necessary to ascertain whether this flow rate could be used during the integrated vrSPE-SPE purifications. The design of the vrSPE-SPE device (Figures 4 and 5) requires that two flow streams (from vrSPE and teardrop-shaped channels) must combine to result in a flow rate of 5 µL/min at the chitosan phase. To achieve this, a volumetric flow rate of 2.5 µL/min was used for both the elution step of the vrSPE, and delivering chitosan load buffers through the teardrop-shaped channels. Previous work by Reedy et al.[29] utilized an optimal volumetric flow rate of 15 µL/min for the vrSPE elution step. It was necessary to establish whether a flow rate of 2.5 µL/min, in comparison to 15 µL/min, would result in the same extraction efficiency and elution profile shape when DNA was purified on the single-phase vrSPE (as this device was used for method developments that were then implemented on the vrSPE-SPE device). A large volume sample (500 µL) containing hgDNA was loaded onto the single-phase vrSPE device and extracted as

described in the *SPE Elution Profile* section of the *Materials and Methods*. Ten 2 µL fractions collected during the final elution step (at 2.5 µL/min) were analyzed with a fluorescence assay to determine the quantity of DNA in each elution fraction. Figure 7 demonstrates that a reproducible elution profile and shape is still obtained with a corresponding extraction efficiency of 41.5 (±1.5)%, which is statistically similar (t-test, 95% confidence interval) to that previously reported, 34.7 (±4.0)%[29] (Chapter 2), at a flow rate of 15 µL/min. This demonstrates that a flow rate of 2.5 µL/min can be used effectively for the elution of DNA from the vrSPE phase with no adverse affects seen on the shape of the elution profile.

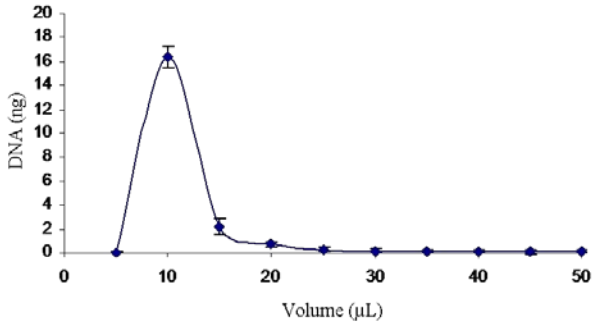

Figure 7. Elution profile (n = 3) of prepurified hgDNA using the single-phase vrSPE device with an elution flow rate of 2.5 µL/min and a corresponding extraction efficiency of 41.5 (±1.5)%.

In addition to optimizing the flow rates used during purification, it was important to determine how long each phase needed to be conditioned prior to loading the DNA sample onto the device. The optimal condition time had previously been investigated and determined for the vrSPE phase with silica-coated paramagnetic particles (Chapter 2) but the optimal condition time for the chitosan phase had not yet been explored. As previous work with a chitosan solid phase had used a 10 min condition step with 10 mM MES, pH 5[27], this condition time was investigated first. Following the extraction procedure

described in the *Materials and Methods*, the vrSPE phase was conditioned with 6 M GuHCl, pH 6.1 for 10 min followed by the chitosan SPE phase for 10 min with 10 mM MES, pH 5 at 5 µL/min. A large volume sample containing prepurified hgDNA was then purified using the vrSPE-SPE device and the amount of DNA in each elution fraction determined with a fluorescence assay. Figure 8 shows the elution profile that resulted with larger error bars and an EE of 17.8 (±1.0)%, which is lower than the hypothesized EE for the vrSPE-SPE device, ~25%. Additionally, the error bars that were seen in Figure 8 were also larger than expected and were thought to be a result of the insufficient conditioning of the chitosan phase.

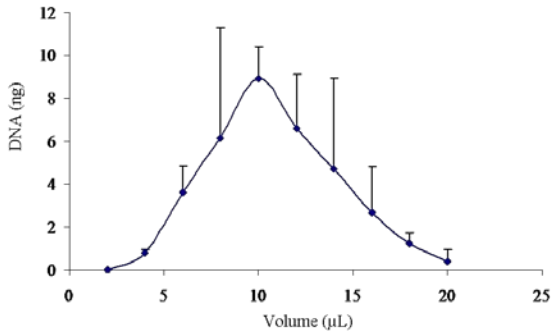

Figure 8. Elution profile (n = 3) of prepurified hgDNA purified using the vrSPE-SPE device with a 10 min condition of the chitosan phase within the SPE channel resulting in an extraction efficiency of 17.6 (±1.0)%.

It was hypothesized that increasing the conditioning time for the chitosan phase may be necessary to provide a higher EE, as it had already been seen that varied conditions (i.e., flow rate) were necessary for efficient DNA purification using chitosan. To investigate this, an extraction was performed where the chitosan phase was conditioned for 30 min prior to loading a 500 µL solution containing hgDNA onto the vrSPE-SPE device. DNA was then extracted as described in the *Elution Profile and Optimization of Extraction Conditions* section of the *Materials and Methods*. Ten 2 µL

fractions collected during the final elution step were analyzed with a fluorescence assay to determine the quantity of DNA in each elution fraction. The elution profile in Figure 9A was generated from three replicate extractions on this chip and the reproducibility seen in the shape and extraction efficiency [28.1 (±1.3)%]. Additionally, the improved extraction efficiency over that obtained with a 10 min chitosan conditioning step [17.8 (±1.0)%], which was found to be statistically different based upon a t-test (95% confidence interval, p = 0.05), demonstrates that a 30 min condition of the chitosan phase is optimal for the functionality of the device. Furthermore, the reproducibility and EE seen is indicative of sufficient mixing through the herringbone region. Previous work using a different device design has shown that insufficient mixing of eluate and loading buffer for a second SPE phase leads to irreproducibility in elution profile shape and DNA recovery (data not shown).

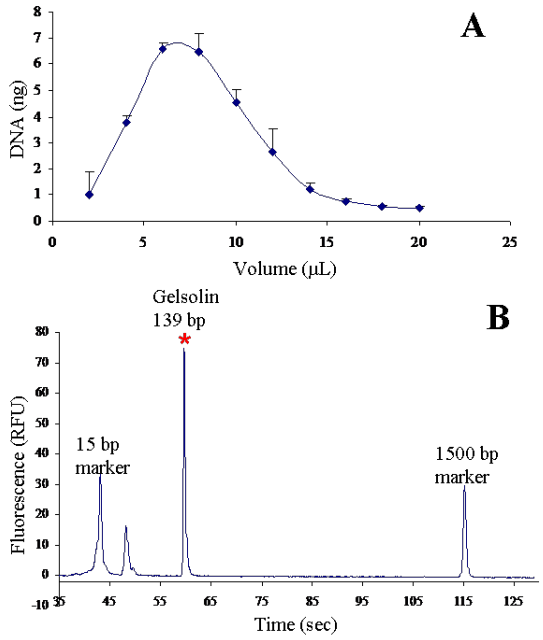

Figure 9. A) Elution profile (average of n = 3) from the extraction of prepurified hgDNA using the vrSPE-SPE device. B) Electropherogram of an elution fraction from the extraction of prepurified hgDNA using the vrSPE-SPE device. A 139-bp amplicon, representative of a fragment of the gelsolin gene, is present.

It should be noted that 80% of the eluted DNA is recovered in the first 10 µL, providing a ~50-fold reduction (Table 1) in the volume containing the vast majority of the DNA, and a 14-fold concentration enhancement when compared to the original sample. The volume decrease and concentration enhancement provided by the vrSPE-SPE device presents an advantage over other SPE methods that often provide little or no volume reduction and/or concentration enhancement. For example, conventional spin-column DNA extraction procedures typically reduce sample volume by 3.1-fold and provide a 5-fold enhancement in concentration. Previously developed single-domain chip-based extractions (µSPE) provide roughly comparable parameters, with a 5-fold concentration enhancement and a slightly improved volume reduction of 12.5-fold[2, 6]. This highlights the advantages of the vrSPE-SPE dual-domain extraction device over conventional tube-based extraction and chip-based µSPE methods. Compared to the single-phase vrSPE method, the volume reduction provided by the vrSPE-SPE device is equivalent (50-fold) while the overall concentration enhancement is slightly less (14-fold) than that obtained using the single-phase vrSPE method (15-fold). This is due to broadening of the elution profile seen with the use of chitosan as a solid phase[27] in the SPE domain of the vrSPE-SPE device. However, the concentration effect in each elution fraction is greater using the vrSPE-SPE method, showcasing a distinct advantage over the single-phase vrSPE method for integration with downstream PCR processing. In addition to enhanced elution fraction concentration, this dual-phase vrSPE-SPE approach has the advantage over single-phase vrSPE for potential seamless integration with downstream sample processing steps such as PCR due to the more PCR-compatible use of aqueous chemistry in the second stage of the vrSPE-SPE process. The reagent used to bind

nucleic acids to silica, GuHCl, which is also a PCR inhibitor, is flowed out R3 (Figure 5A) during vrSPE-SPE so as not to interfere with the second aqueous SPE process. In the single-phase vrSPE method, the GuHCl is flowed through the device to the outlet reservoir directly before nucleic acid elution, therefore, residually remaining present in elution fractions and causing PCR inhibition of the first elution fractions.

	EE (%)	Volume Reduction
Conventional	~70-80	3.1
µSPE	~40-50	12.5
vrSPE	34.7 (±4.0)	50
vrSPE-SPE	28.1 (±1.3)	50

Table 1. Extraction efficiencies and volume reductions seen with conventional (i.e. silica spin columns), µSPE, vrSPE, and vrSPE-SPE purification methods.

3.3.5 Amplification of hgDNA from vrSPE-SPE Purification

After demonstrating reproducibility of elution profiles obtained after fluorescence analysis using the vrSPE-SPE method for DNA extraction, further proof-of-principle experiments were conducted to determine whether DNA purified using the device was PCR-amplifiable. A 500 µL sample containing prepurified hgDNA was loaded onto the dual-phase volume reduction device and elution fractions were amplified off-chip using primers specific for amplification of a fragment of the gelsolin gene, a gene whose product is known to play an important role in regulating the length of filaments involved in cell structure, apoptosis, and cancer.[30, 31] Figure 9B shows the successful amplification of a 139-bp fragment of the gelsolin gene from a representative elution fraction. These results further indicate that sufficient mixing of the eluate and chitosan binding buffer occurred in the herringbone region using a representative biological sample. If

insufficient mixing of the two solutions occurred in the HB region of the device, little or no DNA would bind to the chitosan at the pH of the Tris buffer/eluate solution, and little to no DNA would then be released from chitosan during elution, likely resulting in reduced or failed downstream PCR amplification.

3.3.6 vrSPE-SPE Purification of hgDNA from Dilute Whole Blood and a Blood Stain

Once it had been established that prepurified DNA in large volume samples could be extracted, it was necessary to evaluate the ability of the device to extract DNA from a much more complex sample - whole blood – applicable to both the forensic or clinical arenas. To simulate a forensic sample solubilized from a surface using a large eluate volume, a 500 µL volume containing 4 µL of whole blood (0.28 ng/µL DNA determined from the white blood cell count of the sample) was loaded onto the vrSPE-SPE microdevice and purified elution fractions were collected for amplification with AmpFℓSTR® COfiler® amplification kit. The amplified product was then separated and analyzed on an ABI 310 Genetic Analyzer. Figure 10A shows a representative full STR (short tandem repeat) profile (one of n = 3 repeats) that resulted from the on-chip purification of this large volume, dilute blood sample. All 7 loci are present and 'callable' (using a 50 RFU threshold[32]) with all peaks above 500 RFU. In contrast, an STR profile obtained after extraction using a standard µSPE device and low-volume protocol, where 40 µL of the same load sample was loaded, shows that a full profile cannot be obtained (Figure 10B). These results illustrate that the lack of ability to load the entire 500 µL sample in a timely manner (~30 min for the dual-phase vrSPE-SPE device; would require ~2 hr using µSPE),[11, 17] causes a full STR profile to not be

attainable with the µSPE device. This limits the use of µSPE as a purification process in an integrated device for DNA analysis of whole blood. The poor performance of the µSPE device in comparison to the vrSPE-SPE device for the purification of DNA from whole blood is most likely due to the complex nature of this biological sample. Blood contains components other than DNA, e.g., proteins, cellular debris, and heme, which compete for binding sites on silica, leaving less available for DNA binding, and are known to inhibit PCR[21]. Utilizing the vrSPE-SPE device instead, even in the presence of these binding competitors and inhibitors, an amplifiable amount of DNA was successfully purified from whole blood. The two-phase vrSPE-SPE system provides the advantage of having a larger DNA binding capacity in the first stage of the device compared to a silica bed in a µSPE device. This larger capacity, in addition to a second purification domain in the vrSPE-SPE device, lessens interference from binding competitors and inhibitors present in a sample. The reproducible results above have proven the vrSPE-SPE method a viable alternative for the forensic and clinical communities for large volume, dilute whole blood purification.

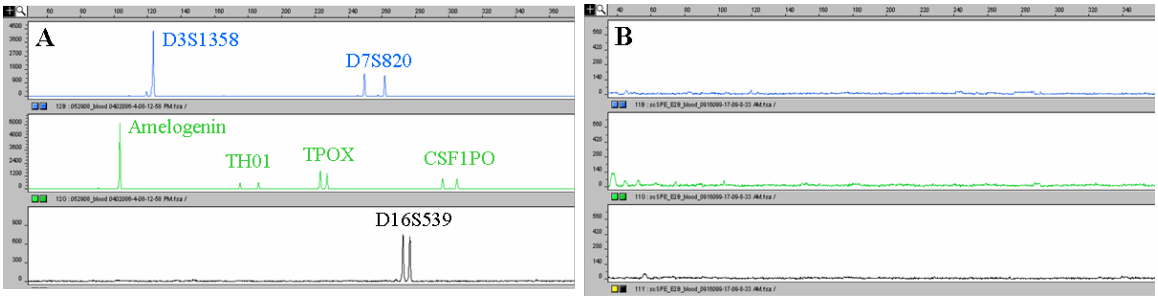

Figure 10. A) STR profile (representative of n = 3) resulting from extraction of hgDNA from a dilute, large volume whole blood sample on the vrSPE-SPE device. All loci are present. B) STR profile (representative of n = 3) after extraction of hgDNA from a dilute, large volume whole blood sample on a standard µSPE device.

To further test the vrSPE-SPE method, a more forensically-relevant sample type was used, blood stains. Biological stains are commonly encountered in forensic

casework, i.e., found at crime scenes and, as previously stated, can require milliliters (~0.5-2 mL) for solubilization or removal from a surface or substrate. To evaluate the vrSPE-SPE method, a dried 6 µL blood stain was prepared and the DNA purified from the sample using the vrSPE-SPE device. The purified DNA was amplified using the COfiler® PCR amplification kit and separated/detected using an ABI 310 Genetic Analyzer. Preliminary results from this sample type demonstrate that a full (7 of 7 loci) STR profile (Figure 11) was obtained, indicating that the vrSPE-SPE method can be used for more forensically-relevant sample types, such as blood stains. Due to the large volume necessary for removal of the sample from the cotton surface, processing with the vrSPE-SPE method is ideal because of the large volumes the device can accommodate, allowing for a shorter analysis time in comparison to µSPE methods. Additionally, as previously mentioned, the large binding capacity of the vrSPE phase ensures sufficient binding sites for the DNA as other sites may be occupied by cellular and extracellular material, i.e. heme, present in the blood sample. These results again demonstrate that the vrSPE-SPE device is a reliable purification method for the forensic community.

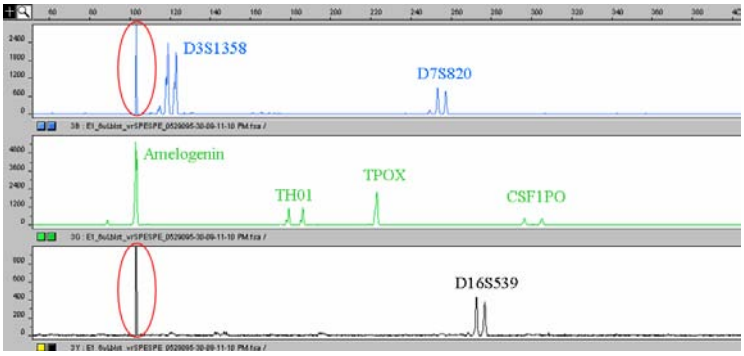

Figure 11. Full STR profile resulting from extraction of hgDNA from a blood stain using the vrSPE-SPE device. Peaks circled in red are due to pull-up from Amelogenin.

3.3.7 vrSPE-SPE Purification of hgDNA from Dilute Semen

The vrSPE-SPE method was next evaluated for the purification of DNA from dilute semen - another biological fluid commonly encountered in forensic cases, particularly those involving the analysis of sexual assault evidence. A 500 µL load solution into which 1.5 µL semen had been diluted (resulting in a final DNA concentration of 0.53 ng/µL) was loaded onto the device to simulate the volume of a sexual assault sample solubilized from a collection device (typically a cotton-tipped swab) used to collect the sample from the victim. The sample was processed using the vrSPE-SPE method, and elution fractions amplified using the AmpFℓSTR ® COfiler® kit prior to PCR product separation using an ABI 310 Genetic Analyzer. Figure 12A shows a full STR profile that was obtained after on-chip vrSPE-SPE purification of a large volume, diluted semen sample. A different sample (different semen donor) prepared in an identical manner was loaded onto a standard µSPE device, using the low volume protocol (40 µL) typically used with this device, and the resulting STR profile shown for comparison (Figure 12B). A full STR profile [all alleles callable (peak height above 50 RFU)] was also attainable after purification of a lower volume sample of DNA from semen using a standard µSPE device. As seen in this case, an equivalent STR profile resulted after purification of DNA from semen using either the vrSPE-SPE or a standard µSPE device. These results can be attributed to the decreased number of inhibitors present in semen samples in comparison to more complex blood samples, as used above.[21] It is more advantageous to use the vrSPE-SPE device when extracting DNA from more complex samples with larger masses of protein and cellular debris or inhibitors present, which will bind to the phase and reduce the number of binding sites

available for DNA[16], and is shown and detailed later when DNA from samples containing indigo, a PCR inhibitor, is successfully extracted. Importantly, however, there is no disadvantage to using the vrSPE-SPE device for less complex samples such as semen, as the results in Figure 12A, B demonstrate. These results further exemplify the spectrum of sample types that the vrSPE-SPE microdevice can accommodate. The concentration enhancement and volume reduction achieved with the vrSPE-SPE device is more conducive to integration with downstream analyses, unlike other μSPE methods that provide less concentration enhancement and volume reduction. Additionally, because of the small footprint of the microfluidic architecture, multiple vrSPE-SPE processes could be carried out in parallel in a forensic laboratory using either singular devices or perhaps a multiplexed device design, permitting higher sample throughput. Previously developed dual-stage purification devices have been more cumbersome to use due to larger footprints[16], rendering it more difficult to use more than one device at a time and impossible to multiplex into an easy to use, single device. The smaller footprint and potential for integration of the vrSPE-SPE method with microchip PCR[3, 18, 33] and microchip electrophoresis (ME) would further enhance the processing of evidence, allowing for complete sample analysis on a single device.[34]

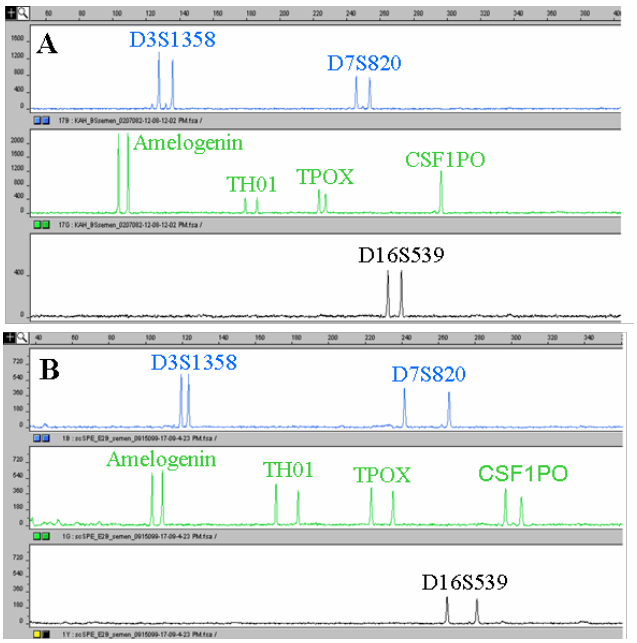

Figure 12. A) STR profile (representative of n = 3) resulting from extraction of hgDNA from a diluted, large volume semen sample on the vrSPE-SPE device. B) STR profile (representative of n = 3) resulting from extraction of hgDNA from a diluted, large volume semen sample on a standard µSPE device.

3.3.8 vrSPE-SPE Purification of hgDNA from a Semen Stain

Forensic samples encountered at a crime scene are typically in the form of a stain on a particular fabric or surface. To demonstrate the effectiveness of vrSPE-SPE analysis on a semen stain solubilized from a surface, better representing a mock crime scene sample, a dried semen stain (10 µL of semen) cut as a 0.25 x 0.25 cm square from a white cotton swatch was immersed in 6 M GuHCl, pH 6.1, loaded onto the vrSPE-SPE device, and processed using the large volume method, with the resulting purified DNA again amplified using the AmpFℓSTR® COfiler® amplification kit prior to analysis on the ABI 310 Genetic Analyzer. A full STR profile was obtained (Figure 13A) with all 7 loci present, and callable, demonstrating the effectiveness of the vrSPE-SPE method for the purification of DNA from a mock forensic sample. A sample prepared in an identical

manner was loaded onto a standard μSPE device, using the low volume protocol (40 μL), and also resulted in a full STR profile (Figure 13B). Although successful, a significant decrease in the peak height (RFU) of each of the alleles from the μSPE purified sample in comparison to the vrSPE-SPE purified sample was seen. This effect in peak height difference may be more pronounced when samples that contain a lower quantity of DNA template are encountered, potentially resulting in a blank STR profile. This demonstrates the superiority of the vrSPE-SPE method over low volume methods (μSPE) for the purification of DNA from mock forensic samples that require solubilization from surfaces resulting in a diluted, large volume sample. Along the same lines, a disadvantage typically seen with conventional evidential processing methods of sexual assault samples or diffuse stains, is the large volume of ~500 μL required to solubilize the samples from the substrate to ensure the greatest quantity of nucleic acids present in the solubilized sample. This large volume cannot be processed in a timely manner, and so only a small volume of the solubilized sample is analyzed. The results in Figure 13A demonstrate that use of the vrSPE-SPE device facilitates the processing of large volume samples, providing a solution to this commonly-encountered problem of large volume forensic samples. In addition, the vrSPE-SPE method is designed primarily for removal of inhibitors, *reagent*- and *sample-based* (indigo dye, which is discussed later), and for dilute, large volume samples for forensic applications. Therefore, although a minimal decrease in efficiency is seen (see Table 1), as would be expected by doing two purifications with their associated combined loss, the demonstrated removal of both *reagent*- and *sample-based* inhibitors is of greater value to the forensics community, as it results (regardless of the smaller amount of DNA present) in more amplifiable DNA and

therefore full STR profiles from the type of samples that are commonly-encountered in casework analysis.

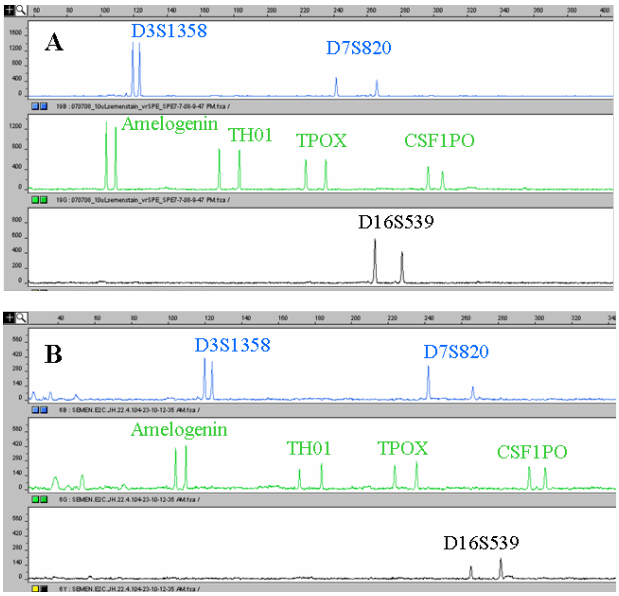

Figure 13. A) STR profile (representative of n = 3) resulting from extraction of hgDNA from a semen stain on the vrSPE-SPE device. B) STR profile (representative of n = 3) resulting from extraction of hgDNA from a semen stain on a standard µSPE device.

3.3.9 vrSPE-SPE for Removal of Indigo Dye

Perhaps the greatest advantage of the vrSPE-SPE method is the ability to remove potential PCR inhibitors that would not be as effectively removed using a standard microfluidic purification technique, such as those previously developed in our lab[2, 6] and by others[7]. To illustrate this, a sample spiked with indigo dye was processed using the vrSPE-SPE device as well as vrSPE and µSPE devices. Indigo is a PCR inhibitor commonly found in blue jeans, and has been included in studies testing the capability of different conventional STR amplification kits to amplify DNA in the presence of common PCR inhibitors.[24] Results from this work by Wang et al.[24] indicate that amplification of prepurified DNA samples using the AmpFℓSTR ® Identifiler® kit, which amplifies 16 loci, will result in a full profile with indigo dye concentrations up to 3 mM.

At 3 mM, loci drop out occurs and only partial profiles are attainable. The vrSPE-SPE integrated device was used to purify DNA from samples with concentrations of indigo dye $\leq$ 8 mM present in a 500 µL load solution containing 918 nL whole blood and amplified off-chip with the Identifiler® PCR amplification kit. A full STR profile with all 16 loci present and callable above 50 RFU resulted (Figure 14A) from all concentrations of indigo tested. The same load solution was loaded onto a single-phase vrSPE device as well as a µSPE device. With the vrSPE device, DNA from blood samples containing < 8 mM indigo dye were amplified using Identifiler®, and full STR profiles resulted. However, at a concentration of 8 mM indigo dye, allelic drop out occurred (circled in Figure 14B) leaving only a partial STR profile. Although this single-phase vrSPE device was able to remove a substantial concentration of indigo dye from the inhibited blood sample, an improvement in STR results obtained was seen when using the vrSPE-SPE device at the same indigo concentration. When 40 µL of the same sample containing 8 mM indigo was processed using the lower volume µSPE technique, all STR profiles resulted in a maximum of 9 callable alleles (of 29 expected alleles) above a 50 RFU threshold (Figure 14C), most likely due to the insufficient removal of indigo dye from the sample [not due to differences in extraction efficiency (EE); see Table 1]. These results demonstrate that the vrSPE-SPE device outperforms both vrSPE and µSPE, allowing for effective removal of PCR-inhibitory indigo dye from a sample due to the secondary, orthogonal chitosan phase that was utilized and essential in the removal of all indigo dye. During the vrSPE-SPE extraction, the indigo dye binds to the first silica phase (vrSPE) and is then co-eluted with the DNA from the phase. The indigo dye then flows through the secondary chitosan phase unretained due to the charge similarities between the

chitosan phase and dye at a pH of 5 (binding pH for DNA to chitosan) allowing for successful removal of all indigo dye from the purified sample. Additionally, in this study, the limit of indigo dye which it can accommodate and successfully remove has not yet been reached and is already almost 3x the concentration that can be tolerated in commercial STR amplification kits (e.g., Qiagen) without any purification. Use of the vrSPE-SPE device would greatly assist the forensic community, who commonly encounters samples inhibited by indigo dye found on material such as blue jeans, in removing PCR inhibitors from DNA samples and would allow for conclusive results to be obtained from such samples. Additionally, the vrSPE-SPE method could be extended to the removal of additional inhibitor types such as humic acid and calcium[35] and could demonstrate similar or even improved results. This is early evidence that a two-stage orthogonal purification can not only provide the benefit of handling large volume samples, but also improve results obtained when working with various types of inhibited samples.

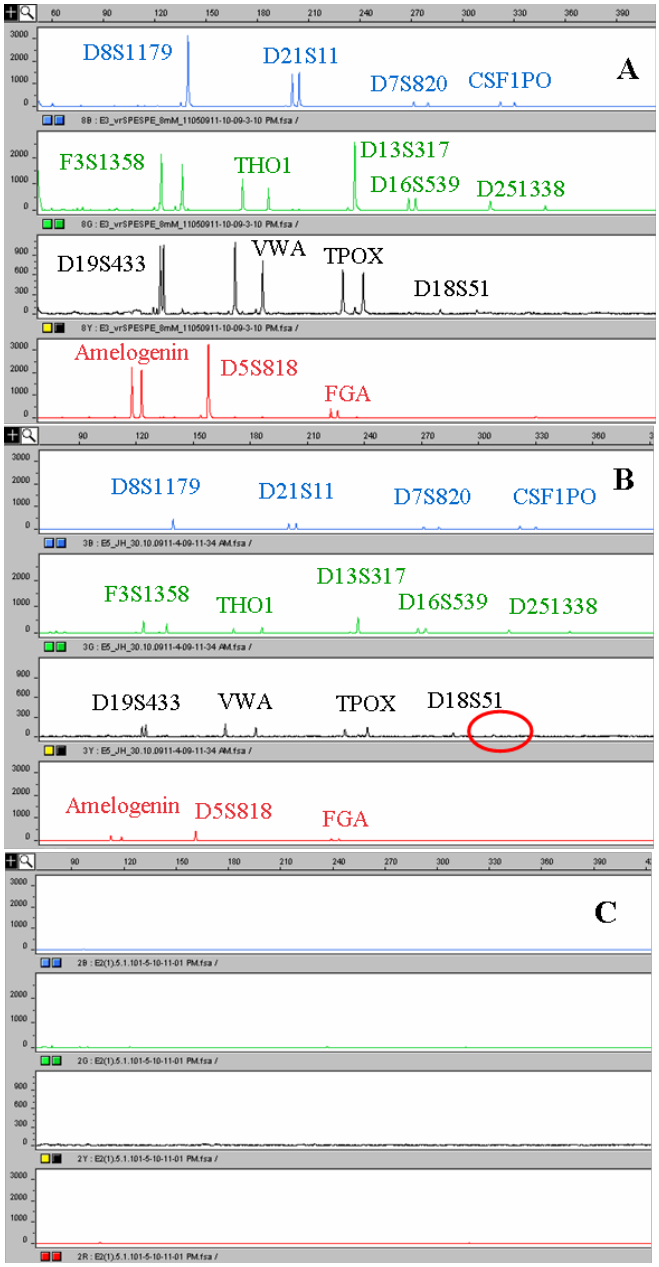

Figure 14. Comparison of STR results of amplified DNA that was purified from a diluted, whole blood sample spiked with indigo dye using three microfluidic devices. A) STR profile (representative of n = 3) resulting after extraction of hgDNA from a diluted, large volume whole blood sample containing 8 mM indigo using the vrSPE-SPE device. B) STR profile (representative of n = 3) resulting from extraction of hgDNA from a diluted, large volume whole blood sample containing 8 mM indigo using the single-phase vrSPE device. Allelic dropout is indicated by circle. C) STR profile (representative of n = 3) resulting from the extraction of hgDNA from a diluted, large volume whole blood sample containing 8 mM indigo using a standard µSPE device.

3.4 Conclusions

The use of a microfluidic device for the on-chip volume reduction, purification, and subsequent off-chip amplification of hgDNA from biological samples has been proven reliable and successful in this chapter. Dye studies were used to demonstrate the complete and sufficient mixing of reagents on the vrSPE-SPE device between two orthogonal SPE processes. A 14-fold concentration enhancement and 50-fold volume reduction was achieved through use of the vrSPE-SPE device. The extraction of hgDNA from diluted whole blood, blood stain, diluted semen, and a semen stain was shown, demonstrating the expanse of forensic and samples the integrated vrSPE-SPE device is capable of handling, and that the greater volume reduction and concentration effect achieved with the vrSPE-SPE device compared to other μSPE devices allows for successful off-chip STR amplification from samples that would otherwise be too dilute. Perhaps most advantageous, the vrSPE-SPE device was shown to more effectively remove inhibitory concentrations of indigo dye from blood samples compared to a single phase vrSPE or μSPE device. Both the silica and chitosan SPE domains are critical to the vrSPE-SPE device, allowing for rapid processing of large volume diluted samples as well as removal of PCR inhibitors. The first, larger silica bed allows for faster flow rates for rapid sample concentration, while the second chitosan bed allows for purification of nucleic acids free from reagent- and sample-based PCR inhibitors. Future work will involve application to additional sample types and other PCR inhibitory compounds that may be present in forensic, biological samples. A multiplex design containing multiple vrSPE-SPE systems will also be investigated, as parallel vrSPE-SPE processes run simultaneously would greatly increase evidential or clinical sample throughput.

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4. Development of Modular and Integrated Microfluidic Systems for DNA Identification Using Short Tandem Repeat Analysis

Microfluidic technology has been utilized in the development of a modular and an integrated system for DNA identification through STR (short tandem repeat) analysis, and was completed with a four and two person team, respectively. Development of the modular system allowed for a reduction in total analysis time from 6 hrs (conventional analysis) to less than 3 hrs. Results demonstrate the utilization of only microfluidic devices for the purification, amplification, separation and detection of 9 loci associated with a commercially-available miniSTR amplification kit commonly used in the forensic community. First, DNA from buccal swabs purified in a microdevice was proven amplifiable for the nine miniSTR loci via infrared (IR)-mediated PCR (polymerase chain reaction) on a microdevice. Microchip electrophoresis (ME) was then demonstrated as an effective method for the separation and detection of the chip-purified and chip-amplified DNA, with results equivalent to those obtained using conventional separation methods on an ABI 310 Genetic Analyzer. The 3-chip system presented here demonstrates development of a modular, microfluidic system for STR analysis, allowing for user-discretion as to how to proceed after each process during the analysis of forensic casework samples.

The partially integrated system involved a valveless SPE-PCR microfluidic device that was also developed for STR analysis of DNA from a biological sample. In contrast to the modular system which uses three separate devices, this device consists of fluidically connected SPE and PCR domains. Purification of DNA from buccal swabs and amplification using the integrated device is shown with a full STR profile (16 loci)

resulting. The 16 loci Identifiler® multiplex amplification was also performed using IR-PCR. Use of the integrated device provided an ~80-fold and 2.2-fold reduction in sample and reagent volumes consumed, respectively, as well as an ~5-fold reduction in overall analysis time in comparison to conventional analysis. Results indicate that the SPE-PCR system can be used for many applications requiring genetic analysis, and the future addition of microchip electrophoresis (ME) to the system would allow for the complete processing of biological samples for forensic STR analysis on a single microdevice. Development of both the modular and integrated microfluidic devices will be discussed in depth in this chapter.

4.1 Introduction

DNA identification via STR analysis is currently used in the forensic community to identify a victim or perpetrator of a crime, establish paternity, or identify remains.[1] The conventional analysis involves extraction and purification of DNA from a biological sample, which is then amplified in a multiplex PCR reaction. This multiplexed analysis works to statistically-reduce the probability of the analyzed DNA belonging to more than one individual based on the high discriminatory power of STRs for human identification[2] (Table 1). To perform amplification of STRs, numerous kits are commercially-available that allow for amplification of anywhere up to 16 loci (containing STRs) within the genome with the Identifiler® kit amplifying 16 loci (Table 1). The majority of kits available, i.e. Identifiler®, result in amplicon lengths anywhere from 90-475 base pairs (bp) and are used for amplifying purified DNA from a variety of biological samples (i.e., blood, semen). The inherent downfall of these kits is observed when degraded or

compromised samples are encountered, which often occurs in forensic casework, resulting in allelic or locus dropout (i.e., loss of loci in the amplified sample) of large bp amplicons due to the degraded nature of the DNA. To combat this problem, miniSTR kits have been developed by companies such as Applied Biosystems, including MiniFiler™, which use primers designed to anneal closer to the STR region than primers from other forensic STR amplification kits (Figure 1). This allows for amplification of shorter fragments, which increases the likelihood of obtaining a full DNA profile from degraded, forensic casework samples.[1] Frequently, MiniFiler™ is used in tandem with the Identifiler® kit where amplification is carried out with Identifiler® first and, if a partial (less than 16 loci) profile results, MiniFiler™ is used to hopefully obtain the number of loci needed to upload the STR profile to CODIS.

	Number of STR Loci Amplified	**Probability Match**
COfiler®	7	2.0×10^{-7}
Profiler®	10	9.0×10^{-11}
MiniFiler™	9	1.97×10^{-10}
Identifiler®	16	7.2×10^{-19}

Table 1. STR PCR amplification kits with the number of STR loci each kit amplifies and the associated probability of matching another individual's profile. Probability match values from Butler, J. M. *Journal of Forensic Science* **2006**, *51*, 253-265 and Nakamura, S., Murakami, C., Maeda, K., Kobayashi, M., Irie, W., Wada, B., Hayashi, M., Sasaki, C., Furukawa, M., Kurihara, K. *Forensic Science International: Genetics Supplement Series* **2009**, *2*, 19-20.

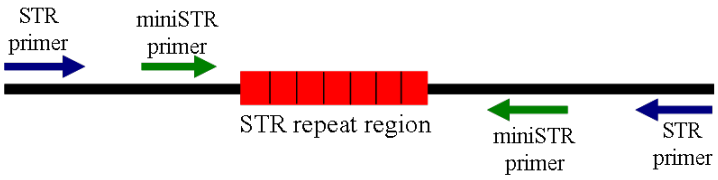

Figure 1. Schematic of the binding of longer STR fragment primers and miniSTR primers. MiniSTR primers bind closer to the repeat region resulting in a shorter amplified product.

4.1.1 Modular, Microfluidic STR Analysis System

Conventional STR analysis is a very time-consuming process, with substantial time allocated for solid phase extraction (SPE) (~1 hr), DNA quantitation (2-3 hr), PCR (~3.5 hr), and separation of PCR product (45 min/sample). The lengthy time required for STR analysis continues to contribute significantly to the backlog of DNA evidence which exists today.[3] A more rapid method for STR analysis, that also consumes a smaller volume of reagents, would greatly benefit the forensic community who currently utilize this technology. In the field of microfluidics, advances in speed of analysis and reduction of reagent consumption, which in turn decreases the overall cost per analysis, are well-known advantages. The application of microfluidic technology to STR analysis would expedite the process allowing for timelier sample analysis, while decreasing the backlog of samples. Additionally, microfluidic devices allow for sample analysis within a closed system, reducing sample transfer and centrifugation steps which could introduce contamination to the sample being processed. These advantages make the transition to a microfluidic STR analysis system a logical step.

Previous work has detailed the use of microfluidic devices for the silica-based solid phase extraction[3-6], amplification by PCR[4-7], and electrophoretic separation (ME)[8, 9] of genetic material, each individually and as a fully-integrated process, which is discussed later.[10] The advantages microfluidic technology can bring are quite clear in regards to STR analysis. Due to the reduced thermal mass of a microfluidic device, PCR via IR-mediated heating (IR-PCR) allows for much sharper temperature transitions, and therefore an ~2-fold reduction in analysis time, compared to conventional PCR. IR-PCR utilizes a focused heating source and cooling fan to perform more rapid temperature

cycling. This, in combination with the smaller volumes used in microfluidic devices compared to conventional PCR reaction volumes provides a substantial decrease in overall reaction time.[11, 12] Also, a 9-fold reduction in time is seen with the use of ME as opposed to conventional separation methods. The reduction in sample and reagent volumes necessary for analysis is inherent to microfluidic technology and, in combination with the greatly-reduced analysis time and potential utility of microdevices as a modular analysis system, will lend a distinct advantage to the forensic community. Specifically, as less sample and reagents volume are required for analysis when using microfluidics, a decrease in the cost of analysis per sample will occur, so a greater number of samples can be analyzed at the same cost. Throughput of sample processing in forensic labs will also increase due to the inherent decrease in analysis time seen with microfluidics, as less time is required for each analysis, helping to decrease the backlog of samples.

There are distinct advantages to both an integrated STR analysis microdevice, which are discussed later, and a modular-based microfluidic system. A modular system allows the forensic analyst a choice with how to proceed during each step of sample processing, which can be crucial to the successful analysis of forensic casework samples. The greatest advantage of a modular system would be the discretion the forensic analyst/examiner is allowed to have, which is standard in current forensic casework analysis, to determine the optimal method of analysis (i.e., adjustments to amplification protocols) for each step of the process depending upon what is known about the sample (e.g., whether it be degraded, pristine, or contain little DNA). Each process would be performed on separate microdevices so the analyst would also have the ability to take into account characteristics specific to each sample (conditions it was exposed to or found in)

and use that knowledge to determine whether a larger quantity of DNA (i.e. combine several elution fractions), from the microchip purification, needs to be added to the microchip PCR amplification master mix to obtain a successful result.

The work presented in the first portion of this chapter describes a modular, microfluidic STR analysis system. First, the reproducibility and characterization of the microfluidic solid phase extraction method is shown for the purification of DNA from buccal swab lysate. The use of microfluidic (μ) IR-PCR is demonstrated for miniSTR amplification of microchip-purified DNA from a buccal swab, followed by both conventional and microchip-based separation and detection. Finally, the complete STR analysis, from DNA purification and amplification to separation of PCR products is described with the use of three microfluidic devices, one for each process, in series. This demonstrates the first entirely microchip-based STR analysis to date, with an ~2.8-fold reduction in total analysis time.

4.1.2 Integrated Microfluidic STR Analysis System

The development of a forensic genetic micro total analysis system (μTAS[13]) requires the integration of multiple sample processing steps including SPE, PCR, separation and detection, all miniaturized on a single microdevice. Numerous single process microdevices have been developed, with only select focus on integration of multiple processes. One example of a totally integrated microfluidic system for genetic analysis was developed by Easley et al.[10], demonstrating sample-in, answer-out capability for single-plex amplification and detection for *B. anthracis* and *B. pertussis*. Most work toward development of an integrated system, specifically for STR analysis, has pertained

to the separation and detection of PCR products[14-19] and integration of PCR with ME[14, 20]. Some work has been completed for the integration of SPE with PCR, but the method still used conventional thermocycler heating and cooling, therefore, not harnessing the time-reduction potential of microfluidics.[21] As a result, it is important to continue the development of methods for integration of sample preparation (SPE) with the latter processes.

Silica has been well-characterized as a reproducible, reliable solid phase for the purification of both DNA[3-6, 13, 25-27] and RNA[22] from biological sources in a microfluidic device for clinical and forensic purposes. Previous work has detailed the first example of integrated sample purification utilizing silica as a solid phase followed by amplification on a single microdevice for forensic analysis.[23, 24] However, this work involved the use of a conventional thermocycler for PCR and relied on conventional thermal cycling times, therefore, not resulting in any decrease in analysis time. By incorporating non-contact, IR-PCR on a microdevice,[15, 31, 32] sample processing could be made significantly more rapid. Therefore, integration of both SPE and IR-PCR on a microfluidic device would provide a robust system for reproducible and more rapid STR analysis.

The experiments detailed below describe the first integration of SPE and PCR on a single device, utilizing non-contact, IR-mediated PCR for a multiplex amplification using commercially-available forensic STR amplification kits. Initial characterization of the SPE domain of two generations of an integrated device is shown, demonstrating the experimental timing necessary to trap the ideal elution fraction inside the PCR chamber of the device for amplification. The integration of SPE and PCR on a single device is first demonstrated for the analysis of miniSTRs from buccal swab samples using the

commercially available MiniFiler™ amplification kit. Optimization studies were performed to improve the device design, as well as to decrease amplification time using different DNA polymerases, and finally, SPE and IR-mediated PCR were performed on a single device, demonstrating the successful amplification of sixteen loci using the Identifiler® STR amplification kit in ~50 min. With further optimization and inclusion of microchip electrophoresis for separation and detection of the PCR products, a complete microfluidic system for STR analysis will soon be a reality, providing a fully-integrated, portable genetic analysis system for the forensic community.

4.2 Materials and Methods

4.2.1 Reagents

Hyperprep silica beads (15-30μm) were purchased from Supelco (Bellefonte, PA). Silica beads (5-15 μm) were purchased from Fuji Silysia Chemical Ltd (Aichi, Japan). Guanidine hydrochloride (GuHCl), 2-amino-2-(hydroxymethyl)propane-1,3-diol-HCl **(**Tris-HCl), isopropanol (IPA), ethylene diamine tetraacetic acid disodium salt (EDTA), hydrochloric acid, sodium hydroxide, urea, Takara SpeedSTAR™ HS DNA Polymerase, Fermentas PyroStart™ 2X Fast PCR Master Mix, and 2-(4-morpholino)-ethane sulfonic acid (MES) were purchased from Fisher (Fairlawn, NJ). Potassium chloride was purchased from Mallinckrodt Chemical Company (Paris, KY). SigmaCote® and N-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS) were purchased from Sigma-Aldrich (St. Louis, MO). Quant-iT™ PicoGreen® DNA reagent was purchased from Invitrogen (Carlsbad, CA). AmpFℓSTR® MiniFiler™ and Identifiler® amplification kits were purchased from Applied Biosystems (Foster City, CA). Human

genomic DNA (hgDNA) was purified from whole blood (University of Virginia School of Medicine from fully deidentified residual clinical specimens) in-house. Buccal swabs were obtained from anonymous donors through a University IRB-approved protocol. All solutions were prepared in Nanopure water (Barnstead/Thermolyne, Dubuque, IA). LPA-co-DHA (linear polyacrylamide copolymerized with dihexylacrylamide) was a gift from the lab of Dr. Annalise Barron.

4.2.2 Microchip Preparation

Microdevices were fabricated with borofloat glass (Telic Company, Valencia, CA) using standard photolithographic techniques[25]. The resulting channel dimensions of the SPE device (Figure 2A) were 1.5 cm effective length, 225 μm deep, a top width of 650 μm, and a bottom width of 200 μm. A 1.1-mm-diameter diamond-tip drill bit (Crystallite Corp., Lewis Center, OH) was used to drill access holes at both ends of the channel. A borofloat glass cover plate was cut to fit the device and thermally bonded to the etched bottom plate. The distance from the top of the weir to the cover plate was ~15-20 μm. Following fabrication, silica beads, 15-30 μm, were suspended in water and packed against the weir in the channel using vacuum. The channel was filled with new silica beads prior to each extraction.

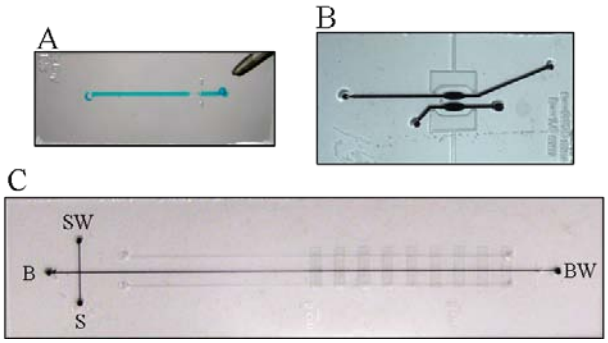

Figure 2. A) Microfluidic solid-phase extraction (SPE) device. B) Microfluidic polymerase chain reaction (PCR) device. C) Microfluidic electrophoresis (ME) device.

The PCR device (Figure 2B) channel dimensions were 200 µm deep with a 500 nL chamber volume. An additional chamber (reference chamber) was etched with the same dimensions parallel to the PCR chamber to allow for temperature monitoring during IR heating. A window, 100 µm deep, was etched around the PCR and reference chamber to reduce thermal mass after bonding each device to a glass cover plate. Access holes, 1.1 mm in diameter, were drilled at both ends of the channels. After fabrication, and prior to each amplification, the device was dried and passivated with SigmaCote®.

The channel dimensions for the ME device (Figure 2C) were 50 µm deep with widths of 50 µm and 150 µm at the bottom and top of the channel, respectively. A cross-tee structure was used with 0.6 mm sample (S), sample waste (SW), and buffer (B, buffer; BW, buffer waste) arms, and a 7.5 cm separation channel. Alignment channels were etched parallel to the separation channel. Access holes, 1.1 mm in diameter, were drilled at the end of each channel and cover plates were thermally bonded. The channels of the separation chips were prepared as previously described[26], but briefly were exposed to 1 M HCl for 30 min, rinsed with H_2O, and then exposed to poly(N-hydroxyethylacrylamide) (pHEA) for 30 min. The pHEA was then removed from the channels by vacuum. The proper Z-axis height was determined by filling the alignment

channel with 10 mM fluorescein solution. The Z-axis height was adjusted to maximize fluorescence signal from the chip at 540 nm while keeping the signal at a minimum at 700 nm where there is little fluorescence signal. A nitrogen pressure line (80 psi) was used to fill the channels with 4% (w/v) LPA-co-DHA while 100 µL of 7 M urea/1X TTE (49 mM Tris, 49 mM TAPS, 2 mM EDTA) buffer was added to the SW, B, and BW reservoirs, and the sample added to the S reservoir.

The resulting channel dimensions of the first generation integrated SPE-PCR device (Figure 3A) were: SPE – 1 cm effective length, 200 µm deep with a line width of 150 µm; side arm – 200 µm deep, waste arm – 50 µm deep, PCR – 200 µm deep with an ~500 nL chamber volume. An additional chamber (reference chamber) was etched with the same dimensions parallel to the PCR chamber to allow for temperature monitoring during infrared (IR)-mediated heating. A 1.1-mm-diameter diamond-tip drill bit was used to drill access holes at the SPE inlet, side arm (SA), and waste arm (WA) while a 0.7-mm-diameter diamond-tip drill bit was used to drill access holes for the outlet. A borofloat glass cover plate was cut to fit the device and thermally bonded to the etched bottom plate. The distance from the top of the weir to the cover plate was ~5-20 µm. A window, 100 µm deep, was etched around the PCR chamber to reduce thermal mass after bonding each device to a glass cover plate. After fabrication, and prior to each amplification, the device was dried and passivated with SigmaCote®. For SPE, 30 µm silica beads were suspended in water and packed against the weir in the channel using vacuum. When 15 µm beads were used, a frit of 30 µm beads, ~1 mm in length, was first packed against the weir before filling the remaining portion of the channel with 5-15 µm

silica particles. The channel was filled with new silica beads prior to each extraction to eliminate carry-over between samples.

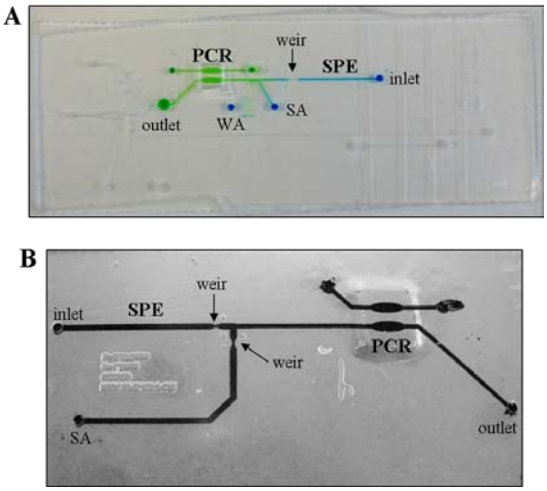

Figure 3. A) First generation SPE-PCR device design. B) Second generation SPE-PCR device design.

The second generation integrated SPE-PCR device (Figure 3B) channel dimensions were 225 µm deep with a bottom width of 200 µm for both the SPE and side-arm channels. The SPE channel and side-arm channel had an effective length of 1.5 cm and 1.8 cm, respectively. The channels surrounding the PCR chamber were 200 µm deep, with a bottom width of 75 µm and 1.5 cm in length on each side of the 500 nL PCR chamber. A 500 nL reference chamber was etched with a channel of 4 mm for thermocouple insertion on the sample outlet end of the device, and a 5 mm channel on the opposite side. A window, 100 µm deep, was etched around the PCR and reference chamber to reduce thermal mass after bonding each device to a glass cover plate. Access holes, 1.1 mm in diameter, were drilled at the ends of all the channels. After fabrication, and prior to each amplification, the device was dried and the surface passivated with SigmaCote®. The SPE channel was packed with new 30 µm silica beads prior to each extraction to eliminate carry-over between samples.

4.2.3 Apparatus

The microchip-solid phase extraction (SPE) apparatus consisted of a SP100i model syringe pump (WPI, Sarasota, FL) with a 250 μL Hamilton gastight syringe (Hamilton, Las Vegas, NV). The syringe was connected to the microchip using PEEK™ tubing and mini-tight fittings (Upchurch Scientific, Oak Harbor, WA).

The non-contact IR mediated-PCR system was built in-house as previously described in Easley, et al.[27, 28] and aluminum foil placed over the chambers was used to enhance heating of solution.

The ME system was based on the design used in previous work[19, 26]. The excitation source consisted of a multi-line argon ion laser (457, 488, 514 nm primary lines) from Dynamic Laser (Salt Lake City, UT). Steering mirrors were used to direct the incident beam to a 5x-beam expander (Edmund Optics, Barrington, NJ) and then to a first-order mirror. The beam was directed upwards through a 525 nm short pass (SP) dichroic mirror (Omega Optical, Brattleboro, VT) and into the rear aperture of a 40x LD Acroplan, 0.6 NA objective (Zeiss, Thornwood, NY), where it was then focused onto the microchannel. The emission line was collected through the same objective and reflected off the dichroic filter towards the acousto-optic tunable filter (AOTF). The excitation beam was spatially filtered first through a 5 mm diameter iris to match the diameter of the collimated beam exiting the objective; it was then focused with a 160 mm focal length lens onto the AOTF with another iris for further spatial filtering prior to the AOTF crystal. Downstream, the diffracted beams were collected and focused onto the photomultiplier tube (PMT) with two 35 mm achromatic lenses with a beam block placed

between them to spatially filter the undiffracted zero-order beam. Prior to reaching the PMT, the emission light passed through a 530 nm longpass filter (Omega Optical) to filter any stray excitation light that may have reached the detector. The detector was a side-on model PMT from Hamamatsu (model R3896, Bridgewater, NJ).

The output signal from the PMT was fed into a lock-in amplifier (LIA) which used a 1.2 kHz clock to modulate the electronic signal and the AOTF controller in order to modulate the optical signal prior to the PMT. The circuit also had an 80 Hz low-pass (LP) filter with 4-fold gain. The output from the LIA was fed into a 100 Hz LP filter with 10x gain (Avens, Plumsteadville, PA) which was then interfaced with the computer via a PCI data acquisition (DAQ) card (National Instruments, Austin, TX).

4.2.4 Conventional Solid Phase Extraction

Conventional SPE was performed using a Qiagen QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) according to the manufacturer's specifications.

4.2.5 Microchip Capacity Study Procedure

Solid phase extraction microdevices were filled with silica beads and conditioned with 6 M GuHCl, pH 6.1 for 10 min at 7.0 μL/min. A load solution containing hgDNA (30 ng/μL) prepared in 6 M GuHCl, pH 6.1 was loaded onto the device at 7.0 μL/min while 2 μL fractions were collected in PCR tubes for fluorescence analysis. Collected fractions were analyzed using a PicoGreen® fluorescence assay and a NanoDrop 3300 Spectrofluorometer.

4.2.6 Microchip Solid Phase Extraction Procedure for the Modular Microfluidic System

Solid phase extraction microdevices filled with silica beads were conditioned with 6 M GuHCl (pH 6.1) for 10 min at a flow rate of 7.0 μL/min prior to each extraction. The extraction procedure consisted of pressure-driven load, wash, and elution steps, each performed at a flow rate of 7.0 μL/min. First, buccal cells were eluted from a cotton swab (cells allowed to dry on swab for ≥24 hrs prior to elution) by vigorous mixing in 1 mL solution of 6 M GuHCl, pH 6.1 for 1 min. This solution was vortexed for 1 min, and 134 μL removed and diluted to 1 mL with 6 M GuHCl, pH 6.1. This solution was vortexed for 15 sec and 29.1 μL of this solution containing buccal cells was loaded onto the silica bed. A 70 μL wash of 80% IPA (80/20 (v/v) IPA/water) was flowed over the silica bed to remove any unbound material, proteins, or other potential PCR inhibitors. Finally, DNA purified from the buccal cell solution was eluted from the silica with water, and 1 μL fractions were collected in PCR reaction tubes for subsequent fluorescent or PCR analysis.

4.2.7 Microchip Solid Phase Extraction Procedure for the Integrated SPE-PCR System

The solid phase extraction domain of the first generation integrated SPE-PCR microdevice filled with silica beads was conditioned with 6 M GuHCl, pH 6.1 for 10 min prior to each extraction. All steps in the extraction procedure with the first generation SPE-PCR device were performed at a flow rate of 5.23 μL/min, except for the elution step which was performed at 2.5 μL/min. Buccal cells were eluted from a cotton swab by vigorously mixing in 1 mL of 6 M GuHCl, pH 6.1 for 1 min. This solution was vortexed

for 1 min, and 400 µL aliquoted and diluted up to 1 mL with 6 M GuHCl, pH 6.1. The resulting solution was vortexed for 15 sec and ~30 µL of this solution containing buccal cells was loaded onto the SPE domain. A 52 µL wash of 80% IPA (80/20 (v/v) IPA/water) was flowed over the silica bed to remove PCR inhibitors, cellular debris, and protein. The elution procedure consisted of the following steps: 2X PCR buffer was flowed in from the side arm (2.5 µL/min) and through the PCR chamber for 1 min 10 sec. The side arm flow was stopped, and 2X PCR buffer was flowed through the SPE inlet (2.5 µL/min) until 1 µL emerged from the waste arm [the PCR chamber outlet was simply closed off using PDMS (polydimethylsiloxane) press-sealed over the outlet]. At this point, the side arm syringe was again started (the PDMS was removed to allow flow through the outlet) and twenty 1 µL elution fractions were collected for fluorescence analysis. For integrated SPE-PCR analysis, water was flowed through the side arm during the load and wash at 5.23 µL/min with all flow exiting through the outlet. The waste arm and PCR chamber were rinsed with PCR master mix to eliminate any PCR-inhibiting compounds prior to elution. MiniFiler™ reaction mix was then flowed (through the SPE inlet) until 1 µL exited the outlet and the side arm was then flowed (containing PCR master mix) in addition to the SPE inlet simultaneously (at a 1:1 ratio) until 1 µL exited the outlet (until the optimal fraction of eluted DNA was located and held in the PCR chamber using PDMS press-sealed over the outlet).

With the second generation integrated SPE-PCR device, a slight variation of this procedure was performed. The flow rate of the condition, load, and wash step was increased to 7 µL/min, due to the larger channel dimensions of this device, as well as the flow rate of water through the sidearm during these steps. The silica bed was conditioned

for 10 min with 6 M GuHCl, pH 6.1 prior to each extraction. Cells from a buccal swab were eluted in 980 µL of 6 M GuHCl, pH 6.1, and 20 µL Proteinase K (20 mg/mL), incubated in a water bath at 56 °C for 10 min, and then 15.4 µL of the swab eluate was loaded onto the device. A 70 µL wash of 80% IPA was performed following the load. To elute the DNA, the flow rate of both the SPE and sidearm was also reduced to 2.5 µL/min (as performed on the first generation SPE-PCR device). MiniFiler™ or Identifiler® reaction mix was flowed through the SPE bed to elute the DNA at a 1:1 ratio with water simultaneously flowing through the sidearm while 1 µL fractions were collected at the outlet for fluorescence analysis. For integrated SPE-PCR analysis using this second generation device, all the steps of the procedure were kept the same as described above, except PCR master mix was flowed through the sidearm during the elution. In order to trap the optimal fraction within the PCR chamber during the elution, both the SPE and SA were flowed until 4.5-4.75 µL had been collected from the outlet, and both flows were then stopped.

4.2.8 Fluorescence Detection

Fluorescence detection was performed on a NanoDrop 3300 Fluorospectrometer (NanoDrop, Wilmington, DE) using a PicoGreen® fluorescence assay.

4.2.9 Non-contact IR-mediated PCR Procedure for the Modular Microfluidic System

Samples that were purified as described above in *Microchip Solid Phase Extraction Procedure* were added to a master mix developed in-house containing AmpFℓSTR® MiniFiler™ primers and reaction mix. The master mix was made to 5 µL

final volume, containing 2 µL MiniFiler™ reaction mix, 1 µL MiniFiler™ primers, 0.6 U/µL AmpliTaq Gold®, 0.4 µL H_2O, and the 1 µL elution fraction. This mix of PCR reagents and purified DNA was loaded into the PCR sample chamber while 1X PCR buffer was loaded into the temperature reference chamber. A thermocouple (Model T-240C, PhysiTemp Instruments, Inc., Clifton, NJ) was inserted into the reference chamber, and mineral oil was placed over each open reservoir to prevent sample evaporation. The filled PCR microchip was then placed on a stage seated over the IR lamp of the IR-PCR system. Thermal cycling was performed using the following protocol: 95 °C for 10 min (initial denaturation), 35 cycles of 94 °C for 5 s/59 °C for 120 s/72 °C for 60 s, followed by 72 °C for 10 min (final extension). PCR products were separated and analyzed on an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) or using the ME system previously described.

4.2.10 Non-contact IR-mediated PCR Procedure for the Integrated SPE-PCR System

IR-PCR was initially performed using an in-house developed master mix containing MiniFiler™ primers and MiniFiler™ reaction mix in a 1:1 ratio, and 0.12 U/µL AmpliTaq Gold® DNA polymerase. For PCR tests, this mixture was mixed 1:1 with template DNA, and for integration, was flowed in a 1:1 ratio with eluting DNA. Cycling conditions consisted of: 94 °C for 11 min (initial denaturation), 32 cycles of denaturing at 94 °C for 5 s/annealing at 59 °C for 120 s/extension at 72 °C for 60 s, followed by 72 °C for 10-20 min (final extension). When the amplification was reduced to 1.5 hours, the AmpliTaq Gold® was increased to 0.36 U/uL in the master mix, and the anneal and extension were reduced to 10 s each. The final extension was also reduced

down to 1 min. For rapid Identifiler® amplification, the master mix recipe consisted of 1X PyroStart™ Fast PCR Mix, 2 µL Identifiler® primers, 0.125 U/µL SpeedSTAR™ HS DNA Polymerase, and 0.5 mg/mL BSA combined in a 1:1 ratio with template DNA. Cycling conditions: 94 °C for 60 s (initial denaturation), 32 cycles of denaturing at 94 °C for 5 s/annealing at 59 °C for 10 s/extension at 72 °C for 20 s, followed by 72 °C for 60 s (final extension). PCR products from all amplifications were separated and analyzed on an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

4.2.11 Microchip Electrophoresis Procedure

Samples were desalted using Microcon® YM-30 centrifuge filters (Millipore, Billerica, MA) by adding 100 µL H_2O, sample, and 3 µL sizing standard to the filter and centrifugation for 5 min. The filter was inverted and centrifuged again for 2 min to collect the retentate into a vial containing formamide.

The separation microdevice was placed on the stage so the detection point was 6.5 cm from the cross-tee. Electrodes were placed in the reservoirs and injection voltages of -185 V at the S reservoir and 185 V at the SW reservoir were applied for 135 s. A pullback scheme was used for the separation voltages. Separation voltages were as follows: S and SW 65 V, B -125 V, and BW 2050 V. PCR products were detected as previously described in the thesis of Dr. Daniel Marchiarullo[26] and above in *Apparatus* for the ME system.

4.3 Development of the Modular, Microfluidic STR Analysis System

4.3.1 Defining the Capacity of the SPE Microdevice for hgDNA

Prior to use of the microchip SPE method for the extraction of DNA from buccal swab lysate, it was necessary to determine the capacity of 30 μm silica for binding hgDNA in this specific SPE device. Establishing the capacity of the phase for hgDNA would determine whether sufficient sample can be loaded onto the device, resulting in enough DNA recovered from the phase for downstream forensic STR amplification, which, to perform optimally, requires an input DNA amount of 0.25 – 0.75 ng.[29] Along the same lines, the recovery of sufficient quantities of DNA also depends upon the number of binding sites available to DNA, as the cellular debris present in the sample will also occupy some available binding sites. A hgDNA (30 ng/μL) sample in 6 M GuHCl, pH 6.1 was loaded onto the SPE microdevice with 2 μL fractions collected and subjected to fluorescence analysis to quantify the DNA in each fraction. From this, a breakthrough curve was generated (Figure 4A) and a first derivative approximation was fit to the data. The inflection point (that corresponds to the capacity of the silica phase) was 290 (± 35) ng, which is greater than that shown with past SPE devices[30] due to the larger channel dimensions (650 μm width vs 425 μm), and, therefore, increased amount of silica phase packed in the channel. A capacity of ~290 ng DNA is sufficient for the purification of DNA from buccal swab lysate (~780 cells/μL, determined by counting using a hemacytometer, with 6.25 pg/cell[2]), as it has been shown previously that the silica used in this work does not permit 52% of proteins present in the sample to bind during the load step[31]. Due to this, an adequate number of DNA binding sites should be left for DNA binding, ensuring enough DNA for downstream amplification.

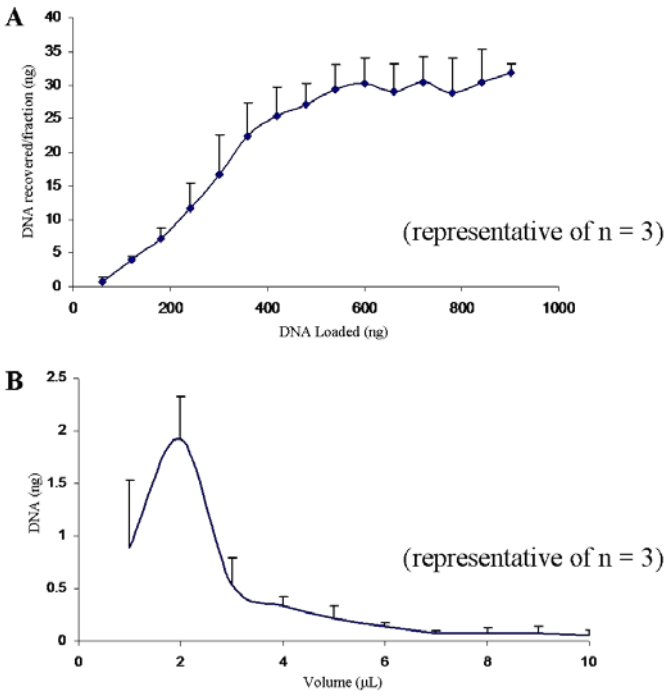

Figure 4. A) Breakthrough curve for capacity study of 30 µm silica for human genomic DNA using the µSPE device. B) Elution profile of DNA extracted from buccal cells using the µSPE device.

4.3.2 Microchip-based SPE of Buccal Swab Lysate

Elution profile studies were performed to provide insight into the amount of DNA that could be recovered from the silica phase after purification of a sample containing buccal cells. It was also important to determine whether the DNA would elute from the device in a concentrated fraction containing a large enough mass of DNA for downstream microchip PCR amplification using a MiniFiler™ amplification kit, which requires a specific mass range of DNA for optimal performance. This was investigated specifically because previous work performed on this SPE device with buccal swab lysate as the sample type had shown that the extracted nucleic acid (specifically RNA in the work referred to here) eluted in a broader peak (~5 µL) than seen with the extraction of pre-purified RNA (~ 2 µL).[32] It needed to be determined that if a broader elution peak also

occurred after purification of DNA from buccal swabs in this work, that the optimal mass of DNA for amplification would still be present in the peak of the elution curve. Additionally, as the focus of this work was to develop a modular, forensic DNA analysis system that would allow for analyst discretion, it was important to establish what quantities of DNA could be recovered using this method as the analyst could then select what range of elution fraction(s) to collect in order to meet the requirements of the secondary analysis they would perform.

The reproducibility of the microchip-based method for purification of DNA from buccal cells can be seen in the elution profile displayed in Figure 4B where ~5 ng of DNA was recovered from the extraction (n = 3). As expected, based on the previous studies, the DNA purified from buccal swab samples elutes in a broad peak, over the span of ~3-4 μL. This amount of DNA, specifically in the most concentrated fraction which contained ~2.5 ng, would then equate to ~250 pg of DNA that would be present in the chamber for microchip PCR (based upon the fact that the 2.5 ng DNA in 1 μL of eluate will be collected and diluted 5-fold in PCR master mix, and then 2-fold, as only 500 nL will fill the PCR chamber volume). This is a sufficient mass of DNA for downstream PCR analysis as the recommended input mass of DNA for a MiniFiler™ amplification is 250-750 pg[29]. For the microchip-based IR-PCR experiments that follow, the second microliter of eluate, which contains the 2.5 ng of eluted DNA, was, therefore, used.

4.3.3 Translation of STR Amplification to the Microscale

The MiniFiler™ PCR reaction involves a multiplex amplification of 8 different loci and the sex marker amelogenin. Due to the complexity of the amplification, it was

important to first explore whether DNA could be amplified using the MiniFiler™ kit on a microfluidic device utilizing IR heating, prior to attempting amplification of DNA extracted via μSPE. Buccal swab samples were conventionally purified using a commercially-available DNA extraction kit. After amplification of the purified DNA via IR-PCR on a microdevice, PCR products were separated and detected using an ABI 310 Genetic Analyzer. A full STR profile (all loci present) was obtained from microchip PCR amplified buccal swab DNA (Figure 5A) with the PCR time being 130 min. Previous work has demonstrated the versatility of microchip PCR to be used not only for a single-plex amplification[27] but also for an octo-plex amplification in as few as 27 minutes.[33] In this work a nona-plex amplification (8 core loci and amelogenin) was performed in 130 minutes. This more lengthy time was necessary due to the complexity of the MiniFiler™ amplification compared to the multiplex amplifications shown in previous work. First, because it is sold as a kit, primer concentrations for each specific loci cannot be adjusted during translation of the amplification to the microscale. Also, not only must all nine loci amplify to demonstrate success, but inter- and intra- peak balance and complete adenylation is also important to consider in forensic STR analysis, again making the MiniFiler™ amplification more complex.

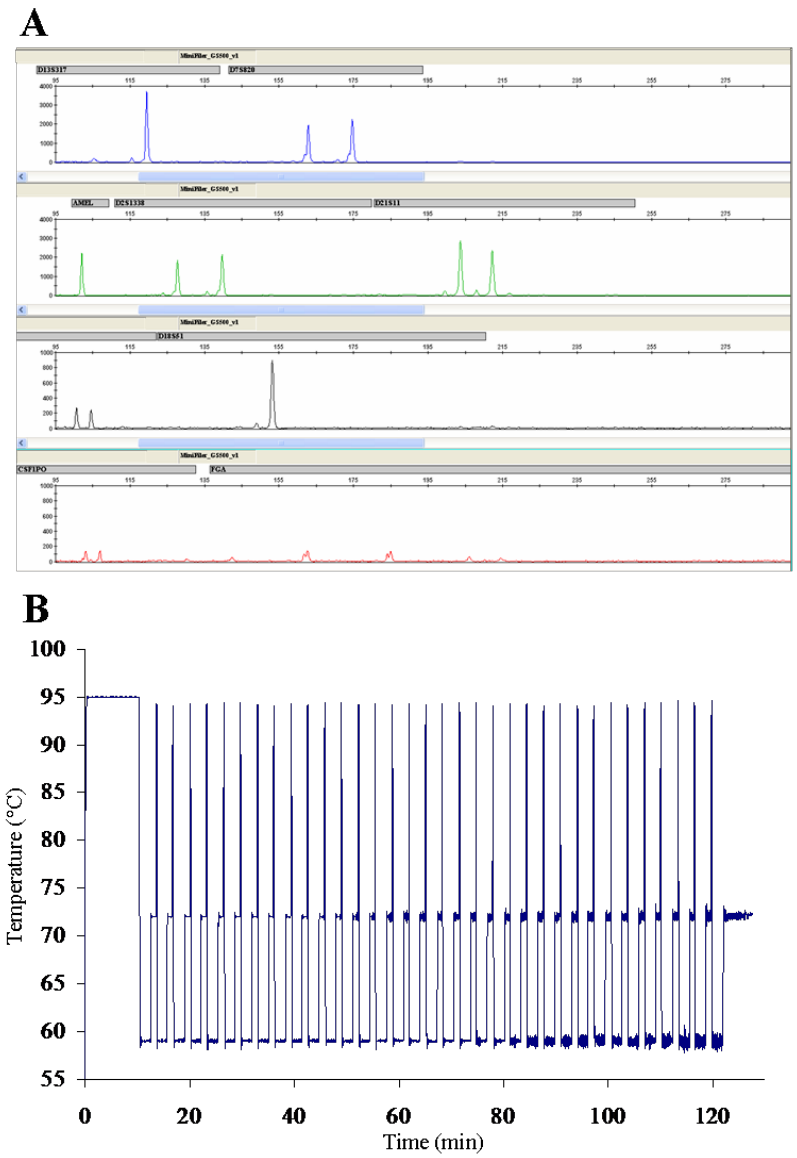

Figure 5. A) Full STR profile resulting after conventional DNA extraction from a buccal swab, microchip IR-PCR MiniFiler™ amplification, and conventional separation. B) Thermal cycling profile generated using the IR-PCR system and μPCR device.

The results presented in Figure 5A detail the first use of non-contact IR-mediated heating for the microchip-based nona-plex miniSTR amplification of DNA. The development and use of IR-mediated heating for PCR on a microdevice provides an advantage for miniSTR analysis over conventional thermal cycling methods due to the substantial, ~2-fold, decrease in analysis time achieved. Inherent to the use of

microdevices for PCR, this reduction in analysis time is largely due to the reduced thermal mass of the microfluidic PCR device, as well as more rapid temperature transitions compared to conventional thermal cyclers. These rapid transitions are evident in the thermal cycling profile obtained from the IR-mediated PCR system (Figure 5B) which shows the temperature profile of the nona-plex amplification over the 130 min.

Once initial studies had been completed to determine the success and reproducibility of the μIR-PCR nona-plex amplification, the next logical step was to test the amplification using microdevice-purified DNA. The fraction containing the highest concentration of DNA extracted from buccal cells using microchip SPE (predetermined to be fraction 2), was added to PCR master mix and amplified in a microchip using IR-mediated heating. The first successful STR profiles, with all loci (8 core and amelogenin) present, from buccal cells extracted and then amplified sequentially on two separate microdevices utilizing non-contact IR-mediated heating for PCR amplification are presented (Figure 6), with STR profiles shown from two different individuals. A representative STR profile is shown for each individual and reproducible results were obtained (complete STR profiles) from n = 3 amplifications of DNA extracted using microchip SPE and amplified using IR-PCR for both individuals. These results demonstrate that the microchip SPE and microchip PCR methods, when used in conjunction, are reproducible and reliable run-to-run (n = 3 for each individual), as well as sample-to-sample (reproducible across samples from two different individuals). The sequential use of μSPE and μPCR allows for a 2-fold reduction in analysis time over conventional SPE and PCR methods, demonstrating the advantage of a microfluidic-based system for STR analysis. Additionally, the use of two separate microdevices for

sample processing allows for an analyst's judgment to decide the quantity of sample loaded onto the microdevice for purification and whether the same, additional, or less of the eluted DNA needs to be added to the microchip amplification, depending upon whether the sample is degraded or not (as here, a non-degraded sample was investigated).

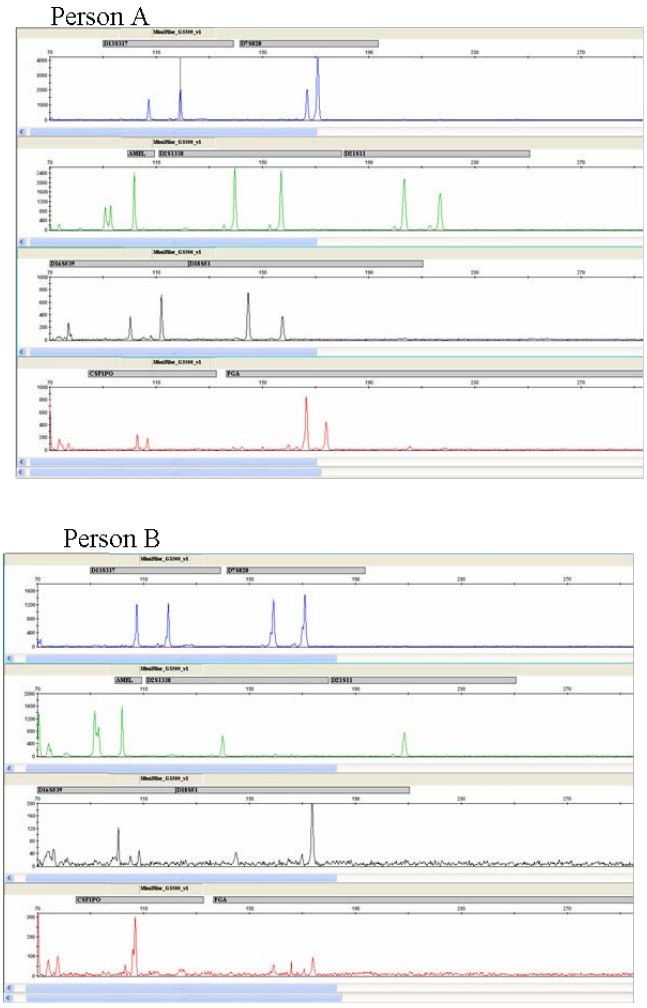

Figure 6. Full STR profiles resulting from microchip SPE, microchip IR-PCR MiniFiler™ amplification, and conventional separation from two different individuals. A single representative STR profile is shown for each person, though n = 3 replicates were performed for each individual.

4.3.4 Optimization of Microchip Electrophoresis (ME)

Prior to complete analysis using a three-chip process, it was necessary to further develop the separation and detection method on a microdevice. Optimization with respect to the polymer and separation conditions were completed and described in detail in the thesis of Dr. Daniel Marchiarullo.[26]

4.3.5 Sequential Microchip SPE, Microchip IR-PCR, and Microchip Electrophoresis

As the development of a modular system for complete processing of a biological sample progressed, the next logical step was to test all three single processes involved in the complete STR analysis method on separate microdevices sequentially. First, a buccal swab sample was purified using the μSPE method. A 1 μL fraction containing 2.5 ng of DNA (fraction 2) was then combined with MiniFiler™ reagents for amplification using μPCR. After the DNA sample had been amplified, the PCR product was removed from the PCR device and separated by microchip electrophoresis.

A raw microchip STR profile (Figure 7A) can be compared to the raw ABI-based conventional separation of the same sample (Figure 7B). All MiniFiler™ amplicon peaks (9 loci) present in the ABI trace were detected in less than 5 minutes on the microchip - a nearly 9-fold decrease in separation time compared to conventional STR separations. With ME performed in combination with microchip-based SPE and PCR, the total analysis time for generating an STR profile from a buccal swab sample was reduced ~3-fold (from 7.2 hours to 2.5 hours) from the conventional sample processing time. This success of the modular microchip STR analysis system is clearly demonstrated in these results. A buccal swab sample, although usually non-degraded and used as a comparison

or database sample and not usually processed with MiniFiler™, was used throughout this work for proof-of-principle. However, this work still lays the foundation for future optimization and use of a modular, microchip STR analysis system in the forensic sector.

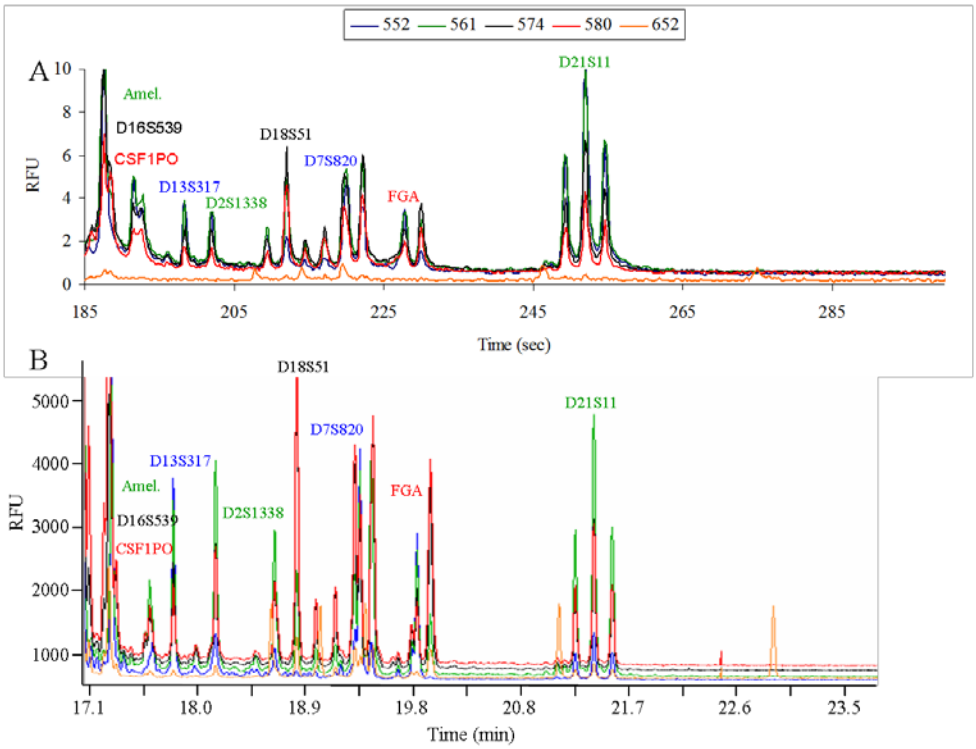

Figure 7. A) Raw data from microchip electrophoresis performed after microchip SPE and microchip IR-PCR MiniFiler™ amplification of DNA from a buccal swab. B) Raw data from ABI 310 Genetic Analyzer separation performed after microchip SPE and microchip IR-PCR MiniFiler™ amplification of DNA from a buccal swab.

4.4 Development of an Integrated, Microfluidic SPE-PCR STR Analysis System

As discussed above, previous work has been completed towards the integration of SPE-PCR on a single microdevice for genetic analysis. Initial progress showed application of the integrated device for detection of anthrax and amplification of a portion of the gelsolin gene. Although this work also used IR-PCR, providing rapid thermal cycling, only single-plex amplifications were completed.[28] A next-generation SPE-PCR device was then developed for forensic STR analysis using commercial amplification

kits, COfiler® and Profiler®. Although this showed application to multiplex amplifications, the number of loci amplified was only 7 and 10.[21] This required that both amplifications be completed in order to have the 13 core CODIS loci. Additionally, a conventional themocycler was used for heating and cooling, resulting in slower temperature transitions and, therefore, longer amplification times. This demonstrates the need for an integrated SPE-PCR method capable of amplifying a greater number of loci, and demonstrated success with other useful STR amplification kits, such as MiniFiler™, for degraded sample types.

4.4.1 First Generation Device Design and Elution Profile Studies

In order to achieve optimal amplification of all STRs, a sufficient quantity of template DNA must be present in the PCR reaction. In addition, while these devices will be single use and disposable when utilized for forensic applications, for research purposes, the devices were cleaned and reused. Consequently, a solid phase first needed to be selected for the SPE domain of the integrated device that would provide the mass of DNA necessary for downstream PCR, while also allowing for simple device preparation and easy removal from the device after each extraction. Both 15 and 30 µm silica beads were tested as a solid phase in the first generation SPE-PCR device as described in *Microchip Solid Phase Extraction Procedure for the Integrated SPE-PCR System* in *Materials and Methods*. DNA was extracted using each phase (n = 3 each) on the device from buccal cells eluted from a cotton swab and fluorescently analyzed to determine the DNA content in the elution fractions collected. It can be seen in the elution profiles in Figure 8 that, although 15 µm beads should have a higher capacity for DNA than 30 µm

beads based upon the ratio of surface area to volume, both the 15 (Figure 8A) and 30 (Figure 8B) µm silica result in a statistically similar (± one standard deviation) recovery of DNA in the elution. This can be likely attributed to the protein from the buccal swab lysate binding to the phases during the load step (which has been previously shown to occur by Tian et al.[31]), blocking binding sites on the silica and, therefore, hindering the binding of DNA, so the assumed advantage of a higher surface area to volume ratio allowing for more binding of DNA to the 15 µm beads is not seen.

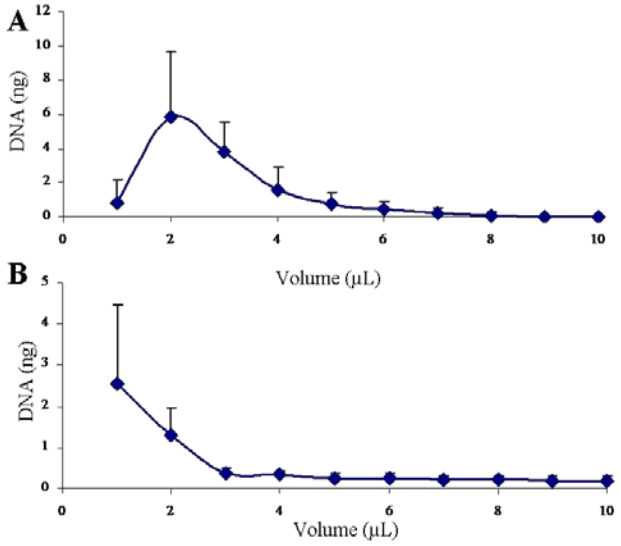

Figure 8. Elution profiles from the first generation SPE-PCR device. A) Elution profile of DNA from a buccal swab sample on the SPE-PCR device using 15 µm silica. B) Elution profile of DNA from a buccal swab sample on SPE-PCR device using 30 µm silica. Both bead sizes provided similar recoveries of DNA.

Based upon these results and because the 30 µm silica was easier to unpack from the devices than 15 µm silica, the 30 µm phase was chosen for all future studies. Additionally, use of microchip SPE and 30 µm silica provided a concentration enhancement of 3.75-fold in comparison to the starting buccal swab lysate. The elution profiles resulting from investigations into which phase would be optimal for further studies not only solidified the selection of 30 µm silica as the optimal phase, but also

indicated the optimal elution fraction for capture within the PCR chamber during the integration of SPE-PCR using this chip design. Figure 8B depicts that the elution fraction that contained the largest quantity of DNA is fraction 1, or the first microliter of the elution. However, residual IPA (a PCR inhibitor) from the wash step, pushed out from the SPE bed during elution, and is typically present in the first elution fraction (as shown by Easley, et al[30]) inhibiting PCR, so fraction 2 was chosen as the ideal fraction for amplification. To achieve a 1:1 ratio of DNA to master mix (which was determined to be optimal in tube studies – data not shown), both the SPE elution buffer and PCR master mix must be flowed at the same volumetric flow rate. The optimal timing of the elution and mixing of PCR master mix must also result in fraction 2 of the eluted DNA in the chamber (leaving ~250 pg DNA in the chamber). To achieve this timing during integration of SPE and PCR on this device, a total of 1 μL eluate should be collected from the device outlet.

4.4.2 Integration of SPE-PCR with First Generation Device Design

Microfluidic devices provide the optimal setting for forensic genetic analysis, as points of contamination are reduced due to the closed environment in the microdevice throughout purification and amplification. Furthermore, the development of an integrated microfluidic device capable of DNA purification and PCR amplification can provide the next advancement in genetic analysis for the forensic community as the microfluidic platform would allow for both processes to be carried out in a shorter time period, therefore, increasing the sample throughput of forensic laboratories. These devices also consume smaller reagent volumes than conventional analysis, which decreases the cost-

per-analysis. Most importantly, because of the small footprint of microfluidics they are ideal for being developed into portable technology which further decreases the time required for analysis.

Following determination of the optimal solid phase for use within the integrated device, the integration of SPE with non-contact IR-mediated PCR was performed on the first generation SPE-PCR device with a MiniFiler™ amplification. SPE was performed using 30 µm silica, as described in *Materials and Methods*, for the extraction of DNA from buccal swab eluate. Upon trapping fraction 2 in the PCR chamber, along with the appropriate master mix, MiniFiler™ amplification was performed as described in *Materials and Methods*. Separation and detection of resulting PCR products was performed on an ABI 310 Genetic Analyzer. The successful integration of DNA extraction and amplification on a single microdevice can be seen in the STR profile in Figure 9. A total of 7 core loci and the sex marker amelogenin were amplified – with the dropout [lack of presence of the amplicon(s) for a locus] of one locus, D16 (circled). Although not a complete MiniFiler™ profile, these results still demonstrate the first on-chip integration of SPE-PCR for the amplification of miniSTRs using non-contact IR-mediated heating. STR profiles were obtained from the analysis of multiple buccal swab samples (n = 3, data not shown) and demonstrate the reproducibility of the system as 8 loci were again amplified with dropout of locus D16. This type of allelic dropout has been observed when a decreased amount of DNA template is available for amplification.[34] This is the case in the results shown in Figure 9, as the greatest quantity of DNA available for amplification in the PCR chamber is ~250 pg, which is at the lower limit of template optimized by the manufacturer for the MiniFiler™ kit, and is only

achieved if that precise fraction and, therefore, volume (only ~500 nL) is trapped in the chamber .[29] The combination of this lower amount of template in the chamber, literature reported dropout, and difficulty in integration of these processes on a microdevice with precise flow control, add to the difficulty and problems seen with the dropout of D16. Although allelic dropout was seen, the PCR amplification was reduced from a conventional time of 3.5 hours to 2.5 hours, by utilizing the faster temperature transitions of the IR-PCR system, as well as by reducing the denature time from 60 s to 5 s, and the final extension from 45 min to 20 min. The 1.6-fold reduction in total analysis time here (for both SPE and PCR) from that required for conventional methods provides a clear advantage over conventional sample purification and amplification systems. The SPE-PCR device developed also uses 40-fold less sample volume than conventional SPE and PCR analysis, making it advantageous in forensic cases where sample may be limited. The SPE portion of the device provides a 3.75-fold concentration enhancement, which is also important for forensic cases where template within the starting sample may be dilute. Also, the method uses 1.4-fold less volume of reagents than conventional analysis making the device cost effective for the forensic community.

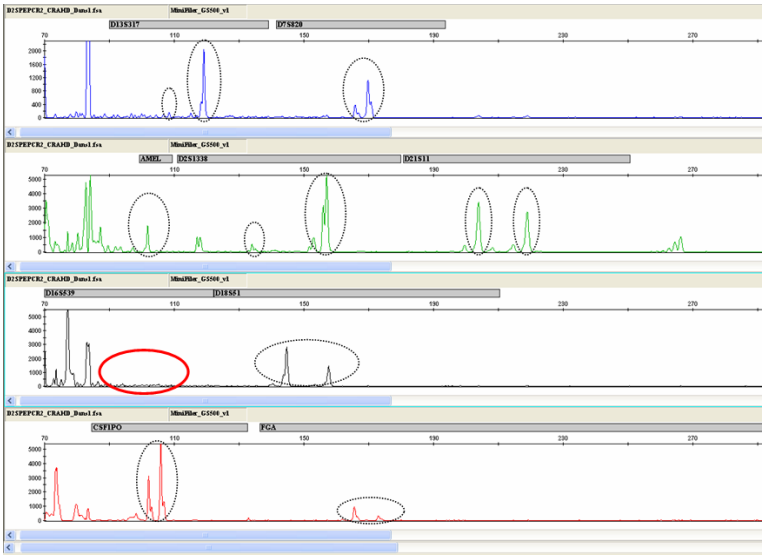

Figure 9. MiniFiler™ STR profile resulting from a 1:1 ratio mixture of buccal swab DNA template to PCR master mix after integration of SPE-PCR utilizing IR-PCR on the first generation SPE-PCR device. Eight of nine MiniFiler™ loci (circled in black dashed line) are present after a 2.5 hour amplification with the missing D16 loci circled in red.

4.4.3 Development and Optimization of a Second Generation SPE-PCR Microdevice

One approach to improving the results of the MiniFiler™ amplification involved in the integration of SPE with PCR where 8 (7 core and amelogenin) of 9 loci were present (Figure 9), eliminating the allelic dropout, was to increase the mass of DNA available for amplification. This was investigated as only ~250 pg would be present within the PCR chamber, which is at the lower limit recommended by the manufacturer for MiniFiler™[29]. To test this theory, the integrated chip was redesigned to incorporate larger channel dimensions for the SPE domain (Figure 3B). A larger channel can hold a greater number of 30 µm silica beads, allowing for an increase in DNA binding capacity, and increase in DNA bound and recovered, therefore, increasing the template in the PCR chamber closer to 0.5 ng (mid-range of that recommended by the manufacturer, 0.25-0.75 ng[29]). Additionally, previous work has shown that with an increase in channel

dimensions (and increase in the quantity of phase packed in the device), the DNA elutes in a larger volume and a broader peak.[32, 35, 36] This would be beneficial as it increases the window, and volume range, containing concentrated DNA that can be trapped within the PCR chamber for amplification. Larger channel dimensions, and, therefore, a larger cross-sectional area, also allow for a faster volumetric flow rate to be used (still maintaining the same linear flow rate optimized for silica-based extractions[37]), decreasing total analysis time. Elution studies were first performed to ensure the increased DNA capacity achieved through larger channel dimensions would also increase the mass of DNA recovered. DNA was purified from buccal cells using the second generation SPE-PCR device, and a fluorescence assay performed to determine the mass of DNA in each elution fraction. In the elution profile that resulted [Figure 10 (n = 3)], it can be seen that an increased DNA recovery is indeed achieved through the use of the second generation device. The majority of the DNA elutes in the 5-6th uL (containing ~5 ng), which makes the timing of trapping the maximum amount of DNA in the PCR chamber easier, as there is a 2 μL window which contains ~4 – 5 ng/μL. This provides a much larger window of timing than with the previous generation device, which had a 1 μL window that only contained ~2.5 ng. The shape of the elution profile from the second generation device indicates that a sufficient mass of DNA (0.25-0.75 ng) is available if anywhere from the 5th-7th μL (corresponding to 2-5 ng/μL), of the elution profile shown in Figure 10, is trapped within the chamber [providing anywhere from 0.5-1.25 ng of DNA within the PCR chamber (well within the recommended range)]. This is again an advantage to using this chip design over the first generation SPE-PCR device, where the majority of

the DNA eluted in the first microliter, limiting the number of opportunities to trap a sufficient amount of DNA for amplification.

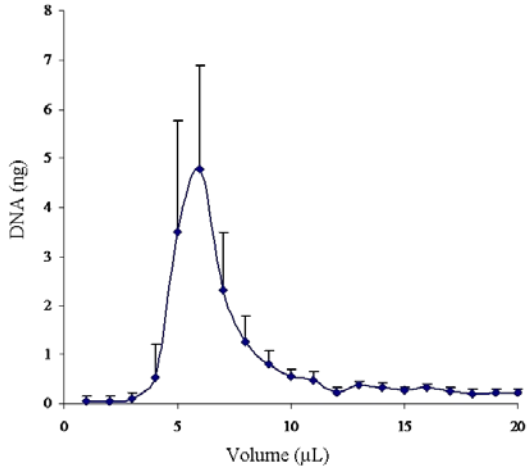

Figure 10. Elution profile of DNA from a buccal swab sample on the second generation SPE-PCR device using 30 µm silica.

4.4.4 Microchip PCR Optimization for Faster STR Analysis

Another approach that was investigated to improve the MiniFiler™ results was to further optimize the PCR. Although the previous PCR recipe had been shown successful on a PCR microdevice, little optimization was completed to obtain this recipe, including little done to ensure optimal functionality on an integrated SPE-PCR microdevice. Additionally, a major hindrance to the timely processing of DNA evidence is the lengthy analysis times for each step. By performing SPE on a microdevice, the time for DNA purification is reduced 4.5-fold (from 60 min down to < 15 min). The lengthiest process though, is the PCR amplification, which conventionally requires 3.5 hours. An overall decrease of 1 hour in PCR time alone was seen by moving to microchip-based PCR, harnessing the advantage of faster temperature transitioning inherent to the use of the IR-PCR system. To further reduce the time required for the MiniFiler™ amplification, each

hold time in the thermal cycling protocol was further reduced. The initial denaturation of 11 min was kept consistent to ensure the proper length of time for heat activation of the AmpliTaq Gold® DNA polymerase used in this amplification. The denature, anneal, and extension steps were reduced to 5 s, 10 s, and 10 s, respectively, from the conventional times of 60 s, 120 s, and 60 s. The final extension was also reduced from 45 min to 1 min. After performing a microchip PCR MiniFiler™ amplification using these reduced thermal cycling times, the resulting STR profile (representative of n = 3), shown in Figure 11A, was complete with all 9 loci (8 core and amelogenin) present. Although adenylation (+A/-A) issues with the amplification can be seen (split peaks in electropherogram indicated by red box), it is outweighed by the decrease in amplification time from the 3.5 hour conventional MiniFiler™ amplification to 1.5 hours, a decrease of 2 hours. With the successful amplification demonstrated here, resulting in a complete STR profile, the next logical step was to test the integration of SPE and PCR on the second generation SPE-PCR device, which provided a greater recovery of purified DNA, incorporating the faster amplification times.

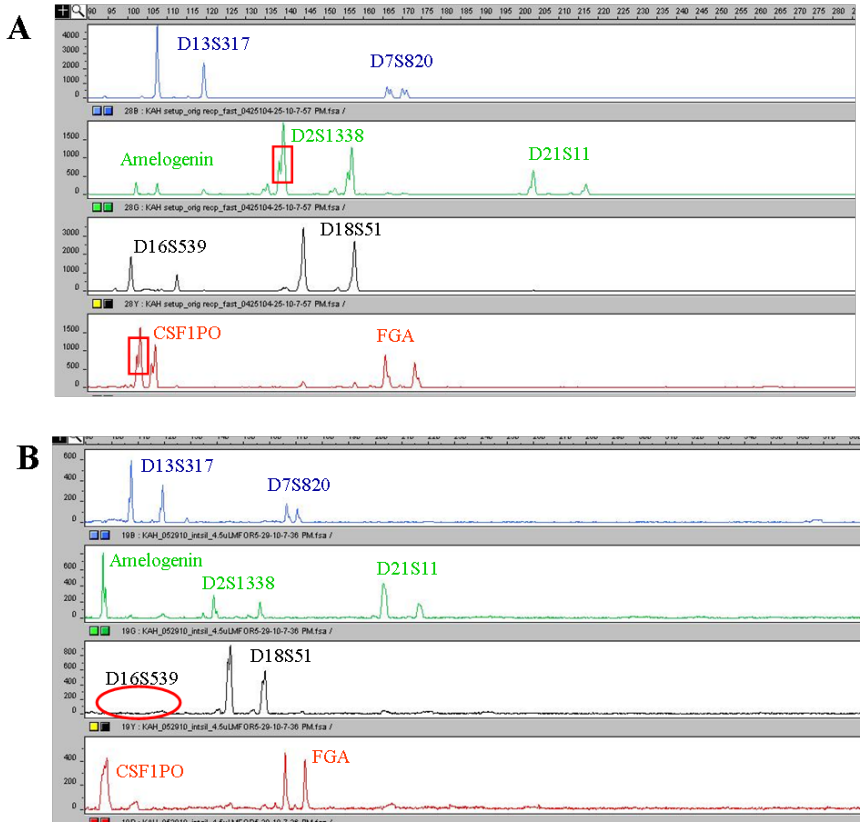

Figure 11. A) Complete MiniFiler™ STR profile from buccal swab DNA template performed in 1.5 hours on a microfluidic PCR device utilizing IR-PCR. Split peaks due to +A/-A indicated by red box. B) MiniFiler™ STR profile resulting after integration of SPE-PCR utilizing the second generation SPE-PCR device. Eight of nine MiniFiler™ loci are present with the missing D16 loci circled.

4.4.5 Integration Using Second Generation SPE-PCR Microdevice with Fast MiniFiler™ Amplification

The integration of purification of DNA from a buccal swab with PCR amplification on a single device was performed, as described in *Materials and Methods*. The ~6th µL of the elution curve, containing the maximum mass of eluted DNA, was trapped within the PCR chamber after elution (~1.25 ng), and the MiniFiler™ amplification performed (PCR time: 1.5 hours). A representative STR profile (of n = 3) which resulted (Figure 11B) contains 8 (7 core and amelogenin) of 9 MiniFiler™ loci,

with D16 missing (circled). It is interesting to note that this is the same locus that was missing from the STR profile resulting from the integration of SPE-PCR using the first generation device. The dropout of this locus has previously been reported to have one of the largest dropout rates, even when the authors used an increased injection time, 10 or 15 s in comparison to 5 s (manufacturer's protocol), during separation and detection of PCR products.[34] The combination of the repeat occurrence of the D16 locus dropping out in the results presented, and those in the literature, demonstrate the difficulty often seen when multiple processes are integrated into a glass microchip environment. To determine whether these issues were characteristic for this system with STR analysis, another commercial forensic STR kit, Identifiler®, was investigated.

4.4.6 Microchip PCR Optimization of Amplification Using the Identifiler® kit

Due to the incomplete STR profiles resulting after MiniFiler™ amplification during the integration of SPE-PCR, another kit commonly used for forensic human identification was investigated. First, a PCR master mix was developed incorporating the Identifiler® kit primer set with other reagents, including a mixture of different DNA polymerases which would allow the amplification time to be further reduced. Identifiler® consists of primers for 16 loci, including amelogenin. Many forensic labs will use Identifiler® first for identification, and then go to MiniFiler™ to gain additional information such as larger loci, that are overlapping loci amplified in both the MiniFiler™ and Identifiler® kits, that may not be amplified by Identifiler® (as MiniFiler™ amplifies shorter fragments). The results from both kits are then combined to provide a higher power of discrimination for human identification.

Currently, the same DNA polymerase (AmpliTaq Gold®) is commonly used in all forensic amplification procedures, even though faster polymerases are now available. This polymerase has an extension rate of 2-4 kb/min (the number of bases added to the growing DNA chain per minute held at the extension temperature) and 50-60 base processivity (the number of nucleotides that are added to the growing chain of DNA per association/disassociation of the polymerase with the DNA).[5] Faster enzymes on the market, such as PyroStart™ Fast PCR Master Mix (40 nuc/s) and SpeedSTAR™ HS DNA Polymerase (100 nuc/s), could provide up to a 3-fold increase in speed of amplification over AmpliTaq Gold® (~33 nuc/s). In combination with the use of faster thermal cycling technology, this could allow for a great decrease in amplification time and make DNA typing a more rapid process. To enhance the rapidity of the Identifiler® amplification, a new master mix recipe was developed using a mixture of these two fast polymerases (described in *Materials and Methods).* The master mix optimization for rapid amplification was based upon work completed by Vallone et al.[38], which utilized both of the aforementioned fast polymerases. Some adjustments were made to the recipe for successful translation to the microdevice, as that completed by Vallone et al. was performed in a tube, which has a smaller surface area to volume ratio than that of a microdevice. This can be problematic due to the increased potential of master mix components adhering to the channel walls. Due to this, the first component changed was the addition of BSA (bovine serum albumin), as it has been shown by others to be beneficial for passivation[39]. The ratio of master mix components to solution containing DNA template was also adjusted from 1:3.4 to 1:1, so more DNA template would be trapped in the PCR chamber during integration.

Due to the faster extension rates of the two polymerases chosen, less time is needed at each step during thermal cycling. The initial denaturation was reduced from 11 min to 1 min, the denature, anneal, and extension reduced to 5 s, 10 s, and 20 s, respectively, and the final extension reduced to 1 min. Test amplifications (n = 3) were performed on a PCR microdevice utilizing the IR-PCR system, and in the resulting STR profile (Figure 12), a complete profile, with 16 of 16 loci amplified and present, is shown, which demonstrates the first 16-plex amplification on a microdevice. Thermal cycling was completed in 45 min, which is an ~5-fold decrease from conventional amplification time. These results suggest promise that an Identifiler® analysis of a buccal swab, integrating SPE with PCR on a single device, could be completed in ~1 hour analytical time.

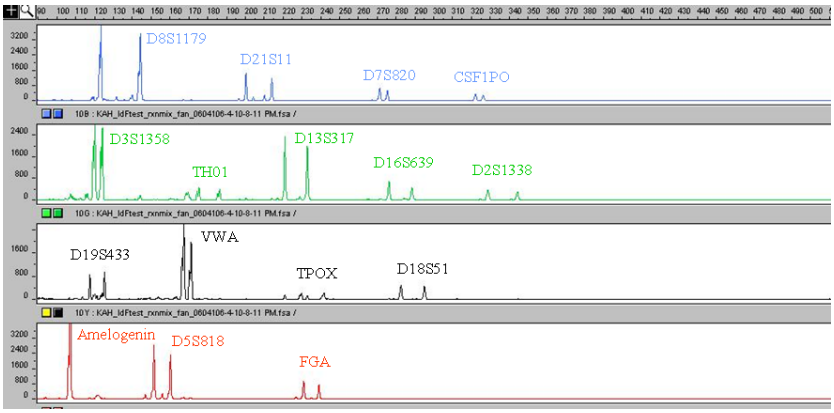

Figure 12. Identifiler® STR profile resulting from a 1:1 ratio mixture of buccal swab DNA template to PCR master mix on a microfluidic PCR device utilizing IR-PCR. Rapid PCR was performed using a mixture of fast DNA polymerases, resulting in amplification of 16 Identifiler® loci in 50 min.

4.4.7 Identifiler® Analysis of a Buccal Swab Sample Using Integrated, Second Generation SPE-PCR Microdevice

With a goal of performing rapid STR analysis using a microfluidic device for the integration of SPE-PCR, resulting in a complete STR profile, a buccal swab sample was processed using the second generation SPE-PCR microdevice. The new PCR master mix, incorporating faster polymerases with the Identifiler® primer set, was flowed through the sidearm of the SPE-PCR device during the elution phase of SPE in a 1:1 ratio with the eluting DNA. Tests of different volumes of eluate collected from the outlet indicated that collecting 4.5 μL, effectively trapping the ~6^{th} μL in the PCR chamber, resulted in consistent, reproducible STR profiles. In Figure 13, a representative STR profile from one of the integrations performed demonstrates the success of the SPE-PCR method. A complete Identifiler® profile is seen, with all 16 loci present and callable above a threshold of 50 RFU. This clearly demonstrates the effectiveness of the microfluidic method for the integration of SPE-PCR for STR analysis. When evaluating an STR profile, it is important to consider not only the presence of amplified alleles, but also the quality of that amplification, including: whether signal intensity is above a set threshold for all expected loci, whether there is comparable signal intensity between loci (interlocus peak balance), heterozygote balance (intralocus peak balance), absence of incomplete adenylation products, absence of non-specific amplification products, and absence of significant stutter artifacts. Further optimization of the method is necessary to achieve these characteristics, as the locus-to-locus balance especially could be improved. However, the method developed here is a workable foundation for future optimization studies and will lead to the development of a device for complete STR analysis, from

DNA purification to microchip electrophoresis for PCR product separation and detection, which will make rapid STR analysis a reality for use in forensic laboratories. Although further optimization is necessary, the results presented show the first demonstration of integrated SPE-PCR using IR-mediated heating for the amplification of 16 forensic STR loci in ~1 hr. Not only is this a large decrease over the time that is required for conventional analysis, which would increase the sample throughput of forensic laboratories, but also provides a completely closed environment, from the lysate to amplified PCR product, which is ideal for forensic genetic analysis.

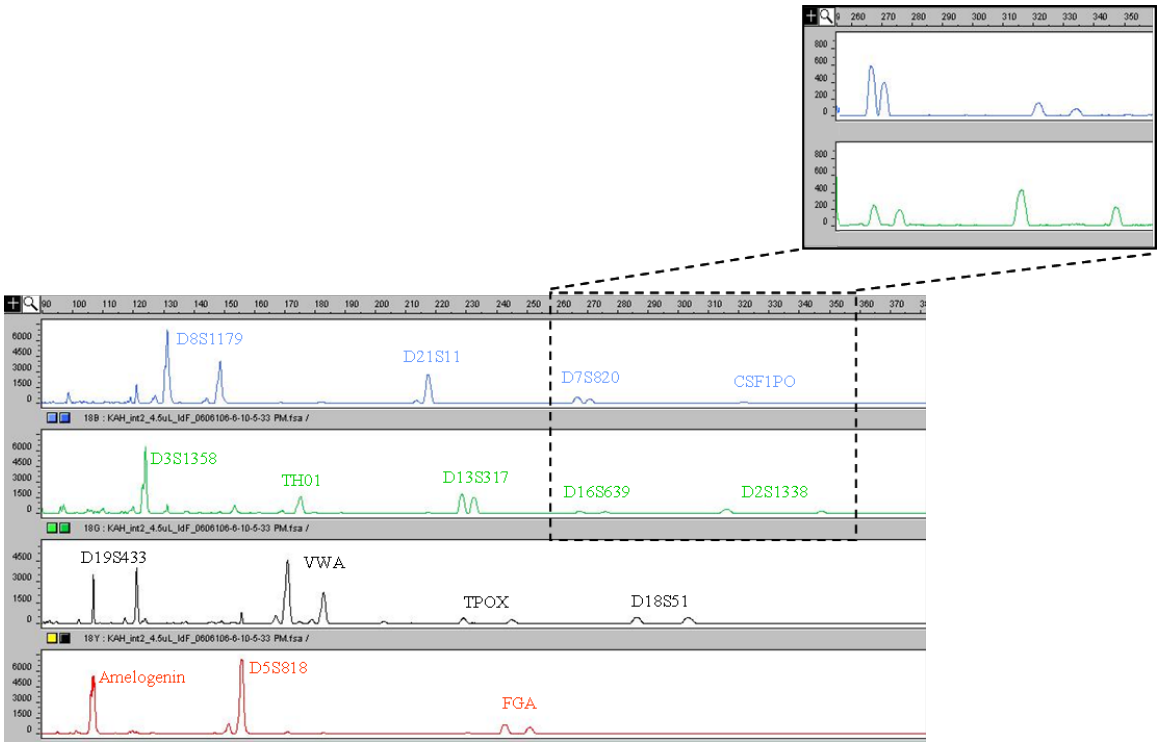

Figure 13. Complete Identifiler® STR profile resulting from integration of SPE-PCR on the second generation SPE-PCR device. DNA was purified from a buccal swab and a rapid Identifiler® amplification performed in a total analysis time of ~1 hour.

4.5 Conclusions

With this first successful development of a modular, microfluidic STR analysis system come numerous advantages for the forensic community. Not only does it provide a more rapid DNA analysis and decreased cost-per-analysis compared to using conventional processing techniques, but it also allows for analyst discretion in determining the best way to proceed, depending upon information known or learned about the condition of the sample. Buccal swab samples from two separate individuals were processed, highlighting the versatility and reproducibility of the method. A complete STR analysis from DNA extracted from a buccal swab sample was performed in 2.5 hours, demonstrating an ~3-fold reduction in total analysis time compared to conventional methods. The strides made with this modular three-chip system for STR analysis provide the ideal system for processing of forensically-relevant samples in a timely and cost-effective manner. Future optimization will involve demonstrating use of the modular system for processing a broader spectrum of samples and the use of other commonly used forensic STR amplification kits.

In addition to the modular system, the first integration of SPE and PCR on a single microdevice utilizing non-contact IR-mediated heating for an Identifiler® STR amplification was detailed in this chapter. Rapid STR analysis was demonstrated, highlighting the advantages inherent to microchips, by utilizing fast DNA polymerases to decrease total analysis time to ~1 hour. Future work will involve further testing and optimization of the new SPE-PCR device design and PCR amplification conditions in addition to the integration of microchip electrophoresis into the device to provide the

sample in-answer out capability of a microchip-STR genetic analysis system for the forensic community.

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5. Solid Phase Extraction of DNA from Biological Samples in a Post-based, High Surface Area Poly (methyl methacrylate) (PMMA) Microdevice

This chapter describes the performance of poly (methyl methacrylate) (PMMA) microfluidic DNA purification devices with embedded microfabricated posts, functionalized with chitosan. PMMA is attractive as a substrate for creating high surface area (SA) posts for DNA capture because X-ray lithography can be exploited for extremely reproducible fabrication of high SA structures. However, this advantage is offset by the delicate nature of the posts when attempting bonding to create a closed system, and by the challenge of functionalizing the PMMA surface with a group that invokes DNA binding. Methods are described for covalent functionalization of the post surfaces with chitosan that binds DNA in a pH-dependent manner, as well as for bonding methods that avoid damaging the underlying post structure. A number of geometric posts designs are explored, with the goal of identifying post structures that provide the requisite surface area without a concurrent rise in fluidic resistance that promotes device failure. Initial proof-of-principle is shown by recovery of prepurified human genomic DNA (hgDNA), with real-world utility illustrated by purifying hgDNA from whole blood and demonstrating it to be PCR-amplifiable.

5.1 Introduction

As previously described in Chapters 1-4, polynucleic acid purification is the precursor to DNA analysis of any type. Solid phase extraction (SPE) using a silica solid phase has become the most common method and is easily adaptable to microdevices[1-10] A wide variety of silica phases have been developed for microfluidic DNA purification

ranging from packed silica beads[7] to fabricated silica posts[6]. Most microfluidic DNA extraction methods, including those mentioned, are performed in glass microchips. In the case of fabricated silica posts, it is often difficult to consistently bond glass covers to the top of glass posts (with small areas for bonding) without damaging the posts, due to the requisite bonding pressures. These difficulties imply that glass devices are labor-intensive and thus, costly, discouraging their use as disposable devices for point-of-care applications. Moreover, silica-based extraction requires the use of reagents (e.g. guanidine or isopropyl alcohol) that can inhibit downstream processes such as PCR.

The inherent challenges associated with glass microfabrication has spurred efforts to develop devices with alternative materials, notably polymers (polyolefin, polycarbonate, epoxy, PMMA[11-15]) that are amenable to low cost fabrication approaches. The integration of silica-based SPE into polymeric devices offers fabrication advantages and yields attractive extraction performance, but existing approaches still require chaotropes and/or organic reagents that interfere with downstream processes. Alternatives to silica-based extractions, such as carboxy-coated surfaces that use poly (ethylene glycol) (PEG) or tetra (ethylene glycol) (TEG) for DNA binding[14] still utilize other organic reagents (i.e. ethanol) that lead to similar concerns regarding inhibition of downstream processing. Moreover, the use of alcohols with polymers creates additional challenges relating to the deleterious effects of alcohols on polymer surfaces and interface integrity.

While each of the polymer-based microdevices presented here represent valuable steps towards a single-use plastic microdevice for SPE, none have met the optimal criteria: a device that *(i)* demonstrates efficient extraction of DNA from complex

biological samples, *(ii)* avoids the use of PCR-inhibitory reagents used in silica-based purifications, *(iii)* exhibits a highly reproducible solid phase, *(iv)* yields surfaces that are easily modified and robust, and *(v)* is amenable to scalable cost-effective fabrication (i.e., enabling disposable devices).

In this chapter, a polymeric device for DNA purification is presented which meets these requirements by utilizing an extraction method, previously described by Cao *et al.*[16], based on pH-induced DNA binding to a chitosan-based phase.[16] Chitosan, the partially deacetylated form of chitin, was shown to bind DNA in a pH-dependent manner: charge-charge interactions bind the DNA to the protonated chitosan at pH 5; neutralization of the charge at pH 9 results in DNA release. Purification of nucleic acids using the chitosan-based phase has previously been adapted to the microchip for SPE of DNA from blood and RNA from buccal swabs and resulted in PCR and reverse transcriptase PCR (RT-PCR) amplifiable product.[16, 17] This phase has been shown to be reproducible and is completely aqueous (eliminates PCR-inhibitory reagents necessary for traditional silica-based purifications), making the method more compatible with PCR integration.[7, 8]

The work presented in this chapter describes the combination of the chitosan-based purification chemistry with a PMMA microfluidic, fabricated using the LIGA [Lithographie, Galvanoformung, and Abformung (Lithography, Electroplating, and Molding)] process that exploits X-ray lithography to create the high surface area, complex 3-D structures (pillars). The high aspect ratio of the pillars necessitated development of a procedure for bonding PMMA to PMMA which *(i)* would not destroy the complex 3-D structures within the channels but *(ii)* would create a bond which can

withstand the pressure of syringe-driven flow. The most advantageous device design was determined from six possibilities. Recovery of DNA using the aqueous, chitosan-based extraction method from both prepurified DNA and whole blood, a common clinical sample, is also demonstrated, proving the utility of the device for clinical applications. This work demonstrates the foundation/first steps for developing a fully integrated genetic analysis plastic microdevice.

5.2 Materials and Methods

5.2.1 Reagents

2-(4-morpholino)-ethane sulfonic acid (MES, enzyme grade), hydrochloric acid, sodium hydroxide, *Taq* DNA polymerase, 10X PCR buffer, dNTPs, and $MgCl_2$ were purchased from Fisher (Fair Lawn, NJ). 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Trizma Base, 99.9%), low molecular weight chitosan (chitosan oligosaccharide lactate), ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate, 1,2-dichloroethane anhydrous (99.8%), and bovine serum albumin were purchased from Sigma (St. Louis, MO). Potassium chloride was purchased from Mallinckrodt Chemical Company (Paris, KY). Triton X-100 for molecular biology was purchased from Fluka (St. Lous, MO). Proteinase K was purchased from Qiagen® (Valencia, CA). Quant-iT™ PicoGreen® dsDNA reagent, an intercalating fluorescent dye, was purchased from Invitrogen™ (Carlsbad, CA). Ethanol was purchased from AAPER Alcohol and Chemical Company (Shelbyville, KY). DNA primers for amplification of a fragment of the gelsolin gene were purchased from MWG-Biotech, Inc. (High Point, NC). Purified human genomic

DNA was obtained through in-house purification from whole blood. All solutions were prepared in Nanopure water (Barnstead/Thermolyne, Dubuque, IA).

5.2.2 Microdevice Fabrication

Microdevices were fabricated by HT Microanalytical, Inc., using the LIGA process that exploits X-ray lithography. Glass was used as the base substrate onto which a layer of titanium was deposited. PMMA posts were then fabricated by exposing a PMMA layer that was bonded to the titanium (Figure 1) to X-rays. The exposed portions were then developed, resulting in a 1 cm long channel with a width of 800 µm and depth of 50 µm (Figure 2A, B). PMMA cover pieces were fabricated with two 1 mm reservoirs drilled for the inlet and outlet.

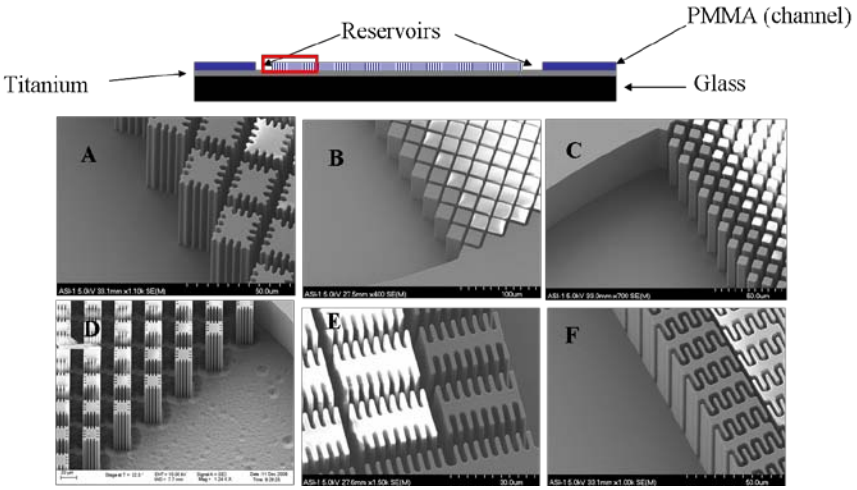

Figure 1. Schematic of device design without a bonded PMMA cover piece (top). Dimensions: 1 cm long channel, 800 µm wide and 50 µm deep. Red box indicates location of SEM images A-F. A. Device design A with dimensions: 14 µm square posts with ~3 µm extensions and 4 µm distance between posts. B. Device design B with dimensions: 22 µm square posts with 4 µm distance between posts. C. Device design C with dimensions: 8 µm square posts with 4 µm distance between posts. D. Device design D with dimensions: post structure similar to Design A but with 17 µm between each post. E. Device design E with dimensions: 6µm x 30 µm posts with 8 µm extensions. F. Device design F with dimensions: 6µm x 30 µm posts with 5 µm extensions.

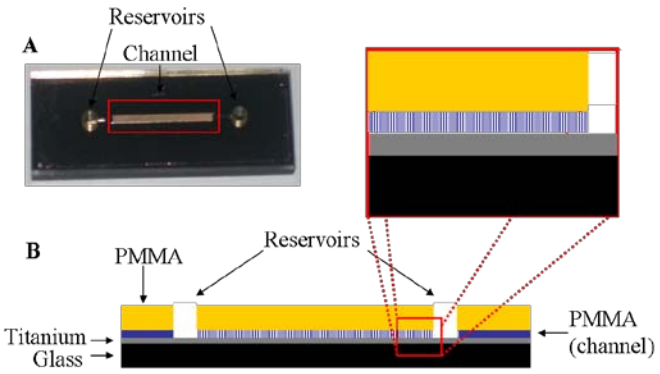

Figure 2. A. Bonded PMMA microdevice. B. Schematic of bonded PMMA microdevice with each layer of device labeled. Expanded image is of a bonded device with a focus on the microstructures.

5.2.3 SPE Apparatus

Hamilton (250 µL) gas-tight syringes (Hamilton, Reno, NV) were connected to the microdevice through 0.25 mm i.d. PEEK™ tubing and mini-tight fittings (Upchurch, Oak Harbor, WA). The PEEK™ tubing was connected to the microdevice by press-fit into the inlet reservoir. Pressure driven solution flow was achieved with a SP100i syringe pump (WPI, Sarasota, FL). All experiments were performed at a flow rate of 1.6 µL/min.

5.2.4 Device Bonding

Bonding of the PMMA cover to the channel containing the PMMA posts was achieved using a method modified from that of Lin *et al.*,[18] which uses a low azeotropic solvent for bonding. The cover and channel were held together and 1-2 µL of 20% 1, 2-dichloroethane (DCE), by weight, 80% ethanol solution (EtOH/DCE solution) was pipetted along one contact edge and allowed to wick in. Manual pressure, by hand, was applied for 5 min. The same volume of the EtOH/DCE solution was applied to the other

contact edge of the substrates followed by 5 min of pressure. Glass slides along with C-clamps were tightened down (just to the point where the chip and slides remained stationary without sliding) to both sides of the substrates and allowed to sit for ≥ 2 hrs. The bonded device was removed from the clamps and water was flowed through the device for 15 min.

5.2.5 Chitosan Functionalization

Functionalization of the PMMA was based upon a method reported by Witek, *et al.*[14] using ethylenediamine. The reported procedure was altered for this work using chitosan, and functionalization was achieved by first flushing water through the device for 15 min, followed by exposure to UV light (254 nm, Mineralight Model UVGL-25, Upland, CA) for 30 min to create carboxylate groups on the surface of the PMMA.[14, 19-21] Exposure to UV light prior to each coating was found to be unnecessary (data not shown). Approximately 50 µL of 10 mM chitosan/10 mM N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC)/100 mM K_2HPO_4, pH 7 coating solution, prepared fresh for each coating, was flowed into the device manually with a syringe. The solution was allowed to incubate in the chip for 5 min. An additional 50 µL of coating solution was manually flowed onto the chip. The chip was submerged in 10 mL of the coating solution and vortexed at 1200 rpm for ≥ 11 hrs. The device was removed from the coating solution and water was flowed (by syringe and syringe pump) through the device for 30 min.

5.2.6 Sample Preparation

Human genomic DNA (hgDNA) was purified in-house from whole blood obtained from the University of Virginia School of Medicine (from fully deidentified residual clinical specimens). To prepare lysed whole blood samples, a volume of blood containing 500 ng of DNA [calculated from the known white blood cell (WBC) count of the whole blood and the assumption of 6.25 pg DNA/WBC[22]] was added to 20 µL of 20 mg/mL proteinase K (Qiagen) and 50 mM MES pH 4.24 to bring the volume up to 500 µL. The solution was vortexed for 1 min and incubated in a water bath at 56 °C for 10 min.

5.2.7 Chitosan Stripping

Following each extraction on a chitosan-coated PMMA device, the chitosan phase was stripped by flowing 1 M NaOH through the device for 1 hr. The device was then flushed with water for 1 hr.

5.2.8 SPE Procedure for DNA Purification Using an Unfunctionalized PMMA Device

A device that had never been coated with chitosan was used and first conditioned with 10 mM MES, pH 5 for 10 min. A load solution containing 40 ng hgDNA in 25 µL 10 mM MES, pH 5, was then flowed into the device, and five 5 µL fractions were collected from the outlet. The channel was then washed with 10 µL of 10 mM MES, pH 5 while five 2 µL fractions were collected. The DNA was then eluted from the phase by flowing 10 mM Tris/50 mM KCl, pH 9, and ten 2 µL fractions were collected. An additional 100 µL of 10 mM Tris/50 mM KCl, pH 9 was flushed through the device as a

rinse. All flow rates were 1.6 µL/min. All fractions collected were stored for later fluorescence analysis.

5.2.9 SPE Procedure for DNA Purification Using a Chitosan-Coated and Stripped PMMA Device

Devices were conditioned with 10 mM MES, pH 5, for 10 min. A 25 µL load solution containing 10 ng hgDNA in 10 mM MES, pH 5, was loaded onto the device and five 5 µL fractions were collected. The phase was washed with 10 µL of 10 mM MES, pH 5 and five 2 µL fractions were collected. DNA was eluted by flowing elution buffer, 10 mM Tris/50 mM KCl, pH 9, through the device and collecting ten 2 µL fractions. All fractions were stored for fluorescence analysis.

5.2.10 Procedure for Binding Capacity Studies

Devices that had been coated with chitosan were conditioned with 10 mM MES, pH 5, for 10 min at 1.6 µL/min. A load solution containing hgDNA (1, 2, 5, or 10 ng/µL) in 10 mM MES, pH 5, was continually loaded onto the device, while twelve or fifteen 2 µL fractions were collected. Each fraction was then stored for fluorescence analysis to quantitate the amount of DNA in each.

5.2.11 SPE Procedure for DNA Purification from Whole Blood

A lysed whole blood sample was prepared as described above in *Sample Preparation*. A chitosan-coated device was conditioned with 50 mM MES/1 % Triton X-100, pH 4.24, for 10 min. Sample (25 µL) was then loaded onto the device and five 5 µL

fractions were collected. The phase was washed with 10 µL of 10 mM MES, pH 5, and five 2 µL fractions were collected. The DNA was eluted from the phase with 10 mM Tris/50 mM KCl, pH 9, and ten 2 µL fractions were collected. All fractions were stored for later fluorescence or PCR analysis.

5.2.12 Fluorescence Analysis

All fractions collected were prepared for analysis using a PicoGreen® intercalating dye, fluorescence assay.[23] The samples were analyzed using a NanoDrop 3300 Fluorospectrometer (NanoDrop, Wilmington, DE).

5.2.13 PCR Analysis

Extracted DNA elution fractions were added to PCR master mix containing 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.8 µM forward and reverse primers (5'-AGTTCCTCAAGGCAGGGAAG-3' and 5'-CTCAGCTGCACTGTCTTCAG-3') for a portion of the gelsolin gene, 1X PCR buffer, 10 µg/mL BSA, 0.5 units/µL *Taq* DNA polymerase, and autoclaved water up to 25 µL. Samples were amplified using standard PCR protocols developed in lab and a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA) for thermal cycling. The protocol used an initial denaturation step of 95 °C for 2 min, followed by 35 cycles of 95 °C, 30 s/64 °C, 30 s/72 °C, 30 s, and a final extension of 72 °C for 2 min. DNA 1000 Series II kit and a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA) were used for separation and analysis of the PCR products.

5.3 Development of a PMMA SPE Microdevice

5.3.1 Development of Device Bonding

In order to produce devices that can withstand the pressure of the syringe-driven flow, at a flow rate of 1.6 μL/min, a bonding method is required to form a tight PMMA-PMMA bond without damaging the intricate post structures that form the backbone of the solid phase. It is important to first note the basis for the selection of 1.6 μL/min as the volumetric flow rate required. Previous work in the Landers lab had determined the optimal linear flow rate, and, therefore, volumetric flow rate (calculated by incorporating the cross sectional are of the microchannel), for DNA purification on a chitosan solid phase to be 0.67 mm/sec (as discussed in Chapter 3). Based upon this, the optimal volumetric flow rate was calculated to be 1.6 μL/min. Initial bonding attempts employed epoxy (a thin layer applied to the PMMA cover followed by bonding to the channel) to bind the top PMMA cover to the channel that contained the PMMA fabricated post structures. This method resulted in only partial bonding at the tops of the posts, as was evident when black dye was flowed through the device (Figure 3A). Black printer dye, used for easy visualization of bonded regions, can be seen in Figure 3A where the tops of the posts are not bonded to the top PMMA cover piece (*i.e.*, the dye flows over the posts). Only posts that are bound to the cover piece are seen when dye was flowed through the channel. Although the posts remained intact with this method, the bonding was not capable of withstanding the pressure generated by the syringe-driven flow, as evidenced by leaking between the contact edges of the bonded surfaces.

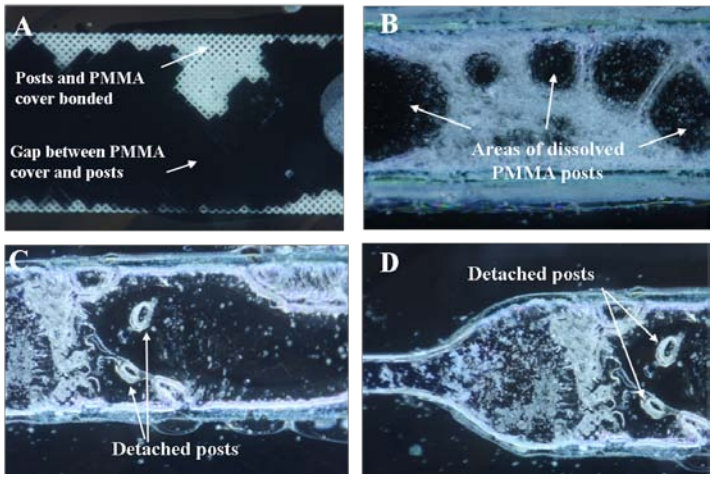

Figure 3. Images from devices bonded using epoxy (A) which resulted in insufficient bonding or solvent bonding (B, C, and D) which resulted in destruction of post structures.

Bonding using a sacrificial layer (agarose and photoresist) as described previously[24], solvent vapor (hexanes and chloroform) with applied pressure[25], or solvent coating (isopropanol, acetone, and chloroform) with applied pressure were all tested. Both sacrificial layer and solvent vapor with pressure resulted in non-bonded (over the entire surface of the chip) chips (data not shown), while solvent coating with applied pressure resulted in damage to the 3-D microstructures (Figure 3B, C, and D). Damage to the posts is clear in Figures 3B–D where posts have been detached from the bottom of the channel or dissolved, leaving gaps within the channel that significantly reduce the separation efficiency of the device by dramatically lowering the surface utilized during extraction. This clearly demonstrates that previous methods that successfully bond plastic devices are unsuitable for devices with post structures.

A device bonding method was then devised by modifying that described by Lin *et al.*[18] where bonding was completed at room temperature using a low azeotropic solvent. In that work, the authors characterized the bonding method and showed it successful for bonding channels as wide as 3 mm without causing the channel to collapse. Additionally,

the bonding method was shown to have a 17-fold greater bonding strength than conventional thermal bonding techniques. For bonding of the devices in this work, the volume of bonding solution was adjusted to ~20 μL of the DCE/EtOH mixture, from the 2-3 drops (equivalent to ~50-75 μL) suggested by Lin *et al.*, due to the delicate nature of the complex 3-D structures present in the channel. This volume resulted in some damage to the post structures (Figure 4A). In an attempt to account for this, and to prevent excess solvent from contacting the posts in the devices used in this work, the volume of solvent was decreased to 1-2 μL, and pressure was applied with C-clamps as before. This method resulted in a portion of the device being unbonded (Figure 4B). By applying five minutes of directed pressure (handheld) before switching to C-clamps, fully bonded devices were achieved with intact post structures (Figure 5A – C).

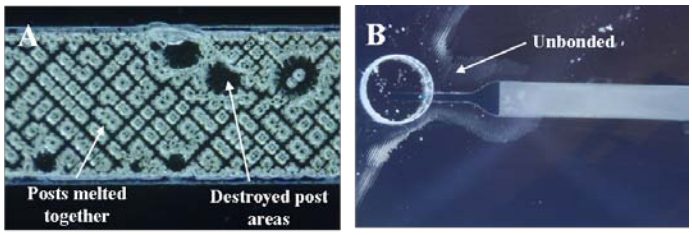

Figure 4. Images showing development of bonding method where 20 μL of DCE/EtOH solvent was used (A) destroying some of the post structures or where 1-2 μL was used but with too little pressure, resulting in lack of bonding near the reservoirs (B).

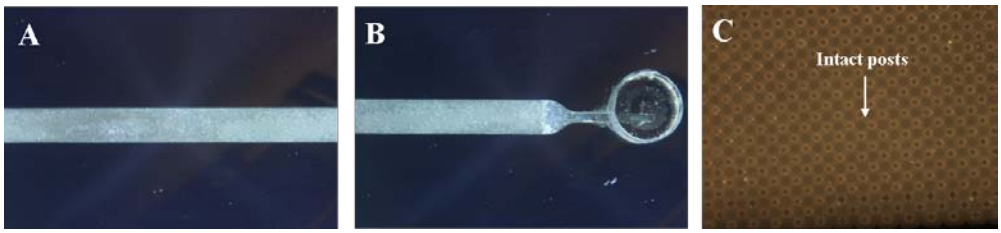

Figure 5. Optimal bonding method using 1-2 μL of DCE/EtOH solvent with manual (handheld) and C-clamp applied pressure. Complete bonding around the channel and reservoirs was obtained (A, B) without damage to the post structures (C).

Following bonding, a 50 µm deep, 1 cm long, and 800 µm wide channel with microscale posts resulted (Figure 2A, B). The strength of the bonding was then tested by flowing water through the device at 1.6 µL/min. For design D, the device remained bonded with no observable leak of solution from the device (Table 1). In contrast, the other designs came unbonded and solution began to leak between the cover and PMMA channel piece upon flowing water through the device. This demonstrated that the devices, using the developed bonding procedure, can withstand a head pressure, as calculated by collaborator Dr. Matthew Begley, (Table 1) of 0.8 kPa (head pressure generated with design D) whereas at 27.2 kPa (head pressure generated with design C) the bond no longer holds. This led to the selection of design D for all subsequent studies - this selection is described in depth in the next section. The adjustments made to the bonding procedure provided a method for bonding PMMA to PMMA for devices that contain 3-D microstructures that are susceptible to damage when exposed to excessive solvent, heat and/or pressure

Device Design	Head Pressure (kPa)	Withstood Pressure During Flow
A	41.6	No
B	62.4	No
C	27.2	No
D	0.8	Yes
E	32	No
F	360	No
No – Device bond failed and solution leaked from the device.		

Table 1. Head pressure of each device design, calculated by collaborator Dr. Matthew Begley, and whether each was capable of withstanding the pressure generated during syringe-driven flow (denoted by yes and no).

5.3.2 Selection of Device Design

The effectiveness with which DNA can be extracted using the PMMA microdevices depends not only on the surface chemistry, but also on the design of the device. The device must have ample surface area for functionalization with chitosan to ensure sufficient binding sites for DNA, and a design that provides minimal back pressure during syringe-driven flow. Six post designs were fabricated in order to determine the optimal design with regard to both surface area and flow. The surface area and largest aperture were determined for each design, as well as for 30 and 15 μm beads packed in a channel (1 cm x 200 μm x 200 μm) (Table 2), to allow for comparison to previously described methods for DNA extraction.[1, 4, 7, 8, 26, 27] The device design chosen was based upon a number of factors including flow resistance through the device, and surface area for binding. The chosen design D (as previously mentioned) has a similar surface area (0.63 cm^2) to that of 30 μm beads (0.67 cm^2), which would allow for sufficient quantities of DNA to bind to the phase, resulting in adequate DNA for ensuing analyses. Another factor that led to the selection of design D was the aperture diameter of 17.1 μm, significantly larger than the other designs at ~2-4 μm. The larger aperture of design D leads to less flow resistance through the channel when using pressure-driven flow. Head pressure calculations were completed for each design, as previously mentioned, and show that less flow resistance occurs with design D during syringe-driven flow at 1.6 μL/min (Table 1). It was determined that design D resulted in the lowest head pressure, 0.8 kPa at a flow rate of 1.6 μL/min, compared to 27.2 to 360 kPa for the other designs considered (Table 1). Flow of water through each device design was attempted at 1.6 μL/min and the effect on the device was observed. It was found that designs A, B,

C, E and F resulted in solution leaking from the side of the device, an indication that the PMMA bond failed under the pressure conditions associated with that flow rate. Thus only design D, with the lowest calculated head pressure and largest aperture, was found to be amenable to separations using syringe-driven flow at the rate required for efficient purification (Table 1), 1.6 µL/min. Design D was, therefore, chosen as the most advantageous design for remaining studies (Figure 6).

Sphere Diameter (µm)	Chamber SA/V ratio (cm^2/cm^3)	Total chamber SA including chamber walls (cm^2)	Largest diameter inscribed circular aperture (µm)
30	1480	0.67	4.64
15	2960	1.26	2.32
Micropost Designs			
Micropost Design A	3007	1.29	4
Micropost Design B	3363	1.44	4
Micropost Design C	2218	0.98	4
Micropost Design D	**1281**	**0.63**	**17.1**
Micropost Design E	3737	1.58	4
Micropost Design F	4028	1.69	2

Table 2. Surface and aperture dimensions of each PMMA device design, in addition to 30 and 15 µm silica for comparison.

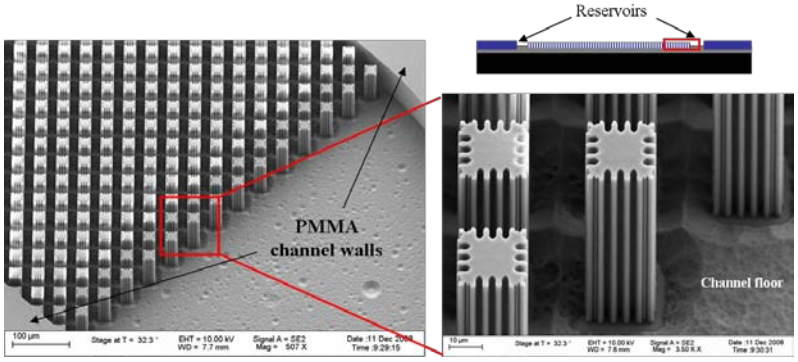

Figure 6. SEM images of optimal device design D.

5.3.3 Extraction of hgDNA on Chitosan-coated PMMA Device

Prior to functionalizing the PMMA surface to yield a surface chemistry that would allow for DNA binding (chitosan), the interaction of DNA with the PMMA microstructures of the channel was examined during the three steps that comprise an

extraction – load, wash, and elution. Typically, during the load step, DNA in the biological sample binds to the solid phase, while unbound cellular and extracellular material is removed during the wash step. The purified DNA is then eluted from the solid phase during the elution step. A solution containing 40 ng hgDNA (the mass of DNA yielded from ~5700 cells) in 25 µL 10 mM MES, pH 5 was loaded onto the device (after the device had been thoroughly rinsed for ~1 hr with deionized water at 1.6 µL/min) and the extraction performed as described in *SPE procedure for DNA Purification Using an Unfunctionalized PMMA Device* in *Materials and Methods*. DNA, detected by fluorescence using a commercial intercalating reagent (PicoGreen®), flowed unretained through the device containing an underivatized surface during the load step (Figure 7A), demonstrating that little to no DNA was binding to the PMMA micropost structures. Residual DNA from the load was recovered during the wash step with 10 mM MES, pH 5. No DNA was recovered during the elution step where 10 mM Tris/50 mM KCl, pH 9 was infused through the device. This clearly demonstrates that the PMMA microdevice requires functionalization with a DNA binding phase, such as chitosan.

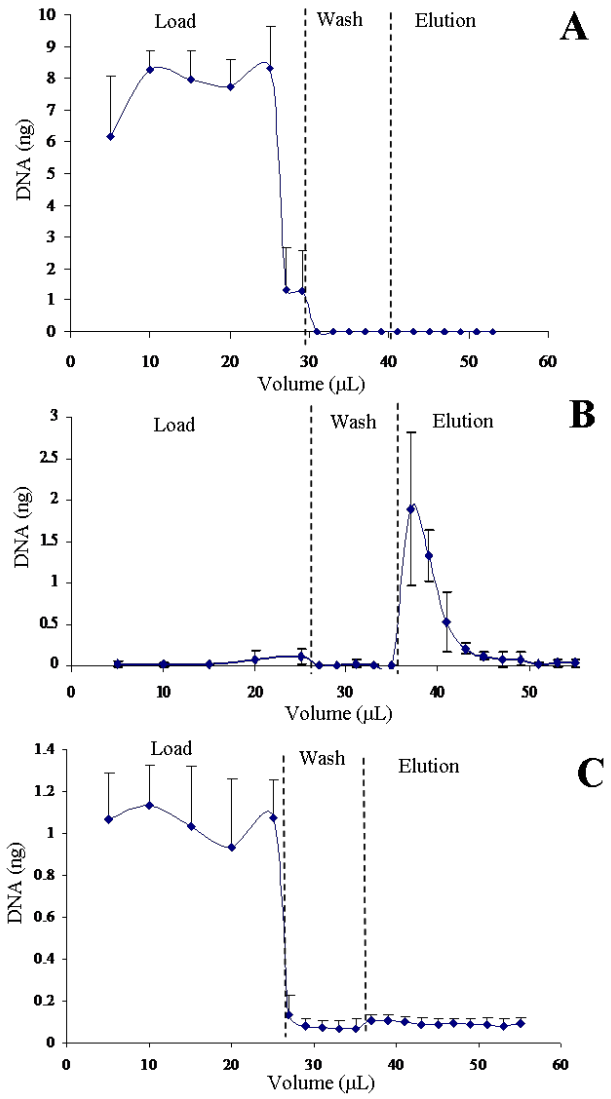

Figure 7. A. Extraction profile from the purification of hgDNA (40 ng) on a PMMA device not coated with chitosan (n = 3). B. Extraction profile from the purification of hgDNA (10 ng) on a PMMA device coated with chitosan which resulted in an extraction efficiency of 47.8 (±9.3)% EE (n = 5). C. Extraction profile from the purification of hgDNA (10 ng) on a chitosan-coated device that had been stripped with 1 M NaOH post-extraction to remove the chitosan phase (n = 4).

After determining that functionalization was necessary in order to reversibly bind and purify DNA using the PMMA device, a method developed for coating PMMA with chitosan was developed and tested. As described in the *Materials and Methods*, prior to functionalization of the PMMA, the device is exposed to UV light for 30 min. This has

been previously reported to successfully generate carboxylate groups, which have a pKa of ~4[28], on the surface of the PMMA.[14, 19-21] Once these groups are generated, a conjugation technique can be employed to link the carboxylates to amines. The linkage between the PMMA and chitosan (the amine) in this work is completed using a zero-length cross-linker, EDC, which has been previously reported for linking UV-exposed PMMA to an amine containing compound[14], and was used as a basis for the method developed here. Following incubation of the UV-exposed PMMA, chitosan, and EDC, an amide bond is formed between the PMMA and chitosan resulting in a solid phase that can be used for reversible binding of DNA (Figure 8).[14, 29] A device that had been coated with chitosan as described in *Chitosan functionalization* in *Materials and Methods* was used to purify hgDNA (10 ng). After loading the sample, the device was washed with 10 mM MES, pH 5, and the DNA eluted with 10 mM Tris/50 mM KCl, pH 9. Fractions collected were analyzed using a fluorescence assay as described in *Materials and Methods*. The resulting extraction profile shows that DNA was efficiently bound to the chitosan phase during the load, and recovered during the elution, resulting in an extraction efficiency of 47.8 (±9.3) % (n = 5) (Figure 7B). This data demonstrates that a PMMA device can be coated with chitosan and provide a reproducible, efficient extraction of hgDNA. These results were obtained with multiple devices, demonstrating chip-to-chip reproducibility of the method. This represents the first use of a PMMA microdevice with chitosan functionalization for the extraction of DNA. Functionalization of glass microdevices using chitosan for the extraction of DNA has previously been shown by Cao *et al*.,[16] but plastic (PMMA) microdevices provide the inherent advantage

of being cost effective when in large scale production, thus moving toward a completely disposable, integrated device for clinical or on-site analysis.

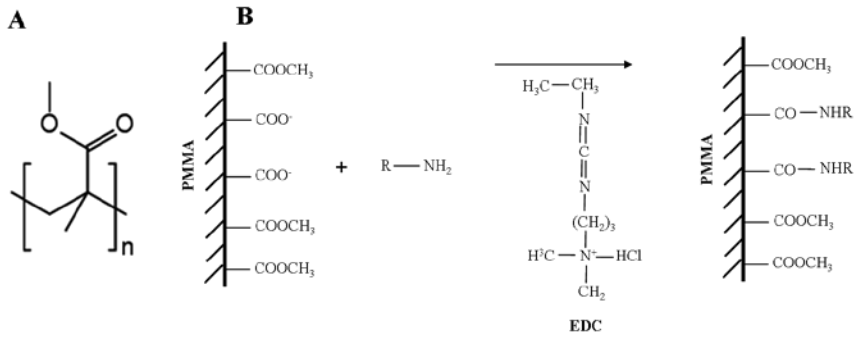

Figure 8. A. Structure of PMMA. B. Following exposure of the PMMA surface with UV light carboxylate groups are formed. Chitosan is then linked to these groups, forming the solid phase for reversible binding of DNA, using EDC as a cross-linker. Adapted from http://en.wikipedia.org/wiki/Poly%28methyl_methacrylate%29.

5.3.4 Chitosan Stripping and Extraction of Stripped PMMA Device

As these devices are not yet mass produced in quantities to allow for single-use disposability, the chitosan-coating needed to be stripped from the PMMA posts following the extraction of DNA to ensure that no DNA was carried over between multiple extractions on the same device. The multiple use of each device will be eliminated and changed to single-use when the devices become commercialized, but for research purposes the devices needed to be used for multiple extractions. The removal of the chitosan phase post-extraction (chitosan stripping) was performed by flowing 1 M NaOH through the device for 1 hr, followed by water for 1 hr. Sodium hydroxide was chosen for stripping due to the fact that it can hydrolyze the amide bonds that are formed between the PMMA and chitosan during the coating procedure, resulting in carboxylic acid groups on the PMMA surface.[30] A concentration of 1 M was chosen to balance

sufficient hydrolysis of the amide bond, while preserving the fragile post structures, which are susceptible to highly concentrated solutions.

The efficiency of the base hydrolysis of the amide bond was determined to ensure that all chitosan had been removed, thus reestablishing the binding sites for recoating with chitosan for the next extraction. The efficiency of hydrolysis was determined by loading 10 ng of hgDNA onto the stripped PMMA device in 10 mM MES, pH 5, washing with 10 mM MES, pH 5, and eluting the DNA in 10 mM Tris/50 mM KCl, pH 9. Figure 7C shows the extraction profile that resulted, demonstrating that all DNA passed through the device, unbound, during the load. This profile is like that of the underivatized device (Figure 7A) and clearly shows the absence of DNA binding. As further confirmation that no DNA bound during the load, no DNA is recovered during the elution step. These results demonstrate that 1 M NaOH is an effective method for removal of chitosan from the PMMA surface, returning the device to its original state where no DNA binding is seen.

It was then necessary to determine whether a device that had undergone chitosan stripping could be then be recoated with chitosan, and successfully purify DNA. Extractions of hgDNA were performed on devices that had undergone chitosan stripping and then recoated. This resulted in a reproducible elution profile with an extraction efficiency of 47.8 (±9.3)% (Figure 7B), demonstrating that the chitosan stripping and coating procedure provides a reproducible solid phase generation method that results in the reproducible extraction of DNA. It also shows that the number of times a device is recoated has little effect on the performance, as the extraction profile shown in Figure 7B represents extractions on a device which had undergone multiple cycles of stripping and

recoating. However, the coating process would benefit from further optimization, as the error bars seen in Figure 7B are most likely due to a lack of a complete homogeneous coating on the surface of the posts. Another variable was also investigated alongside stripping of the chitosan from the PMMA surface – UV exposure. As previously described in the *Materials and Methods*, prior to coating the PMMA device with chitosan, the device is exposed to UV light to generate carboxylate groups on the surface of PMMA. Following coating, purification, and stripping, the PMMA surface consisted of carboxylic acid groups which eliminated the need to UV expose the device prior to each coating (Figure 8), as the functional groups necessary for coating were present. Purifications of hgDNA were performed as described in the *Materials and Methods*, with and without further UV exposure. Preliminary results from this showed that the additional UV exposure before each coating was not necessary, as both resulted in similar shape and extraction efficiency (data not shown).

5.3.5 Extraction of hgDNA from Whole Blood

Although successful and reproducible extraction of prepurified hgDNA on the PMMA devices has been shown, the application of the device to commonly encountered clinical samples, such as whole blood, would provide a more stringent measure of the method. A lysed whole blood sample was loaded (25 μL of a 1 ng/μL solution; ~4000 WBCs) onto a chitosan-coated device. The device was washed with 10 mM MES, pH 5, and DNA eluted in 10 mM Tris/50 mM KCl, pH 9. Fractions collected were analyzed using a fluorescence assay and an extraction profile was generated (Figure 9A, n = 5 with EE of 9.9 (±5.0)%). The extraction profile shows reproducibility from sample-to-sample

and chip-to-chip despite the fact that multiple devices and blood donors were used. In addition to demonstrating reproducibility, sufficient quantities of DNA were recovered in a concentrated fraction during the elution for subsequent PCR analysis. The concentration of DNA recovered makes this DNA extraction method amenable to integration with on-chip downstream PCR. An additional advantage of the chitosan-coated PMMA device is the low protein binding nature of chitosan,[16] which allows for the method to be applicable to complex biological samples, such as the whole blood demonstrated here, that contain variable, and often large quantities of protein.

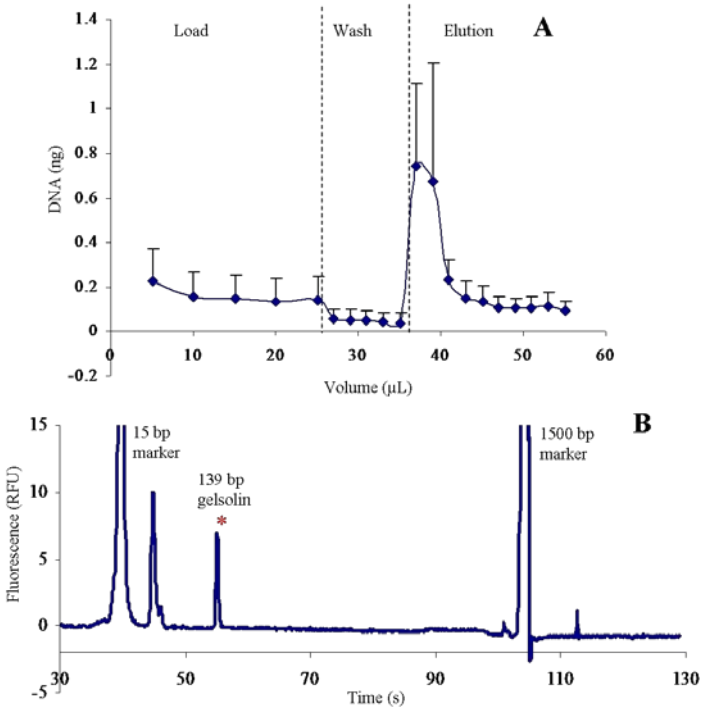

Figure 9. A. Extraction profile from the purification of hgDNA from whole blood using a chitosan-coated PMMA device (n = 5). B. Electropherogram from PCR amplification of a representative elution fraction of DNA purified from whole blood using a chitosan-coated PMMA device (n = 3). Successful purification is indicative by 139-bp amplicon for a portion of the gelsolin gene.

5.3.6 Amplification of Extracted hgDNA from Whole Blood

Although sufficient quantities of hgDNA were eluted from the chitosan-coated PMMA device for PCR analysis, the suitability of this DNA for PCR amplification was unknown. To ensure that the extracted DNA was indeed PCR-amplifiable, an extraction of hgDNA was performed by loading 25 µL of lysed whole blood (sample prepared as described in *Sample Preparation* in *Materials and Methods*) onto the device. The phase was washed with 10 mM MES, pH 5, and DNA eluted with 10 mM Tris/50 mM KCl, pH 9. The elution fractions collected were then amplified for a portion of the gelsolin gene, whose gene product is known to play an important role in regulating the length of filaments involved in cell structure, apoptosis, and cancer[31, 32], as described in *PCR Analysis* in *Materials and Methods*. PCR products were separated and analyzed on an Agilent 2100 Bioanalyzer. An electropherogram of a representative elution fraction is shown in Figure 9B, where a 139-bp amplicon, indicative of the gelsolin gene fragment, is present. This confirms that not only can sufficient quantities of hgDNA for subsequent analysis be extracted using the device, but the extracted DNA is of high quality, allowing for successful PCR analysis to be completed. This method provides a clear advantage as the extraction is completely aqueous, eliminating the use of PCR-inhibitory reagents (guanidine and isopropanol) which are required in silica-based extraction methods. Additionally, the chitosan phase used has been shown to be low protein binding, increasing the number of binding sites available for DNA on the phase, and increasing the quantity of DNA recovered. This advantage is not seen with silica-based purifications, where both the DNA and protein present in the sample bind the phase during the load, inherently decreasing the sites available for DNA[26].

5.3.7 DNA Binding Capacity Studies

Although the chitosan-coated PMMA device has been shown successful for the purification of hgDNA from whole blood resulting in PCR-amplifiable DNA, it was important to investigate the DNA binding capacity of the device to determine whether the method can be improved upon, resulting in a larger quantity of DNA available for subsequent amplification. To determine the DNA binding capacity, a hgDNA sample (1 ng/μL) was continuously loaded onto the device while fractions were collected and subsequently analyzed to quantitate the amount of DNA in each. From this, a breakthrough curve was generated which is seen in Figure 10. Similar to the analysis completed in Chapter 2, a first derivative of the data in Figure 10 has an inflection point which corresponds to the volume of load solution where DNA no longer binds to the phase. From this it was determined that the capacity was 13 (±7) ng. A breakthrough curve was also generated, experimentally, for binding of hgDNA from varying load sample concentrations of 2, 5, and 10 ng/μL (Figure 10). After taking the first derivative of the data in Figure 10, the binding capacities were determined to be 35 (±5), 60 (±7), and 130 ng (preliminary results) for the load sample concentrations of 2, 5, and 10 ng/μL, respectively. It can be seen from the increasing binding capacity at each concentration (Figure 11), that binding of DNA to chitosan is concentration-dependent. This trend is also clear from the linear relationship between the load concentration and capacity seen in Figure 11 (inset, R^2=0.9885). This could possibly be due to incomplete removal of chitosan during the stripping process even though DNA from prior extractions is sufficiently removed, which is clear from the consistent extraction efficiencies obtained

from devices that had undergone multiple purifications. Additionally, if 100% of the chitosan groups are not removed, when the device is recoated the total number of binding sites (amino groups on chitosan) would increase, further increasing the capacity. A similar trend has previously been observed by Lavertu, *et al.*[33], who saw an enhanced DNA binding to chitosan with an increase in amine groups [increase in amine to phosphate (DNA) ratio].

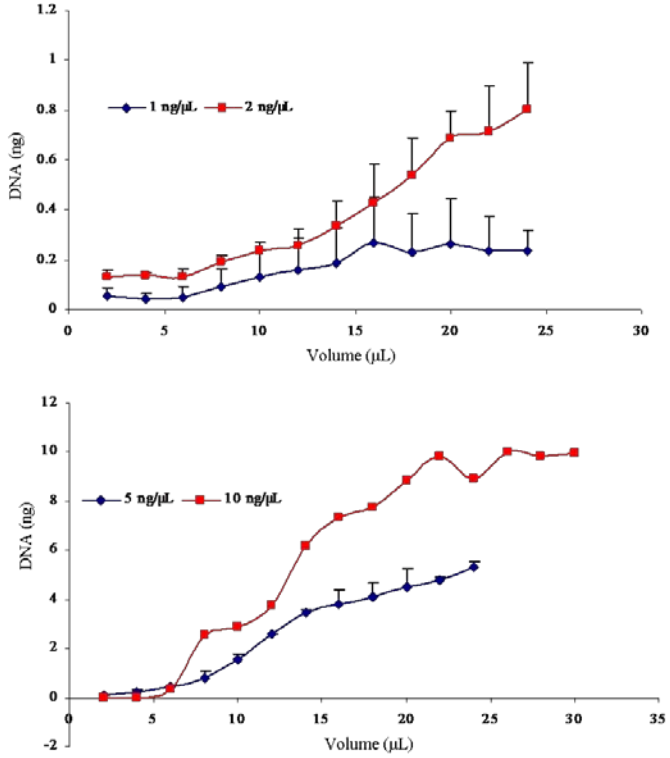

Figure 10. Breakthrough curves generated for DNA binding capacity studies on a chitosan-coated PMMA device. Load sample concentrations of 1 (n = 3), 2 (n = 3), 5 (n = 2), and 10 ng/µL (n = 1) were tested. A first derivative of the data were completed to determine the binding capacity at each concentration.

As previously stated, it was important to determine the capacity for DNA binding to further characterize the method, and also provide insight into additional optimization that can be completed. As seen in Figures 10 and 11, the capacity was determined to be 13 (±7) ng for a load solution containing 1 ng/µL hgDNA. The same concentration of

load solution was used, and 25 μL (25 ng) loaded, in the purification of DNA from whole blood (Figure 9), where an extraction efficiency of 9.9% resulted. It is clear that the capacity of the phase was exceeded with this quantity loaded, although no adverse affects on the success of the purification were seen, which accounts for the lower extraction efficiency observed. The result could be improved in the future by loading a more concentrated sample, as Figure 11 clearly shows an increased capacity and, therefore, potential higher recovery when a more concentrated load sample is used. These results also show that further optimization of the coating procedure is necessary. This could be completed by incorporation of N-hydroxysulfosuccinimide (sulfo-NHS) during the coating procedure. Sulfo-NHS is known to increase the stability of the intermediate, which reacts with the amine to form the amide bond, formed between the EDC and carboxylate groups. It does so by increasing the half-life of the intermediate to hours, allowing more time for amide bond formation.[29] Future work will involve incorporation of sulfo-NHS into the coating procedure to potentially increase the number of binding sites available, making the method applicable to samples containing a lower quantity of DNA template.

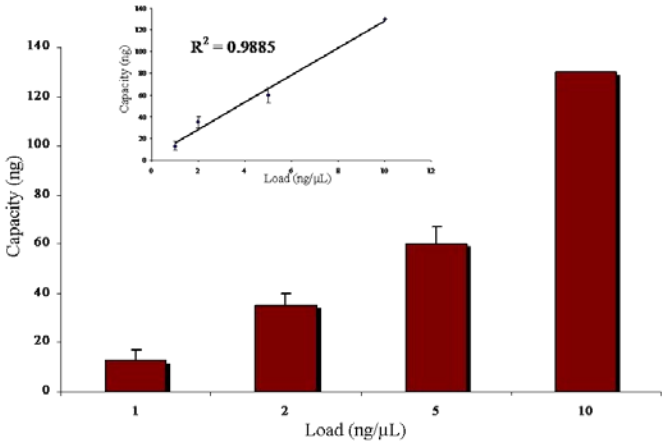

Figure 11. DNA binding capacities of a chitosan-coated PMMA device at load sample concentrations of 1, 2, 5, and 10 ng/µL. It was determined that the binding capacity is concentration-dependent. Inset demonstrates the linear relationship between the load concentration and capacity of the phase with an $R^2 = 0.9885$.

5.4 Conclusions

The development of a plastic microdevice capable of extraction of hgDNA from whole blood was demonstrated, making the device viable for numerous clinical applications, as whole blood is a common clinical sample. By utilizing chitosan-based purification technology for reversible DNA binding, PCR-inhibiting reagents, including guanidine hydrochloride and isopropyl alcohol, were eliminated. The DNA was also eluted in PCR buffer, making the extracted DNA PCR-ready and amendable to integration with PCR in efforts to develop a micro total analysis system (µTAS)[34]. The results shown in this chapter provide the next step towards a point-of-care, single-use device for use in clinical settings, such as physician offices. The desirable advantages of reduced analysis time and reagent consumption are achieved with this microdevice and technique. Decreased wait time for analysis could be vital for patients for whom delayed treatment can be disastrous. The results in this chapter also demonstrated sample-to-

sample and chip-to-chip reproducibility owing to the fabrication process and functionalization method.

Future work will include optimization of the device coating with chitosan to determine whether greater quantities of DNA can be extracted from biological samples. Additionally, developments towards a μTAS[34] will be investigated by integration with PCR on the plastic microfluidic device.

5.5 References

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6. Conclusions and Future Directions

6.1 Overarching Conclusions and Future Directions of Microfluidic Sample Preparation

This dissertation described effort exerted towards the development and optimization of microfluidic systems for sample preparation of forensic biological samples, with an emphasis on processing large volume samples that cannot be readily processed with other microfluidic methods. The work detailed in Chapter 2 described the development of a microfluidic solid phase extraction method for the purification of large volume, forensic biological samples. The successful optimization of the vrSPE method allowed for a 50-fold sample volume reduction and 15-fold sample concentration enhancement, which is an improvement over previous μSPE and conventional SPE methods. To demonstrate the breadth of applications, a range of samples including blood and heat- and UV-degraded blood and semen stains were processed using the method, all resulting in full, forensic STR profiles. Furthermore, the first demonstrations of microfluidic purification of mitochondrial DNA from blood and blood stains, and genomic DNA from bone were shown successful with the vrSPE method. This showed that microfluidic sample preparation is not solely limited to small volume samples, genomic DNA, or pristine samples, but can be used successfully for a wide-array of sample types. Although mtDNA purification was successful, during the purification process, mitochondrial and genomic DNA were simultaneously purified, as the silica phase used also binds genomic DNA under the conditions used for mtDNA. This means that binding sites for mtDNA on the phase are occupied by genomic DNA. Although most samples being analyzed for mitochondrial DNA contain little genomic DNA, it

would be beneficial if a solid phase could be tailored specifically to the nucleic acid of interest, in this case, for the sole binding of mtDNA. Even though both types of DNA contain the same charge-to-mass ratio, there are numerous factors that could be investigated, and potentially allow for an increased binding of mtDNA over genomic DNA. For example, within the mtDNA genome are repeat sequences, such as $(AC)_5$ (five repeats), which is in most individuals, or C-stretches (C-rich stretches). By exploiting this commonality, a probe, complimentary to these sequences, could be attached to a solid phase allowing for hybridization of these sequences to the probe, and an enhanced binding of mtDNA. Similar technology was previously developed by Toriello, et al.[1], but used oligonucleotide probes for hybridization of genomic DNA PCR products and not mtDNA or pre-PCR DNA. Therefore, a method that would allow for the simultaneous extraction and selection of mtDNA before PCR would be beneficial, as a secondary hybridization, like that developed by Toriello, et al.[1], would be unnecessary. Additionally, because of the smaller size of the mtDNA genome (~16,569 bp), separation based upon size (i.e. size exclusion chromatography), could potentially be used to separate the mtDNA from genomic DNA. This could allow for improved results from samples that are ancient or highly-degraded, which is often the sample type used for mtDNA analysis.

Chapter 3 demonstrated further development of the vrSPE device described in Chapter 2 by integrating the method with a secondary, orthogonal, purification method. The dual-phase purification system was found to be advantageous for the removal of the PCR inhibitor indigo dye, used to dye blue jeans, which is commonly encountered in forensic casework samples. The dual-phase system was shown to outperform low

volume μSPE and vrSPE in the removal of indigo, with both resulting in partial STR profile results, while the dual-phase device provided complete STR results. It was hypothesized that the method could also be used for the removal of other PCR inhibitors, such as humic acid or calcium, which are often encountered in forensic samples. Little work, except for that described in Chapter 3, has been completed using microfluidic solid phase extraction for the removal of extracellular inhibitors that are often present in biological samples. Continued optimization in this area would allow for the extraction method, and possibly solid phase, to be tailored to the particular inhibitor suspected to be present in the sample. This type of customization for each particular sample could greatly benefit the forensic community, as the analyst would be able to choose a phase, and method, that is designed for either the analyte or the extracellular material that must be removed before further processing, possibly improving the results obtained from challenging sample types.

Chapter 3 also described the potential of microfluidics to be multiplexed, where up to 16 analyses could be completed simultaneously on a single microdevice. Currently, macro-scale robotic instrumentation, such as the BioMek workstation in combination with the DNA iQ™ system, exists that allows for processing of numerous samples at once (96-well plate). Although this has helped to increased sample throughput, if a method such as the vrSPE-SPE device were to be multiplexed, it would increase sample throughput not only due to the number of simultaneous runs, but also due to the shorter analysis time inherent to microfluidics. This type of advancement would provide the throughput needed in forensic labs, as the backlog of samples to be processed increases every year with the reported number reaching close to 100,000 in 2008.[2] Additionally,

the robotic systems developed thus far allow for very few method adjustments depending upon the sample type encountered, whereas a microfluidic method which allows for easy interchanging of solid phases, could be tailored to the sample being processed, providing improved results. With the developments that have been made in robotic, automated platforms, it is foreseeable that future directions of microfluidic SPE not only include multiplexing but also automation, decreasing the amount of user time required, and, therefore, increasing the sample throughput of forensic laboratories.

Development of a microfluidic, forensic genetic analysis system, both in a modular and integrated form, is presented in Chapter 4. The modular system, which used three microfluidic devices for SPE, PCR, and ME, provided a 2-fold decrease in analysis time, when compared to conventional analysis. Additionally, it allowed for user/analyst intervention and discretion, which may be necessary when processing forensic casework samples. An integrated, microfluidic genetic analysis device was also developed that integrated SPE and PCR using IR-mediated heating. Processing of buccal swab samples was completed, and complete STR results using Identifiler™ (amplified 16 loci) were obtained in < 1 hr, a 5-fold reduction in analysis time.

Chapter 5 details the development and use of a PMMA microfluidic device for DNA purification. Plastic, such as PMMA, microdevices provide the distinct advantage of low-cost fabrication, allowing for disposability after a single-use. In chapter 5, a bonding method was developed that could not only withstand syringe-driven flow necessary for purification, but also ensure that the microposts structures, unique to this device, remained intact for later functionalization with a completely aqueous purification method. Successful application of the device to purification of DNA from whole blood, a

clinically- and forensically-relevant sample, was shown. Future developments in microfluidic DNA purification will not only be focused on plastic devices, but also application to a more wide variety of sample types.

6.2 Microfluidic Forensic STR Analysis

Microfluidic forensic STR analysis systems were presented in Chapter 4, both in modular and integrated form. Both methods have distinct advantages that would benefit the forensic community. The modular system allows the analyst to determine the optimal protocol to use for each device, depending upon the sample type, and its condition, being analyzed. This type of system may be more easily integrated into a forensic laboratory setting, as each process is separate and allows for protocol changes, under the analyst discretion, a commonality in forensic labs. This method also allows for laboratories to validate each process separately (i.e., μSPE, μPCR, ME) and begin use in a step-wise fashion, depending upon what area each particular lab foresees as a bottleneck for sample processing. Additionally, with each process performed in separate microdevices, automated platforms, for each individual step, could be developed that would decrease the amount of user time required and would also provide the opportunity for multiplexing, either with the same biological sample undergoing different analyses (i.e., different STR amplifications) or different samples undergoing the same process.

On the other hand, the integrated SPE-PCR device developed in Chapter 4 would provide a much faster analysis time and reduce the points of entrance for contaminants, due to it being a closed system, as all the processes are performed on one device. This would benefit the forensic community in decreasing the continuously growing backlog of

samples. Additionally, because the processes are performed on one device, the method is amenable to portability, which would allow for on-site analysis, further decreasing the analysis time. Although this type of on-site analysis has the potential to revolutionize the field of forensic genetic analysis, it could be a slow process for the method to gain large and widespread usage. In order for this to be accomplished, the method would have to require a lower cost-per-analysis from what is currently required. Additionally, it would be beneficial for reagents for each process to be included within each device, further decreasing opportunities for entrance of contaminants, a concern when not in a clean laboratory environment, and decreasing the expertise required to complete on-site analysis. Although the forensic community may be cautious of the results provided by an on-site analysis, it would provide presumptive results, which can generate leads in a shorter time period, and later be confirmed in a laboratory setting. Even if this type of technology does not gain immediate, widespread field use, a number of advantages still exist if used within a laboratory setting, i.e., decreased analysis time.

6.3 Plastic Microfluidic Devices

Chapter 5 described the development of a plastic microdevice for solid phase extraction. Recently, focus has been placed on transitioning from glass to plastic microdevices due to the lower costs of fabrication, as the plastic devices can be fabricated in bulk from a mold which is not as easily achieved with glass devices, and, therefore, disposability of the devices after a single-use. In Chapters 2-4 of this thesis, analyses were completed in glass devices which required cleaning after each extraction, amplification, or separation. If plastic devices were used, no cleaning would be

necessary as the device would be disposed of after a single-use. Not only does this remove the time required for cleaning, but it also eliminates any concern of carry-over or contamination from previous samples, an absolute requirement in forensic and clinical analysis. Additionally, higher fabrication costs are often associated with glass devices which eliminate the ability to dispose of the device after a single-use, which would be possible with plastic devices due to lower fabrication costs. Transitioning of sample processing methods to plastic devices provides the steps needed towards developing the quintessential, disposable, integrated genetic analysis device that could revolutionize the forensic and clinical fields.

6.4 Summation

The work described in this thesis demonstrated development of microfluidic methods for the purification of DNA from biological samples. Purification of DNA, using a volume reduction SPE method, from large volume, forensic biological samples was shown, along with the first demonstrated purification of mtDNA from blood stains and genomic DNA from human bone. The greatest advantage of the vrSPE method was seen when integration with a secondary, orthogonal purification method was completed, allowing for removal of a common PCR inhibitor, indigo dye. Development of microfluidic, forensic STR analysis systems, modular and integrated, was demonstrated in chapter 4 to provide a significant reduction in required analysis time. The next steps towards developing a disposable, plastic microfluidic device was shown in chapter 5, where a SPE plastic device was developed and successfully employed for DNA purification from whole blood. All the work described in this thesis for sample

processing on microfluidic devices lays the foundation, and method development, to greatly benefit the forensic and clinical fields by providing reliable and expedited analysis techniques that could revolutionize sample analysis. Future directions include investigation of solid phase modifications, to selectively bind analytes of interest (mtDNA or remove other PCR inhibitors), continued optimization of each technique to provide the essential integrated genetic analysis systems for the forensic and clinical fields.

6.5 References

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