Final Report
(Revised)

Closed System DNA Purification for Degraded, Compromised Evidence in Microfluidic Devices

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Abstract

Acquiring a DNA profile from large volume highly degraded or compromised forensic samples is particularly challenging sample type to be interfaced with a microfluidic system. Previous microfluidic devices utilizing a silica-based solid phase have been successful at purifying DNA from complex biological samples such as whole blood, however due to their small cross-sectional areas cannot operate at fast enough flow rates to make the processing of mL volumes a feasible option. The development of a volume reduction solid phase extraction (vrSPE) device employs a large cross-sectional area, enabling flow rates capable of handling a 0.5 mL sample in just ~30 minutes was realized. The large solid phase allows for greater binding sites on the silica surface, capable of binding the DNA but also the binding of PCR inhibitors, removing them from the eluate allowing for successful DNA profiling. In addition the vrSPE device reduces, and thus concentrates the DNA found in large volume samples often encountered throughout an investigation. Such samples include whole blood and semen stains, which need to be solubilized from the substrate prior to processing, diluting the DNA to concentrations in the order of 0.1 ng/µL. The vrSPE device was utilized to purify degraded whole blood and semen stains that were exposed up to 80°C for three months or subjected to UV light for the equivalent of 8 months and 16 days in LA sunlight, resulting in full STR profiles in each instance. The extraction of bone was also demonstrated, a particularly difficult sample type due to the demoralization of the bone; a partial profile of 15 of 16 loci was produced. Once nuclear DNA is too degraded for STR amplification, mitochondrial DNA (mtDNA) can be used to identify persons. mtDNA is particularly susceptible to contamination, therefore an especially suitable sample type for the closed system microfluidic platform. The vrSPE device successfully purified and extracted amplifiable mtDNA from a severely heat degraded whole blood stain. The solid phase was also demonstrated to effectively remove inhibitors from the sample, specifically 35 µg of humic acid which is found in soil, outperforming current methods which see allelic dropout at 27 µg. The addition of a downstream µSPE phase, using chitosan-coated silica particles providing completely aqueous chemistry, further enhanced the removal of indigo dye – a common sample

contaminant found in blue jeans. STR profiles produced with the dual phase device were superior to those from the vrSPE channel alone, whereas a single µSPE channel failed to produce any peaks. This second phase offers two key advantages over one vrSPE alone – further removal of inhibitory material and increased concentration of the sample, especially resonant with severely diluted samples. Once the vrSPE method was fully established and demonstrated to be applicable to a plethora of sample types, fabrication of further devices in either a multi-channel or multiplex format were devised. For ease of fabrication and increased reproducibility between multiple channels, poly (methyl methacrylate) PMMA, was chosen as the new substrate for these devices. Following verification that PMMA operated as well as the previous glass substrate devices, a multiplex extraction of four separate dilute whole blood samples from four individuals was performed, resulting in full STR profiles. These results show the vrSPE technology is a successful method for processing a wide variety of large volume, degraded, inhibited biological samples in a timely, cross-contamination free manner.

Executive Summary

The aims of this grant were to develop a volume reduction solid phase extraction (vrSPE) microchip capable of accommodating large volumes. The device was then to be applied to degraded and compromised samples as well as the purification of mitochondrial DNA. The device was also to be demonstrated in a multiplexed format for as few as two samples. Finally devices were to be field tested in a forensic laboratory using non-probative evidentiary samples.

A vrSPE glass device was conceived and optimized using 8 µm superparamgantic silica particles as the solid phase. This allowed for copious binding sites within the phase, ensuring that contaminants would not occupy sufficient binding sites to prohibit the binding of DNA. Equally, the device results in a 50-fold reduction in sample volume, concentrating the DNA, enabling successful PCR. The effectiveness of the device was demonstrated initially with dilute whole blood samples, with only 140 nL of blood in 500 µL required to produce a full STR profile. These results demonstrate the effectiveness of the vrSPE method at removing protein and cellular debris found in biological samples.

Degraded samples are often encountered at crime scenes that have been exposed to heat or UV light and often require large volumes to remove the biological stain form the substrate. Heat degraded blood and semen stains were exposed to 56°C for either one or three months, with a further set exposed to 80°C also for one or three months. Each of the four samples were extracted and amplified producing full STR profiles. Blood and semen stains on cotton cloth were subjected to UV for 56 min which equates to 4 months and 8 days outside. Using the vrSPE device, full STR profiles were obtained for these highly degraded samples. Doubling the UV exposure time to 1 hour and 52 minutes degraded the samples further, however a full STR profile was also obtained for both the blood and the semen stain.

A key benefit to the large solid phase is the large number of binding sites available, not only for DNA but also for contaminants in the sample that would inhibiter downstream PCR processing. Humic acid, found in soil, was spiked into dilute whole blood samples to highlight the ability of the vrSPE device to remove inhibitors. Previously reported work had allelic dropout when 27 µg of humic acid was present in the sample. vrSPE outperformed this by demonstrating a full STR profile when 35 µg of humic acid was present in a 500 µL dilute whole blood sample.

Forensic analysis of bone samples required large volumes for the demineralization, normally in the order of 3 mL, therefore an ideal sample type for processing with vrSPE. By following the AFDIL demineralization process, the device was able to purify the sample, resulting in a partial profile of 15 of 16 loci.

Mitochondrial DNA (mtDNA) processing is particularly susceptible to contamination; therefore a microdevice for the processing of this sample is a favorable choice as microfluidics offers a closed system. Following successful extraction of mtDNA form a dilute whole blood sample, a blood stain was then created to be more representative of a true forensic sample. The DNA was degraded by being incubated at 80°C for 3 months and was successfully amplified following processing with the vrSPE device.

Adding a second orthogonal phase downstream to the characterized vrSPE channel create the opportunity for the sample to go through two purification and concentration steps. Using a µSPE channel with a chitosan-coated silica phase, an all aqueous chemistry follows the vrSPE phase, allowing for further downstream processes to be integrated. In addition, the application of a second phase not only provides binding site of a different chemistry but also concentrates the extracted DNA sample further, enhancing PCR by providing a greater mass of template.

Following optimization of the device, initially with prepurified hgDNA and then with blood and semen stains, the success of the device over a single vrSPE alone was demonstrated with the

removal of the contaminant of indigo dye. Indigo dye is commonly found in forensic casework resulting from a sample being collected from blue jeans and is a known PCR inhibitor and must be removed from the sample prior to genetic profiling. Up to a concentration of 8mM, the indigo dye was successfully removed by both vrSPE alone and the vrSPE-SPE device; however the dual phase gave an improved STR profile, demonstrating that either further indigo dye was removed, or the further concentration of the sample was significant.

Fabrication of these vrPSE devices used glass as the substrate – an expensive material that uses dangerous chemicals for the creation of the microfluidic channels (e.g. HF). To curtail these issues, vrSPE devices were created using the common polymer poly (methyl methacrylate), PMMA as an alternative substrate, providing an inexpensive, fast and reproducible method of fabrication. These devices operate with the same procedure as the glass devices, and upon comparison where shown to provide the same recovery of DNA.

The reproducibility of the fabrication method – creating the channels through laser ablation - leant itself well to the utilization of the PMMA substrate for the creation of multi-channel and multiplexed devices. These new devices enables faster processing of such large volumes, will providing greater binding sites to remove inhibitory substances. The success of the multiplexing vrSPE was demonstrated with simultaneous extraction of dilute whole blood from four individuals, demonstrating high throughput of the vrSPE device without cross contamination, each with full STR profiles.

Detailed Technical Report

I. Introduction

DNA profiling is now a process synonymous with forensic casework and the development and application of new technologies to deliver evidence with increased sensitivity, throughput and ease of use are significant tools for the investigation. Microfluidic devices present numerous advantages to current forensic analyses, including low reagent consumption and reduction in analysis time, combined with the ability to deliver a closed sample in-answer out multi-process system within a single device[1]. Additionally, these devices require less sample template, enabling preservation of sample for further analysis when there is limited quantity during forensic casework.

A challenging forensic sample type for microfluidic platforms are those collected from surfaces or stains, which may involve large volume (milliliters) of a dilute sample for processing. Further, diluting samples with PCR inhibitory contaminates reduces their impact on the purified, extracted DNA, therefore increasing the discrimintory power of the laboratory result with full, callable STR profiles. The challenge of macro-to-micro interfacing has been addressed by the development of volume reduction solid phase extraction (vrSPE), where a large SPE phase was designed to accept sample volumes 10-fold larger than traditional SPE devices[2, 3].

II. Materials and Methods

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). Potassium chloride was purchased from Mallinckrodt Chemical Company (Paris, KY). Triton

X-100 for molecular biology was purchased from Fluka (St. Lous, MO). λ–phage DNA was purchased from USB (Cleveland, OH). 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). 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). AmpFlSTR® 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).

Microdevice Fabrication

Glass microdevices were fabricated using borofloat glass (Telic Company, Valencia, CA) and standard photolithographic techniques.[4] The PMMA devices were fabrication through laser ablation using a VersaLASER® system 3.50 from Universal Laser Systems (Scottsdale, AZ). The channels were ablated into 1.5-mm-thick Poly(methyl methacrylate) (PMMA) purchased from McMaster-Carr (Santa Fe Springs, CA).

vrSPE and vrSPE-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; the PMMA multiplexed flow was controlled by four Aladdin-1000 syringe pumps (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. Following a given extraction, the glass channels were rinsed with 2M HCl to prevent DNA contamination.

Fabrication of Chitosan-coated Phase

Chitosan-coated silica beads were fabricated as described in Hagan, et al.[5] 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.

Sample Preparation - vrSPE

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 $\mu W/cm^2$). 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. **The samples used for the study were prepared in a manner that is similar to those prepared for conventional processing. No 'extra care' was provided for the samples over and above that normally given in a casework lab setting, and many of the samples were prepared at VDFS by qualified forensic scientists.**

Sample preparation – vrSPE-SPE

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. **Again, the samples used for the study were prepared in a manner that is similar to those prepared for conventional processing. No 'extra care' was provided for the samples over and above that normally given in**

a casework lab setting, and many of the samples were prepared at VDFS by qualified forensic scientists.

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. First, the sample was loaded in 6 M GuHCl, pH 6.1 and then eluted with 1X TE 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.

SPE Procedure, Dual-phase vrSPE-SPE

A silica and chitosan packed vrSPE-SPE microdevice 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 16A). 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 16A) 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 16).

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.

Fluorescence Detection

Once elution fractions had been collected, a commercially developed PicoGreen® fluorescence assay[6] 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.

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.

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.

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).

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.

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.

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.[7] The samples were then separated and analyzed using

an ABI 310 Genetic Analyzer. **The quality of the produced STR profiles was measured based upon the number of loci present in the resulting STR profile, using a threshold for peak height established by ABI 310 Genetic Analyzer. This quality was then compared against the number of loci reported by others in the literature with similar sample types but with different purification methods.**

III. Results and Discussion

Microdevice Design

Previous microfluidic SPE devices were designed to handle small sample sizes (≤ 100 µL), etched to 200 µm deep and 500 µm wide, operating at a flow rate of 4 µL/min[8]. To accommodate a greater flow rate of 15 µL/min, while maintaining the given relationship for effective nucleic acid binding of

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

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[9], the channel dimension used are 200 µm deep and 1.4 mm wide.

Phase selection

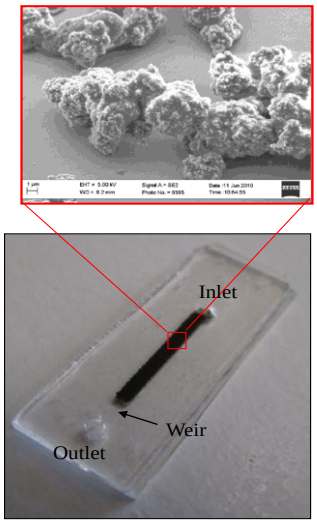

Figure 1. vrSPE device (1 cm to weir, 1 mm line width, 200 µm deep, 20 µm weir depth) packed with MagneSil™ solid phase. SEM image of a MagneSil™ particle is shown enlarged.

Establishing the optimal solid phase capable of both high DNA binding capacity and low protein affinity, while delivering a large surface area for the irreversible adsorption of contaminants was paramount to the success of the device. The efficiency of 30 µm silica and 5-8 µm silica-coated-paramagnetic particles to reversibly bind

prepurified λ-phage DNA was explored; the 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 1**), the latter resulted in a more concentrated, smaller volume eluate. Therefore the silica-coated-paramagnetic particles were chosen as the increased concentration enhancement is favorable for further analysis of the extracted DNA.

The binding capacity of the phase was then addressed, both with prepurified λ DNA and with whole blood to establish protein affinity. A linear relationship between DNA loaded and then recovered for 11.4 µL of whole blood is present, with an exponential relationship present for 400 ng of prepurified λ DNA (**Figure 2**). To establish a breakthrough curve (when the DNA no longer binds

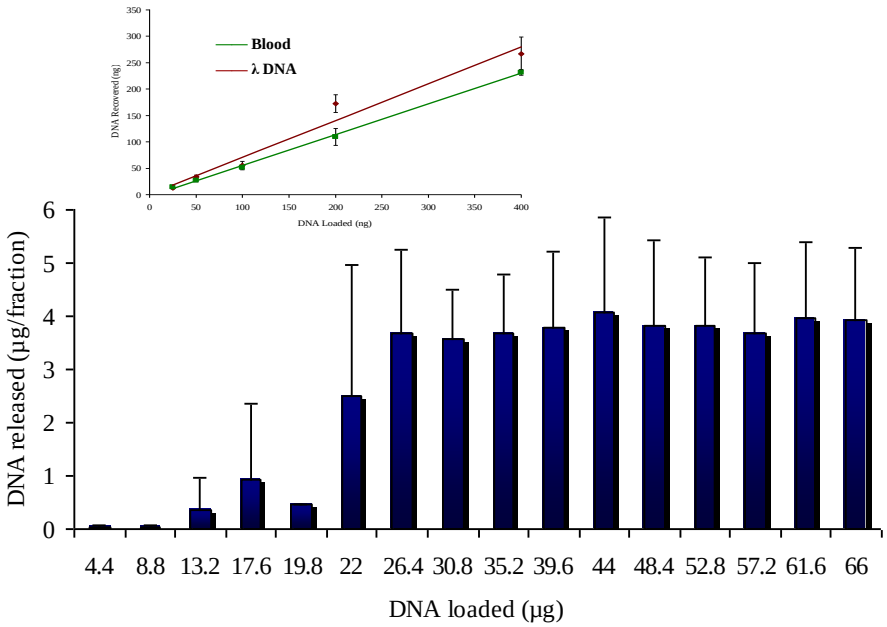

Figure 2. Breakthrough curve indicating binding capacity of the MagneSil™ phase in the vrSPE device for hgDNA, λ–phage DNA (inset), and whole blood (inset).

to the phase as binding sites are occupied) with a large genome, hgDNA was loaded onto the device in a concentrated sample of 0.11 µg/µL. The results show the capacity of the phase to be 50.3 (± 5.6) ng of hgDNA/µg of particles (450 µg of particles loaded into the microdevice) (n = 3).

and separated and analyzed using a Bioanalyzer 2100, which has the capability of providing a semi-quantitative measurement of amplicon concentration. A 5 min 80% IPA wash resulted in a gelsolin amplicon concentration of 0.26 (± 0.03) ng/µL (n = 3) and no wash concentration of 1.41 (± 0.45) ng/µL (n = 3) (**Figure 4A and B**). The removal of a wash step from the procedure, delivers improved STR quality, whilst decreasing processing time.

Extraction of low DNA template and time-degraded samples

Forensic samples with a low template are often created through dilution of the original sample to minimize the effect of contaminants on downstream processes. The vrSPE device demonstrated the successful extraction of a 1:3,500 fold dilution of whole blood (140 nL of template) in a concentrated fraction, resulting in a full (7 of 7 loci) STR profile using the COfiler® amplification kit (**Figure 5**).

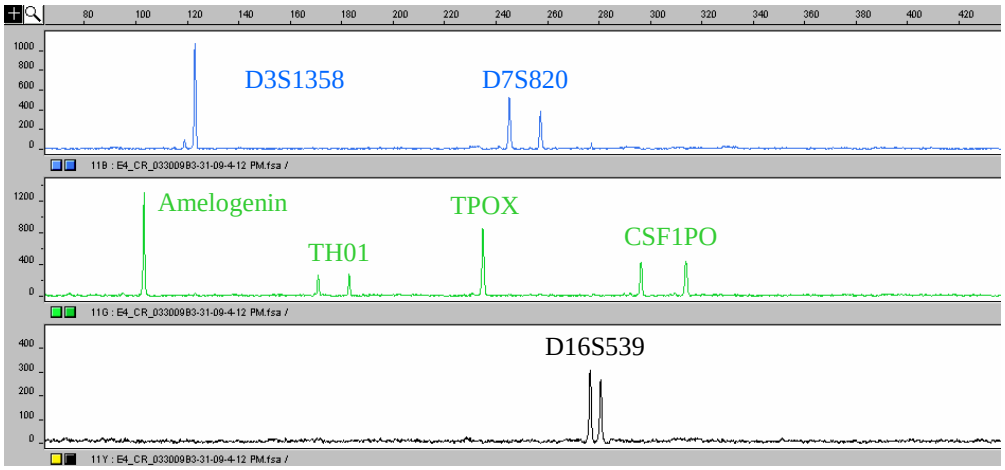

Figure 5. Full STR profile (7 of 7 loci) of DNA extracted from a large volume, low DNA template sample containing 140 nL of whole blood.

When the same particles were utilized in a robotic platform, locus dropout was observed following a 1:1000-fold dilution of blood stored on Whatman paper. Although these sample types are not directly comparable, the impact of the vrSPE device is shown. blood stain on Whatman paper, held at room temperature for 3 years was successfully amplified using the MiniFiler™ amplification kit, resulting

in a full (9 of 9 loci) STR profile (**Figure 6**). **It is noteworthy that the quality of the STR profiles produced by the vrSPE method clearly demonstrate the value of using this purification technique. We did not perform PCR-STR on crude samples, as both literature and experience demonstrate that cellular material and inhibitors cause the PCR to fail. This will be exacerbated by data on indigo-contaminated samples in a later section (Fig. 24).**

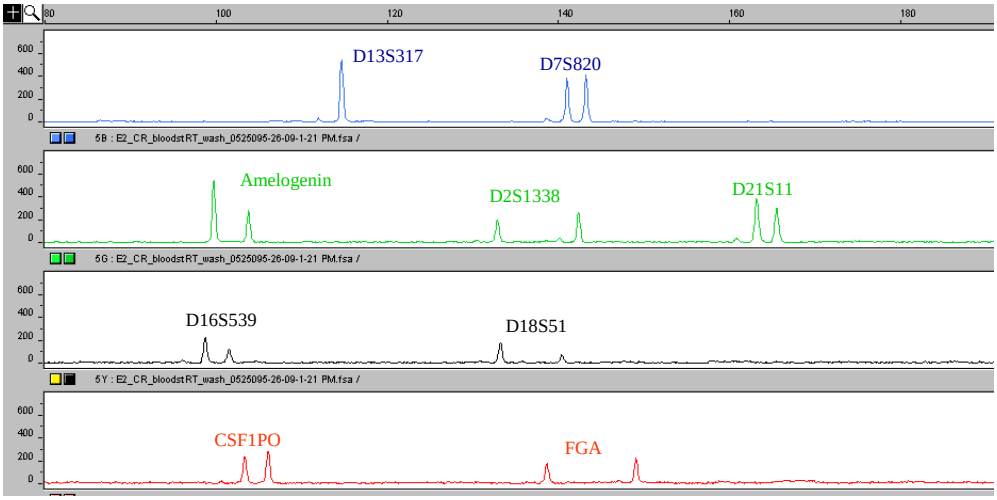

Figure 6. Full STR profile (9 of 9 loci) of DNA extracted from a 3 year old blood stain on Whatman paper that was solubilized/removed from the paper in a large volume (500 µL).

Extraction of DNA from heat-degraded blood and semen stains

Common sample types encountered during forensic investigations are heat-degraded biological stains that require large volumes for solubilization, thus processing. DNA exposure to heat can cause the longer genomic DNA to break, creating shorter 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.[10] 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 7**, 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. These peaks 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. 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.

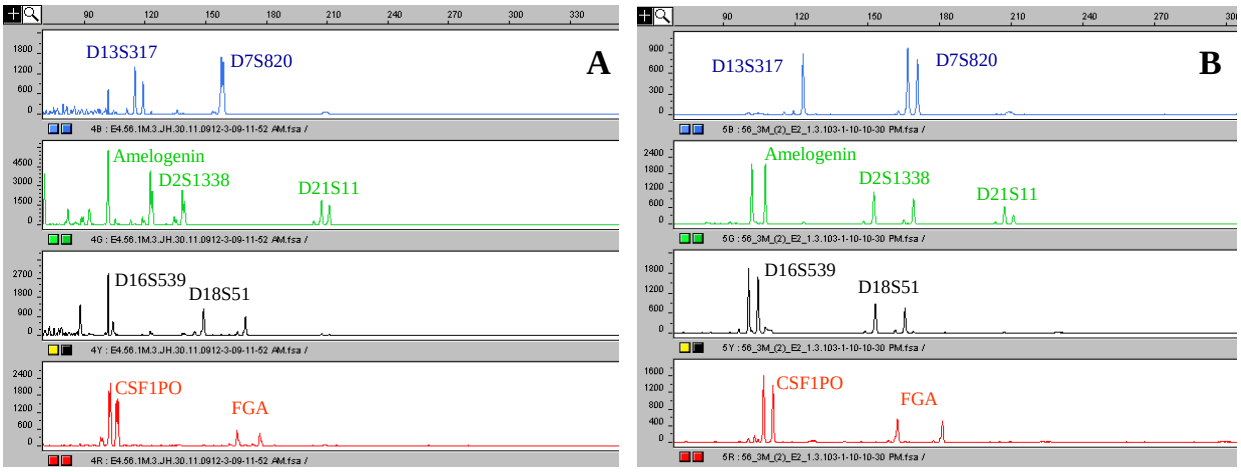

Figure 7. Full STR profiles (9 of 9 loci) of DNA extracted from blood stains on Whatman paper incubated at 56 °C for 1 (A) and 3 (B) months that was removed from the paper using a large volume lysis solution (500 µL).

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 8,** n = 3). It is important to note that spurious peaks (allelic drop-in) were again seen, 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.

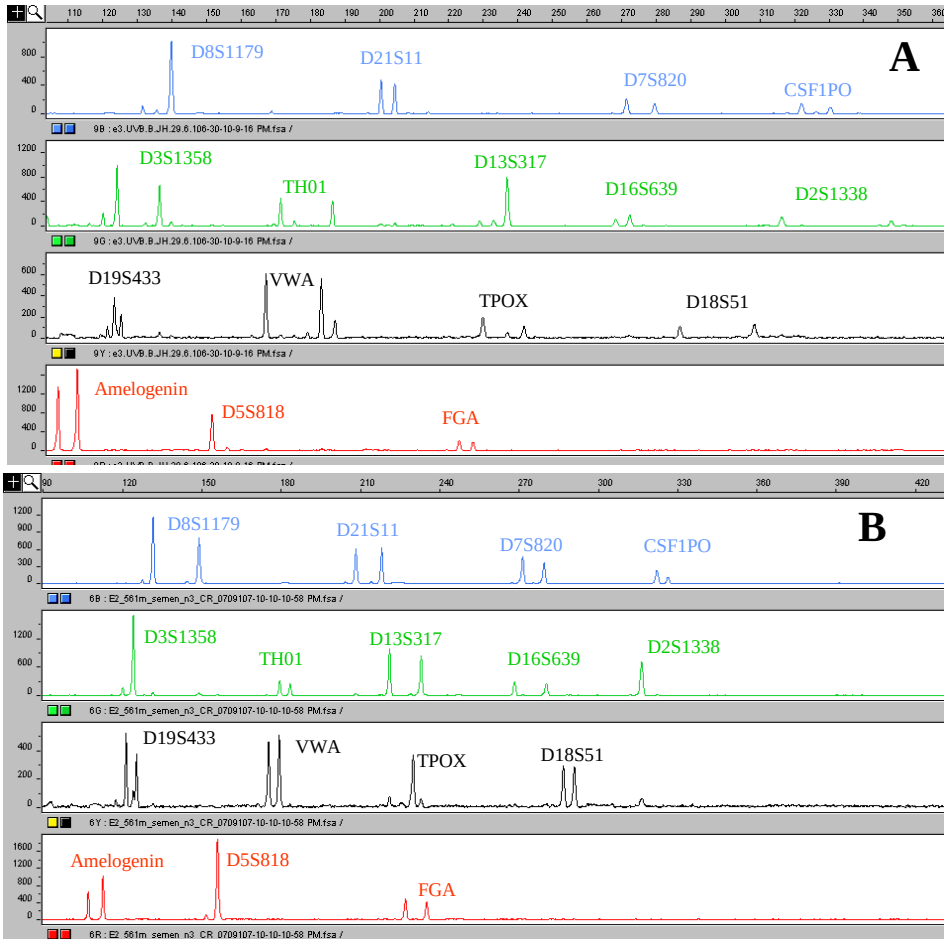

Figure 9. Full STR profiles (16 of 16 loci) of DNA extracted from blood stains on cotton incubated at 56 °C for 1 (A) and 3 (B) months that was removed from the cotton using a large volume lysis solution (500 µL).

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 requires 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[11]. 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[12] 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). Approximately 25% of the blood and semen stains were removed from the cotton in a large volume, the DNA was extracted using the vrSPE method and 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 10A**, n = 3) and semen (**Figure 10B**, n = 3) stains. Therefore several processes and analyses can be performed on a single sample, allowing for more definitive STR identification. This demonstrates the successful application of a microfluidic extraction method for UV-degraded samples.

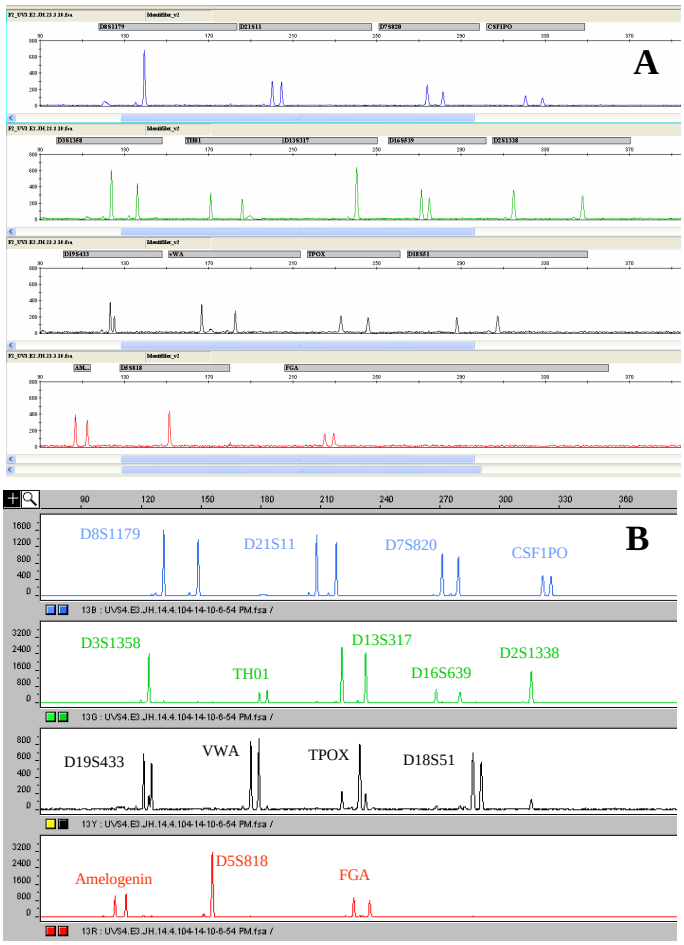

Figure 10. Full STR profiles (16 of 16 loci) of DNA extracted from blood (A) and semen (B) stains on cotton exposed to UV light for 56 min. The stains were removed from the cotton surface using a large volume lysis solution.

To further explore the limits of UV-degraded samples that can be purified with the vrSPE method of blood and semen stains that were exposed to 1 hr 52 min of UV light (8 months and 16 days). The degradation of the DNA requires amplification with Minifiler® kit; resulting in full (9 of 9 loci) STR profiles (**Figure 11**). For the first time a microfluidic platform is able to process these large volume samples, where the closed-system nature of the device is ideal to prevent additional contaminants affecting an already degraded sample. The successful demonstration of the vrSPE

method extracting amplifiable DNA from severely compromised samples, highlights the applicability of the device in mainstream forensic processing.

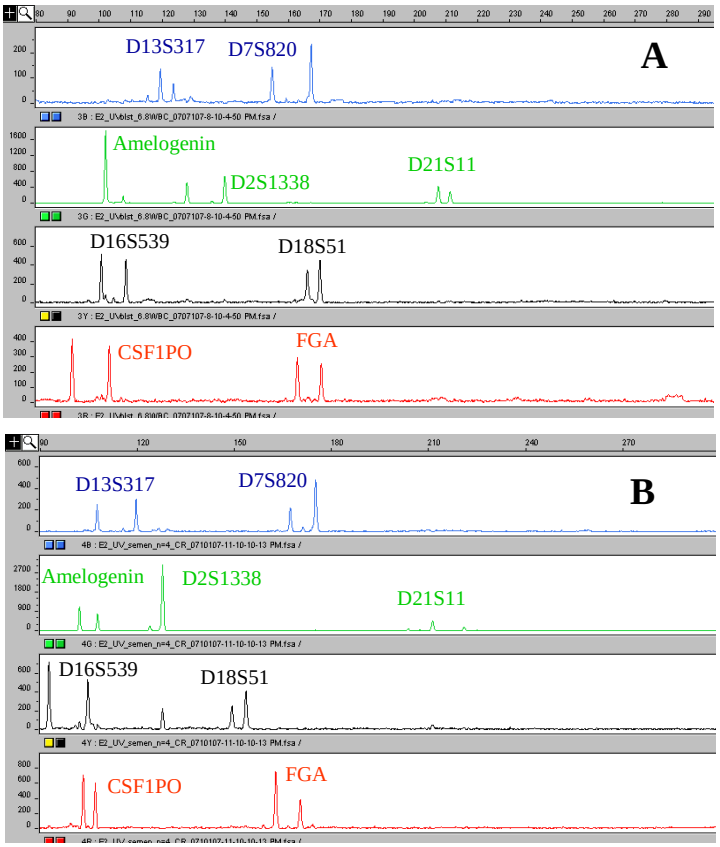

Figure 11. Full STR profiles (9 of 9 loci) of DNA extracted from blood (A) and semen (B) stains on cotton exposed to UV light for 1 hr 52 min. The stains were removed from the cotton surface using a large volume lysis solution.

vrSPE for the removal of Humic Acid

In addition to the evaluating the capability of the vrSPE method for purification of DNA from degraded stains, samples containing the contaminant humic acid were also purified using the microfluidic method. Humic acid is commonly found in soil and is known to be a PCR inhibitor, which forensic biological samples often contain. Conventional extraction methods, such as low volume purification columns or low volume microfluidic methods, often have difficulty removing this inhibitor. The vrSPE method provides a distinct advantage over these methods, having a larger volumetric throughput and increased binding capacity. The ability to process a larger sample volume allows the biological sample can be diluted to a larger volume, therefore, diluting the inhibitor present in the sample and reducing the possible impact it has on post-extraction analysis. The increased

development towards purification of DNA from a bone sample on a microdevice.

Extraction of Mitochondrial DNA from Whole Blood and Degraded Blood Stains

Often forensic samples are degraded to the point where nuclear DNA typing is challenging, leading to mitochondrial DNA (mtDNA) analysis as an alternative. Contamination is a great concern in mtDNA processing; therefore microdevices offer an ideal closed system environment for their analysis. Further, most mtDNA samples are of a large volume[7], making vrSPE and ideal tool to process such samples in a time efficient manner.

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. 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. MtDNA was successfully extracted on-chip and amplified off-chip from dilute whole blood, shown by the 459bp amplicon (**Figure 14A**).

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. The resulting mtDNA product can be seen by the presence of the 271 bp amplicon [**Figure 14B** (n = 3)]. These results illustrate the first successful extraction of mtDNA from whole blood and from a degraded blood sample using a microfluidic device.

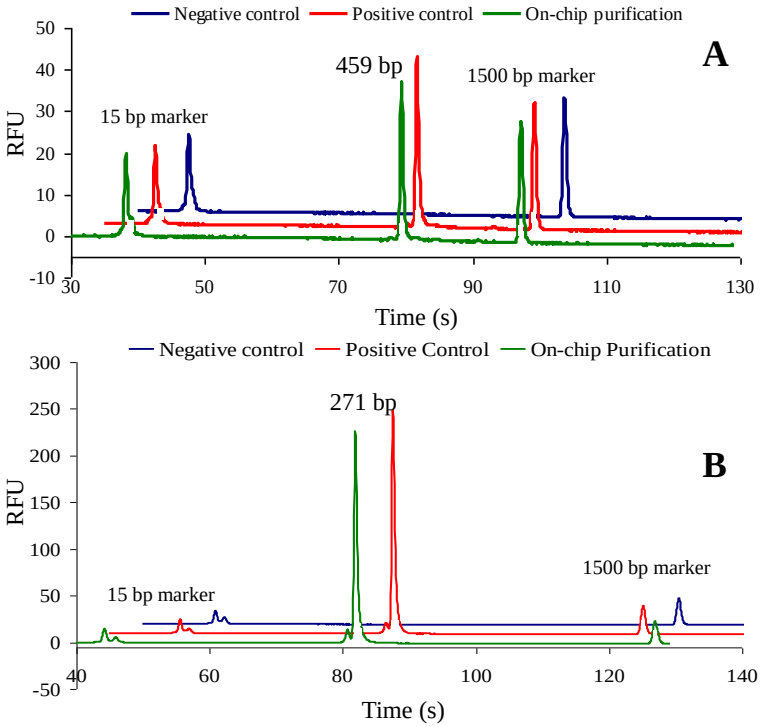

Figure 14. Electropherograms indicating successful amp of a portion from the HV1 region of the mtDNA genome after on-chip vrSPE of mtDNA from (A) dilute whole blood (HVI primers from VDFS) and (B) a degraded blood stain (MPS 2B or PSII primers from AFDIL).

Dual-domain Microchip Theory

Most commercially-developed products for purification of DNA use only one type of binding chemistry, mainly silica. 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. Further, by adding a μSPE channel, the volume reduction achieved by a single vrSPE process will be increased, allowing for future downstream integration in a microfluidic device. A totally aqueous method[14] utilizing chitosan-derivatized silica beads to reversibly bind DNA in a pH-dependent manner was chosen as the second phase as the aqueous chemistry avoids PCR-inhibitory reagents found in silica-based purification methods (guanidine hydrochloride and isopropyl alcohol).

Dual-domain Microchip design

The success of the vrSPE method for the processing of large volume forensic samples could be further concentrated and purer by interfacing the domain with a second SPE phase. Successful binding of the DNA to the second chitosan phase requires a pH of ~5, therefore the eluate from the first phase (pH=8) needs to be lowered by mixing this with the µSPE loading buffer together. They are brought together by a teardrop (TD) design, where the loading buffer is mixed to the eluate from both sides, reducing the diffusion distance (**Figure 15**).

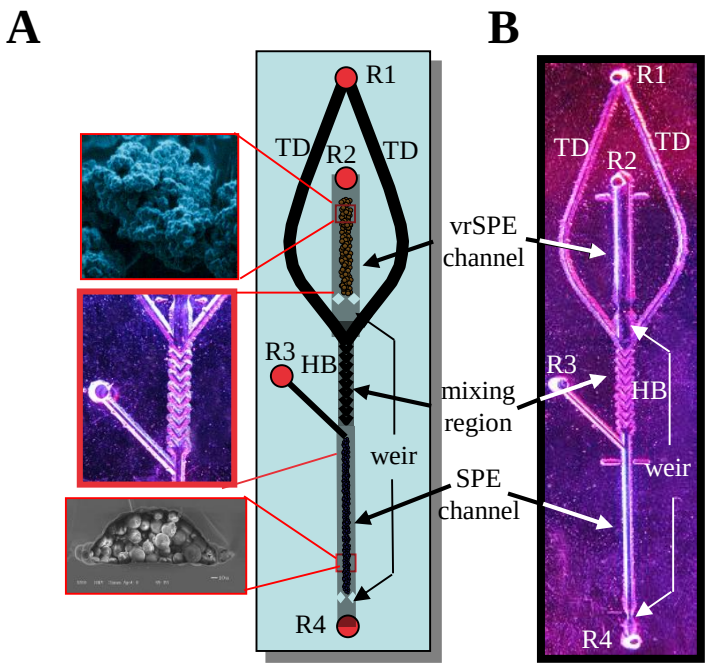

Figure 15. A) Schematic drawing of vrSPE-SPE device depicting the MagneSil™ phase in the vrSPE channel, herringbone mixing structure, and chitosan phase in the SPE channel (right). Enhanced views of MagneSil™[26] phase (top left), herringbone mixing region (middle left), and a packed SPE channel (bottom 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.

Complete on-chip mixing is achieved with a herringbone region, devised to induce turbulent flow (**Figure 16).** 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.[15] The µSPE channel is placed downstream from the herringbones, with channel dimensions of 500 µm wide, 200 µm deep and 1cm long.

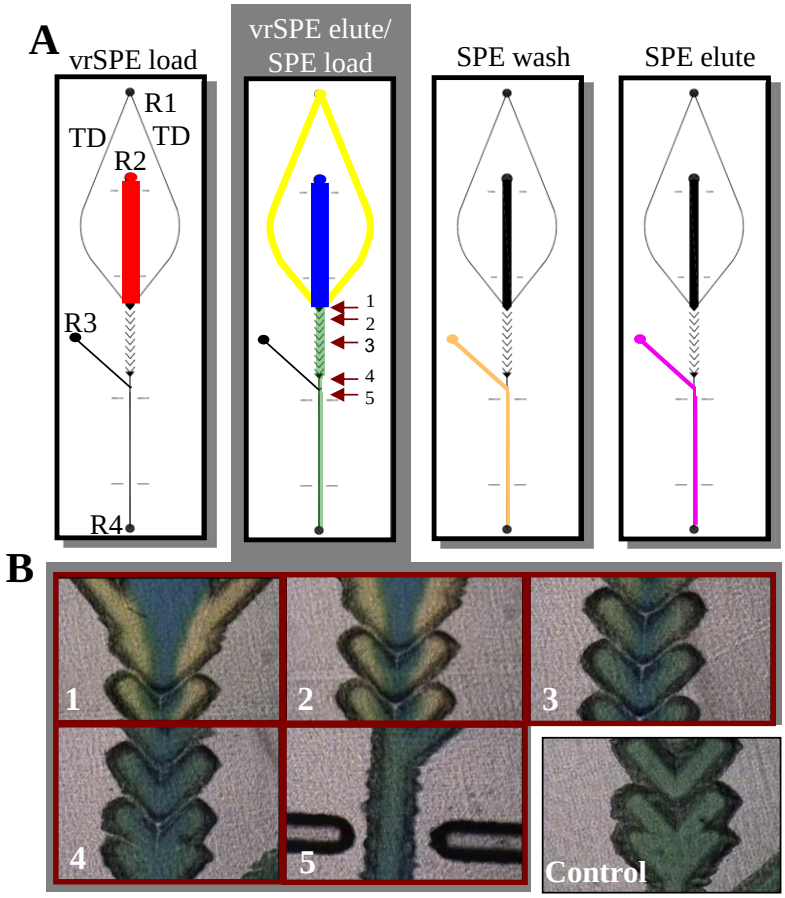

Figure 16. vrSPE-SPE device protocol and evaluation of mixing in herringbone region. A) vrSPE-SPE device protocol where sample is infused through R2 (*vrSPE load*). Nucleic acids are then eluted from vrSPE via R2 while load buffers for SPE are simultaneously infused through R1 and the teardrop (TD) structures (*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.

Optimization of vrSPE-SPE flow rate conditions

Previous work using chitosan-coated silica beads for DNA purification in a microdevice in the Landers lab demonstrated that a volumetric flow rate of 5 µL/min was optimal for DNA binding. However as previously demonstrated the optimal elution flow rate for the vrSPE phase is 15 µL/min, however this would need to be reduced to 2.5 µL/min as the 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. Demonstrating the effectiveness of eluting the DNA from the vrSPE phase at 2.5 µL/min, a large

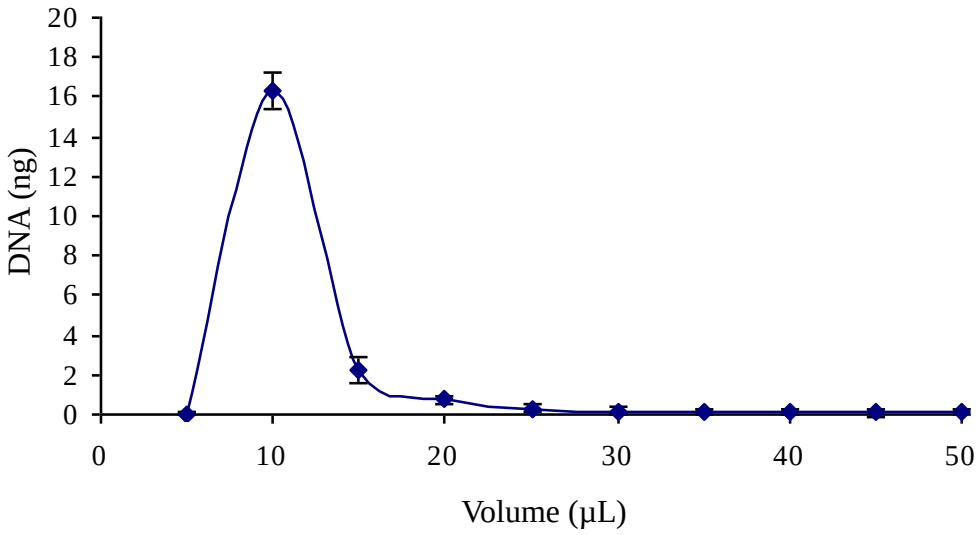

Figure 17. Elution profile (n = 3) of prepurified hgDNA using the single-phase vrSPE device with an elution flow rate of 2.5 µL/min and a corresponding extraction efficiency of 41.5 (±1.5)%.

volume sample (500 µL) containing hgDNA was loaded onto the single-phase vrSPE device and extracted. 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)%[16] , 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 effects seen on the shape of the elution profile (**Figure 17**).

vrSPE-SPE Extraction Efficiency and Elution profile

Extraction efficiency for DNA on a SPE device using chitosan as a solid phase is typically 47 (± 4.2)%[5] while an efficiency of 34.7 (± 4.0)% is seen using the vrSPE device for purification of DNA.[17] These studies suggested that an integrated microdevice combining these two orthogonal processes would result in an overall extraction efficiency of ~25%. An extraction was performed where the chitosan phase was conditioned for the optimized 30 min prior to loading a 500 µL solution containing hgDNA onto the vrSPE-SPE device. DNA was then extracted and analyzed with a fluorescence assay to determine the quantity of DNA in each elution fraction. The elution profile demonstrates this chip design and functionality is reproducible, seen in both the shape and extraction efficiency [28.1 (±1.3)% (n=3)] (**Figure 18**). Further this represents successful mixing of the eluate and the loading buffer, and thus the binding of the DNA to the chitosan surface.

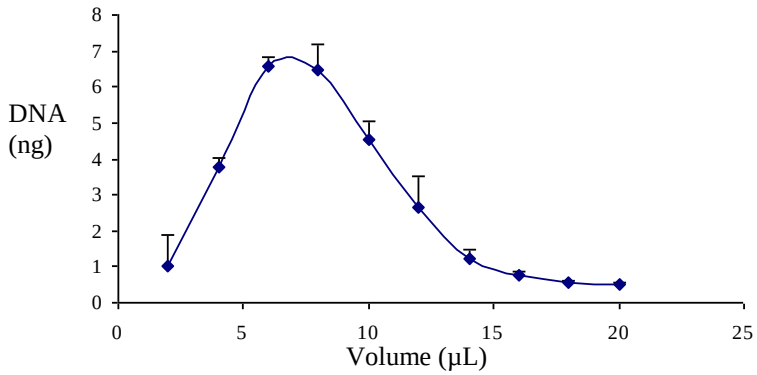

Figure 18: Elution profile (average of n=3) from the extraction of prepurified hgDNA using the vrSPE-SPE device.

These elution profiles show 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

conducted to determine whether DNA purified using the device was PCR-amplifiable. A 500 µL sample containing prepurified hgDNA was loaded onto the device and elution fractions were amplified off-chip using primers specific for amplification of a fragment of the gelsolin gene, whose product is known to play an seminal role in regulating the length of filaments involved in cell structure, apoptosis, and cancer.[20, 21] The 139-bp amplicon corresponds to a fragment of the gelsolin gene from a representative fraction, demonstrating the DNA recovered from the device is PCR amplifiable (**Figure 19**).

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

Following verification with prepurified samples, the device was evaluated using a relevant forensic sample type – 4 µL (150ng) of whole blood in 500 µL was loaded onto and subsequently extracted from the device. Amplification of the DNA with the AmpFℓSTR® COfiler® kit resulted in a full (7 of 7 loci) STR profile (**Figure 20).**

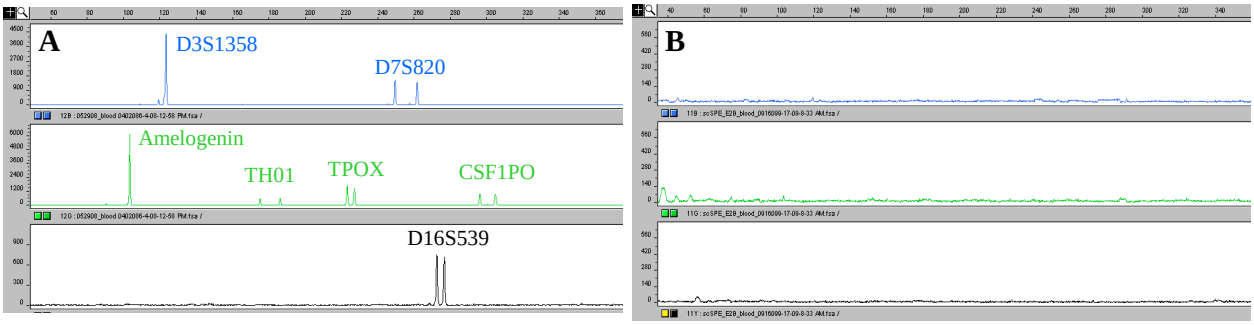

Figure 20. A) STR profile (representative of n = 3) resulting from extraction of hgDNA from a dilute, large volume whole blood sample on the vrSPE-SPE device. All loci are present. B) STR profile (representative of n = 3) after extraction of hgDNA from a dilute, large volume whole blood sample on a standard µSPE device.

Further, a 6 µL blood stain on cotton was prepared, representing a large-volume forensic sample. Amplification with COfiler® kit again resulted in a full (7 of 7 loci) STR profile (**Figure 21**).

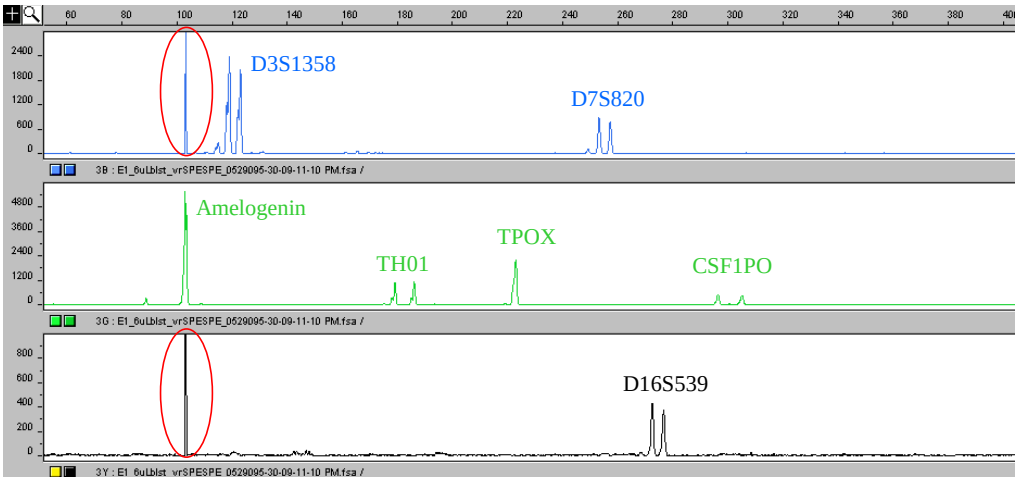

Figure 21. Full STR profile resulting from extraction of hgDNA from a blood stain using the vrSPE-SPE device. Peaks circled in red are due to pull-up from Amelogenin.

vrSPE-SPE Purification of hgDNA from Dilute Semen and a Semen Stain

A further sample type often encountered in large-volume forensic samples is semen, found often in sexual assault cases, was evaluated using the vrSPE-SPE device. A 500 µL sample with 1.5 µL (265ng) of semen was loaded and the DNA subsequently extracted and amplified using the COfiler® kit, resulting in a full (7 of 7 loci) STR profile (**Figure 22**). This volume of semen was chosen to simulate the amount of template expected in a typical collected sample from a victim on a

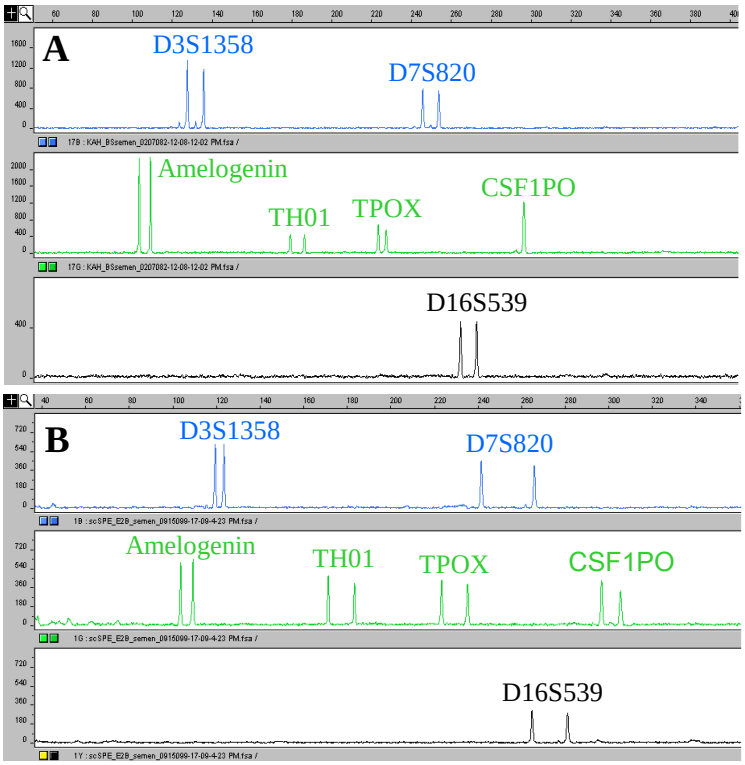

Figure 22. A) STR profile (representative of n = 3) resulting from extraction of hgDNA from a diluted, large volume semen sample on the vrSPE-SPE device. B) STR profile (representative of n = 3) resulting from extraction of hgDNA from a diluted, large volume semen sample on a standard µSPE device.

cotton-tipped swab.

To test the dual-phase device against a better representative sample type, a dried semen stain (10 µL of semen) cut as a 0.25 x 0.25 cm square from a white cotton swatch was prepared and fully immersed in 6M GuHCl, pH 6.1, for processing via vrSPE-SPE. The extracted DNA was amplified using the COfiler® kit, resulting in a full (7 of 7 loci) STR profile (**Figure 23**).

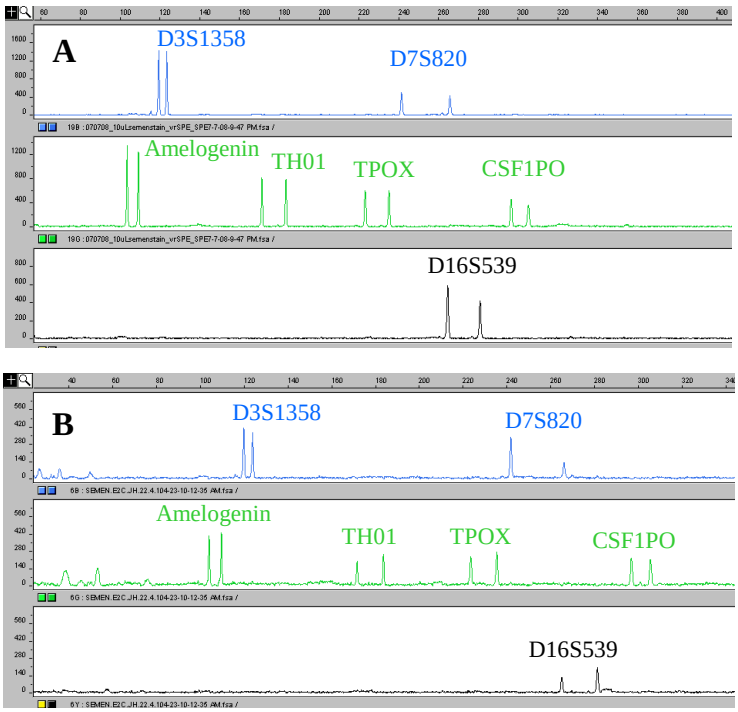

Figure 23. A) STR profile (representative of n = 3) resulting from extraction of hgDNA from a semen stain on the vrSPE-SPE device. B) STR profile (representative of n = 3) resulting from extraction of hgDNA from a semen stain on a standard µSPE device.

vrSPE-SPE for Removal of Indigo Dye

The large binding site capacity, coupled with the ability to handle large-volumes is the greatest advantage to the vrSPE device at removing PCR inhibitors, which is only intensified with the addition of the chitosan phase. 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.[22] Results from this work by Wang et al.[22] 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 ≤ 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 (16 of 16 loci) STR profile resulted (**Figure 24A**) 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 24B**) 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) (**Figure 24C**), 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.

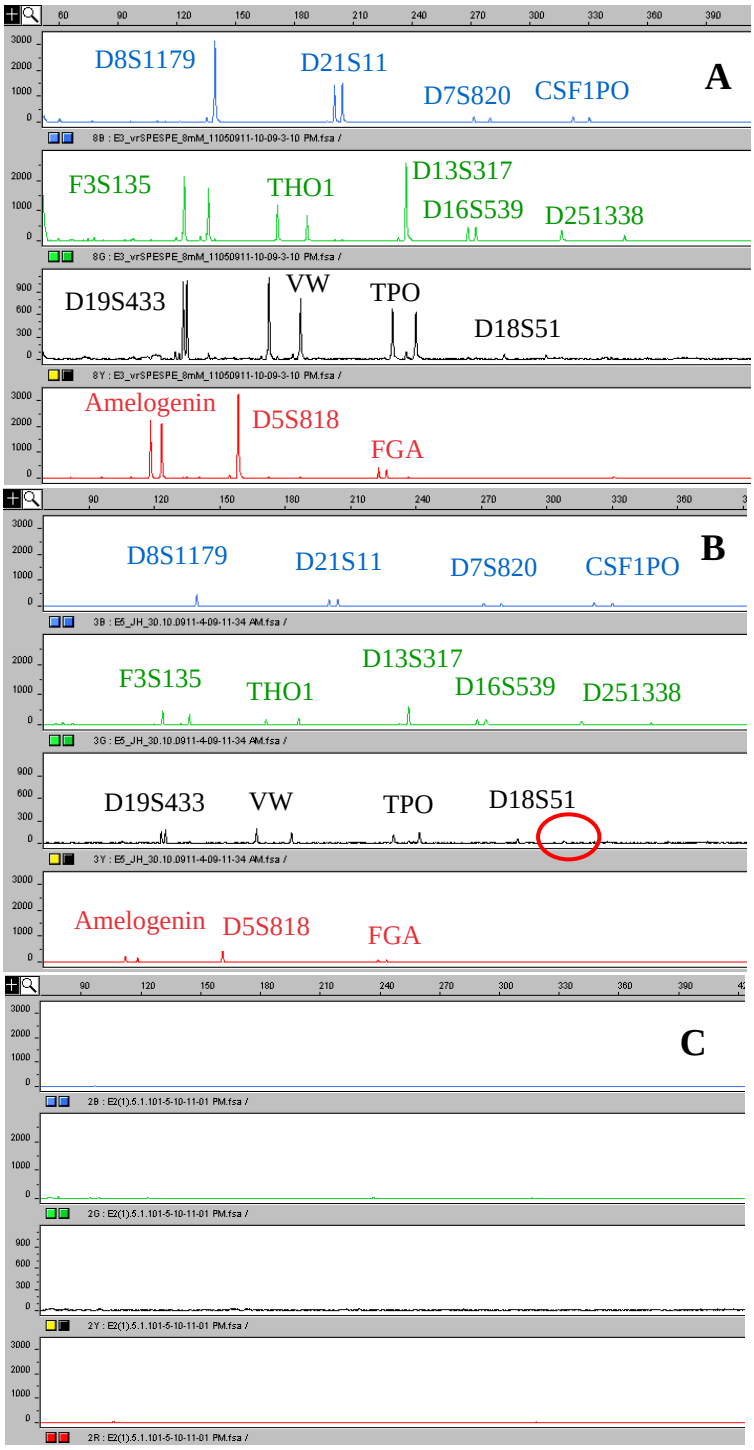

Figure 24. Comparison of STR results of amplified DNA that was purified from a diluted, whole blood sample spiked with indigo dye using three microfluidic devices. A) STR profile (representative of n = 3) resulting after extraction of hgDNA from a diluted, large volume whole blood sample containing 8 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.

Development of PMMA vrSPE Devices

The work described thus far have used microdeivces fabricated glass, an expensive, time consuming process that utilizes hazardous chemicals (e.g. HF). The use of plastic substrates, however, (e.g., poly (methyl methacrylate); PMMA) circumvents these fabrication issues. In

from a 500 µL sample containing DNA at a concentration of 100 pg/µL. Amplification with the Idnetifiler® kit resulted in a full (16 of 16 loci) STR profile (**Figure 26B**).

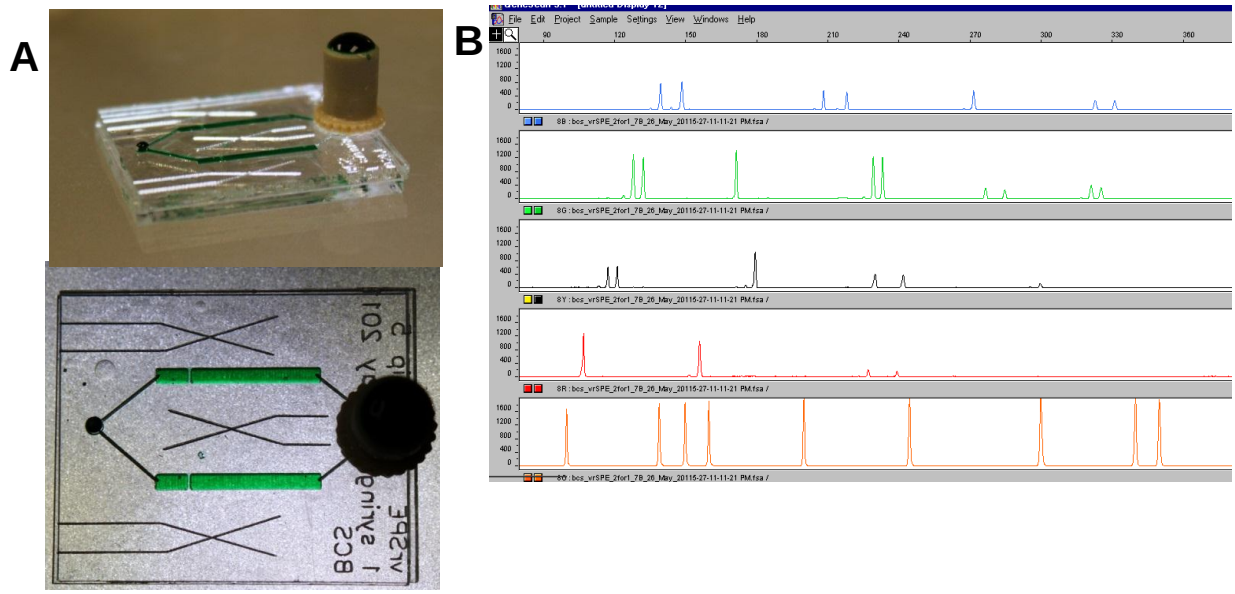

Figure 26: PMMA vrSPE designs to reduce analytical time or increase throughout of large volume samples. A) Pictures a a devices using a duplex phase to extract and purify DNA from a sample, with the channels filled with green food dye for illustration. B) An full STR (16/16 loci) using the Identifiler® amplification.

4-Plex PMMA vrSPE Extraction of Whole Blood

Fabrication and successful functionality of the multi-channel device shows that the creation of a multiplexed device in PMMA was feasible, allowing for four separate samples to be purified and extracted simultaneously from complex matrices (e.g. whole blood) (**Figure 27A**). The extraction of whole blood represents the first biological sample purified and extracted using a PMMA vrSPE microdevice, from 4, 500 µL samples of 60 ng of whole blood diluted to 120 pg/µL. The successful, cross-contamination-free of four sample is demonstrated by the amplification of a full (16 of 16 loci) STR profile using the Idenifiler® kit (**Figure 27B**). This represents the first multiplexed vrSPE extraction on a microdevice.

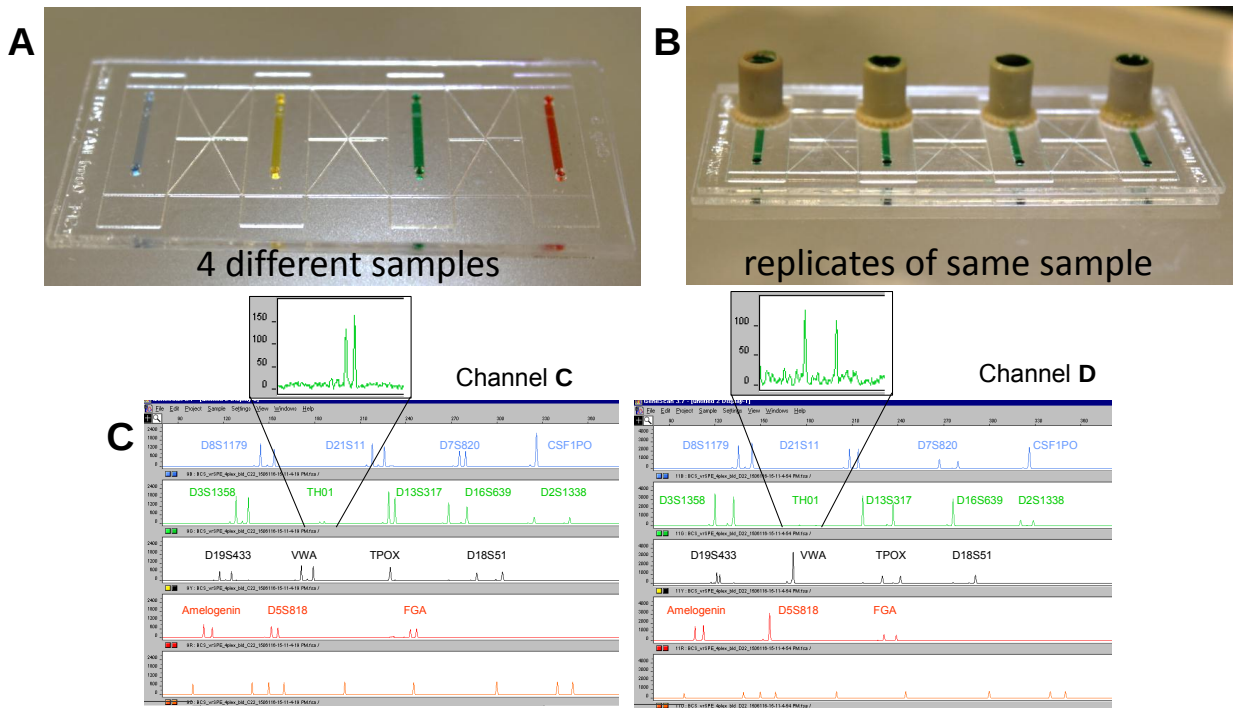

Figure 27: Pictures of a multiplex PMMA vrSPE microdevice. Allows for the simultaneous extraction and purification of four separate samples (A) or the simultaneous extraction froma single source allowing for replicates of the sample ina timely manner. C) Two examplare full (16/16 loci) Identifier® STR profiles from simultaneous extraction of whole blood from 4 separate individuals.

Dissemination

Oral Presentations

Carmen R. Reedy, Briony C. Strachan, Josh J. Higginson, Joan M. Bienvenue*, Susan A. Greenspoon, James P. Landers. DNA purification on microfludic devices for large volume forensic biological samples. Presented at 61th Annual American Academy of Forensic Sciences Meeting, 2008, Washington, D.C.

Hagan, K., Reedy, C., Strachan, B., Meier, W., Ferrance, J., Landers, J. A 2-D Microchip-based Process for Volume Reduction and Purification of Total Nucleic Acids. Presented at 60th Annual American Academy of Forensic Sciences Meeting, 2008, Washington, D.C.

Poster Presentations

Reedy, C., Higginson, J., Landers, J.P. Microfluidic Volume Reduction Solid Phase Extraction of Compromised and Low DNA Template Forensic Samples. Proceedings of 14th International Conference on Miniaturized Systems for Chemistry and Life Sciences (μTAS), 2010, Groningen, Netherlands.

Reedy, C., Higginson, J., Landers, J.P. Microfluidic Volume Reduction Solid Phase Extraction of Compromised Forensic Samples. Presented at National Institute of Justice Conference, 2010, Arlington, VA.

Reedy, C., Bienvenue, J., Bhatri, N., Coletta, L, Greenspoon, S., Ferrance, J., Landers, J.P. Volume Reduction Solid Phase Extraction of Large Volume, Dilute Whole Blood Samples. Presented at National Institute of Justice Conference, 2009, Arlington, VA.

Reedy, C., Hagan, K., Strachan, B., Meier, W., Bienvenue, J., Ferrance, J., Landers, J. Development and Application of a 2-D Microchip-Based Purification and Volume Reduction of Nucleic Acids. Presented at 18th International Association of Forensic Sciences Triennial Meeting, 2008, New Orleans, LA.

Reedy, C., Hagan, K., Strachan, B., Meier, W., Ferrance, J., Landers, J. A 2-D Microchip-Based Process for Volume Reduction and Purification of Total Nucleic Acids. Presented at 22nd International Symposium on Microscale Bioseparations and Methods for Systems Biology, 2008, Berlin, Germany.

Publications

Reedy, C., Bienvenue, J., Coletta, L., Strachan, B., Bhatri, N., Greenspoon, S., Landers, J. Volume *Reduction Solid Phase Extraction of DNA from Dilute, Large-Volume Biological Samples*. Forensic Sci. Int. Genet. 2010, 4, 206-212.

Reedy, C., Hagan, K., Strachan, B., Higginson, J., Greenspoon, S., Ferrance, J., Landers, J. *2-D Dual-domain Microchip-based Process for Volume Reduction Solid Phase Extraction of Nucleic Acids from Dilute, Large Volume Biological Samples*. Analytical Chemistry 2010, 82 (13), 5669-5678.

Reedy, C., Higginson, J., Landers, J. Microfluidic Volume Reduction Solid Phase Extraction of Compromised and Low DNA Template Forensic Samples. *Proceedings of 14th International Conference on Miniaturized Systems for Chemistry and Life Sciences (μTAS),* 2010, Groningen, Netherlands.

Reedy, C., Higginson, J., Coble, M., Greenspoon, S., Landers, J.P. Microfluidic Volume Reduction Solid Phase Extraction of Compromised Forensic Samples. Manuscript in Preparation.

Reedy, C. *DNA Purification on Microfluidic Devices with a Focus on Large Volume, Forensic Biological Samples*. Ph. D defense – University of Virginia – October, 2010

Patents

Landers, J., Ferrance, J., Reedy C., Hagan, K., and Bienvenue, J. Large Volume Reduction Nucleic Acid Extraction. United States Provisional Patent Application Serial No. 61/030,788. Filed on February 22, 2008.

Concluding Remarks

Discussion of Findings

This study involved the design and development of several microdevices suitable for the purification and extraction of large volume complex, degraded and contaminated samples likely to be encountered in a forensic investigation. Sample types successfully extracted on-chip and amplified off-chip include blood and semen stains on cotton cloth that were degraded either by heat or by UV exposure. Additionally, dilute whole blood samples spiked with common PCR inhibitors that included humic acid and indigo dye, were amenable to purification yielding a PCR-amplifiable DNA, outperforming other methods. Furthermore, with the vrSPE device we were able to demonstrate the first examples of microdevice extraction of bone and mitochondrial DNA, which are particularly challenging samples to purify and amplify. To increase throughput of the microfluidic system, we exploited the ease with which plastic (PMMA; PlexiGlass) devices could be fabricated to create multi-channel and multiplexed vrSPE devices. This contrasts more elegant devices that we have devised (see *Lab on a Chip* 11(9):1603-11, 2011) where PMMA posts were fabricated into the devices as the solid phase – unfortunately, the eccentric fabrication method required made the prohibitively expensive (>$150 per chip). In the current report, PMMA was shown to be as effective as the traditional glass substrate (some 20+ published papers from my lab established the methods for glass chip extraction; see *Appendix* at end of report), allowing for the simultaneous extraction of four distinct dilute blood samples without contamination. These results meet three of the aims of this proposal, demonstrating a microfluidic device can be used more effectively than current methods to process large volume forensic samples. The forth aim - on-site testing of these devices - is currently being explored with three different laboratories and the details of that testing defined.

Advancements Beyond Previously-devised SPE Methods

A number of notable advancements were made during this funding period. A high-level summary of these is provided below.

Chip-SPE does not require an alcohol wash step: We found that an IPA or ethanol wash was not absolutely necessary to remove PCR inhibitory compounds such as heme, which is known to co-elute with DNA if not removed prior to elution, inhibiting downstream PCR. Eliminating this step has discrete advantages, increasing the amount and quality of the recovered DNA, reducing processing time and a lower likelihood of PCR inhibition from washing buffers (Fig. 3 and 4).

Development of a single phase vrSPE microchip: This allows, for the first time, large-volume samples to processed using microfluidic technology by interfacing the macro and micro scales effectively. This was achieved through microdevice design, development of a new solid phase, optimization of flow rate, studies of optimal eluting buffer and characterization of the DNA quality that was extracted.

Development of a two phase vrSPE-SPE microchip and protocol: This invokes the use of a second 'orthogonal' phase together with the standard silica phase in a single device to produce PCR ready DNA using aqueous chemistry. Although complex, effective mixing of the eluted DNA and loading buffer for the second SPE phase was achieved. In addition, this second phase also removed additional inhibitors (e.g., indigo dye), producing full STR profiles when all other SPE microdevices failed, including vrSPE (Fig. 24).

Forensically-relevant samples were evaluated on vrSPR microchips: These included heat-, UV- and time-degraded-samples that were successfully demonstrated to yield quality STR profiles, highlighting the versatility of microfluidics in the extraction of forensic DNA. Of particular note was the demonstration of mtDNA and bone extraction using the vrSPE device

– both being a first in the microfluidic community – impossible with previously-devised SPE methods. In addition, development of vrSPE-SPE allows for the first time successful removal of 8M indigo dye, a PCR inhibitor found in blue denim, further demonstrating the performance of microfluidic SPE over conventional methods.

Utilizing PMMA for Microfluidic vrSPE Device: These were developed using PlexiGlass material, as opposed to glass, to allow for inexpensive, rapid fabrication of disposable devices – a shift in microfluidics during the funding period. This shift, although not stated in the proposal, was essential to maintain the device at the forefront of current technology. Through this shift, our devices can now be fabricated for pennies, creating single-use, disposable devices for the first time.

Multiplexed Microfluidic vrSPE Device: Using the newly developed PMMA microdevices , optimization of a 4-plex device (that will be trialed at VDFS) was successfully demonstrated to extract DNA from four individuals simultaneously, with no evidence of cross-contamination between samples. Such devices are suitable in forensic laboratories as a bench-top alternative to an expensive robotic platform.

Benefit over existing methods*:* The original basis for the study was to compare this vrSPE technology to that developed at VDFS for processing large volume samples on a robotic platform. A number of the samples processed using the vrSPE method were prepared by VDFS and ran on their instrumentation for a direct comparison. The vrSPE method was shown to, in some cases, outperform the conventional robotic method at VDFS [1] (i.e., heat- and time-degraded blood stains on Whatman filter paper, Figs. 7 and 8). Additionally, the vrSPE method was compared against work completed by Ballantyne for UV-degraded methods and was again shown to outperform the conventional processing method used by those authors. Based on discussions with our collaborators, the particular niche a 4- or 6-plex microfluidic device would fill is not competition with a robotic methodology, but

as a bench-top low sample number (high sample volume) extractor, with the 'closed nature' of the system being a valuable asset.

Comparisons were also completed against other microfluidic SPE technology, specifically for the inhibitor studies were the vrSPE is shown to outperform the microfluidic SPE methods that had been previously developed in the our lab (see Fig. 24).

The purpose of this work was not to re-invent microfluidic SPE, but rather allow microfluidics to move beyond microscale volume samples typically used and accept macroscale volumes for the first time. Designing and optimizing such a device made the technology, while similar to previous µSPE devices, stand apart from that reported in 2006. As a result of the NIJ funding of the project, vrSPE now presents the potential to make the processing of large volume, dilute samples a feasible method within the forensic community by reducing the analytical time required and delivering STR profiles of high quality that were not microfluidically possible prior to this device. The closed-system nature of the microfluidic platform is ideal for forensic casework as it limits handling steps and thus contamination.

Future Prospects

To implement vrSPE-based microdevicies into forensic laboratories, on-site testing will be conducted to demonstrate the reproducibility of these devices in different settings and to also allow for side-by-side comparison to current laboratory methods. The PMMA microdevices will be used to demonstrate their application for degraded samples e.g., blood stains exposed to heat and UV light simultaneously to more accurately represent a sample exposed to sunlight. This will aid in verification that the PMMA substrate can equal the performance of the glass devices for degraded samples. Other sample types will be investigated including touch DNA samples,

Further designs with multi-channels will be explored to increase the throughput of the method (8-, 16- and 32-channel devices) to make these competitive with robotic processing systems. In many respects, performance of these devices surpasses current methods for the purification and extraction of DNA for this specific class of samples. In terms of broader application to DNA extraction in general (degraded and non-degraded samples) only when higher-plexed devices are defined are they likely to become cost-effective as injection-molded devices.

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Appendix

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