Final Report

Separation of Sperm and Epithelial Cells in a Microfluidic Device: An Automated Method for High Efficiency, High Purity Separations

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Abstract

The proven utility of forensic DNA evidence has increased the demand for DNA analysis services. Although conventional DNA analysis techniques are effective, they are time-consuming and laborious, which contributes to the overall cost and to an overwhelming backlog of forensic casework samples with possible biological evidence. Research efforts have focused on the development of more rapid and efficient analytical methods using microdevices to reduce the time and cost of forensic analysis as well as the magnitude of the existing casework backlog. Successful microfluidic methods for separating sperm and epithelial cells have previously been demonstrated by our laboratory.[1, 2] These methods circumvent a time-consuming step in DNA analysis of sexual assault evidence, the conventional differential extraction procedure. The presented research will focus on a separation technique utilizing acoustic trapping (Acoustic Differential Extraction; ADE), which was developed for the isolation of male and female fractions from sexual assault evidence. The acoustic trapping microdevice consists of piezoceramic microtransducers imbedded into a printed circuit board layer, which serves as the bottom of a microchannel, and a glass fluidic layer containing the microchannel structure. Upon application of an alternating voltage to the microtransducers, a standing ultrasonic acoustic wave is formed within the microchannel, generating pressure minima in the center. Particles and cells, in a size- and density-dependent manner, are drawn to these minima by acoustic forces generated from the standing wave; the magnitude and direction of the forces are dictated by the physical characteristics of the specific particles or cells.

The ADE system has been optimized to immobilize sperm cells, while free DNA (primarily from epithelial cell lysate) will not be trapped. A biological sample containing a mixture of sperm and epithelial cell lysate is infused through the microfluidic channels and transported over the transducers. Upon activation of the ultrasound, sperm cells are retained in the acoustic trap, while free DNA is directed towards the female DNA outlet. Laminar flow valving is used to redirect the flow towards the male outlet, and the ultrasound is terminated, resulting in the release of sperm cells from the acoustic trap and subsequent movement of cells towards the male outlet. The male and female products from the ADE system were collected from their respective outlets and analyzed off-chip; DNA purification, amplification, and separation were performed using conventional laboratory methods. The results show that highly purified male and female fractions can be obtained within the ADE microdevice.

Executive Summary

Genetic analysis of mixed profile DNA samples obtained from vaginal swabs is a well-established technique in the investigation of sexual assault and rape cases.[3-5] To obtain independent short tandem repeat (STR) profiles of both the victim and the perpetrator, it is necessary to separate the male and female components of the recovered genetic material. The current separation protocol, differential extraction (DE) is a well-established technique; however, it is one of the most time-consuming sample preparation steps of sexual assault evidence analysis, contributing to a substantial forensic casework sample backlog.[6] In addition, conventional DE requires extensive sample handling, and often results in inefficient separation of female DNA from the male fraction.[7, 8] Research efforts have focused on the development of more rapid and efficient analytical methods to reduce the time and cost of forensic analysis. Microfluidic technology provides the opportunity to automate forensic DNA sample processing, and all forensic genetic analysis steps have been demonstrated on microdevices.[9-11] Techniques performed on microchips are advantageous because they can be integrated with other analytical steps on a single microfluidic device.[12] The laborious, time-consuming centrifugation and wash steps of conventional DE are not easily amenable to a microdevice; therefore, the development of a microfluidic device for the separation of recovered biological material from rape case evidence is of interest.

Trapping based on acoustic forces offers a simple, efficient way of retaining particles in a microfluidic system. This report focuses on the development and characterization of an acoustic differential extraction (ADE) microfluidic device. ADE

relies on acoustic trapping of sperm cells in the presence of epithelial cell lysate (which is unretained). Laminar flow valving (LFV) was utilized to direct the male and female fractions to separate outlets. Preliminary results showed suboptimal purity of the male fraction after ADE separations.[13] Improvements in fluidic control and trapping on the ADE device enabled efficient separation of mixed biological samples, resulting in independent male and female DNA profiles.

The ADE microdevice consists of piezoceramic microtransducers imbedded into a printed circuit board layer, which serves as the bottom of a microchannel, and a glass fluidic layer containing the microchannel structure. The depth of the microfluidic channels was determined based on the number of desired pressure nodes (trapping sites) in the channel upon activation of the transducer. The fluidic layer conducting the cell suspension was enclosed by an air-backed miniature transducer. Regardless of the number of desired pressure nodes, the thickness of the reflector layer above the channel corresponded to an odd number of quarter wavelengths [$(2n + 1)\lambda/4$], as calculated by the speed of sound in borosilicate glass.

ADE was performed in a sealed microdevice. A waveform generator was used to actuate the transducers with a sinusoidal signal at the specified frequency and amplitude. Samples and buffer were infused into the chip using pressure-driven flow from syringe pumps. Polytetrafluoroethylene (PTFE) tubing was utilized to connect the microdevice to the buffer and sample syringes. Outlets were comprised of capillaries [250 μm ID, bare or dynamically-coated with poly-*N*-hydroxyethylacrylamide (PHEA)] or 0.3 mm ID PTFE tubing. Samples consisted of prepurified human genomic DNA, semen diluted in

phosphate buffered saline (PBS) solution, epithelial cell lysate, or a combination of sperm and epithelial cell lysate to simulate a sexual assault sample. PBS or conventional DE buffer was used for hydrodynamic focusing and laminar flow valving. During the sample infusion, the ultrasound was activated at a specific frequency and voltage using the waveform generator. Hydrodynamic focusing was used to direct the infused sample towards the center of the transducer to ensure a high trapping efficiency, while sample was infused through the sample inlet. Cells were trapped above the transducer in the pressure nodes of the standing wave, while the unretained material (female lysate) was directed towards the female outlet using laminar flow valving (LFV). During the wash step, the sample infusion was terminated; the ultrasound remained activated, allowing the sperm cells to be levitated in the acoustic trap, while the focusing buffer perfused the trapped cells and removed any residual lysate from the microchannel and outlet tubing. Upon completion of the washing, LFV was used to direct flow towards the male outlet, and the ultrasound was deactivated, resulting in a release of the sperm cells and subsequent collection in the male outlet. Microcentrifuge tubes were attached to each outlet for sample collection. DNA extraction of the ADE fractions was completed using commercially-available purification kit. Real time quantitative PCR (qPCR) was utilized to determine the quantity of human genomic (hg)DNA in the ADE fractions. To assess DNA purity, short tandem repeat (STR) profiles were generated using conventional PCR amplification and separation techniques. STR profiles were obtained from the male sperm cell and female buccal epithelial cell donors, and compared with those obtained

from ADE separations in order to determine purity. Purity was calculated based on the peak area ratios of the male and female contributors for all seven STR loci.

ADE was initially demonstrated using a mock sexual assault sample consisting of 10 μm polystyrene beads to simulate sperm cells and Evans Blue Dye to simulate female epithelial cell lysate. Upon infusion of the mixed sample, the ultrasound was activated, and the polystyrene particles were trapped in the acoustic standing wave over the transducer; the unretained dye was directed to the female outlet using LFV. After sample infusion, sample flow was terminated, and the channels were washed with buffer to remove any inadvertently-trapped Evans Blue Dye from the trapped particles, as well as residual dye from the microchannel. LFV was then implemented such that the direction of flow was switched to the male outlet. The ultrasound was then deactivated to give a controlled release of the microbeads towards the male outlet channel. It was originally concluded that LFV could be used to effectively separate sperm and epithelial cell lysate on the ADE microdevice; however, closer inspection of the still photomicrographs revealed a potential female DNA contamination source. During the ADE wash step, as sample flow was terminated, the infusion of focusing buffer unintentionally "pulled" sample (including residual blue dye) from the sample inlet. This "pull" of sample continued during the fluidic control and release steps, and blue dye was directed towards the male outlet, indicating a potential cause of female DNA contamination in the male ADE fractions.

The suspected source of female DNA contamination in male ADE fractions was verified by initial attempts at separating sperm cells from epithelial cell lysate. ADE of

mock sexual assault samples was performed as previously described; samples collected from the female outlet during sperm cell trapping and from the male outlet during the release were subsequently analyzed off-chip using commercially-available purification and STR amplification kits. The data obtained from these experiments consistently resulted in poor separation efficiencies. Comparison of the profiles from the sperm cell donor with the male ADE fraction indicated recovery of sperm cell DNA; however, the presence of additional alleles consistent with the epithelial cell donor signified poor purity (55% or less) of the male fraction. The data showed that additional efforts towards improving fluidic control were necessary to eliminate female lysate contamination of the male ADE separation product, and to demonstrate better purity levels.

To prevent unwanted sample infusion during the wash and release steps, the experimental conditions were modified so that the sample was withdrawn from the device by the sample syringe (at a lower flow rate relative to that of the focusing buffer). Different colored dyes were used in the initial characterization of the modified sample withdrawal method. Sample was infused through sample inlet, while hydrodynamic focusing was applied. The sample and focusing buffer were directed towards the female outlet using LFV. During the wash step, withdrawal from the sample inlet (1 μL/m) effectively stopped the sample from entering the center channel, and the focusing buffer effectively removed residual sample from the remainder of the channel, including the female outlet. LFV valving was implemented to simulate fluidic control prior to the release step of ADE. The results show that LFV was not adversely affected by sample withdrawal, as no sample was observed entering the male outlet. The reliability of the

modified fluidic control method was further assessed using prepurified DNA and subsequent quantitation of the collected fractions. A sample consisting of prepurified hgDNA used to simulate female epithelial cell lysate was infused through the sample inlet and hydrodynamically focused; the ultrasound was not initiated. LFV was used to direct the flow of the DNA towards the female outlet. After sample infusion, the sample flow was switched to withdraw from the sample inlet (1 μL/m), and the channels were washed with focusing buffer while maintaining flow towards the female outlet. Once the wash step was completed, the outlet flow was switched, such that the net flow was directed towards the male outlet. After each step the eluted fractions were collected from each outlet, and total genomic DNA of each sample was measured using a qPCR assay. The results show that fractions collected from the female outlet after sample infusion contained the majority of the recovered DNA; DNA was not detected in fractions collected from the female outlet, and more importantly, the male outlet, after the release step. The results verify that the use of sample withdrawal during the wash and release steps eliminates a source of contamination, as it ensures that sample (containing epithelial cell lysate) would not be "pulled" into the device and towards the male outlet during the release of the trapped material (sperm cells). Similar studies using pre-purified hgDNA were performed to optimize the sample withdrawal flow rate (while incorporating hydrodynamic focusing); it was determined that 0.5 μL/m sample withdrawal was more suitable for future experiments, as it provided consistent recoveries of hgDNA in the fractions collected from the female outlet after sample infusion.

To ensure that the ADE microdevice was suitable for the separation of mock sexual assault samples, acoustic trapping conditions of sperm and epithelial cell lysate were separately assessed. Lysate trapped in the acoustic standing wave is a potential source of contamination, as it would be released upon termination of the ultrasound and directed towards the male outlet; therefore, it was important to establish that epithelial cell lysate (in the absence of sperm cells) was not trapped. Female epithelial cell lysate was infused through the sample inlet and hydrodynamically focused; trapping was initiated, and LFV was used to direct the flow of the DNA towards the female outlet. The wash step was performed as previously described; once completed, the outlet flow was switched such that the net flow was directed towards the male outlet, and the acoustic trapping was terminated. After each step, the eluted fractions were recovered from each outlet and purified off-chip using a commercially-available extraction kit. Total genomic DNA was measured using a qPCR assay. The results show that fractions collected from the female outlet after sample infusion contained the most DNA, and therefore, the most lysate; DNA was not detected in fractions collected from the female outlet, and more importantly, the male outlet, after the release step, confirming that epithelial cell lysate was not trapped in the absence of sperm cells, (as predicted by acoustic force theory). In addition, the data correlate with the results obtained using pre-purified hgDNA, and further verify the effectiveness of incorporating sample withdrawal to eliminate sample bleed towards the male outlet.

The number of sperm cells captured and released from the acoustic trap, and consequently, the amount of DNA recovered in the male fraction after ADE, is critical to

obtaining a male STR profile. Studies were therefore performed to assess sperm cell trapping and release using the ADE device, without interference from epithelial cell lysate. Preliminary results were obtained with acoustic trapping utilizing a single node fluidic layer; to increase capacity of the trap, a triple node fluidic layer was implemented for the remainder of the experiments. Samples consisting of semen (6, 12, or 24 μL) added to PBS were infused through the sample inlet and hydrodynamically-focused; acoustic trapping, wash and release steps were performed as previously described. The concentrations of DNA recovered from four consecutive runs for each prepared sample was determined via off-chip conventional purification and qPCR. Samples prepared with 6 μL of neat semen provided insufficient concentrations to obtain full STR profiles. For samples prepared with 12 μ L of semen, an increase in DNA concentration was observed over the course of the four trials; samples prepared with 24 μ L of semen provided inconsistent recoveries of DNA. It was concluded that the inconsistent recoveries of DNA obtained from samples prepared with higher volumes of semen were a result of poor release of larger clusters of cells from the outlet tubing. Similar studies were performed with devices containing capillary outlets. Samples consisted of neat semen (6, 12, 24, or 48 μ L) added to PBS; acoustic trapping, wash, and release steps were performed as previously described. The data further support the theory that more acoustically-trapped cells resulted in larger clusters of cells that were not released cleanly from the outlet tubing. In addition, lower numbers of trapped cells, coupled with off-chip conventional purification and amplification techniques, did not provide sufficient DNA for full STR profiles. Sample preparation and trapping conditions for the remainder of

the reported studies were adjusted such that approximately 2000-2400 cells were acoustically trapped and released towards the male outlet. Using the current microchip design, it may be necessary to estimate and measure the sperm content of the sample prior to acoustic separation.

In order to improve the release of biological material from outlets, focusing and LFV buffers were changed from PBS to a conventional DE buffer. Acoustic trapping of sperm cells added to conventional DE buffer was performed. Samples were infused through the sample inlet and hydrodynamically-focused; acoustic trapping, wash, and release steps were performed as previously described. Samples were recovered from the male outlet (250 μm ID capillary) and purified off-chip using a commercially-available extraction kit; conventional amplification and separation of STR loci were performed. The results show that samples prepared in DE buffer consistently provided full STR profiles, indicating that the use of DE buffer was more effective than PBS for the removal of cells from the outlet tubing; therefore, LFV and focusing buffers were changed to conventional DE buffer for all future studies.

Once run-to-run reproducibility of acoustic trapping was established, ADE separations of mock sexual assault samples were performed. Sperm cells were added to female epithelial cell lysate; acoustic trapping, wash, and release steps were performed as previously described. The results indicate that the release of biological material was problematic for samples prepared in epithelial cell lysate, as STR profiles were not obtained. In an effort to further facilitate removal of biological material from the outlets, the capillaries were dynamically-coated with 0.1% (w/v) PHEA, chosen due to its

ability to reduce protein interactions with silica surfaces. Samples were prepared in epithelial cell lysate as previously described. STR profiles of the mixed biological sample prior to ADE, the sperm cell donor, and the female epithelial cell donor were obtained using conventional methods; comparison of the original sample profile with those of the male and female donors indicated that the mixed biological sample was 40 ± 3.9% male. ADE was performed as previously described. Samples were recovered from the male outlet and purified off-chip using a commercially-available extraction kit; conventional amplification and separation of STR loci were performed. Comparison of the resultant profile with those of the male and female donors indicate that the sample is 79 ± 16% male, with seven of the core CODIS STR loci detected. The results show that ADE, performed on a device with coated capillary outlets, provided over 50% enhancement of male purity. However, due to issues with recovery of the separated biological material from the outlets; two out of six of the subsequent trials did not result in STR profiles.

As samples prepared in epithelial lysate did not consistently provide STR profiles, the effects of varying the operating frequency of the transducer on the trapping pattern of particles were investigated. A suspension of particles was infused through the sample inlet with hydrodynamic focusing. Trapping was initiated at 11.6 MHz (the same operating frequency used for previous mock separation studies) and observed using light microscopy; the results show that particles were trapped in a large cluster near the center of the channel, suspended above the transducer. Using the same particle suspension, transducer, and fluidic layer, the frequency was increased to 12.1 MHz. The change in

trapping frequency resulted in a change in the particle distribution; smaller clusters of particles that were spread out over a larger area of the transducer were observed. It was hypothesized that the change in particle distribution would allow for more effective removal of inadvertently-trapped female lysate from the trapped cells, and that the smaller clusters of cells trapped using the higher frequency would be less likely to clog the outlet tubing upon release compared to a single, larger cluster of cells obtained with the lower frequency. To test this, mock sexual assault samples were prepared in epithelial cell lysate. The STR profile of the mixed biological sample prior to ADE was obtained using conventional methods; the sample was determined to be 26 ± 2.0% male. ADE was performed as previously described. Samples were recovered from the male and female outlets (0.3 mm ID PTFE tubing) and purified off-chip using a commercially-available extraction kit; conventional amplification and separation of STR loci were performed. Comparison of the STR profile obtained from the male fraction with those of the male and female donors indicate that the sample is 92 ± 7.9% male, a nearly four-fold enhancement in male purity in comparison with the original sample. All seven core STR loci, devoid of alleles contributed from the female, were detected, allowing for interpretation of the male profile from the original mixture. Comparison of the STR profile obtained from the female fraction with those of the male and female donors indicates that the sample is 5.7 ± 4.7% male. The results suggest that the particle distribution in the acoustic trap may play an important role in the effective separation of sperm and female epithelial cell lysate using the ADE device.

The higher operating frequency and its resultant trapping pattern were applied to ADE separations of a mock sexual assault samples containing lower levels of male DNA. Samples were prepared by addition of sperm cells to epithelial cell lysate generated from a larger number of epithelial cells than used in the previous study. The STR profile of the mixed biological sample prior to ADE was obtained using conventional methods; through comparison of this profile with those of the male and female donors, the sample was determined to be 13 ± 5.9% male. ADE was performed as previously described. Samples were recovered from the male and female outlets, and purified off-chip using a commercially-available extraction kit; conventional amplification and separation of STR loci were performed. Comparison of an STR profile obtained from the male fraction the profile with those of the male and female donors indicate that the sample is 73 ± 17% male, with all seven core STR loci detected – a nearly sevenfold enhancement of male purity compared to the original sample. The STR profile obtained from the female fraction with those of the male and female donors indicates that the sample is 3.1 ± 3.9% male – effectively a pure female profile without presence of male alleles.

The enrichment of male DNA in the sample (as compared to the original mixture), combined with the STR profile obtained from the female donor, allows for interpretation of the male profile from the original mixture. However, the presence of female alleles in the profile obtained from the male outlet indicates a source of female DNA contamination. Since fluidic control was proven to be reliable and reproducible, these results are most likely due to excess female lysate inadvertently trapped within the sperm cell clusters. Further studies may require optimization of the wash step to effectively

remove female DNA and prevent it from contaminating the post-ADE male sample. It is important to note that, while samples collected from the male outlet following subsequent ADE separations of the same sample provided male purities that did not differ considerably from the ones shown; samples collected from the female outlet did not consistently provide STR profiles, indicating that consistent release of female lysate from outlets is an issue.

The results show that highly purified male and female fractions can be obtained with improvements to the ADE technique. It is reasonable to expect that this technique can be integrated with on-chip downstream sample processing steps. To implement ADE into forensic laboratories, further optimization and engineering improvements to the system are required. Developments are currently underway to produce disposable ADE devices; these devices will potentially consist of a sealed channel structure and an external transducer, as opposed to the current system where the transducer is in direct contact with the sample, and will reduce the possibility of sample contamination – a necessity for forensic laboratories. In addition, to obtain more consistent recoveries of DNA after ADE separations, different outlets will be designed and tested. The time savings associated with a fully-integrated analysis system incorporating ADE provides the potential to significantly alter the means by which sexual assault evidence is processed in crime laboratories.

In parallel with the ADE studies, effort was exerted on pre-acoustic trapping processes. Effective utilization of the ADE method or other alternative DE methods relies on efficient elution of sperm cells from the vaginal swab, and the comprehensive

lysis of epithelial cells, all in a timely manner. Regardless of the method utilized for the separation of female epithelial DNA and male sperm cells, the overall effectiveness of the procedure is also dependent on the efficiency with which material can be eluted and recovered from a cotton swab - this is paramount for obtaining an STR profile from a low copy number sample. We have devised a number of improvements over the standard Gill buffer approach used in DE, including the use of enzymatic- and chemical-enhancements to improve the yield of sperm cells removed from the swab. While most of the DE protocols currently in use in crime labs have an associated sperm cell yield of ~40% using Gill buffer and Proteinase K, we have shown that this can be improved substantially [1a].

A two-step method for cell elution and recovery from mock sexual assault samples was developed by our laboratory. The first step involves detergent-mediated elution of intact sperm and epithelial cells from a cotton swab matrix; several detergent types were assessed, and detergent concentrations were investigated for swabs that were dried and stored for various periods of time. The second step involves preferential lysis of epithelial cells that were recovered using the detergent-mediated cell elution method. The effects of detergent and enzyme concentration on cell lysis were evaluated for mock casework samples prepared with various numbers of sperm cells. The results show that the two-step cell elution/preferential lysis method provided nearly twofold improvement in sperm cell recoveries compared to a conventional DE buffer, while providing comprehensive epithelial cell lysis, in a total incubation time of one hour – up to an order of magnitude decrease in incubation time than traditionally performed in some forensic

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[1a] Voorhees, et al., 2006. *J. Foren. Sci.* 51(3):574-79; Voorhees, et al., *J. Foren. Sci.* 52(4):800-5;

laboratories. The improvement in sperm cell recoveries using the SDS elution buffer is ideal for increasing the efficiency of obtaining accurate DNA profiles from samples containing low numbers of sperm cells, while comprehensive epithelial cell lysis is ideal for use in conjunction with the microfluidic ADE platform.

Detailed Technical Report

I. Introduction

Genetic analysis of mixed profile DNA samples obtained from vaginal swabs is a well-established technique in the investigation of sexual assault and rape cases.[3-5] To obtain independent short tandem repeat (STR) profiles of both the victim and the perpetrator, it is necessary to separate the male and female components of the recovered genetic material. The current separation protocol, known as differential extraction (DE), exploits the differential stability of the nuclear membrane of each cell type through the preferential lysis of vaginal epithelial cells. Sperm cells are then separated from epithelial cell DNA using multiple centrifugation and wash steps, allowing independent genetic analysis of male and female DNA. DE, while well-established, is one of the most time-consuming sample preparation steps of sexual assault evidence analysis, contributing to a substantial forensic casework sample backlog.[6] In addition, conventional DE requires extensive sample handling, is difficult to automate, and often results in inefficient separation of female DNA from the male fraction.[7, 8]

Research efforts have focused on the development of more rapid and efficient analytical methods to reduce the time and cost of forensic analysis. Microfluidic technology provides the opportunity to automate forensic DNA sample processing, and all forensic genetic analysis steps have been demonstrated on microdevices.[9-11] Techniques performed on microchips are advantageous because they can be integrated with other analytical steps on a single microfluidic device.[12] The laborious, time-consuming centrifugation and wash steps of conventional DE are not easily amenable to a

microdevice; therefore, the development of a microfluidic device for the separation of recovered biological material from rape case evidence is of interest.

Trapping based on acoustic forces offers a simple, efficient way of retaining particles in a microfluidic system. Recently, an acoustic differential extraction (ADE) microfluidic device was reported[13-15], which relies on acoustic trapping of sperm cells in the presence of epithelial cell lysate (which is unretained). Laminar flow valving was utilized to direct the male and female fractions to separate outlets. Preliminary results showed suboptimal purity of the male fraction after ADE separations.[13] The report describes characterization and advancements in fluidic control and trapping on the ADE device to enable efficient separation of mixed biological samples, resulting in independent male and female DNA profiles.

Methods of Cell Separation

The most common form of sexual assault evidence is swabs taken from the vaginal lining of the victim that are collected after the assault takes place. The swabs typically contain a biological mixture consisting of sperm cells as a minor component in the presence of excess vaginal epithelial cells; in order to obtain independent DNA profiles of the victim and perpetrator, it is necessary to separate the two distinct cell populations. The current protocol used by law enforcement agencies for recovery and separation of biological materials from a cotton matrix involves differential extraction (DE). Conventional DE methods often utilize a dual-purpose buffer that contains proteinase K and an anionic detergent, the combination of which removes sperm and

vaginal epithelial cells from swabs, and selectively lyses the vaginal epithelial cells.[3] Sperm cells are pelleted by centrifugation, and the supernatant containing the epithelial cell DNA is removed, which can be used to generate a DNA profile that is specific to the victim of the sexual assault. The sperm cells are then resuspended in a buffer containing dithiothreitol (DTT), reducing the disulfide bond network in the sperm cell head and allowing the nuclear membranes to be lysed, releasing the male DNA. Ideally, sufficient DNA can be recovered from the pellet to produce a male profile that can be compared to profiles from known suspects or convicted offenders.

DE allows for independent recovery of male and female DNA from vaginal swabs containing mixtures of vaginal cells and spermatozoa, and has been routinely used in forensic laboratories since its introduction. The probability of obtaining an interpretable male DNA profile is reduced when excess female DNA is present, as polymerase chain reaction (PCR) results may be obscured by excess female DNA.[16, 17] Due to the variability in forensic casework, little effort has been made to assess the purity of the sperm cell fraction following conventional DE. To date, the carryover of female DNA into the male fraction using conventional DE has not been reported, however, it is suggested to occur in 40% of samples.[18] In addition, the method is time-consuming, sometimes utilizing an overnight incubation for optimal recovery of DNA[16], although mostly for convenience. Consequently, there is a need to improve conventional DE in a way that improves the purity of the male fraction and, at the same time, enhance the overall speed of analysis.

Cell Separation and Capture on Microfluidic Devices

Research efforts have focused on the development of microfluidic devices to reduce the time and cost of forensic analysis. Microdevices can provide a self-contained, closed system for analysis procedures, diminishing the potential for contamination or loss of sample. Techniques performed on microdevices are particularly advantageous because they provide the opportunity to automate forensic DNA processing through integration of sample preparation steps on a single device.[19-22] Integrated systems that combine several analytical techniques on a single microfluidic device can reduce analysis times, sample handling, and the sample size required for analysis, making them ideal for samples encountered in forensic laboratories.

The laborious centrifugation and wash steps of conventional DE are not easily amenable to a microdevice. Microscale cell separations have been accomplished by various means in the recent past; however, these methods are not without limitations that may preclude them from use in forensic laboratories. For example, the development of microfabricated fluorescence-activated cell sorting (FACS) devices has previously been reported.[23-25] Although this method has high specificity, the technique requires a complicated multilayer structure requiring a number of process steps to fabricate[26], or narrow, shallow channels at the detector,[23, 24] increasing the risk of clogging when biological material is used.[25] In addition, the fluorescent tagging of one (or both) of the cell types is required. Microchip cell separation[27, 28] and capture[29] by dielectrophoresis has been demonstrated, in which cells migrate towards a concentrated electric field

depending upon their physical properties. However, a potential drawback to this technique is the adherence of cells to the microchip substrate at the trapping site.[30]

While there have been numerous approaches for cell sorting demonstrated on microdevices, few have specifically addressed the separation of sperm and female epithelial cells or epithelial cell lysate. Our laboratory demonstrated successful microdevice-based sorting of sperm cells from a mixture of sperm and vaginal epithelial cells.[31] The process exploited the differential physical properties of the cells, resulting in sedimentation and adsorption of epithelial cells to the bottom of an inlet reservoir on the glass microdevice. Subsequent buffer flow through the system caused sperm cells to migrate toward the outlet reservoir while epithelial cells remained in the inlet reservoir, resulting in effective separation of the two cell types. While this technique has recently been integrated with solid phase extraction[2], the cell sorting approach suffers from several shortcomings. The method relies on the recovery of intact sperm and epithelial cells; while this can be achieved using enzymatic[32] and detergent-mediated methods of cell elution[33, 34], the routine method of cell elution in crime laboratories often involves a conventional DE buffer designed to preferentially lyse epithelial cells while sperm cells remain intact. Second, is the potential presence of free DNA from lysed epithelial cells in the sample, although this can be addressed through modification of the microchannel surface.[30] Finally, the capacity of the microchip (~50 μL) is most likely insufficient for the analysis of sexual assault evidence. In forensic casework, the amount of cellular material on the swab is not standardized and, thus, it is imperative to be able to process the entire sample. The conventional method for cell elution from cotton swabs typically

requires 250-500 μL of buffer, whereas the microdevice cannot (in its current form) accommodate this volume.

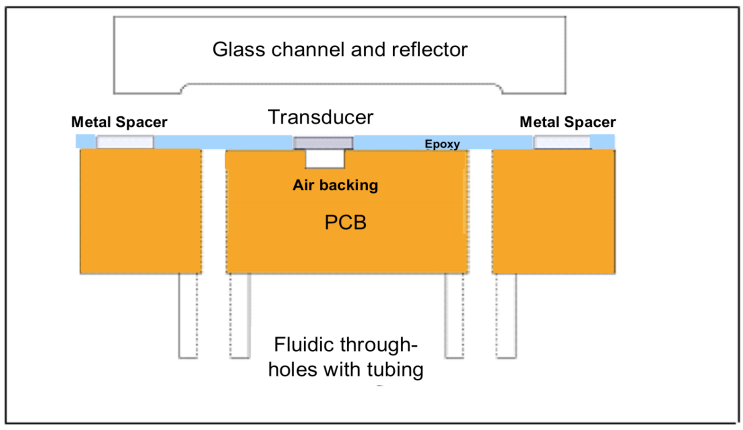
Acoustic Capture of Cells

The use of acoustic forces offers an efficient, alternative method of retaining and manipulating particles in a microfluidic system, and has been reported by several groups. Hawkes et al[35] demonstrated an acoustic device used for enriching yeast cells, while Nilsson[36] and Petersson[37] have manipulated red blood cells in mediums that allow for separation from lipid particles. Hultström et al.[38] investigated the viability and proliferation of adherent cells exposed to ultrasonic standing waves in a microfluidic chip; no direct or delayed adverse effects could be found. An acoustic device for trapping cells was developed by Spengler et al.[39], and has been used to study the physical environment of cells in an acoustic standing wave.[40]

Acoustic particle trapping was recently applied to forensic analysis in the form of acoustic differential extraction (ADE).[13-15] ADE was carried out in a valveless device, consisting of a printed circuit board layer containing a transducer, and a glass layer containing the microchannel structure. Upon activation of an electrical signal to the transducer, a standing ultrasonic wave was formed between the transducer and the glass reflector layer, defining a sperm trapping site. A sample containing sperm cells and female epithelial cell lysate was infused into the device, and sperm cells were trapped in the channel above the transducer, whereas DNA from the epithelial cell lysate was directed towards an outlet reservoir. The immobilized sperm cells were then washed with

buffer to remove any female DNA that was inadvertently trapped. Laminar flow valving was utilized to direct male and female fractions to different outlets. The ultrasound was terminated, resulting in the release of sperm cells from the acoustic trap, movement of cells to a separate outlet, and subsequent isolation of the male fraction.

The work presented here describes the characterization and development of an acoustic differential extraction (ADE) microdevice. It was hypothesized that ADE, once fully-optimized, would enable efficient separations of mixed biological samples, resulting in independent male and female DNA profiles.



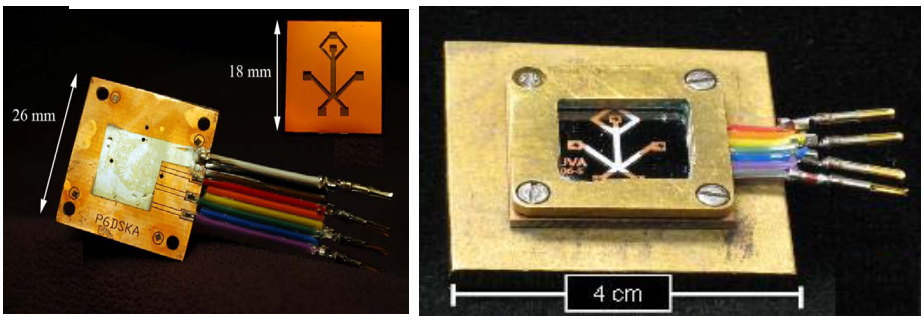


Figure 1: The ADE Microdevice. *Left*. A schematic image showing the components of the ADE system with the PCB holding the transducers and the fluidic access and a separate glass-chip, defining the microfluidic network. *Lower Left.* The device consists of b. a printed circuit board containing piezoceramic transducers, and a fluidic layer (reflector layer) composed of borosilicate glass. *Lower right*. Sealed ADE microdevice in a brass interface.

II. Methods and Materials

Mock Sexual Assault Sample Preparation

Buccal epithelial cells (from a female) were obtained on cotton swabs and stored at room temperature until use. Epithelial cells were eluted from the swab and lysed in a conventional differential extraction buffer as developed by Gill et. al.[41], consisting of 10 mM trizma pH 8 (prepared via titration of trizma-base with trizma-hydrochloride), 10 mM EDTA, 0.1 M NaCl, 2% SDS, and 20 μg/mL proteinase K. A fixed volume of diluted semen was added to the epithelial cell lysate prior to use. All samples were obtained from healthy volunteers, as per IRB HIC approval #10896.

Assembly of Acoustic Trapping Device

The microfluidic device uses an array of miniature lead zirconate titanate (PZT) transducers (920 × 920 × 200 μm, **Figure 1a**) created by dicing a commercial piezoceramic disc. The transducers were mounted on a printed circuit board (PCB) and cast in epoxy as illustrated by **Figure 1b.** Silicone tubings were attached to the backside of the PCB, providing fluidic connections to the ADE system. A borosilicate glass layer with microfluidic channels (**Figure 1c,** reflector layer) was positioned over the transducers, thereby defining the fluidic network of the chip. The glass lid was sealed to the PCB using ultrasonic gel, and the entire assembly was kept in place by a brass fixture (**Figure 1d**), with an observation window allowing for optical monitoring of the trapping sites.

Fabrication of Fluidic/Reflector Layer

Microfluidic channels were created using standard photolithography and chemical etching techniques.[42] The depth of the microfluidic channels was determined based on

the number of desired pressure nodes (trapping sites) in the channel upon activation of the transducer. To create a single pressure node in the center of the channel, the channel depth was set to one-half wavelength (λ) (as calculated by the speed of sound in water/ aqueous solutions) at the operating frequency of the transducer. Alternatively, to create three equidistant nodes within a channel, the channel depth was set to 3λ/2 wavelength. The fluidic layer conducting the cell suspension was enclosed by an air-backed miniature transducer with a thickness of λ/2. Regardless of the number of desired pressure nodes, the thickness of the reflector layer above the channel corresponded to an odd number of quarter wavelengths [(2n + 1)λ/4], as calculated by the speed of sound in borosilicate glass.

Acoustic Differential Extraction Method

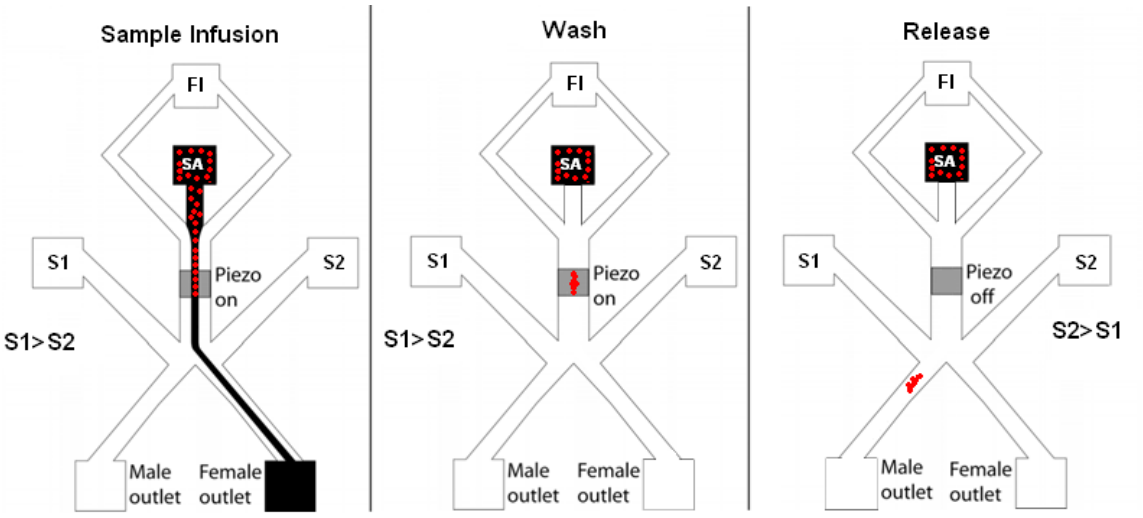


Figure 2: The fluidic design of the ADE-chip. *Left*. Sample infusion: sample is infused through the sample inlet (SA) and hydrodynamically focused by the focusing buffer inlet (FI). Cells (red) are trapped at the transducer upon activation of the transducer, while lysate (black) is unretained. *Middle*. Wash: sample infusion is terminated, and focusing buffer perfuses the trapped cells and removes the residual lysate from the microchannel. *Right*. Upon termination of the standing wave, cells are released and diverted towards the male outlet. The flow ratio of inlets S1 and S2 determines if the sample will go to the male or female outlet.

ADE was performed in a sealed microdevice. The fluidic channels and tubing were evacuated of ultrasound gel using Nanopure water (Barnstead/Thermolyne, Dubuque, IA). An Agilent 33220A waveform generator (Agilent Technologies, Palo Alto, CA) was used to actuate the transducers with a sinusoidal signal at the specified frequency and amplitude. Particle and cell trapping was viewed through an optical microscope (Leitz Orthoplan, Leica, Germany) and recorded to DVD via a CCD camera (Hitachi KP-D20BU, Tokyo, Japan) and DVD recorder (Panasonic DMR T2020, Secaucus, New Jersey). Samples and buffer were infused into the chip using pressure-driven flow from syringe pumps (sample – model # SP 260, buffer – model #SPE 100I; World Precision Instruments, Inc., Sarasota, FL). Polytetrafluoroethylene (PTFE) tubing (Supelco, Bellefonte, PA) was utilized to connect the microdevice to the buffer (0.8 mm ID) and sample (0.3 mm ID) syringes. Buffer was infused using 1 mL glass syringes (Hamilton, Reno, NV), while sample was infused using a 100 µL Hamilton glass syringe. Outlets were comprised of capillaries [250 µm ID, bare or dynamically-coated with poly-*N*-hydroxyethylacrylamide (PHEA)] or 0.3 mm ID PTFE tubing. To coat capillaries with PHEA, bare capillaries were treated with 0.1 M HCl for 15 min, followed by a 15 min water wash. The capillary was then treated with 0.1% w/v polymer solution for 15 min.[43]

Samples consisted of prepurified human genomic DNA (hgDNA) in 1X TE buffer (100 mM Tris, 10 mM EDTA, pH 8.0), semen (6, 12, 24, or 48 µL) diluted in 500 µL phosphate buffered saline (PBS) solution, pH 7.4, epithelial cell lysate, or a combination of sperm and epithelial cell lysate. To determine the number of sperm cells in solution, a portion of semen was diluted, and cells were counted using a hemacytometer. A

conventional differential extraction buffer devoid of the proteolytic digestion agent was used for hydrodynamic focusing and laminar flow valving, unless otherwise indicated. During the sample infusion (**Figure 2a**), the ultrasound was activated at the specified frequency and voltage using the waveform generator. Hydrodynamic focusing, used to direct the infused sample towards the center of the transducer to ensure a high trapping efficiency, was performed by infusion of focusing buffer through the focusing buffer inlet (FI) at 6 µL/min, while sample was infused through the sample inlet (SA) at 1 µL/min. Cells were trapped above the transducer in the pressure nodes of the standing wave, while the unretained material (female lysate) was directed towards the female outlet using a 1:5 (S2:S1) ratio of buffer from the side arm inlets. During the wash step (**Figure 2b**), the sample infusion was terminated. The ultrasound remained activated, allowing the sperm cells to be levitated in the acoustic trap, while the focusing buffer (6 µL/min, 3.5 m) perfused the trapped cells and removed any residual lysate from the microchannel and outlet tubing. Upon completion of the washing, the sidearm flow rate ratio was inverted (5:1, S2:S1), and the ultrasound was deactivated, resulting in a release of the sperm cells and subsequent collection in the male outlet (**Figure 2c**). The released cells and buffer originating from S1, S2, and FI were subsequently collected for 5 minutes. Sample withdrawal (0.5 or 1.0 µL/min) was implemented during the wash and release steps to prevent any sample, and thus, female DNA, from entering the device and contaminating the male fraction. Microcentrifuge tubes were attached to each outlet for sample collection.

Off-chip Analysis of Acoustic Differential Extraction Fractions

DNA extraction of the ADE fractions was completed using a QIAamp® mini spin kit (Qiagen Corp. Valencia, CA). DNA extraction was completed following the manufacturer's specifications in the "Blood and Body Fluid Spin Protocol," with the addition of dithiothreitol (50 mM final concentration; Fisher Scientific, Hampton, NH). The sample was eluted in 40 µL of 'AE buffer,' provided in the kit.

Real time quantitative PCR (qPCR) was utilized to determine the quantity of human genomic (hg)DNA in the ADE fractions; the amplicon was a 63 bp fragment of the TPOX locus. The forward and reverse primers for the autosomal locus were CGGGAAGGGAACAGGAGTAAG and CCAATCCCAGGTCTTCTGAACA, respectively. The TPOX probe (FAM-CCAGCGCACAGCCCGACTTG-TAMRA) was covalently labeled on the 5' terminus with a FAM™ fluorophore (Applied Biosystems, location) and at the 3' terminus with a TAMRA (tetra-methylcarboxyrhodamine) quencher. Probes were synthesized by Applied Biosystems. Primers were synthesized by Invitrogen Corp. (Carlsbad, CA). Fluorescence signal was monitored using a BioRad iQ5 instrument (Bio-Rad Laboratories, Hercules, CA).

To assess DNA purity, short tandem repeat (STR) profiles were generated using conventional PCR amplification and separation techniques. All amplifications were performed using a Perkin Elmer GeneAmp PCR System 2400 thermocycler (Wellesley, MA). STR amplifications were completed using a AmpF*l*STR® COFiler™ amplification kit (Applied Biosystems, Foster City, CA) according to the manufacturer's specifications. The PCR reaction volume for all amplifications was 25 µL, carried out in 0.2 mL PCR

reaction tubes. The COFiler™ amplification kit

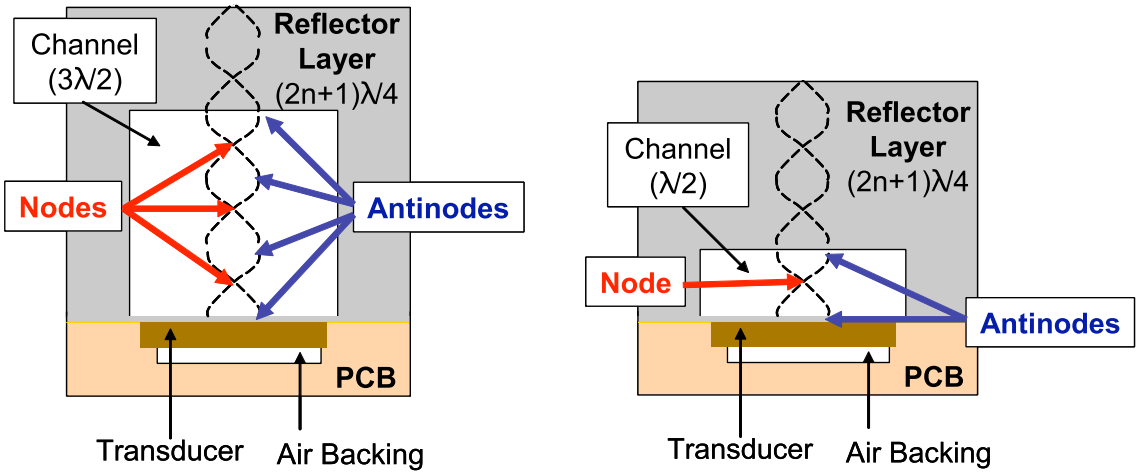


Figure 3: Schematics of the channel design such that acoustic resonators are constructed and standing waves are obtained in the microchannel. *Right*. Single node microchannel: the depth of the channel is 1/2 wavelength (λ) and the thickness of the reflector layer is 5λ/4. b. *Left.* Triple node microchannel: the depth of the channel is 3λ/2 and the thickness of the reflector layer is 3λ/4. Note: drawings are not to scale.

contains primers to simultaneously amplify a portion of the amelogenin gene and 6 STR loci: CSF1PO, D3S1358, TH01, D16S539, TPOX, and D7S820. Cycling conditions for amplifications were as follows: initial denaturation at 95°C for 11 m; denaturation at 94°C, 60 s; annealing at 59°C, 60s; extension at 72°C, 60s, for 28 cycles. Samples were then held at 60°C for 45 m for a final annealing, and were stored at 25°C until removed from the thermocycler. An ABI PRISM® 310 Genetic Analyzer (Applied Biosystems) was utilized for separation of the STR amplification products. Samples were separated using an uncoated capillary (50 µm i.d., 36 cm effective length, 47 cm total length) was filled with POP4 sieving polymer (Applied Biosystems) and maintained at 60°C for the duration of the separation. Separations were performed using the following conditions: 5 s electrokinetic injection (15 kV), 24 min separation (15 kV), and Genetic Analyzer Buffer separation buffer (Applied Biosystems). STR profiles were obtained from the

male sperm cell and female buccal epithelial cell donors, and compared with those obtained from ADE separations in order to determine purity.

III. Results and Discussion

Design and Fabrication Techniques for Effective Trapping

Most acoustic particle trapping and manipulation techniques use standing waves. To set up a standing wave within the ADE microdevice, the depth of the microchannel

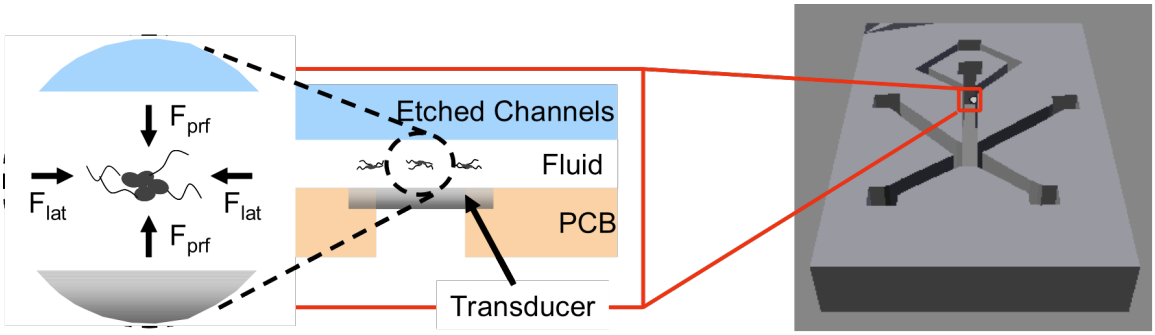


Figure 4: ADE device schematic. Inset: cross-sectional view of the device from the area above the transducer. Sperm cells are shown trapped above the transducer by primary (F_{prf}) and lateral (F_{lat}) forces at the node of the ultrasonic standing wave.

and the thickness of the reflector layer should correspond with the operating frequency of the ultrasound. To achieve a single trapping zone (node) in the center of the channel, the channel depth should correspond to a half wavelength (λ) of the optimal transducer resonance frequency (**Figure 3a**); alternatively, to achieve three equidistant trapping zones (nodes), the channel depth should correspond to $3\lambda/2$ (**Figure 3b**). Regardless of the number of desired trapping zones (nodes), the thickness of the reflector should correspond to an odd multiple of a quarter wavelength. If the optimal channel depth and reflector thickness are not obtained, the system will not be properly tuned (matched) for the optimal resonance frequency of the transducer, and the trapping zones may be located

closer to the glass or transducer surface. Trapping zones close to the microchannel surface or transducer present the potential for cells to interact with the surface, enhancing the possibility for adsorption and negatively affecting release upon deactivation of the ultrasound. In addition, a resonant standing wave will not be properly generated in a system with a mismatched fluidic layer and transducer, resulting in weaker trapping forces, and ultimately loss of cells during sample infusion. Preliminary ADE separations, were performed with single node fluidic layers; subsequent studies were performed with triple node fluidic layers, which provide three times the trapping volume as single node fluidic layers.

Acoustic Force Theory

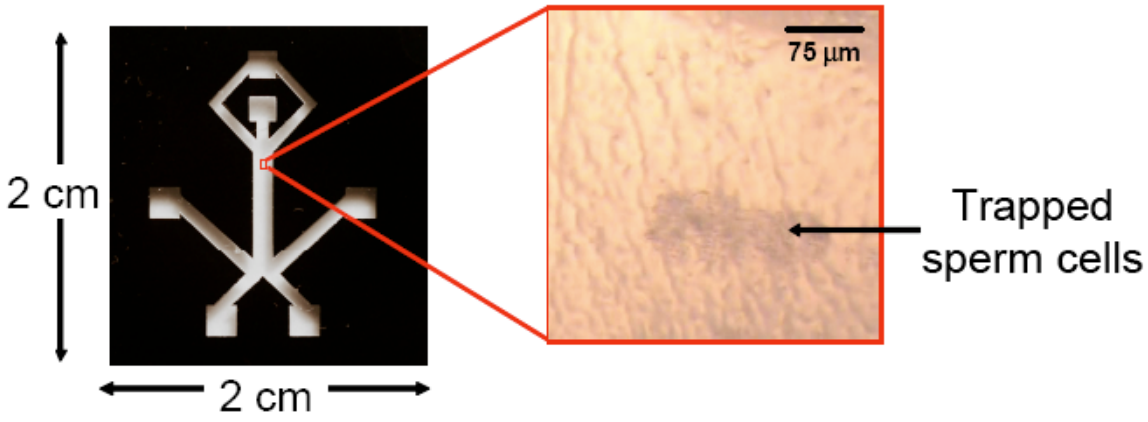


Figure 5: Channel structure of microdevice with the location of the transducer outlined in red. Inset: Photomicrograph of spermatazoa trapped above a microtransducer.

The principles underlying acoustic trapping in a standing wave have been defined in previous work.[44] An object entering an acoustic standing wave will experience forces

acting upon it. The forces acting on the object can be divided into primary, lateral, and secondary forces; all are important when performing particle trapping in a standing wave.[44] The primary radiation forces (F_{prf}, **Figure 4**) act in the direction of the propagating wave and direct the object to either nodes or anti-nodes depending on the material parameters of the object and the surrounding fluid. The equation describing the primary radiation force on a compressible sphere was described by King[45] and Gorkov[46] and is given by **Equation 1:**[47]

$$F = \frac{-\pi \times P_0^2 \times V \times \beta_0}{2\lambda} \times \sin\left(\frac{4\pi \times z}{\lambda}\right) \times \left(\frac{(5\rho_p - 2\rho_0)}{(2\rho_p + \rho_0)} - \frac{\beta_p}{\beta_0}\right) \quad (1)$$

where P_0 is the peak pressure amplitude from the transducer, λ is the wavelength of the standing wave, V is the volume of the particle, z is the particle position in the channel, β_0 and ϱ_0 is the compressibility and density of the medium, and ϱ_p and β_p is the density and compressibility of the particle. The forces will be stronger for larger particle volumes or higher frequencies (shorter wavelengths). The material parameters of the particle and the medium will decide whether the forces will be directed towards the anti-node or the node of the standing wave. Most particles and cells will be directed to the pressure nodes by the primary radiation force. Primary radiation forces do not prevent particles from moving laterally within the nodal plane; lateral forces (F_{lat}, **Figure 4**), keep the particles stationed at stable positions within the nodal plane. These forces act in the direction perpendicular to the wave propagation, and arise due to acoustic streaming and spatial variations in the stationary pressure field.[39] Secondary forces originate from pressure waves reflected on the particles or cells and will create interparticular forces that will

drive the particles to form clusters, helping to stabilize the trapped objects. Trapped particles will therefore form patterns consisting of clusters positioned at or close to the pressure minima; the distribution of the clusters will change depending on the frequency and amount of particles being trapped.[48] **Figure 5** shows the acoustic trapping of sperm in the presence of epithelial cell lysate. Upon activation of the ultrasound at a pre-determined frequency (10.8 MHz, 10 V_{pp} input), an acoustic standing wave was set up within the microchannel (~70 μm) above the transducer; a single trapping zone (node) was generated in the center of the channel. Upon infusion of a mixture of sperm and epithelial cell lysate, the sperm cells were trapped. Termination of the ultrasound released the cells from the trapping site.

Laminar Flow Valving for Fluidic Control

Effective acoustic trapping of cells becomes irrelevant if the effective separation of the male and female fractions cannot be achieved; therefore precise fluidic control is paramount to the success of the ADE system. Numerous methods of fluidic control have been described in the literature, including flow switching[49] and the use of active valves and pumping.[50, 51] One form of active valving, using a PDMS elastomeric membrane as described by Grover et al.,[51] has been shown to be very effective for fluidic control in integrated multiprocess devices.[12, 52, 53] However, for adaptation of the ADE system into forensic laboratories, it was of interest to develop a simple, effective fluidic control technique. The fluidic system chosen for the device incorporated laminar flow valving (LFV), a passive valving technique originally described by Blankenstein et al.[49] This

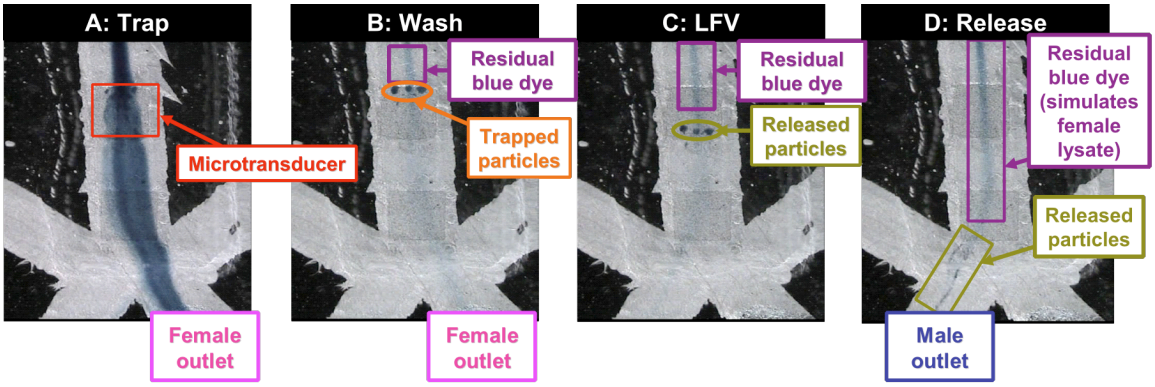


Figure 6: A series of sill photomicrographs of a simulated sexual assault sample consisting of Evans Blue Dye (representing female cell lysate) and blue polystyrene beads (representing sperm cells), demonstrating LFV. 6A. A mixture of dye and microbeads was infused into the device. The beads were trapped in the acoustic wave, while the dye was washed into the female outlet. 6B. After the sample infusion, the sample flow was terminated and the channels and trapped beads were washed with buffer. The infusion of focusing buffer unintentionally "pulls" sample (including residual blue dye) from the sample inlet. 6C. When the wash step was completed, the outlet flow was switched, and the ultrasound deactivated to give a controlled release of microbeads. 6D. The released beads, as well as sample (including residual blue dye) "pulled" from the sample inlet, were directed towards the male outlet.

involves a simple form of valving that exploits laminar flows and does not require physical valves that require actuation. In addition, the use of LFV circumvents the use of PDMS (or other substrates) commonly used in active valves. Laminar flow results in fluid streams traveling smoothly side-by-side without turbulent mixing. The flow profile can be predicted by the Reynolds number (*Re*) which is the ratio of inertial forces to viscous forces. At low *Re* that are typically encountered in microfluidic systems (< 1)[54, 55], viscous forces dominate, resulting in laminar flow; at larger *Re* (~2300), inertial forces dominate, resulting in turbulent flow.[56] LFV is ideal for the ADE system, as it circumvents the use of PDMS or other hydrophobic substrates commonly utilized in active valving, to which biological material may adhere (as a result of its hydrophobic nature). The design of the channel structure, shown in **Figure 2**, allows the flow of unretained female DNA to the female outlet, and released sperm cells to the male outlet, by controlling the ratio of buffer flow from inlets S1 and S2.

Drawbacks of Fluidic Control

The use of LFV for the ADE of sperm and epithelial cell lysate was previously demonstrated using a simulated sexual assault sample, consisting of 10 μm polystyrene beads to simulate sperm cells and Evans Blue Dye to simulate female epithelial cell lysate.[13] Upon infusion of the mixed sample, the ultrasound was activated (10.8 MHz, single node fluidic layer), and the polystyrene particles were trapped in the acoustic standing wave over the transducer; the unretained dye was directed to the female outlet using LFV as shown in **Figure 6A** (S1>S2**, Figure 2**). After five minutes of infusion, sample flow was terminated, and the channels were washed with buffer (FI, as shown in **Figure 2**, but outside of the field of view of **Figure 6A-D**) to remove any inadvertently-trapped Evans Blue Dye from the trapped particles, as well as residual dye from the microchannel (**Figure 6B**). LFV was then implemented (**Figure 6C**) by inverting the ratio of buffer infusion through the two side arm inlets (S2>S1) such that the direction of flow was switched to the male outlet. The ultrasound was then deactivated to give a controlled release of the microbeads towards the male outlet channel (**Figure 6D**). It was originally concluded that LFV could be used to effectively separate sperm and epithelial cell lysate on the ADE microdevice;[13] however, closer inspection of the still photomicrographs revealed a potential female DNA contamination source. During the ADE wash step, as sample flow was terminated, the infusion of focusing buffer from FI unintentionally "pulled" sample (including residual blue dye) from the sample inlet (**Figure 6B**). This "pull" of sample continued during the fluidic control (**Figure 6C**) and

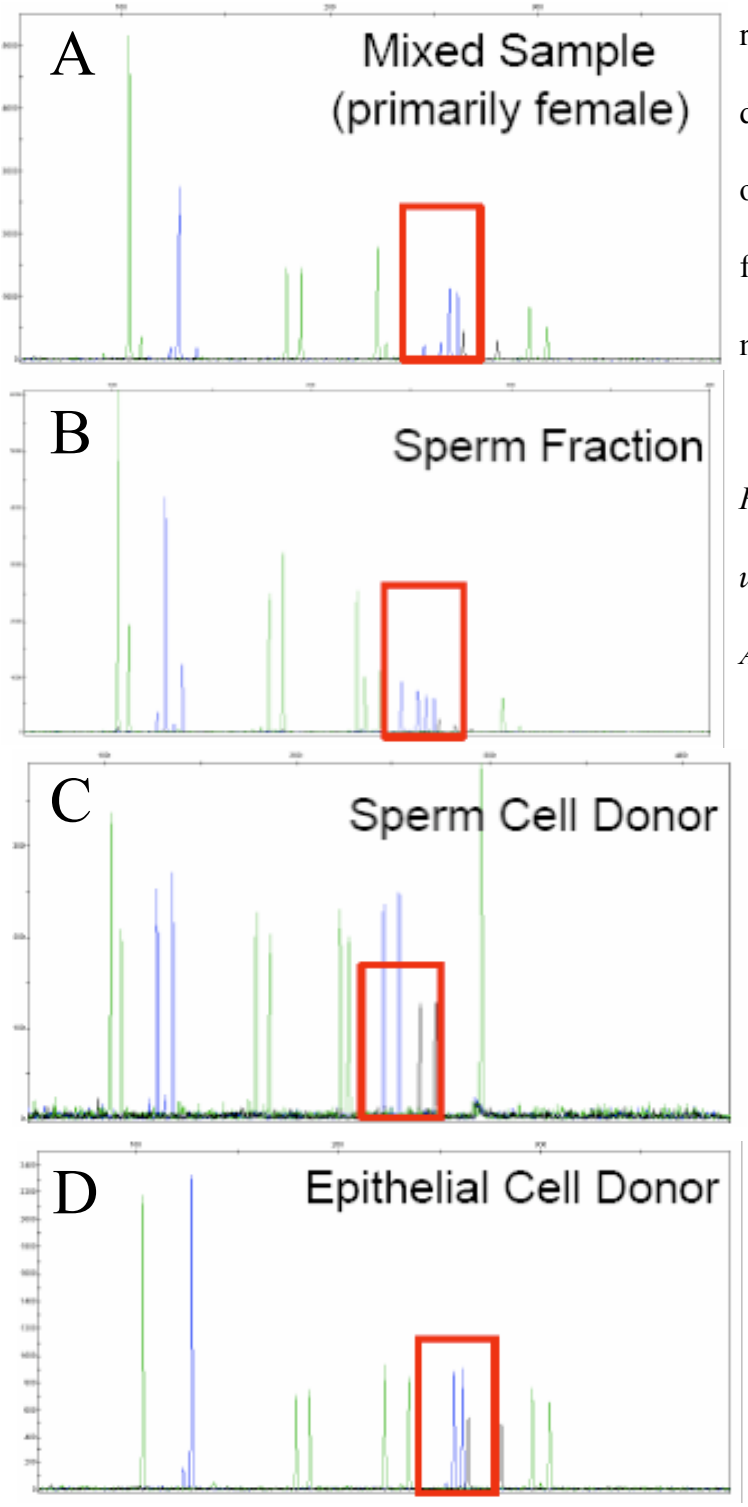


Figure 7: STR profiles generated before and after ADE separations. A. STR profile of sample prior to ADE (~5% male). B. Example of STR profile of sample collected from male outlet after ADE (~55% male; 7/7 loci). C. STR profile of sperm cell donor. D. STR profile of female epithelial cell donor.

release steps (**Figure 6D**), and blue dye was directed towards the male outlet, indicating a potential cause of female DNA contamination in the male ADE fractions.

Preliminary Results: Separations using the ADE Microdevice and STR Analysis

The suspected source of female DNA contamination in male ADE fractions was verified by initial attempts at separating sperm cells from epithelial cell lysate.[13] For these experiments, mock sexual assault samples were created by collecting buccal epithelial cells on a cotton swab and eluting into phosphate buffer (PBS, pH 7.4) with agitation. These cells were lysed using a conventional differential extraction buffer

containing SDS and proteinase K. Semen (6 µL) was then added to the epithelial cell lysate, and infused through SA (5 m) while focusing buffer (PBS) was infused through FI (1.5-2 µL/m). Trapping was initiated, and the sample and focusing buffer were directed towards the female outlet using LFV (S1>S2). Once the sample was infused, sample infusion was terminated, and any remaining epithelial cell lysate was washed from the system by continued infusion of focusing buffer (1.5-2 µL/m) from FI. After washing, LFV was used to direct the flow towards the male outlet (S2>S1), and the transducer voltage was terminated, releasing the sperm cells. Additional buffer flow from FI was used to transport the cells down the center channel. Samples collected from the female outlet during sperm cell trapping and from the male outlet during the release were stored

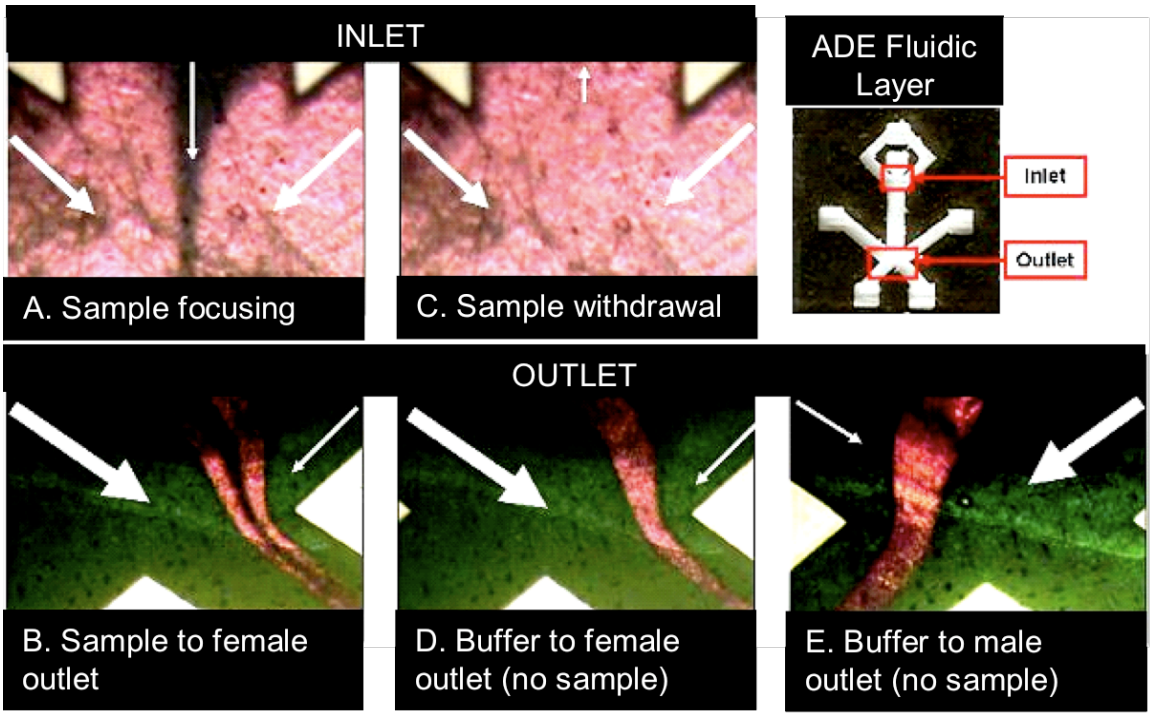


Figure 8. Still photomicrographs showing fluidic control characterization using the modified sample withdrawal method. A. Sample focusing (blue) using focusing buffer (red). B. Sample and focusing buffer directed towards female outlet using laminar flow valving buffers from side arm channels (green). C. Sample withdrawal was implemented, preventing unwanted sample infusion. D. During withdrawal of sample, flow of focusing buffer directed towards female outlet using LFV. E. During withdrawal of sample, direction of flow was switched to male outlet using LFV.

in microcentrifuge tubes; solid phase extraction and conventional STR analysis of samples before (**Figure 7A**) and after ADE (**Figure 7B**) were subsequently performed

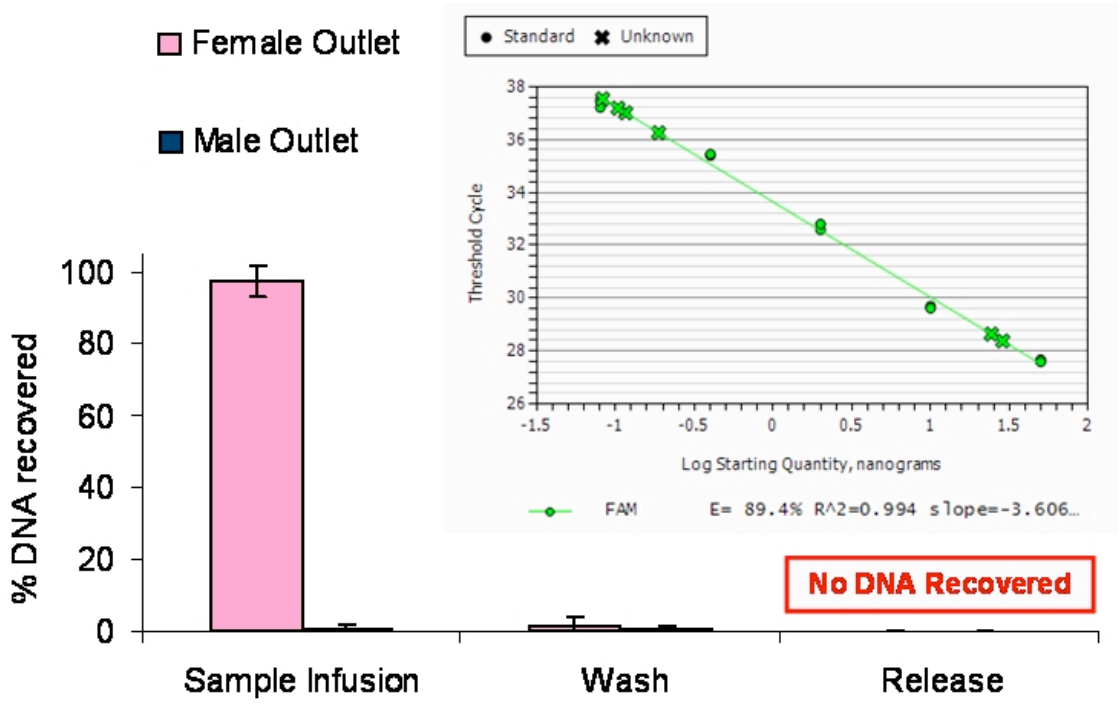


Figure 9. Percentage of hgDNA recovered in male and female fractions obtained from ADE device. Sample: pre-pruified hgDNA (10 ng/µL). LFV was used to direct unretained pre-purified DNA to the female outlet. Fractions were collected after each step and quantified using conventional real-time qPCR methods.

off-chip using commercially-available kits. To illustrate the purity of the collected ADE fractions, STR profiles of the male and female donors were obtained for comparative purposes (using sperm cells and buccal epithelial cells, respectively). Unfortunately, the data (**Figure 7**) obtained from these experiments consistently resulted in poor separation efficiencies. Comparison of the profiles from the sperm cell donor (**Figure 7C**) with the male ADE fraction (**Figure 7B**) indicated recovery of sperm cell DNA; however, the presence of additional alleles consistent with the epithelial cell donor (**Figure 7D**) signified poor purity (55%, as shown in **Figure 7B**, or less) of the male fraction.[13] The

data showed that additional efforts towards improving fluidic control were necessary to eliminate female lysate contamination of the male ADE separation product, and to demonstrate better purity levels.

Improved Fluidic Control: Implementation of Sample Withdrawal

To prevent unwanted sample infusion during the wash and release steps, the experimental conditions were modified so that the sample was withdrawn from the device by the sample syringe (at a lower flow rate relative to that of the focusing buffer). Food coloring was used in the initial characterization of the modified sample withdrawal method. Sample (blue dye) was infused through SA, while focusing buffer (red dye) was infused through FI (6 μL/m); **Figure 8A** shows the resultant focusing of sample by focusing buffer. The sample and focusing buffer were directed towards the female outlet using LFV (green dye, S1>S2, **Figure 8B**). During the wash step, withdrawal from the sample inlet (1 μL/m) effectively stopped the sample from entering the center channel (**Figure 8C**), and the focusing buffer effectively removed residual sample from the remainder of the channel, including the female outlet (**Figure 8D**). LFV valving was implemented to simulate fluidic control prior to the release step of ADE. **Figure 8C** shows that LFV was not adversely affected by sample withdrawal, as no sample was observed entering the male outlet.

The reliability of the modified fluidic control method was further assessed using prepurified DNA and subsequent quantitation of the collected fractions. A sample consisting of prepurified hgDNA (10 ng/μL) used to simulate female epithelial cell lysate

was infused through SA and hydrodynamically focused (PBS, pH 7.4); the ultrasound was not initiated. LFV (S1>S2, PBS, pH 7.4) was used to direct the flow of the DNA towards the female outlet (sample infusion). After 5 minutes of sample infusion, the sample flow was switched to withdraw from SA (1 μL/m), and the channels were washed with focusing buffer while maintaining flow towards the female outlet (wash). Once the wash step was completed, (3 minutes), the outlet flow was switched (S2>S1) for 3 minutes, such that the net flow was directed towards the male outlet (release). After each step (sample infusion, wash, release) the eluted fractions were collected from each outlet, and total genomic DNA of each sample was measured using a quantitative PCR assay (developed in-house). The data (**Figure 9, n=3**) show that fractions collected from the female outlet after sample infusion contained the majority of the recovered DNA (98 ± 4.5%), while fractions collected from the male outlet after sample infusion (0.61 ± 1.1%) and wash step (0.55 ± 0.95%) contained trace amounts. Fractions collected from the female outlet after the wash step contained 1.4 ± 2.5% of the recovered DNA, representative of the residual sample that was present in the microchannel and outlet tubing after the sample withdrawal was implemented. DNA was not detected in fractions collected from the female outlet, and more importantly, the male outlet, after the release step. The results verify that the use of sample withdrawal during the wash and release steps eliminates a source of contamination, as it ensures that sample (containing epithelial cell lysate) would not be "pulled" into the device and towards the male outlet during the release of the trapped material (sperm cells). The sample withdrawal flow rate for these studies (1.0 μL/m) was arbitrarily chosen. The data in **Figure 9** (n = 3) is reported in

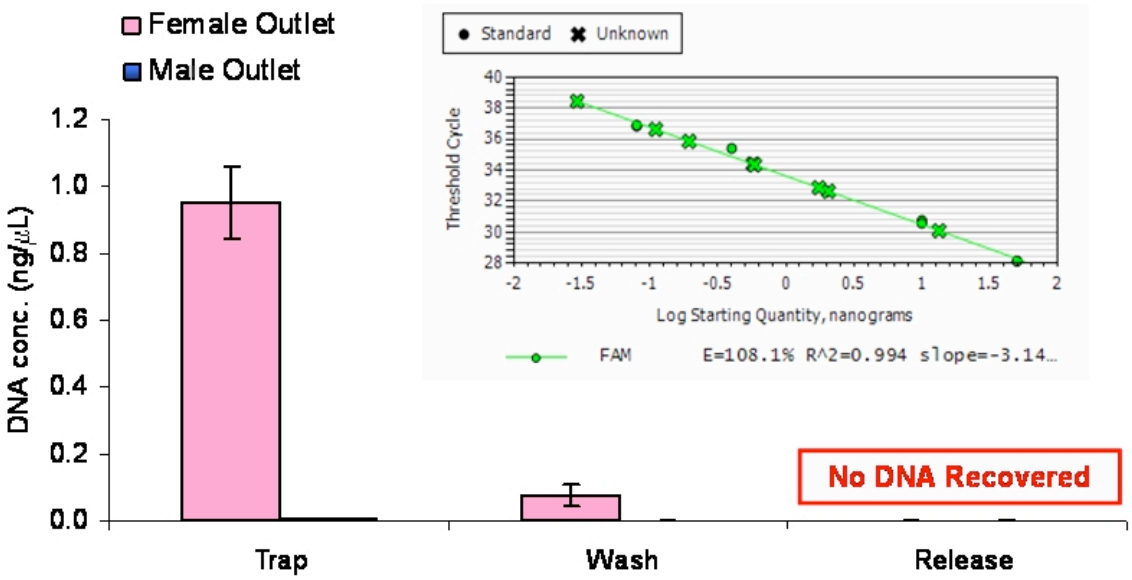


Figure 10. Concentration of hgDNA recovered in male and female fractions obtained from ADE device. Sample: epithelial cell lysate prepared in conventional differential extraction buffer. LFV was used to direct unretained epithelial cell lysate to the female outlet. Fractions were collected after each step; DNA was purified and quantified using conventional \methods.

percent of total DNA recovered, as opposed to total genomic DNA. However, the concentration of DNA recovered from the male outlet decreased over the course of the three trials (data not shown), indicating that the sample withdrawal flow rate was too fast. Similar studies using pre-purified hgDNA were performed to optimize the sample withdrawal flow rate (while incorporating hydrodynamic focusing at 6 μL/m); it was determined that 0.5 μL/m sample withdrawal was more suitable for future experiments, as it provided consistent recoveries of hgDNA in the fractions collected from the female outlet after sample infusion (data not shown).

Assessment of Acoustic Trapping

To ensure that the ADE microdevice was suitable for the separation of mock sexual assault samples, acoustic trapping conditions of sperm and epithelial cell lysate

were separately assessed. Lysate trapped in the acoustic standing wave is a potential source of contamination, as it would be released upon termination of the ultrasound and directed towards the male outlet; therefore, it was important to establish that epithelial cell lysate (in the absence of sperm cells) was not trapped. Female epithelial cell lysate (prepared in conventional DE buffer) was infused through SA and hydrodynamically focused (PBS, pH 7.4); trapping was initiated (10 V_{pp} output, 11.6 MHz), and LFV (S1>S2, PBS, pH 7.4) was used to direct the flow of the DNA towards the female outlet (trap). After 2 minutes of sample infusion, the sample flow was switched to withdraw from SA (0.5 μL/m), and the channels were washed with focusing buffer while maintaining flow towards the female outlet (wash). Once the washing step was completed, (3 minutes), the outlet flow was switched (S2>S1) such that the net flow was directed towards the male outlet, and the acoustic trapping was terminated; samples were collected for 3m (release). After each step, the eluted fractions were recovered from each outlet and purified off-chip using a commercially-available extraction kit. Total genomic DNA was measured using a real-time quantitative PCR assay. The results (**Figure 10**) show that fractions collected from the female outlet after sample infusion contained the highest concentration of DNA (0.95 ± 0.11 ng/μL), and, therefore, the most lysate; DNA was not detected in fractions collected from the male outlet. Fractions collected from the female outlet during the wash step contained 0.18 ± 0.15 ng/μL of DNA, representative of the residual sample that was present in the microchannel and outlet tubing after the sample withdrawal was implemented. DNA was not detected in fractions collected from the female outlet, and more importantly, the male outlet, after the release step – results

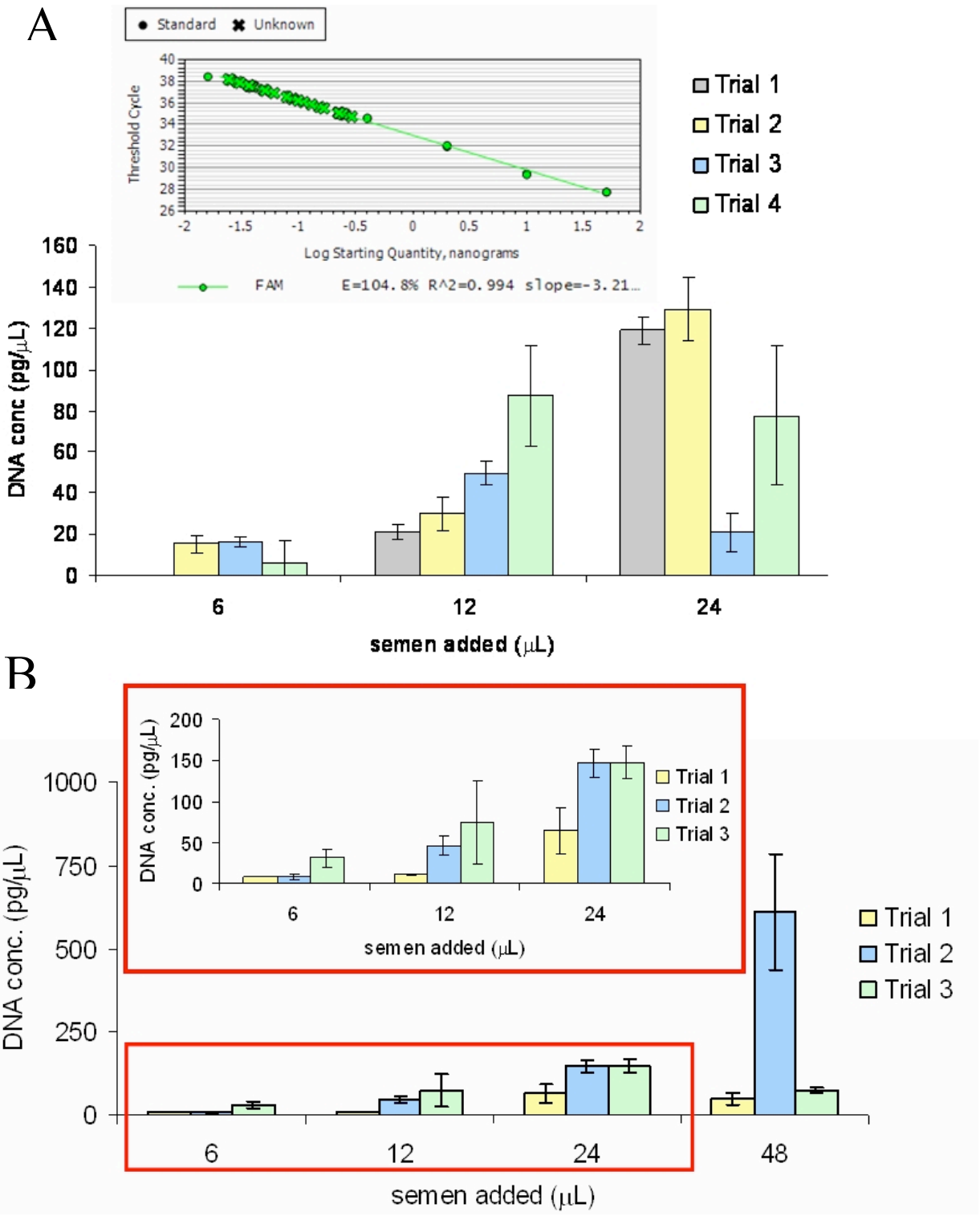


Figure 11. Concentration of human genomic DNA recovered from the male outlet after acoustic trapping of sperm cells. A. 0.3 mm ID PTFE outlet tubing. B. Capillary outlet tubing (250 µm ID). Sperm cells were directed towards the male outlet using LFV. Fractions were collected after release of sperm cells to outlet; DNA was purified and quantified using off-chip conventional analysis methods.

that were consistent with those obtained from studies performed with pre-purified DNA. The results confirm that epithelial cell lysate was not trapped in the absence of sperm cells, as predicted by acoustic force theory; however, these studies do not account for epithelial cell lysate inadvertently trapped in the presence of trapped sperm cells. In addition, the data further verify the effectiveness of incorporating sample withdrawal to eliminate sample bleed towards the male outlet.

The number of sperm cells captured and released from the acoustic trap, and consequently the amount of DNA recovered in the male fraction after ADE, is critical to obtaining a male STR profile. The amount of DNA in a sample is essential for most PCR-based assays, because a narrow concentration works best. For example, the Applied Biosystems' COFiler™ STR kit specify the addition of 1 – 2.5 ng of template DNA (100 – 250 pg/μL for conventional amplifications) for optimal results.[57] Studies were therefore performed to assess sperm cell trapping and release using the ADE device, without interference from epithelial cell lysate. Samples consisting of semen (6, 12, or 24 μL) added to 500 μL PBS, pH 7.4, were infused through the SA and hydrodynamically focused (PBS, pH 7.4); trapping was initiated (8 V_{pp} output, 11.6 MHz), and LFV (S1>S2, PBS, pH 7.4) was used to direct the net flow towards the female outlet. After two minutes of sample infusion, the sample flow was switched to withdraw from SA (0.5 μL/m), and the channels were washed with focusing buffer while maintaining flow towards the female outlet. Once the washing step was completed, (3 minutes), the outlet flow was switched (S2>S1) such that the net flow was directed towards the male outlet, and the acoustic trapping was terminated; samples were collected for 3 m. The eluted

cells were recovered from the male outlet (0.3 mm ID PTFE tubing) and purified off-chip using a commercially-available extraction kit. Total genomic DNA was measured using a quantitative PCR assay. **Figure 11A** shows the concentration of DNA recovered from four consecutive runs for each prepared sample. Samples prepared with 6 μL of neat semen (~960 sperm cells trapped) provided 13 ± 5.6 pg/μL DNA for three of the four consecutive trials (with the exception of the first trial, where no DNA was detected), an insufficient concentration to obtain full STR profiles. For samples prepared with 12 μL of semen (~1920 cells trapped), an increase in DNA concentration was observed over the course of the four trials (21 ± 3.6 pg/μL for the first trial vs. 87 ± 24 pg/μL for the fourth trial). Samples prepared with 24 μ L of semen (~3840 cells trapped) provided inconsistent recoveries of DNA, ranging from 21 pg/μL (trial 3) – 130 pg/μL (trial 2). For these studies, it was assumed that the trapping efficiency [(number of cells captured in the acoustic trap/number of cells infused through the ADE system) × 100] was approximately 100%, as loss of cells during sample infusion/trapping was not observed. In addition, upon termination of the ultrasound, the cells were consistently released cleanly from the trapping site (i.e. cells were not adsorbed to the transducer or glass surface) towards the male outlet, and were observed entering the outlet reservoir. It was therefore hypothesized that the inconsistent recoveries of DNA obtained from samples prepared with higher volumes of semen were a result of poor release of larger clusters of cells from the outlet tubing. Similar studies were performed with devices containing capillary outlets (250 μm ID). Samples consisted of neat semen (6, 12, 24, or 48 μL) added to 500 μ L PBS, pH 7.4; sample infusion, wash, and release of cells were

performed as previously described. The eluted cells were recovered from the male outlet and purified off-chip using a commercially-available extraction kit. Total genomic DNA was measured using a quantitative PCR assay. **Figure 11B** shows the concentration of DNA recovered from three consecutive runs for each prepared sample. Samples prepared with 6 µL of semen (~960 sperm cells trapped) provided 16 ± 13 pg/µL, an insufficient concentration of DNA to obtain a full STR profile. For samples prepared with 12 µL of semen (~1920 cells trapped), an increase in DNA concentration was observed over the course of the three trials (11 ± 1.5 pg/µL for the first trial vs. 74 ± 51 pg/µL for the third trial); a similar trend was observed for samples prepared with 24 µL of semen (~3840 cells trapped; 65 ± 28 pg/µL for the first trial vs. 150 ± 20 pg/µL for the third trial). Samples prepared with 48 µL of semen (~7680 cells trapped) provided inconsistent recoveries of DNA, ranging from 49 pg/µL (trial 1) – 610 pg/µL (trial 2). The data further support the theory that more acoustically-trapped cells resulted in larger clusters of cells that were not released cleanly from the outlet tubing. The data (**Figure 11A, 11B**) also show that lower numbers of trapped cells, coupled with off-chip conventional

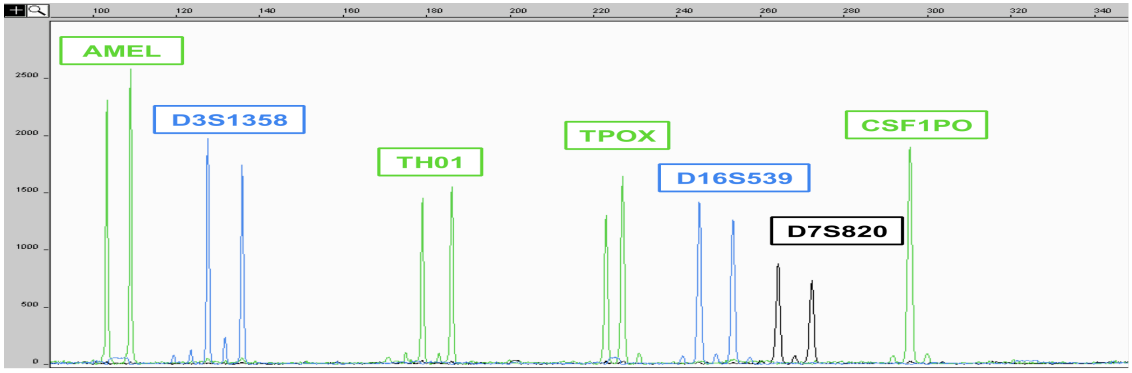


Figure 12: STR profiles of samples collected from the male outlet after ADE. Sample preparation: sperm cells added to conventional DE buffer (full profile – 7/7 loci detected; n = 4). Experimental conditions: sample infusion: 2m, 1 µL/m with 6 µL/m focusing buffer flow (DE buffer), directed towards female outlet; wash: 3m, 6 µL/m (DE buffer) with 0.5 µL/m sample withdrawal; directed towards female outlet; release: 3m, 6 µL/m (DE buffer) with 0.5 µL/m sample withdrawal, directed towards male outlet.

purification and amplification techniques, would not provide sufficient DNA for full STR profiles. Therefore, sample preparation and trapping conditions for the remainder of the reported studies were adjusted such that approximately 2000-2400 cells were acoustically trapped and released towards the male outlet.

Improved Methods for Cell Release

In order to improve the release of biological material from outlets, focusing and LFB buffers were changed from PBS to a conventional DE buffer, the same buffer used to prepare female epithelial cell lysate (devoid of the proteolytic lysis agent). The presence of detergent in the conventional DE buffer was expected to facilitate the release of cells from the outlets. In addition, the use of DE buffer as focusing and LFV buffers allows for a longer release time, as conventional DE buffer is added to post-ADE samples as part of the subsequent off-chip extraction protocol. To test this, sperm cells were added to conventional DE buffer (the same buffer as used for LFV and hydrodynamic focusing). Samples were infused through SA and hydrodynamically focused; trapping was initiated (10 V_{pp} output, 11.6 MHz), and LFV (S1>S2) was used to direct the flow of the DNA towards the female outlet. After two minutes of sample infusion and trapping, the sample flow was switched to withdraw from SA (0.5 μL/m), and the channels were washed with focusing buffer while maintaining flow towards the female outlet. Once the washing step was completed, (3 minutes), the outlet flow was switched (S2>S1) such that the net flow was directed towards the male outlet, and the acoustic trapping was terminated; samples were collected for 5 m. Samples were recovered from the male

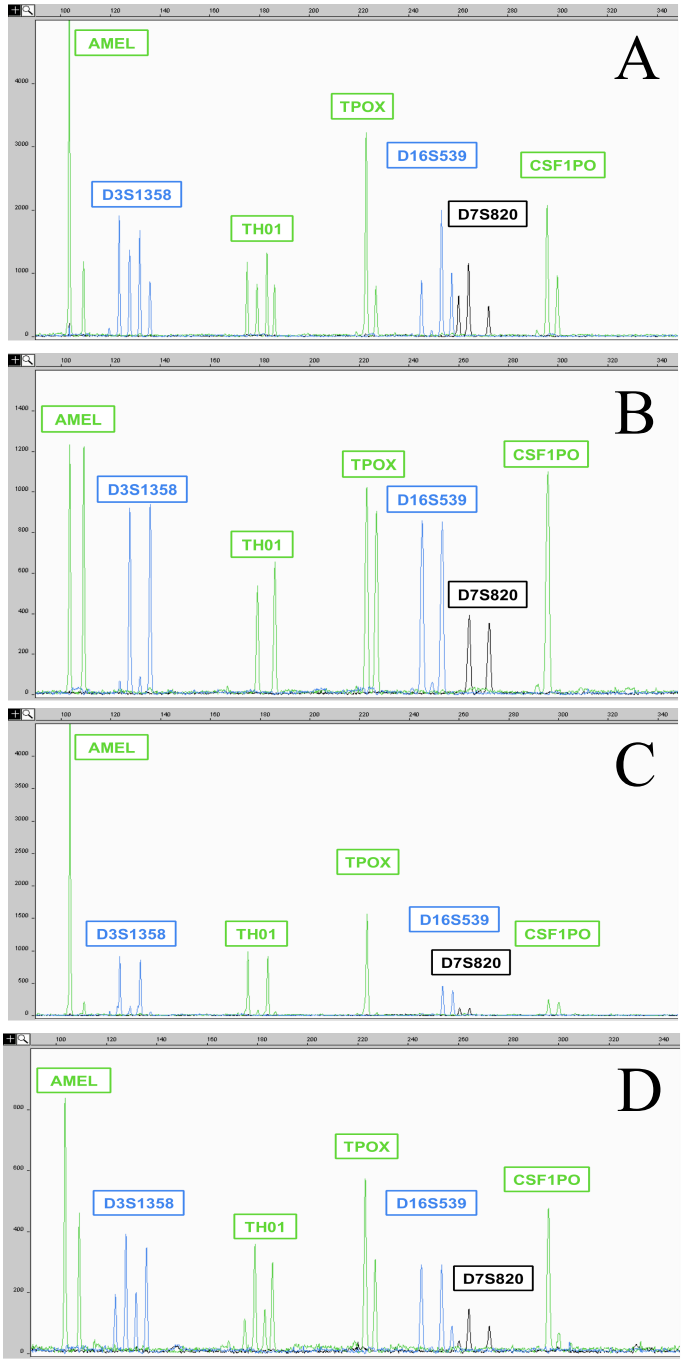


Figure 13: STR profiles generated before and after ADE separations. Male and female outlets were coated with 0.1% (w/v) PHEA prior to separations. Sample preparation: sperm cells added to epithelial cell lysate. A. STR profile of sample prior to ADE (40 ± 3.9% male). B. STR profile of sperm cell donor. C. STR profile of female epithelial cell donor. D. Example of STR profile of sample collected from male outlet after ADE (79 ± 15% male; 7/7 loci). Experimental conditions: sample infusion: 2 m, 1 µ L/m, with 6 µ L/m focusing buffer flow (DE buffer), directed towards female outlet; wash: 3 m, 6 µL/m (DE buffer) with 0.5 µL/m sample withdrawal; directed towards female outlet; release: 3 m, 6 µL/m (DE buffer) with 0.5 µL/m sample withdrawal, directed towards male outlet.

outlet (250 µ m ID capillary) and purified off-chip using a commercially-available extraction kit; conventional amplification and separation of STR loci were performed. The results show that samples prepared in DE buffer consistently provided full STR profiles (**Figure 12**, n = 4, consecutive trials), indicating that the use of DE buffer was effective in the removal of cells from the outlet tubing; therefore, LFV and focusing buffers were changed to conventional DE buffer for all future studies.

The replacement of PBS with conventional DE buffer for focusing and LFV provided consistent, full STR profiles for samples prepared in conventional DE buffer. Once this was established, ADE separations of mock sexual assault samples were

performed. Sperm cells were added to female epithelial cell lysate (prepared in the same conventional DE buffer as used for LFV and hydrodynamic focusing, but with the addition of a proteolytic digestion enzyme); acoustic trapping and separations were performed as previously described. The results indicate that the release of biological material was problematic for samples prepared in epithelial cell lysate, as STR profiles were not obtained (data not shown, n = 4, consecutive trials). In an effort to further facilitate removal of biological material from the outlets, the capillaries were coated with 0.1% (w/v) PHEA. Samples were prepared in epithelial cell lysate as previously described. STR profiles of the mixed biological sample prior to ADE (**Figure 13A**), the sperm cell donor (**Figure 13B**), and the female epithelial cell donor (**Figure 13C**) were obtained using conventional methods; comparison of each locus of the original sample profile with those of the male and female donors indicated that the mixed biological sample was 40 ± 3.9% male. The sample was infused through SA and hydrodynamically focused; trapping was initiated (8 V_{pp} output, 11.6 MHz), and LFV (S1>S2, DE buffer)

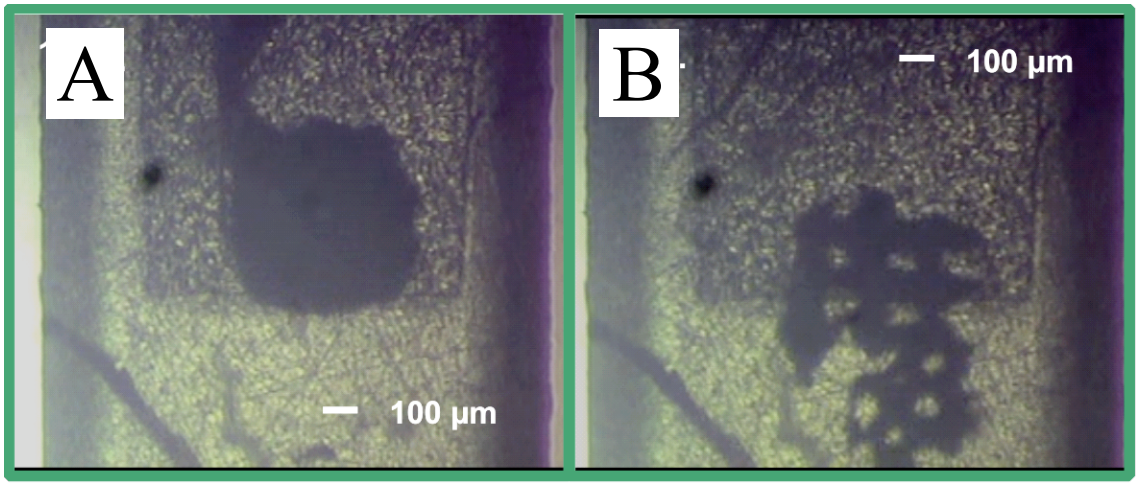


Figure 14:. Particle trapping using ADE device. All panels show the channel area above the transducer (920 µ m width) using light microscopy. Photographs were taken at the moment the transducer was inactivated. a. Trapping performed at 11.6 MHz (same operating frequency as used for ADE separations of mock sexual assault samples). b. After the frequency was changed to 12.1 MHz, the trapping positions changed, and smaller clusters of particles were distributed above the transducer

was used to direct the net flow towards the female outlet. After two minutes of sample infusion, the sample flow was switched to withdraw from SA (0.5 μL/min), and the channels were washed with focusing buffer (DE buffer) while maintaining flow towards the female outlet. Once the washing step was completed, (3 min), the outlet flow was switched (S2>S1) such that the net flow was directed towards the male outlet, and the acoustic trapping was terminated; samples were collected for 5 min. Samples were recovered from the male outlet (250 μ m ID capillary) and purified off-chip using a commercially-available extraction kit; conventional amplification and separation of STR loci were performed. An example of a resulting STR profile is shown in **Figure 13D**; comparison of the profile with those of the male and female donors indicate that the sample is 79 ± 16% male, with all seven core STR loci detected. The results show that ADE, performed on a device with coated capillary outlets, provided over 50% enhancement of male purity. The calculated male purity did not differ considerably from samples recovered in several subsequent ADE trials (data not shown). However, due to issues with recovery of the separated biological material from the outlets; two out of six of the subsequent trials did not result in STR profiles (data not shown).

Acoustic forces: distribution of trapped particles

The trapping position of particles is governed by the local pressure distribution over the transducer, as shown by Lilliehorn et al.[58] Theoretical predictions and previous work using light diffraction tomography demonstrate that the pressure distribution is dependent on the frequency applied to the transducer. **Figure 14** shows the effects of

varying the operating frequency of the transducer on the trapping pattern of particles. A suspension of fluorescent particles (2 µ m) was infused through SA with hydrodynamic focusing. Trapping was initiated and observed using light microscopy; still photomicrographs were taken just as the ultrasound was terminated. **Figure 14A** shows the pattern of particle trapping at 11.6 MHz, the same operating frequency used for assessment of acoustic trapping and separations of mock sexual assault samples; the particles were trapped in a large cluster near the center of the channel, suspended above the transducer. Using the same particle suspension, transducer, and fluidic layer, the frequency was increased to 12.1 MHz. The change in trapping frequency resulted in a change in the particle

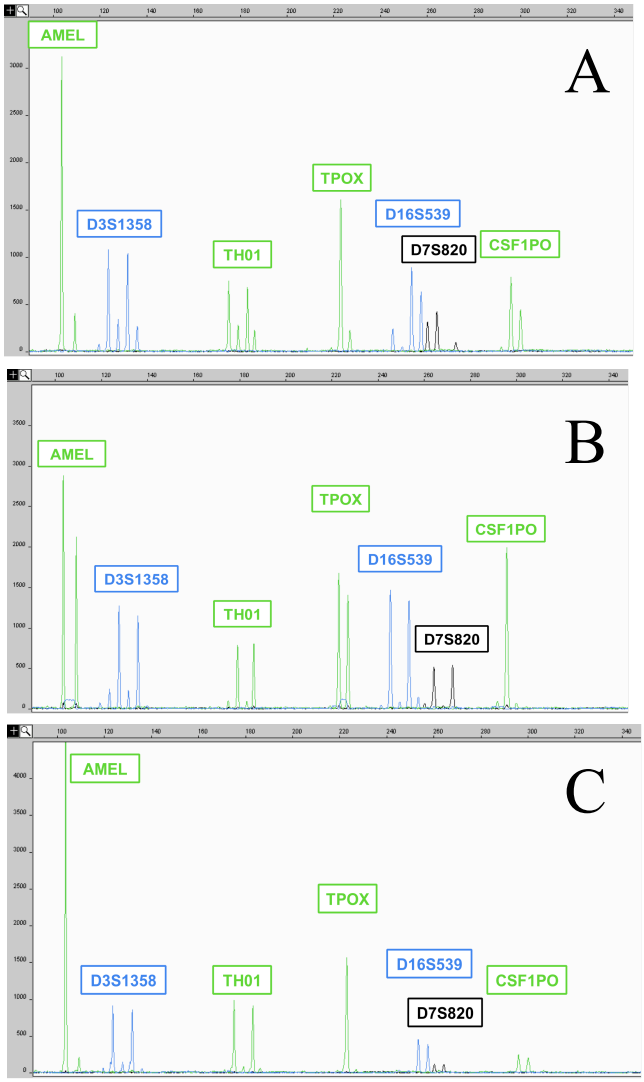


Figure 15: STR profiles generated before and after ADE separations of a mock sexual assault sample. Sample preparation: sperm cells added to epithelial cell lysate. A. STR profile of sample prior to ADE (26 ± 2.0% male). B. STR profile of sample collected from male outlet after ADE (92 ± 7.9% male; 7/7 loci). C. STR profile of sample collected from female outlet after ADE (5.7 ± 4.7% male; 7/7 loci). Experimental conditions: sample infusion: 2 m, 1 µ L/m, with 6 µ L/m focusing buffer flow (DE buffer), directed towards female outlet; wash: 3 m, 6 µ L/m (DE buffer) with 0.5 µL/m sample withdrawal; directed towards female outlet; release: 3 m, 6 µ L/m (DE buffer) with 0.5 µL/m sample withdrawal, directed towards male outlet.

distribution; smaller clusters of particles that were spread out over a larger area of the transducer were observed (**Figure 14B**).

It was hypothesized that the change in particle distribution would allow for more effective removal of inadvertently-trapped female lysate from the trapped cells. In addition, it was anticipated that the smaller clusters of cells trapped using the higher frequency would be less likely to clog the outlet tubing upon release compared to a single, larger cluster of cells obtained with the lower frequency. To test this, mock sexual assault samples were prepared in epithelial cell lysate. The STR profile of the mixed biological sample prior to ADE (**Figure 15A**) was obtained using conventional methods; through comparison of this profile with those of the sperm cell (**Figure 13B**) and female epithelial cell (**Figure 13C**) donors, the sample was determined to be 26 ± 2.0% male. Trapping was initiated (8 V_{pp} output, 12.1 MHz), and LFV (S1>S2, DE buffer) was used to direct the net flow towards the female outlet. The trapping time was adjusted (6 min) such that approximately 2000 cells were trapped. After the sample infusion, the sample flow was switched to withdraw from SA (0.5 μL/m), and the channel above the transducer was washed with focusing buffer (DE buffer) while maintaining flow towards the female outlet. Once the washing step was completed, (2.5 minutes), the outlet flow was switched (S2>S1) such that the net flow was directed towards the male outlet, and the acoustic trapping was terminated; samples were collected for 5 m. Samples were recovered from the male and female outlets (0.3 mm ID PTFE tubing) and purified off-chip using a commercially-available extraction kit; conventional amplification and separation of STR loci were performed. An example of an STR profile obtained from

the male fraction is shown in **Figure 15B**; comparison of the profile with those of the male and female donors indicate that the sample is 92 ± 7.9% male, a nearly four-fold enhancement in male purity in comparison with the original sample. All seven core STR loci, devoid of alleles contributed from the female, were detected, allowing for interpretation of the male profile from the original mixture. In addition, the STR profile obtained from the female fraction is shown in **Figure 15C**; comparison of the profile with those of the male and female donors indicates that the sample is 5.7 ± 4.7% male. The results suggest that the particle distribution in the acoustic trap may play an important role in the effective separation of sperm and female epithelial cell lysate using the ADE device.

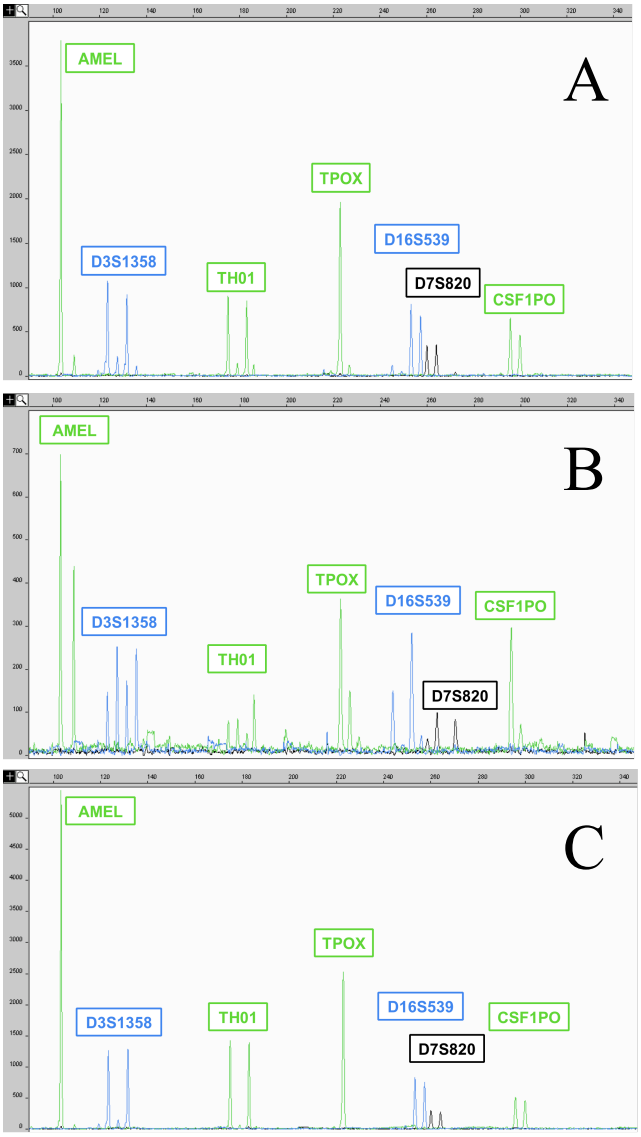


Figure 16: STR profiles generated before and after ADE separations of a mock sexual assault sample. Sample preparation: sperm cells added to epithelial cell lysate. A. STR profile of sample prior to ADE (13 ± 5.9% male). B. STR profile of sample collected from male outlet after ADE (73 ± 17% male; 7/7 loci). C. STR profile of sample collected from female outlet after ADE (3.1 ± 3.9% male; 7/7 loci). Experimental conditions: sample infusion: 2 m, 1 µL/m, with 6 µL/m focusing buffer flow (DE buffer), directed towards female outlet; wash: 3 m, 6 µL/m (DE buffer) with 0.5 µL/m sample withdrawal; directed towards female outlet; release: 3 m, 6 µL/m (DE buffer) with 0.5 µL/m sample withdrawal, directed towards male outlet.

The higher operating frequency and its resultant trapping pattern were applied to ADE separations of a mock sexual assault samples containing lower levels of male DNA. Samples were prepared by addition of sperm cells to epithelial cell lysate generated from a larger number of epithelial cells than used in the previous study. The STR profile of the mixed biological sample prior to ADE (**Figure 16A**) was obtained using conventional methods; through comparison of this profile with those of the sperm cell (**Figure 13B**) and female epithelial cell (**Figure 13C**) donors, the sample was determined to be 13 ± 5.9% male. Trapping was initiated (8 V_{pp} output, 12.1 MHz), and the separation was performed as previously described (6 min sample infusion and 2.5 min wash, sperm cells trapped while unretained lysate directed towards female outlet; 5 m release of sperm cells towards male outlet); the trapping pattern of sperm was again similar to the one depicted in **Figure 14B**. Samples were recovered from the male and female outlets (0.3 mm ID PTFE tubing) and purified off-chip using a commercially-available extraction kit; conventional amplification and separation of STR loci were performed. An example of an STR profile obtained from the male fraction is shown in **Figure 16B**; comparison of the profile with those of the male and female donors indicate that the sample is 73 ± 17% male, with all seven core STR loci detected – a nearly sevenfold enhancement of male purity compared to the original sample. In addition, the STR profile obtained from the female fraction is shown in **Figure 16C**; comparison of the profile with those of the male and female donors indicates that the sample is 3.1 ± 3.9% male – effectively a pure female profile without presence of male alleles.

The enrichment of male DNA in the sample (as compared to the original mixture), combined with the STR profile obtained from the female donor, allows for interpretation of the male profile from the original mixture. However, the presence of female alleles in the profile obtained from the male outlet indicates a source of female DNA contamination. Since fluidic control was proven to be reliable and reproducible, these results are most likely due to excess female lysate inadvertently trapped within the sperm cell clusters. Further studies may require optimization of the wash step to effectively remove female DNA and prevent it from contaminating the post-ADE male sample. It is

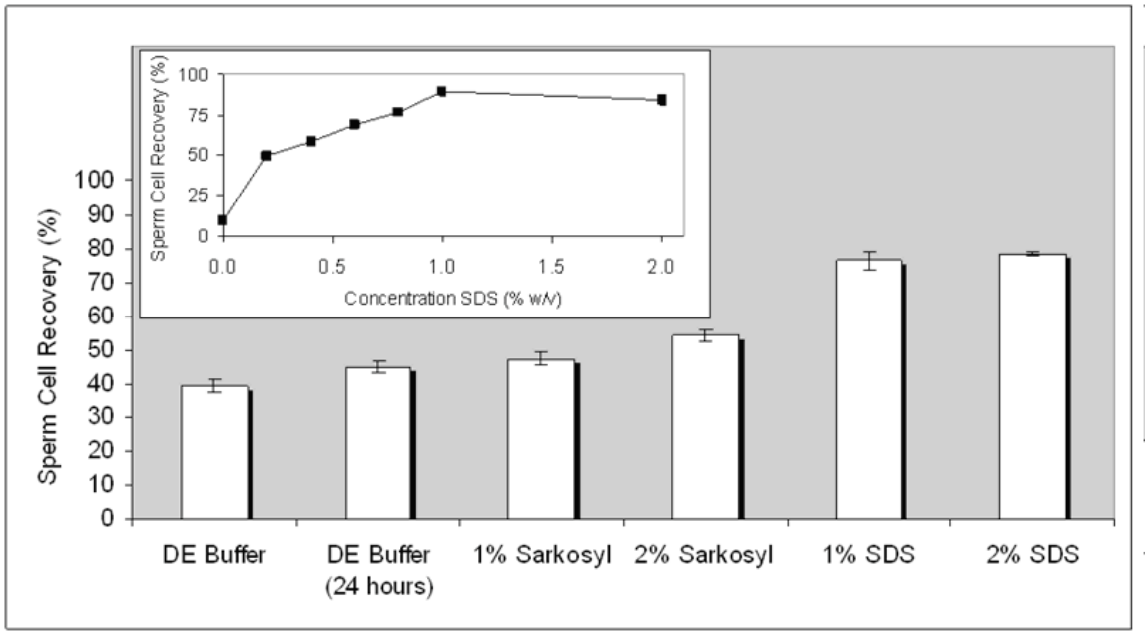


Figure 17: Comparison of sperm cell recoveries from elutions using differential extraction (DE) buffer and anionic detergents. Mock casework samples were dried for 1 week and incubated for 2 h at 42°C (unless otherwise indicated). Inset: Effect of sodium dodecyl sulfate (SDS) concentration on the release of sperm cells from samples dried for 1 week. Samples were incubated for 2 h at 42°C.

important to note that, while samples collected from the male outlet following subsequent ADE separations of the same sample provided male purities (data not shown) that did not differ considerably from the ones shown; samples collected from the female outlet did not

consistently provide STR profiles (data not shown), indicating that consistent release of female lysate from outlets is an issue.

4. Improved Methods for Elution of Cells from Vaginal Swabs

Effective utilization of the ADE method or other alternative DE methods relies on efficient and comprehensive lysis of epithelial cells in a timely manner. Regardless of the method utilized for the separation of female epithelial DNA and male sperm cells, the overall effectiveness of the procedure is also dependent on the efficiency with which material can be eluted and recovered from a cotton swab. The issue is especially important with swab samples containing low numbers of sperm cells, where any loss makes it even more difficult to obtain a profile of the perpetrator. A two-step method for cell elution and recovery from mock sexual assault samples has been developed by our

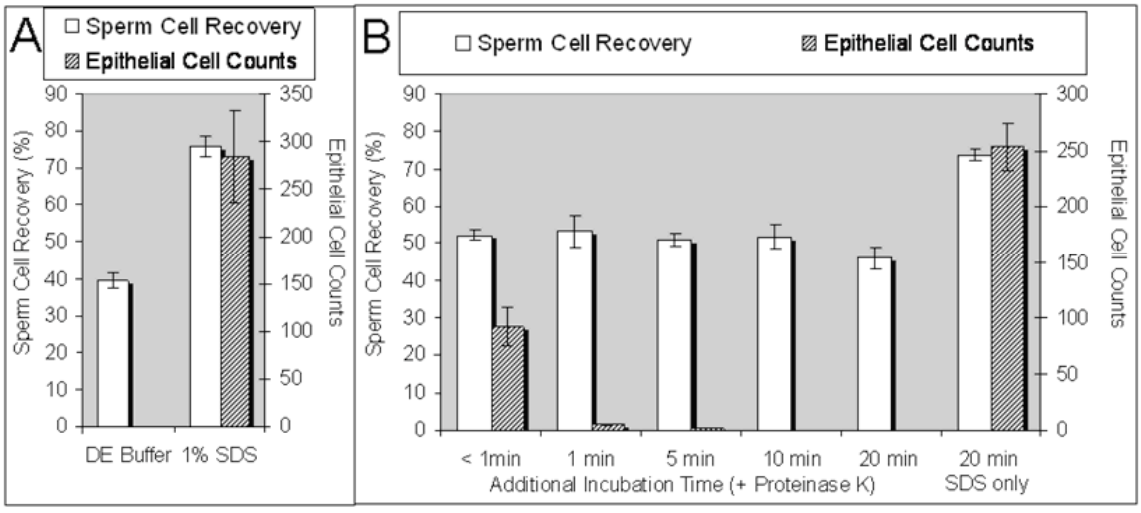


Figure 18: A. Comparison of sperm cell recoveries and epithelial cell counts from elutions using differential extraction (DE) buffer and 1% sodium dodecyl sulfate (SDS). Mock casework samples were dried for 1 week and initially incubated for 2 h. B. Effect of proteinase K on sperm and epithelial cell lysis. Cell solutions obtained from 1% SDS-eluted samples were treated with 20 µg/mL proteinase K and incubated for specified additional incubation times. Control samples remained untreated with proteinase K and were incubated for 20 additional minutes. All samples were incubated at 42°C.

laboratory. The first step involves detergent-mediated elution of intact sperm and epithelial cells from a cotton swab matrix; several detergent types were assessed, and detergent concentrations were investigated for swabs that were dried and stored for various periods of time. The second step involves preferential lysis of epithelial cells that were recovered using the detergent-mediated cell elution method. The effects of detergent and enzyme concentration on cell lysis were evaluated for mock casework samples prepared with various numbers of sperm cells.

A direct comparison of the efficiency of DE with anionic detergents (e.g. SDS, Sarkosyl) for eluting sperm cells from cotton swabs was performed. Mock casework samples, dried for 1 week, were incubated in 1% or 2% (w/v) Sarkosyl, 1% or 2% (w/v) SDS, or conventional DE buffer containing 2% (w/v) SDS, for 2 h at 42°C. Additional samples treated with conventional DE buffer were incubated for 24 hours at the same temperature. **Figure 17** shows that elution with either SDS (1%: 77 ± 2.3%; 2%: 79 ± 0.7%) or Sarkosyl (1%: 47 ± 2.0; 2%: 54 ± 1.8%) alone yielded higher sperm cell recoveries than elution with conventional DE buffer for either 2 or 24 h. This is most likely because conventional DE buffer treatment leads to sperm lysis as well as cell elution; a factor that would be attributable to the non-detergent components of DE buffer. Both concentrations of SDS yielded higher sperm cell recoveries than comparable concentrations of Sarkosyl, indicating that the correct choice of detergent can substantially improve the yield of sperm cells. Finally, the average sperm cell recovery for samples incubated in DE buffer for 24 hours (45 ± 1.5%) was similar to those incubated in DE buffer for 2 h (39 ± 2.1%), indicating that prolonged exposure to the DE

buffer does not considerably enhance sperm cell elution, nor does it provide for greater cell lysis.

It is apparent that the non-detergent DE buffer components cause a reduction in sperm cell recovery. As the most likely culprit is proteinase K, the effect of this component on sperm cell lysis was investigated. Mock casework samples, dried for 1 week, were incubated in DE buffer or 1% SDS for 2 h at 42°C. The average sperm cell recovery for samples eluted in 1% SDS was 76 ± 3.0% (**Figure 18A**, n = 18). To determine the effect of proteinase K on sperm cell lysis, proteinase K was added to aliquots of the SDS-eluted cell solution, and incubated for an additional 0–20 min. The results, presented in **Figure 18B** (n = 3 for each incubation time), show that the addition of proteinase K rapidly decreases the number of sperm cells in solution. After incubation with proteinase K for less than 1 min, the sperm cell recovery decreased to 52% (±1.5%). Even lower sperm cell recoveries were observed for all cell solutions incubated for longer periods in the proteinase K solution (**Figure 18B**), with the results from the DE buffer (39 ± 2.1%, **Figure 18A**) representative of the lower limit after incubation for 20 min. The results after 1 min were revealing, indicating that the majority of sperm cell lysis observed occurred almost immediately after the addition of proteinase K. Differences in sperm cell yield among the different time points were not found to be significantly different Samples without proteinase K, but incubated for an additional 20 min, resulted in recoveries (74 ± 1.4%, **Figure. 18B**) that did not differ from recoveries obtained prior to the additional incubation (**Figure 18A**), showing that proteinase K, rather than additional incubation time, is contributing to sperm cell lysis.

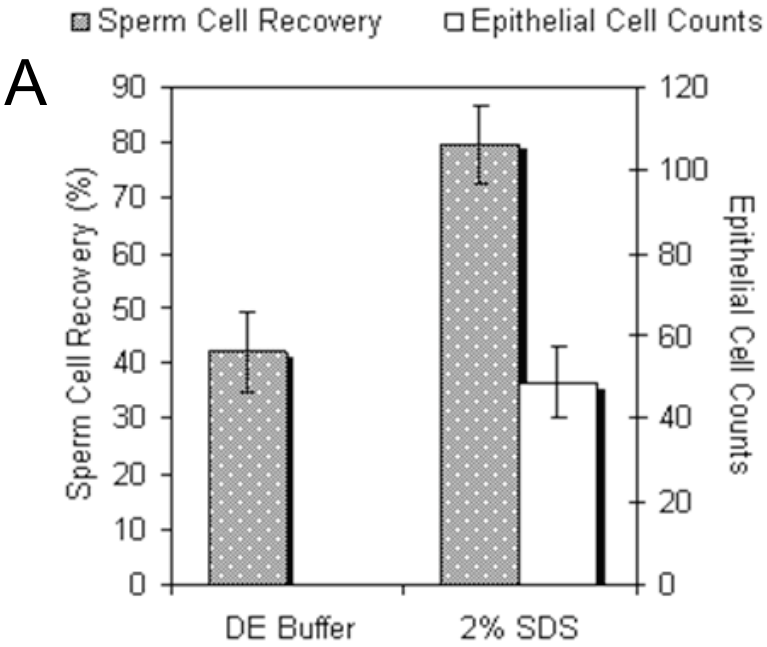
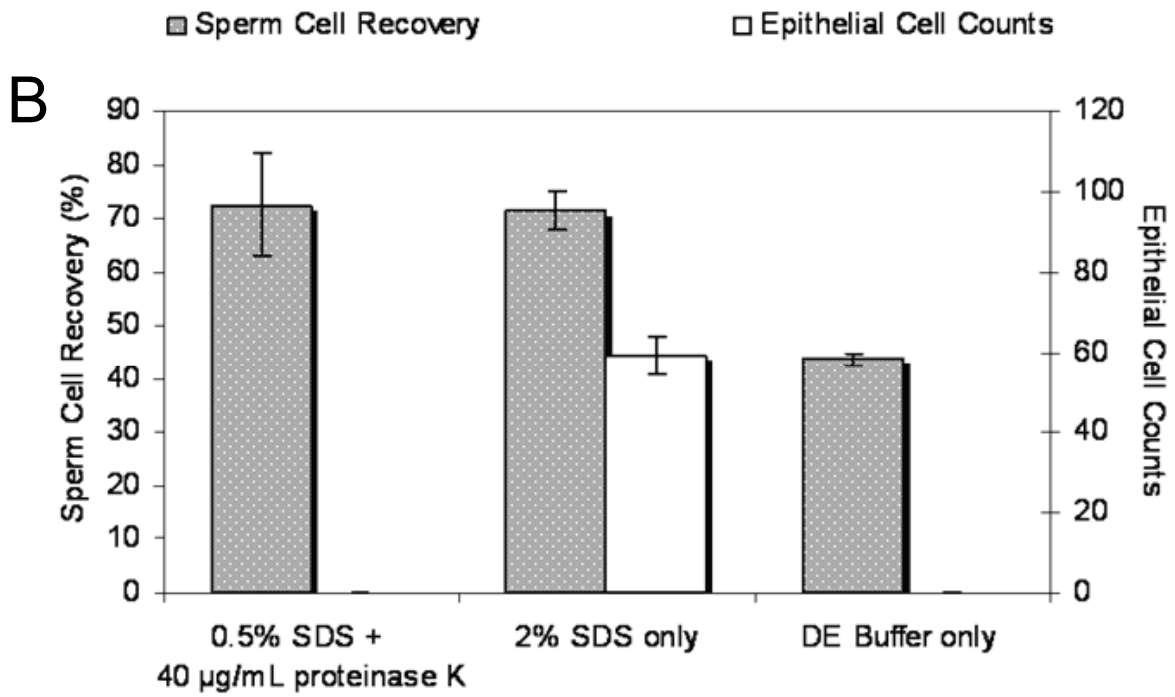


Figure 19: A. Comparison of sperm cell recoveries and epithelial cell counts from elutions using DE buffer and 2% SDS. Mock casework samples were dried for one week and initially incubated for 30 minutes. B. Effect of proteinase K on sperm and epithelial cell lysis. Cell solutions obtained from 2% SDS-eluted samples (Figure 12a) were diluted to 0.5% SDS and treated with 40 µg/mL proteinase K. Control samples remained untreated with proteinase K. All samples were incubated at 42°C for an additional 30 minutes.



Epithelial cell counts obtained from DE buffer-eluted and SDS-eluted samples in the presence and absence of proteinase K are also shown in **Figure 18**. As a result of the heterogeneous nature of epithelial cell adsorption with buccal swab collection, percentage

of recoveries could not be calculated. Intact epithelial cells were recovered following incubation with SDS alone, but were not recovered after sample treatment with DE buffer (**Figure 18A**). Proteinase K has been reported to lyse vaginal epithelial cells[3]; therefore, it was not surprising that epithelial cells were not detected in samples exposed to proteinase K for more than 5 min. Addition of proteinase K to the SDS cell solution aliquots depleted nearly all of the intact epithelial cells after incubation for less than 5 min, and comprehensive epithelial cell lysis was observed after 10 additional min of incubation (**Figure 18B**). The results show that an overnight incubation was not necessary for comprehensive epithelial cell lysis; however, an immediate reduction (~25%) in sperm cell recovery was also observed upon addition of proteinase K (**Figure 18B**).

These results provided the framework for a successful two-step cell elution/ preferential lysis method. Mock casework samples were prepared with neat semen as previously described and dried for ten days. Samples were eluted in 2% (w/v) SDS prepared in MES/Tris buffer, pH 8.0. Cell recoveries were determined and compared with those of samples eluted in conventional DE buffer. **Figure 19A** shows that elution with SDS prepared in MES/Tris buffer, pH 8.0 (80 ± 3.0%) provided a twofold increase in sperm cell recovery than elution with conventional DE buffer (44 ± 1.1%). In addition, intact epithelial cells (49 ± 8.3) were recovered, however these recoveries were less than previously obtained with SDS prepared in water. After the initial 30 minute incubation, the samples eluted in 2% SDS were diluted such that the final concentration of SDS was 0.5%. Previous results (data not shown) showed that increased

concentrations of proteinase K provided greater epithelial cell lysis; therefore, 40 µg/mL proteinase K was added to the diluted samples. The results (**Figure 19B**) show that the addition of proteinase K to the diluted cell solutions provided comprehensive epithelial cell lysis and sperm cell recoveries (73 ± 9.4%) that were similar to those obtained from the initial incubation. Samples without proteinase K, but incubated for an additional 30 minutes, provided epithelial cell recoveries (59 ± 4.6) that were similar to those obtained prior to the additional incubation. To determine the effect of the extended incubation time on cell recoveries, and to provide a direct comparison with the two-step cell elution/ preferential lysis method, samples treated with conventional DE buffer were incubated for an additional 30 minutes (without additional proteinase K); these samples provided similar recoveries (sperm – 44 ± 1.1%, comprehensive epithelial cell lysis) to those obtained from samples incubated for 30 minutes. These results are not surprising, as previous results showed the majority of proteolytic cell digestion occurs within minutes after the addition of proteinase K.[5]

These results illustrate the potential of the two-step procedure as an alternative to the conventional DE procedure in forensic DNA analysis. The results show that the two-step cell elution/preferential lysis method provided nearly two-fold improvement in sperm cell recoveries compared to a conventional DE buffer, while providing comprehensive epithelial cell lysis, in a total incubation time of one hour – with a significantly decreased incubation time over that traditionally performed in forensic laboratories. The improvement in sperm cell recoveries using the SDS elution buffer is ideal for increasing the efficiency of obtaining accurate DNA profiles from samples

containing low numbers of sperm cells, while comprehensive epithelial cell lysis is ideal for use in conjunction with the microfluidic ADE platform.

5. **Dissemination**

Oral Presentations

Evander, M., Horsman, K., Easley, C., Nilsson, J., Laurell, T., Landers, J.P. Using Acoustic Differential Extraction to Enhance Analysis of Sexual Assault Evidence on a Valveless Glass Microdevice. Micro Total Analysis Systems (μ-TAS): The Tenth International Conference on Miniaturized Systems for Chemistry and Life Sciences (Tokyo, Japan) November, 2006.

Horsman, K., Evander, M., Norris, J.V. , Laurell, T., Landers, J.P. "Acousto Differential Extraction: A Novel Alternative to Conventional Differential Extraction." American Academy of Forensic Sciences - (Seattle, WA) February, 2006

Norris, JV, Landers, JP. Acoustic Differential Extraction and Microchip-based DNA Purification, PCR, and Capillary Electrophoresis. First Annual Technological Advances in Human Identification Symposium – (Hampton Roads, VA) April 2008.

Norris, J.V. "Microfabricated Devices for Enhancing Forensic Analysis: Chapter 5 - ADE- a Novel Alternative to Conventional Differential Extraction." Ph.D. defense - University of Virginia - Sept, 2008.

Poster Presentations

Norris, J.V., Evander, M., Horsman, K.H., Green, C.W., Ferrance, J.P., Nilsson, J., Laurell, T., Landers, J.P. Acoustic Differential Extraction on a Microdevice: Assessment of Fluidic Control for Separation of Sperm Cells from Epithelial Cell Lysate. Presented at the Eighteenth International Symposium on Human Identification (annual conference), 2007, Hollywood, CA.

Horsman, K., Evander, M., Norris, J.V. , Laurell, T., Landers, J.P. Applying Microfabrication Technology to Forensic DNA Analysis: Acoustic Differential Extraction for Analysis of Sexual Assault Evidence" American Academy of Forensic Sciences - (San Antonio, TX) February, 2007

Horsman, K., Evander, M., Norris, J.V. , Laurell, T., Landers, J.P. "Applying Microfabrication Technology to Forensic DNA Analysis: Acoustic Differential Extraction for Analysis of Sexual Assault Evidence". University of Virginia Biotechnology Training Program Symposium (Charlottesville, VA) April, 2006.

Publications

Norris, J.V., Evander, M., Horsman-Hall, K.H., Ferrance, J.P. Nilsson, J., Laurell, T., Landers, J.P. Acoustic Differential Extraction for Forensic Analysis of Sexual Assault Evidence. *Anal. Chem.* In preparation. (see draft copy PDF attached)

Horsman, K.M., Bienvenue, J.M., Blaiser, K. and Landers, J.P. 2007. Forensic DNA Analysis on Microfluidic Devices. *J. Forens Sci.* 52(4):784-99.

Norris, J.V., Manning, K., Linke, S.J., Ferrance, J.P., Landers, J.P. Expedited, chemically-enhanced sperm cell recovery from cotton swabs for rape kit analysis. *J Forensic Sci.* 2007; 52 (4):800-5.

Norris, J.V., Cunniffe, H., Ferrance, J.P., Landers, J.P. Improved rape kit evidence analysis: a rapid two-step method for enhanced elution and preferential lysis of cells from cotton swabs. *J Forensic Sci.* In preparation. (see draft copy PDF attached)

Evander, M., Horsman, K., Easley, C., Nilsson, J., Laurell, T. 2006. Using Acoustic Differential Extraction to Enhance Analysis of Sexual Assault Evidence on a Valveless Glass Microdevice. Micro Total Analysis Systems (μ-TAS): *Proceedings of the Tenth*

International Conference on Miniaturized Systems for Chemistry and Life Sciences (Tokyo, Japan) 2:1055-1058.

Horsman, K. *Analytical Advances in Forensic Science: Development of Microfluidic Systems for Analysis of Biological Evidence. Chapter 4 - Acoustic Differential Extraction for Forensic Analysis of Sexual Assault Evidence*. Ph.D. defense - University of Virginia – April, 2007

Norris, J.V. *Analytical Advances in Forensic Science: Improving Sexual Assault Evidence Analysis. Chapter 5 – Acoustic Differential Extraction for Forensic Analysis of Sexual Assault Evidence*. Ph.D. defense - University of Virginia - Sept, 2008.

IV. Concluding Remarks

Discussion of findings

This study investigated development and characterization of microchip acoustic differential extraction (ADE) to separate sperm cells from female epithelial cell lysate for potential application to forensic analysis of sexual assault evidence. Dovetailed with these efforts were studies to enhance the concentration of sperm cells available for ADE purification. The ADE method relies on acoustic trapping of sperm cells in the presence of epithelial cell lysate (which is unretained); to increase capacity of the trap, a triple node fluidic layer was implemented. A passive valving technique (laminar flow valving) was then utilized to direct the male and female fractions to separate outlets. The material recovered following ADE was shown to be amenable to downstream DNA analysis processing steps by conventional means. The results meet the aims of the proposal, and demonstrate that highly purified male and female fractions can be obtained. These

results, showing enhancement of male component from less than 20% to greater than 90% with ADE, confirming that the power housed in acousto-trapping can provide highly purified product from sexual assault evidence. Follow-on studies will need to test the limits of the ADE technology with lower copy number samples in the presence of an abundance of epithelial cell DNA. Consequently, we have been successful in illustrating the potential of ADE as an alternative to the conventional DE procedure in forensic DNA analysis.

Future Prospects

To implement ADE into forensic laboratories, a prototype needs to be built that further optimizes the chip, and engineers a user-friendly system (instrument) that performs robustly and reproducibly in a forensic lab environment. Issues to be addressed in this future research include the following.

Externalizing the Transducer: Funding will be sought to produce disposable ADE devices; these devices would consist of a sealed channel structure and interface with an external transducer that is engineered into the instrument (as opposed to the current system where the transducer is in direct contact with the sample). This modification will obliterate any possibility of sample contamination – *a necessity for forensic laboratories* – and make the devices truly disposable. In addition, to obtain more consistent recoveries of DNA after ADE separations, different outlets will be designed and tested.

Fluidic Control: The passive flow control exploited here, Laminar Flow Valving, was shown effective once a number of significant technical problems were solved. While effective, more robust dynamic fluid control strategies are available and could be put into play to yield a simple, effective and repeatable chip-based ADE process.

Sample Bandwidth and Loading Time: One major advantage to the ADE method is that it will accommodate a large range of sample volumes (up to and including the milliliter range). The amount of cellular material on collected evidence swabs is not standardized, and is typically eluted in 250 – 500 μL of buffer; therefore, it is important to be able to process the entire sample as needed. However, there are limitations to the device in its current form – if the sample is infused at flow rates greater than ~2 μL/m, sperm cells are not exposed to the acoustic trap for a long enough time, and are consequently unretained and directed towards the female outlet. The issue is especially important with swab samples containing low numbers of sperm cells, where any loss makes it even more difficult to obtain a profile of the perpetrator. One possible way to increase the capacity and throughput of the device without significantly increasing the analysis time is to redesign the channel architecture, such that more trapping sites (nodes) are generated, or multiple transducers are actuated in parallel.

Once fully-developed and validated, ADE has numerous advantages over conventional DE, providing the possibility for reducing the backlog of forensic DNA casework that currently exists in criminal laboratories. It is reasonable to expect that this

technique can be integrated with on-chip downstream sample processing steps. The time savings associated with ADE analysis, in combination with the possibility to create a fully automated system, provides potential to significantly alter the means by which sexual assault evidence is processed in crime laboratories today.

V. References

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