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An Affinity-Free, Centrifugal Microfluidic System for Rapid, Automated Differential Extraction

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Introduction

Forensic DNA analysts are routinely asked to evaluate samples from a variety of biological fluids on an assortment of substrates. The outcome of that analysis may play a critical role in narrowing a pool of suspects, determining paternity, or identifying an unknown decedent. One of the most common types of evidence received in a forensic laboratory is a Sexual Assault Evidence Collection Kit (SAECK), which is used to gather biological evidence from victims of sexual assault, battery, rape, and attempted rape. In recent years, much ado has been made concerning backlogged SAECKs. While great strides have been made to reduce historical backlogs (i.e., SAECKs that were never submitted to crime labs for evaluation), room for substantial improvement remains regarding slow turnaround times, which occur for a variety of reasons. As recently as 2014, publicly-funded forensic laboratories reported ~11,000 requests for service that remained uncompleted at yearend, i.e., untested >30 days. To realize a true 30-day turnaround time and, ultimately, eliminate end-of-year backlogs, the *U.S. Bureau of Justice Statistics* estimated that a 73% increase in the number of DNA analysts was required – an unlikely event given that many State and local jurisdictions are struggling to recruit, hire, and retain qualified forensic staff. In essence, it is unlikely that the addition of more analysts will solve this backlog problem; rather improved technology that is automated, lower-cost, and provides enhanced throughput is the solution.

Obtaining a full, single source male DNA profile from a SAECK sample is most often dependent on the extent of release of cells from the evidence substrate and efficient separation of sperm cells from epithelial cells (e-cells) and e-cell DNA. The most widely-used methods for processing these sample types employ Proteinase K (an enzyme) and an anionic detergent that preferentially rupture e-cells, while leaving sperm cells intact. These methods are rooted in the differential extraction (DE) method described by Gill et al. in 1985. While effective and universally used, these traditional DE processes are manually intensive, prone to poor sperm cell DNA recovery, and frequently fail to adequately eliminate non-sperm cell DNA; this, at least in part, contributes to slow laboratory turnaround times. That is, recent forensic literature and anecdotal reporting suggest that sperm cell pelleting followed by simple water/buffer washes is inadequate for the removal of extraneous e-cell DNA, especially in instances where large proportions of e-cells dominate the cell mixture. As such, some labs have incorporated use of nucleases, like DNase I, as a means of removing unwanted e-cell DNA from the sperm cell fraction. Otherwise, little has changed or been modified in the application of the DE process since its original inception. Modifications and improvements to existing DE protocols and methods are described in a review by Cotton and Fisher (2015). Some crime laboratories have adopted mechanized robotic platforms to automate traditional forms of DE. Those labs do report a reduction in hands-on time. Implementation of these large robotic platforms requires substantial financial investment and specialized examiner training. Moreover, there are no substantial differences in the chemistry, execution of the procedure, or enhancement of sperm cell DNA recovery efficiency. Significant, affordable improvement of the DE process will require a combination of changes to both chemistry and technical execution of the automation process. As such, recent research and development efforts for forensic DNA analysis have focused on exploiting micro-total analysis systems (μTAS) for rapid-DNA analysis of reference samples, which do not require DE. That is, these systems are only approved for use with reference samples. Intrinsically, these advances fail to address the specific 2 needs of the forensic science community pertaining to slow processing times, and the DE of backlogged sexual assault cases. Further, it is yet to be determined whether these μTAS or lab-on-a-chip systems ease the growing burden placed on forensic laboratories as a result of swelling state legislative requirements for expanding the collection of reference samples from

offenders and/or arrestees. In comparison, an affordable, automated, self-contained, disposable centrifugal, microfluidic DE device offers an attractive, unconventional approach to standard automation techniques while directly addressing many of the limitations associated with traditional DE methods. Our group specializes in rotationally-driven systems, which, much like the analytical disc originally described by Anderson et al., harnesses the advantages of centrifugal microfluidics (e.g., simplicity, use of low volume, and speed of sample processing etc.). Herein, we propose a rotationally-driven microfluidic system that removes the need for external pumps and valves for fluid movement control, while avoiding the need for large, clunky, and costly robotic platforms for automation.

Accomplishments

What are the major goals and objectives of the project?

Design and develop a simple, inexpensive centrifugally-driven microfluidic disc for forensic differential extraction (DE) that integrates two existing on-chip microfluidic modules for DNA extraction and laser-based closable micro-valving, as well as an existing rotational platform for microfluidic control. This will provide rapid, efficient isolation of the sperm fraction (SF) from non-sperm fraction (NSF) in forensic sexual assault samples.

What was accomplished under these goals?

Major activities

The major activities for this project involved the development of a rotationally-driven polymeric microfluidic device that promotes proper partitioning and sequestration of the perpetrator and victim contributions found in sexual assault evidence. The fluidic architecture of the microdevice and its ability to enable fractionation of the sperm (SF) and non-sperm (NSF) fractions, as well as minimize fluid loss throughout the workflow, was colorimetrically evaluated with saturates dyes. Following architecture validation via dye studies, on-disc sample preparation of mock sexual assault samples with the temperature-controlled differential extraction (TCDE) protocol was validated. Effective isolation of the forensically-relevant non-sperm and sperm fractions was evaluated via quantification of large autosomal, small autosomal, and Y targets using the Quantifiler™ Trio Kit, as well as comprehensive interrogation of the core CODIS and ESS loci with the PowerPlex® Fusion Kit.

1. Design and Fabrication of a Rotationally-Driven Microfluidic Disc for Forensic Differential Extraction

To address the limitations associated with standard DE techniques, a self-contained, rotationally-driven microdevice was developed. The benefits associated with the enclosed platform for DE include reduction of 1) sample and reagent volumes, 2) manual intervention, 3) contamination risk, 4) sample loss, and 5) processing time. The architectural schematic of the microdevice was designed on AutoCAD to emulate traditional DE workflow features, such as pipetting, mixing, centrifuging, and fractionating. Integration of these unit operations into a single device is made possible by the *print-cut-laminate* (PCL) method, that consists of laser-ablating the microfluidic features into common, inexpensive thermoplastic substrates with a CO_2 laser cutter, followed by alignment and lamination with standard office equipment.

Each microdevice (**Figure 1**) consists of five layers, including two outer layers (1 and 5) of clear polyethylene terephthalate (PeT: 101.6 µm, Film Source, Inc., Maryland Heights, MO), two fluidic layers (2 and 4) of clear PeT bound on either side by a heat sensitive adhesive (HSA: 50.8 µm, Adhesives Research, Inc., Glen Rock, PA), and a middle optically-dense layer (3) of black PeT (75 µm, Toray Industries, Inc., Chuo, Tokyo, Japan). Polymethyl methacrylate (PMMA: 1.5 mm thick, McMaster-Carr, Atlanta, GA)

accessory pieces are used to give the chambers the necessary depth/volume and are secured onto the top of a laminated disc with a pressure sensitive adhesive (PSA: 55.8 µm, Adhesives Research, Inc., Glen Rock, PA).

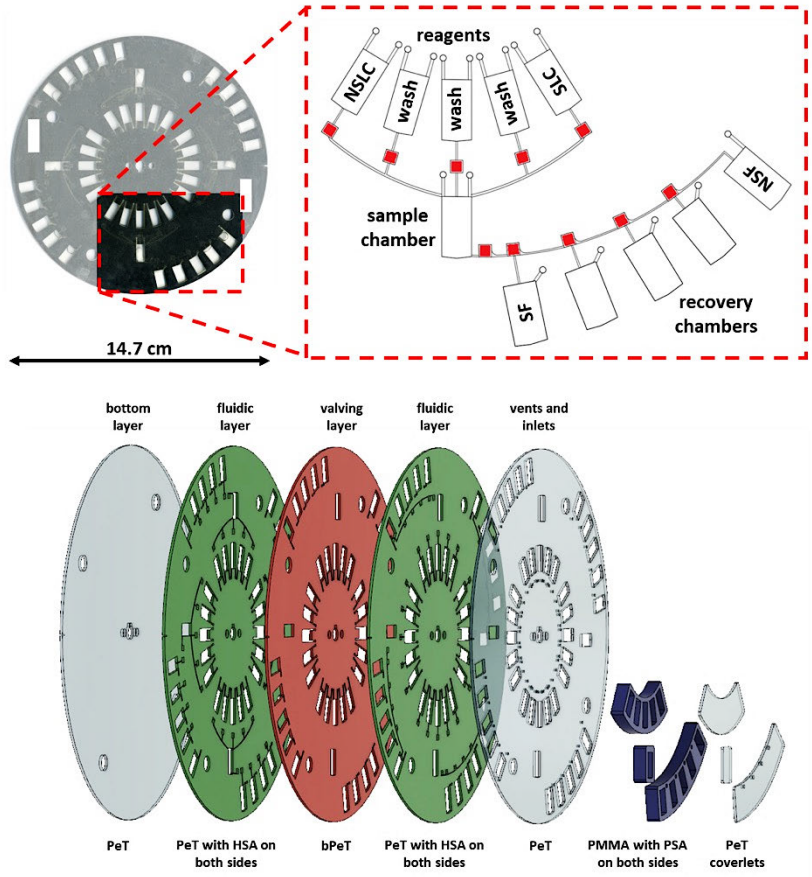

Figure 1. Exploded view (bottom) and architectural schematic (top) of the five-layer disc. Each disc contains five thermally-bonded (180-200 °C) layers of PeT transparency film. The top and bottom layers consist entirely of clear PeT, while layers 2 and 4, bound by HSA, function as microfluidic layers. Layer 3, an optically-dense bPeT layer, serves as the primary valving layer. Microfluidic inlets and vents are cut into the architecture via the same laser-ablation process (CO_2 laser: VLS 3.50, Universal Laser Systems, Scottsdale, AZ) that etches the microfluidic features that mimic a traditional DE workflow. PMMA was used to add depth to the chambers and was attached post-assembly with PSA.

2. On-Disc Differential Extraction Protocol and Valving Strategy

A single device contains four indistinguishable domains, each capable of processing a singular cutting of evidence. The process consists of an initial lysis of epithelial cells, followed by three intermediate wash steps, and a final sperm cell lysis. **Figure 2** details a single processing domain and the fluidic protocol/workflow for the proposed on-disc DE, as well as the associated laser valve positions. Reagents are loaded into the chambers shown in **Figure 2B** and the on-disc DE workflow begins. Sequence 1 (**Figure 2C**) consists of the first valve opening event, which releases the non-sperm lysis cocktail (NSLC, EA1) into the middle sample chamber upon centrifugation at 3,000 rpm. The NSLC is incubated in the sample chamber for ~6 minutes and facilitates the release of non-sperm cell DNA. Upon opening a second downstream laser valve, the eluate and forensically-relevant non-sperm fraction (NSF) is centrifugally-driven into a recovery chamber. Finally, a channel closure event isolates the NSF in the recovery chamber and marks the end of this unit operation. Sequences 2-4, nearly identical, consist of sequential water washing steps (**Figure 2D, E, F**). Sequence 5 comprises the sperm lysis and most forensically-relevant step (**Figure 2G**). The sperm lysis cocktail (SLC, Acrosolv) is driven into the sample chamber, incubated for ~9 minutes, and eluted as the forensically-relevant 'sperm fraction' (SF) into the recovery chamber. Fractions are removed from the disc for downstream analysis by puncturing the PeT coverlet and aspirating the corresponding fluid with a micropipette.

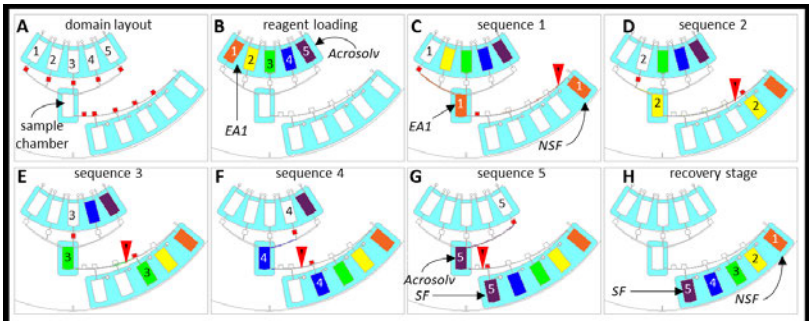

Figure 2. Overview of a single processing domain. (A) Laser valves are represented by red boxes and provide strict flow control and timed release of reagents. (B) Reagent loading. (C) Lysis of non-sperm cells and NSF isolation. (D-F) Wash(es) and isolation of waste fraction(s). (G)

Lysis of sperm cells and SF isolation. (H) Complete on-disc DE with isolated SF, waste(s), and NSF.

3. Evaluating the Architecture of the Prototype Microdevice to Assess Fractionation

Once the microdevice was designed on AutoCAD, fabricated, and an on-disc workflow proposed, a dye study was conducted to verify whether the device worked for its intended DE purpose, i.e., if successful fractionation of the discrete fluid volumes occurs. A solution of colored dye offers a visual way of verifying valving success by emulating molecules that could potentially have a detrimental effect on downstream DE processes – namely PCR – if not removed from the architecture prior to the elution of the final volume and forensically-relevant 'sperm fraction' (SF). Removal of the dye would suggest that these molecules can also be removed from the architecture of the microdevice by simply using the intermediate wash steps. The colorimetric evaluation was completed with a 5 mM Allura red solution in 1X Tris-EDTA buffer. Red dye or water aliquots were added to the proper reagent chamber (**Figure 3, top left**) and the workflow detailed in **Figure 2** was followed (incubations were not performed). Once the workflow was completed, digital images of the devices were captured with a desktop scanner and the saturation of each chamber was determined with the Fiji distribution of ImageJ. Comparison of the mean saturation values of the fluid volumes in the reagent chambers versus the recovery chambers indicates a return to baseline between reagent and recovery chambers 5 (p-value: 0.9924, α: 0.05) (**Figure 3**). This suggests that no residual dye is detectable in recovery chamber 5. In the context of differential extraction, this means that our inexpensive, polymeric, centrifugal device is able to provide intricate fraction isolation and elimination of potential PCR inhibitors prior to ingress in the SF.

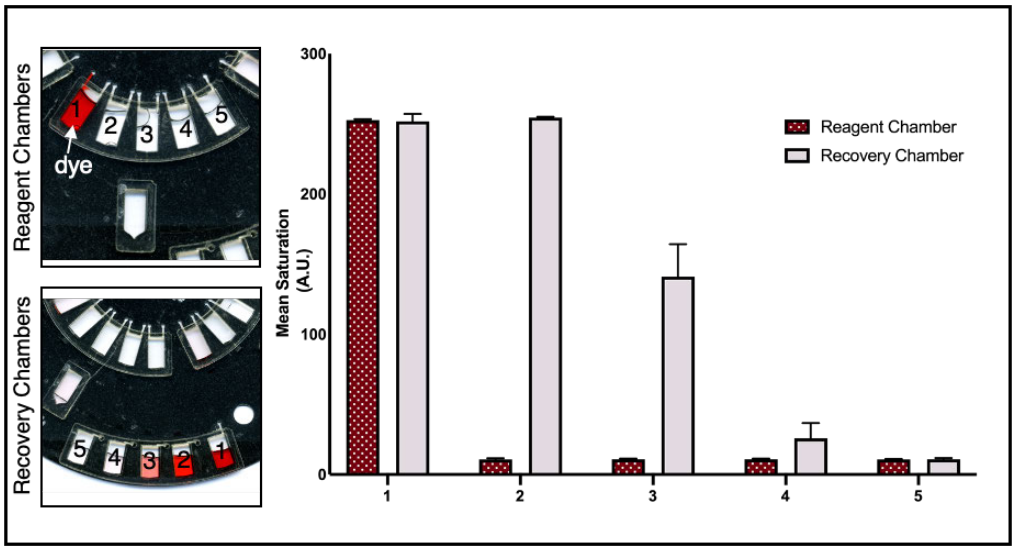

Figure 3. Image analysis to assess the removal of concentrated dye from the disc architecture. Reagents are loaded into the chambers nearer the center of rotation and the workflow described in Figure 2 is followed. Digital images of each processing domain are captured with a desktop scanner prior to on-disc processing, i.e., the 'before' images include dye and water aliquots in the reagent chambers. Similarly, the discs are scanned post-processing, i.e., the 'after' images include the recovered fluid volumes in the recovery chambers, prior to mixing in the sample chamber. The mean saturation values for each reagent chamber is compared to its corresponding recovery chamber, and visual inspection suggests the mean saturation values in the recovery chambers gradually decrease until a return to baseline is observed. (n=3)

4. Evaluating the Architecture of the Prototype Microdevice to Assess Fluid Recovery

Reduced fluid recovery in the downstream chambers could potentially negatively impact the efficiency of the on-disc workflow and its ability to isolate the individual NSF and SF fractions. For that reason, studies were performed to evaluate fluid recovery from the swab chamber into the recovery chambers. This is accomplished by adding known volumes of colored dye into same-sized chambers, capturing digital images of the dye-filled disc, and associating the known volumes with number of pixels by using the aforementioned

Fiji distribution of ImageJ software. A calibration curve (**Figure 4**) of mean pixel count versus volume (μL) enables us to calculate the unknown volumes eluted into the recovery chambers by using the corresponding linear regressions.

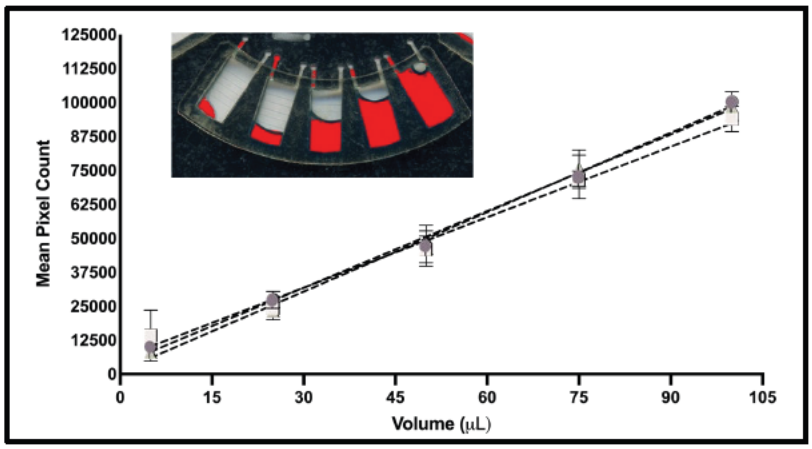

Figure 4. Standard curve(s) of known input volumes for volume recovery assessment in the DE microdevice. Inset: known volumes (5, 25, 50, 75, 100 μL) of colored dye added into the reagent chambers. The red shaded area refers to the number of pixels associated to each known volume. The extrapolated calibration curve enables calculation of unknown volumes eluted into the recovery chambers.

The fluid recovery study was conducted by adding 100 μL of fluid into each reagent chamber, completing the on-disc workflow, and calculating the recovered volume on the corresponding recovery chamber. After completing the proposed on-disc workflow (n=13), the mean pixel count for each recovery chamber was determined with ImageJ image analysis and the associated volume extrapolated by use of the linear regression. Since the input volume per reagent chamber was 100 μL, the *percent volume recovery* per workflow was calculated based on the volumes eluted into the recovery chambers (**Figure 5**). Here, 'workflow' (or 'step number' in the y-axis) refers to each individual step of flowing the volume in reagent chambers 1, 2, 3, 4, or 5 into the sample chamber and recovering it into each corresponding recovery chamber. The fluid recovery in chamber 5 – the SF when the workflow is completed with real or mock sexual assault samples – is, on average, north of 90%.

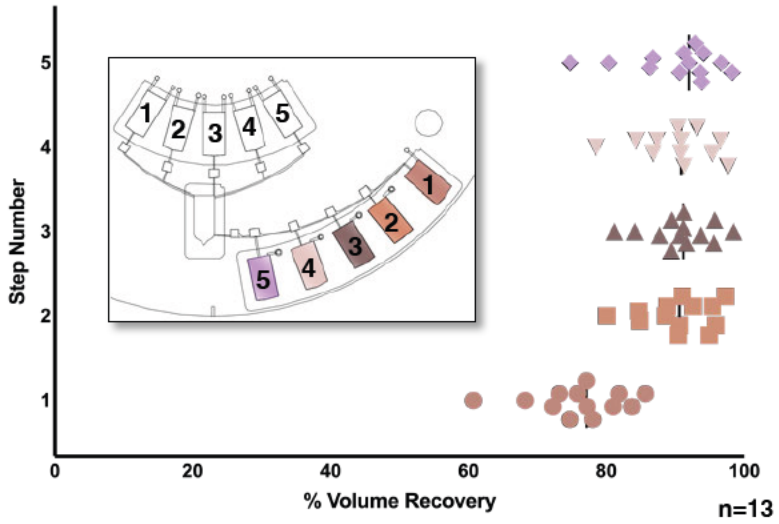

Figure 5. Fluid recovery from the on-disc DE workflow. 100 μL of fluid is added into the reagent chamber and each individual step is performed separately, i.e., the volume of reagent chamber 1 is eluted into recovery chamber 1, the volume in reagent chamber 2 is recovered into recovery chamber 2, etc. Fluid recovery was calculated by computing the average pixel count within the recovery chambers using Fiji ImageJ and extrapolating the values with the proper linear regression. Based on the 100 μL input volume and the obtained recovered volumes, the percent volume recovery for each 'workflow' is calculated.

The colorimetric evaluation of the proposed DE device suggested adequate fractionation and recovery from the sample chamber. However, data derived from dye studies are limited by the assumption that aqueous solutions behave as cellular mixtures would in this system. It follows that further characterization toward an integrated method for microfluidic DE should independently evaluate the performance of the system with

cellular contributions. In the following sections, DNA originating from non-sperm and sperm contributions is measured independently and in mock sexual assault mixtures following on-disc extraction with genetic markers relevant to their requisite contributions.

5. Proposing a Fully Enzymatic Method of Differential Extraction

The conventional process used by forensic laboratories to separate the main cell types present in sexual assault evidence (epithelial and sperm cells) into the discrete non-sperm (NSF) and sperm (SF) fractions is laborious, prone to contamination, and often doesn't yield adequate sperm cell DNA recovery. The microfluidic platform, as mentioned above, addresses several of the issues associated with conventional DE (reduced sample and reagent volumes, manual intervention, contamination risk, and sample loss). However, addressing the disadvantages of the process at a more fundamental level is also of great importance. Although this varies from jurisdiction to jurisdiction, the entire conventional workflow takes from 4-18 hours to complete, at times even north of 24 hours. At the aforementioned 'fundamental level', the reagents used for cell lysis in the conventional process are known PCR inhibitors and can be hazardous to humans. This is due to the nature of the cells present in sexual assault evidence, namely sperm cells, which contain disulfide bonds on their anterior part, or acrosome. To lyse sperm cells, and therefore extract their DNA, the protease enzyme requires assistance in the form of a reducing agent – or DTT – one of the known PCR inhibitors.

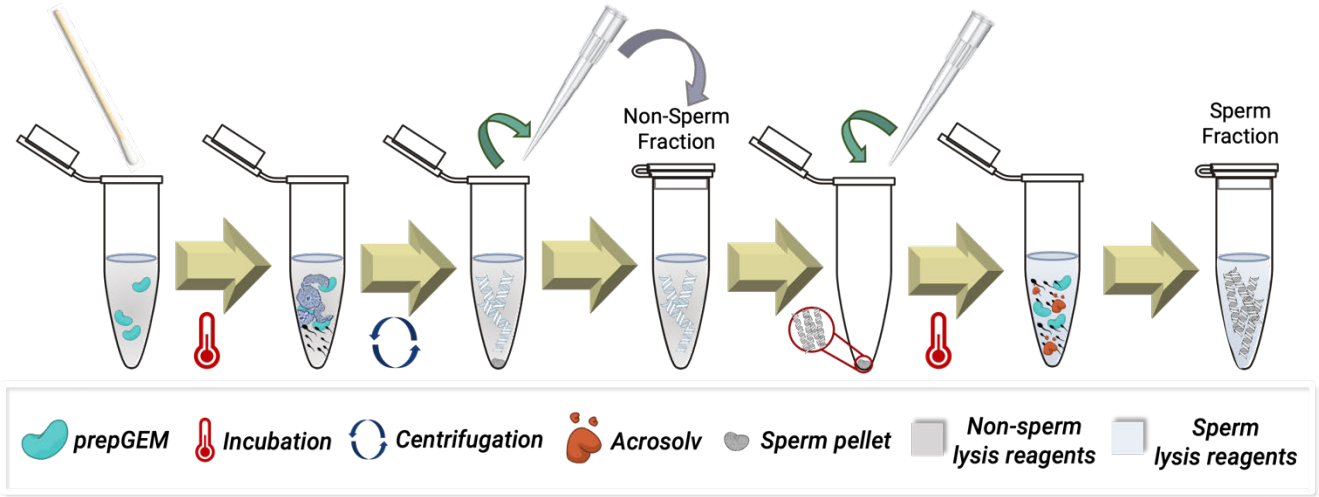

Figure 6. Proposed in-tube fully enzymatic DE workflow. As with the conventional DE process, the first step consists of non-sperm cell lysis with *prepGEM* (MicroGEM) enzyme (incubated at 75°C for 5 min and 95°C for 5 min), followed by a centrifugation step for sperm cell pelleting, recovery of the non-sperm fraction, and reconstitution and incubation of the sperm cells with a mixture of proteases that do not require the use of reducing agents (Acrosolv).

Because quantification of DNA in the NSF and SF is achieved by PCR, a purification step is necessary at the end of the conventional DE process to eliminate those inhibiting reagents. This adds not only to the time of the process, but the cost as well. To that end, we propose a fully enzymatic process of differential extraction that utilizes novel enzymes that do not require the use of reducing agents or any other PCR inhibitors to complete DNA extraction. Compared to the traditional process, this enzymatic extraction takes a matter of minutes to complete, and a purification step is not necessary.

6. Optimization of the Enzymatic Temperature-Controlled Differential Extraction (TCDE)

a. Buffer Selection

We use commercial kits for the proposed enzymatic extraction of biological fluids that offer a variety of buffer options, depending on what type of tissue is being targeted for nucleic acid (NA) extraction. The kit provides protocols for the independent extraction of epithelial cells or sperm cells, but not a DE protocol. Buffer A is recommended for the extraction of NAs from sperm cells, whereas Buffer B is recommended to facilitate extraction of NAs from epithelial cells. We wish to combine these two protocols into a single, comprehensive DE process. We investigated whether using a unique buffer for both processes would be feasible. The objective was to avoid possible incompatibilities between the methods or incompatibilities with downstream processes. Sperm was extracted with the NSCL portion of the process to determine the Y-DNA contribution from non-sperm male cells (e.g., round cells, male epithelial cells, etc.). Following that process, the same sample was extracted with the SCL portion of the process, using Buffer A in one mode of extraction and Buffer B in another mode of extraction. The extracts were amplified with the Quantifiler Trio Kit (ThermoFisher) and C_t values were used to evaluate the extraction success (Y-DNA target). **Figure 7** shows the results. Both buffers are successful in extracting Y-DNA from sperm cells with comparable sensitivity. Since Buffer B is utilized for extraction from epithelial cells, it was selected for the SLC portion of the extraction as well to simplify the process and avoid issues downstream associated with use of multiple buffers.

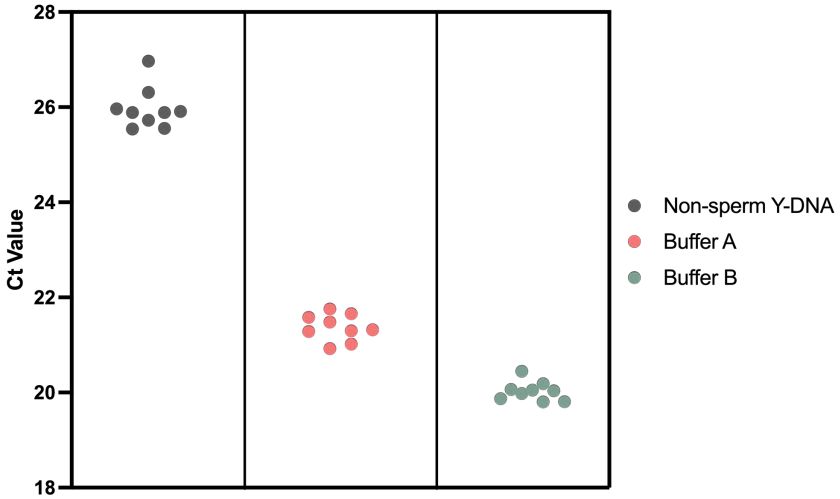

Figure 7. Buffer comparison for enzymatic DE. Comparison of non-sperm male cells extracted with the NSLC only vs. the SLC using both Buffers A and B. The non-sperm Y-DNA provides a baseline of male DNA (round cells, epithelial cells) compared to the DNA from sperm cells. It can be seen that Buffer B performs in a comparable way to the recommended Buffer A for the SLC. Buffer B, however, is recommended for the NSLC, so it's chosen for the SLC too in order to use a unique buffer for the purpose of enzymatic DE.

b. Pelleting Time Study

Centrifugation is an important step in DE as it allows for the pelleting of intact sperm cells so the supernatant (containing the NSF) can be retrieved. The sperm pellet is then resuspended in the sperm lysis cocktail so the cells can be lysed, therefore releasing the sperm cell Y-DNA. To investigate the shortest amount of time necessary to pellet the sperm, all the while maintaining assay sensitivity (i.e., enriching the SF with Y-DNA), we performed a pelleting time study. Liquid mock samples were prepared, and four different DE conditions were tested, including centrifugation times of 20, 15, 10, and 05 minutes. The extracts were amplified with the Quantifiler Trio Kit (ThermoFisher) and C_t values were used to evaluate the extraction success (Y-DNA target). The results of this study are reflected in **Figure 8**.

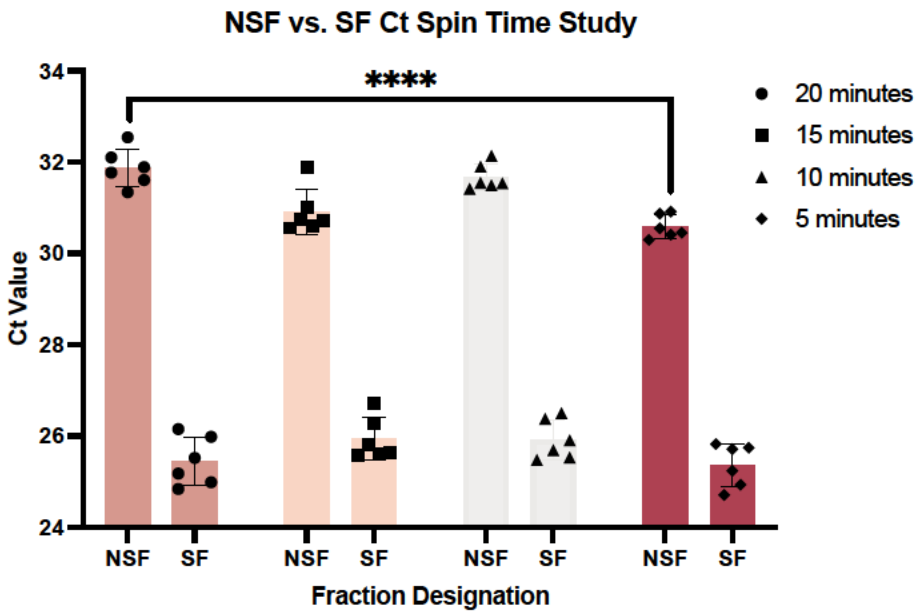

Figure 8. Pelleting time study. Comparison between the NSF and its corresponding SF for different DE centrifugation times (20 – 5 minutes).

The results suggest that there is enrichment of the SF relative to its corresponding NSF within all extraction parameters (different spin times). An unpaired, two-tailed t-test (**Table 1**) shows there's a significant difference between the NSFs when comparing a 20-min spin time vs. s 5-min spin time. However, because the SF is of greater importance and there is no statistical difference between the SFs (as it pertains to amount of Y-DNA) when different spin times are used, we are going to use a 5-min centrifugation step in the interest of time. If issues are found down the line as it pertains to this, they will be addressed.

Table 1. T-test for comparison of Ct mean values between corresponding NSFs and SFs with different spin times.

	NSF		SF	
	P value	Significantly different?	P value	Significantly different?
20 vs. 15 (minutes)	0.0530	no	0.0546	no
20 vs. 10 (minutes)	0.6153	no	0.3430	no
20 vs. 05 (minutes)	<0.0001	yes	0.4410	no

c. Incubation Time Study: Lysis of Non-sperm Cells

The manufacturer protocol for lysis of epithelial cells with this enzymatic method recommends a 5-min incubation at 75 °C (its activation temperature), followed by a 5-min incubation at its denaturing temperature of 95 °C. In efforts to further reduce assay time, we decreased the incubation times of each individual incubation step separately (i.e., the 75 °C step was evaluated at 5, 3, and 1 min, while keeping the recommended 5 minutes for the 95 °C step; then, the 75 °C step was kept as 5 min while incubating at 95 °C for 5, 3, and 1 minute(s)). In this case, we were only looking at the lysis of non-sperm cells, so the incubation times for the sperm cell lysis (SLC) was kept constant and followed manufacturer's instructions.

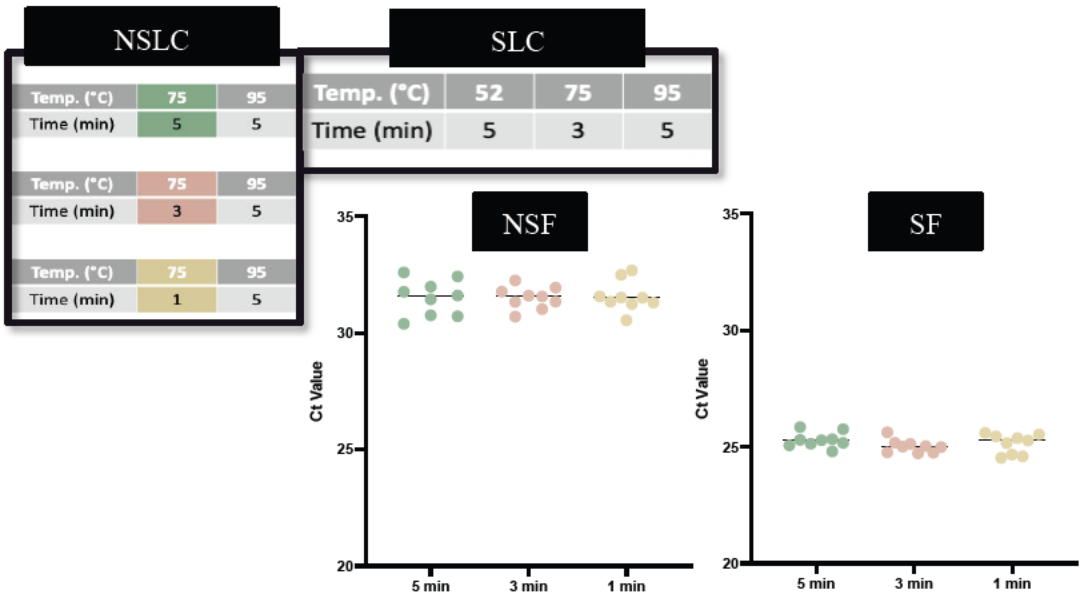

Figure 9. Incubation time study. Comparison between the Ct values for the NSF and its corresponding SF with different incubation times (5, 3, 1 min.) for the 75°C step of the NSLC.

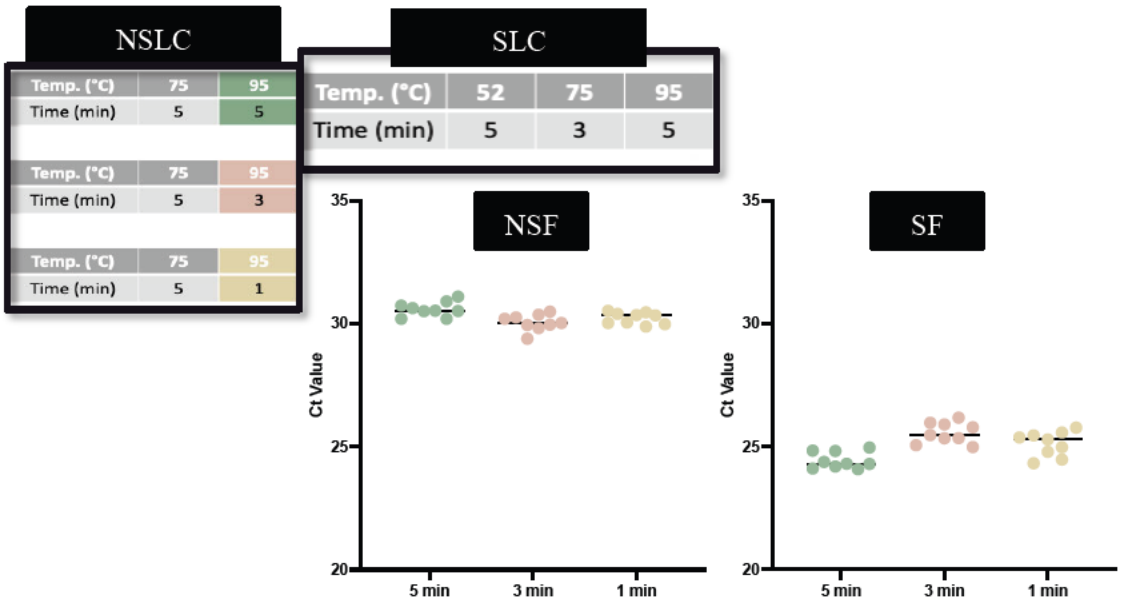

Figure 10. Incubation time study. Comparison between the Ct values for the NSF and its corresponding SF with different incubation times (5, 3, 1 min.) for the 95°C step of the NSLC.

d. Incubation Time Study: Lysis of Sperm Cells

The manufacturer protocol for lysis of sperm cells with this enzymatic method recommends a 5-min incubation at 52 °C (activation temperature of *Acrosolv*, a reagent used to facilitate lysis of sperm heads), followed by a 3-min incubation at 75 °C for enzymatic activation, and, finally, a 5-min incubation at its denaturing temperature of 95 °C. In efforts to further reduce assay time, we decreased the incubation times of each individual incubation step separately (i.e., the 52 °C step was evaluated at 5, 3, and 1 min, while keeping the recommended time for the remaining steps; then, the 52 °C step was kept at 5 min while incubating at 75 °C for 3, and 1 minute(s), etc.). In this case, we were only looking at the lysis of the sperm cells, so the

incubation times for the non-sperm cell lysis (NSCL) was kept constant and followed manufacturer's instructions.

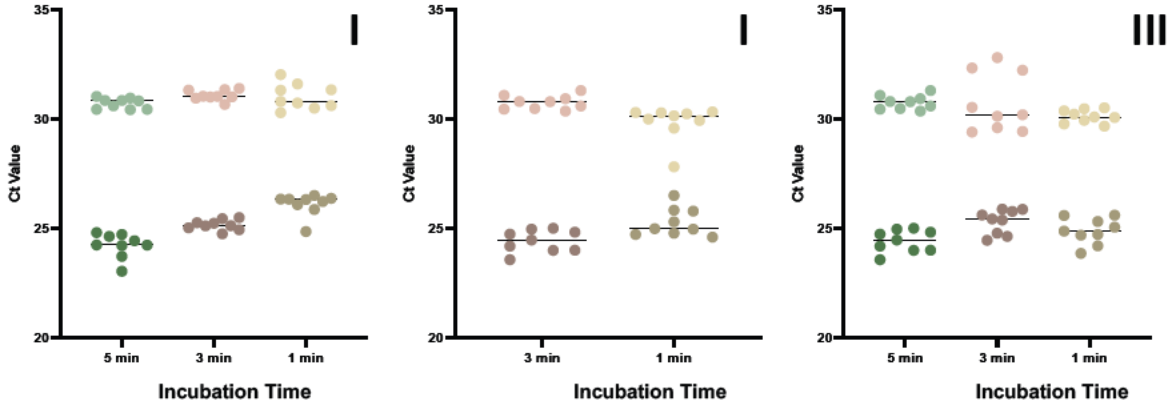

Figure 11. Incubation time study. Comparison between the Ct values for the NSF and its corresponding SF with different incubation times for the SLC. (I) Testing the 52°C step at 5, 3, and 1 min. (II) Testing the 75°C step at 3, and 1 min. (III) Testing the 95°C step at 5, 3, and 1 min.

After each extraction, samples were amplified with the Quantifiler Trio Kit (ThermoFisher) and C_t values were used to evaluate the extraction success (Y-DNA target). The results of this study are reflected in **Figures 9, 10, and 11**. Based on the results, the data in **Table 2** reflect the new incubation times used for the enzymatic extraction.

Table 2. New incubation times for shortened, optimized enzymatic extraction

Non-sperm Lysis		Sperm Lysis	
75°C	1 min.	52°C	3 min.
95°C	1 min.	75°C	3 min.
		95°C	1 min.

7. Comparing the Enzymatic Extraction to a Conventional Process

Depending on the jurisdiction, a conventional process takes anywhere from 4-18h to north of 24h to be completed. To show the value of the enzymatic DE, we extracted the same mock sample using a conventional approach (~24h.) and our optimized enzymatic protocol. Samples were extracted following either the conventional or enzymatic protocol, and either immediately used for downstream processes or stored in a -80 freezer until use. Amplification was performed with the Quantifiler Trio Kit (ThermoFisher) and the amount of DNA in each fraction was calculated according to the standard curve of known concentrations of human DNA. Statistical analysis indicates that the significance in difference between the NSF and SF of the two different modes of extraction is similar.

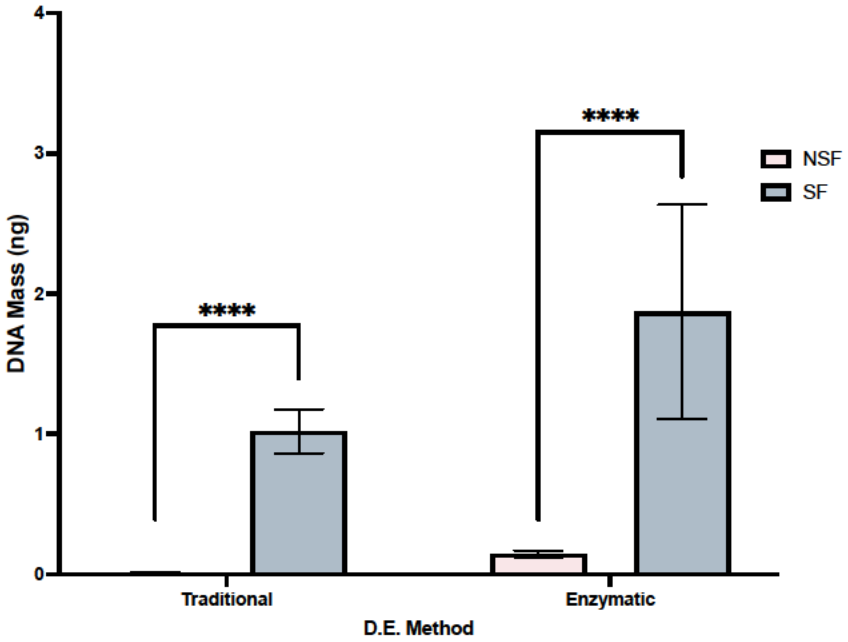

Figure 12. Comparison of traditional (~24h.) DE vs. optimized enzymatic (~25 min.) DE. A conventional DE process that takes ~24h and makes use of hazardous and PCR inhibiting reagents was compared to the optimized, shortened enzymatic method (total time ~25 min.) and the enrichment of the SF with male DNA is statistically comparable between the two modes of differential extraction.

8. Capillary Electrophoresis for STR Profiling

The previous section(s) show a comparison between the 'short' enzymatic DE process and a 'conventional' DE protocol – which we define as processes that use ProK-SDS-DTT chemistries, require long purification steps for the removal of PCR-inhibiting reagents, and can take up to 24h to complete depending on the jurisdiction. Our previous comparison relied on DNA quantification using the Quantifiler™ Trio Kit (ThermoFisher) and showed clear enrichment of the SF with the Y-specific target, which was corroborated with an unpaired t-test of the mean Y-target Ct values in the NSF vs. the corresponding SF (p-values < 0.0001, $\alpha = 0.05$). This successful separation of sperm and non-sperm fractions has the potential to enhance electrophoretic results, particularly when it comes to generating single-source STR profiles for better genetic forensic identifications. Here, we processed extracts for comprehensive interrogation of the core CODIS and ESS loci with PowerPlex® Fusion (Promega). An STR profile was generated from the semen sample used for the optimization studies and served as a 'reference' male profile. Identical mock sexual assault samples containing a 1:1 F:M ratio were subjected to the 'conventional' as well as the 'short' enzymatic DE process. **Figure 13** shows the STR profiles obtained from the SF of the 'conventional' and enzymatic methods, respectively. Both methods of extraction give a profile that is comparable to the male reference profile. Similarly, it appears there is no residual NSF DNA in the SF when using either extraction method. These data show that our simple, rapid, and hazard-free DE approach enriches the SF with sperm DNA to such a magnitude that generation of single-source profiles from Y-DNA is possible.

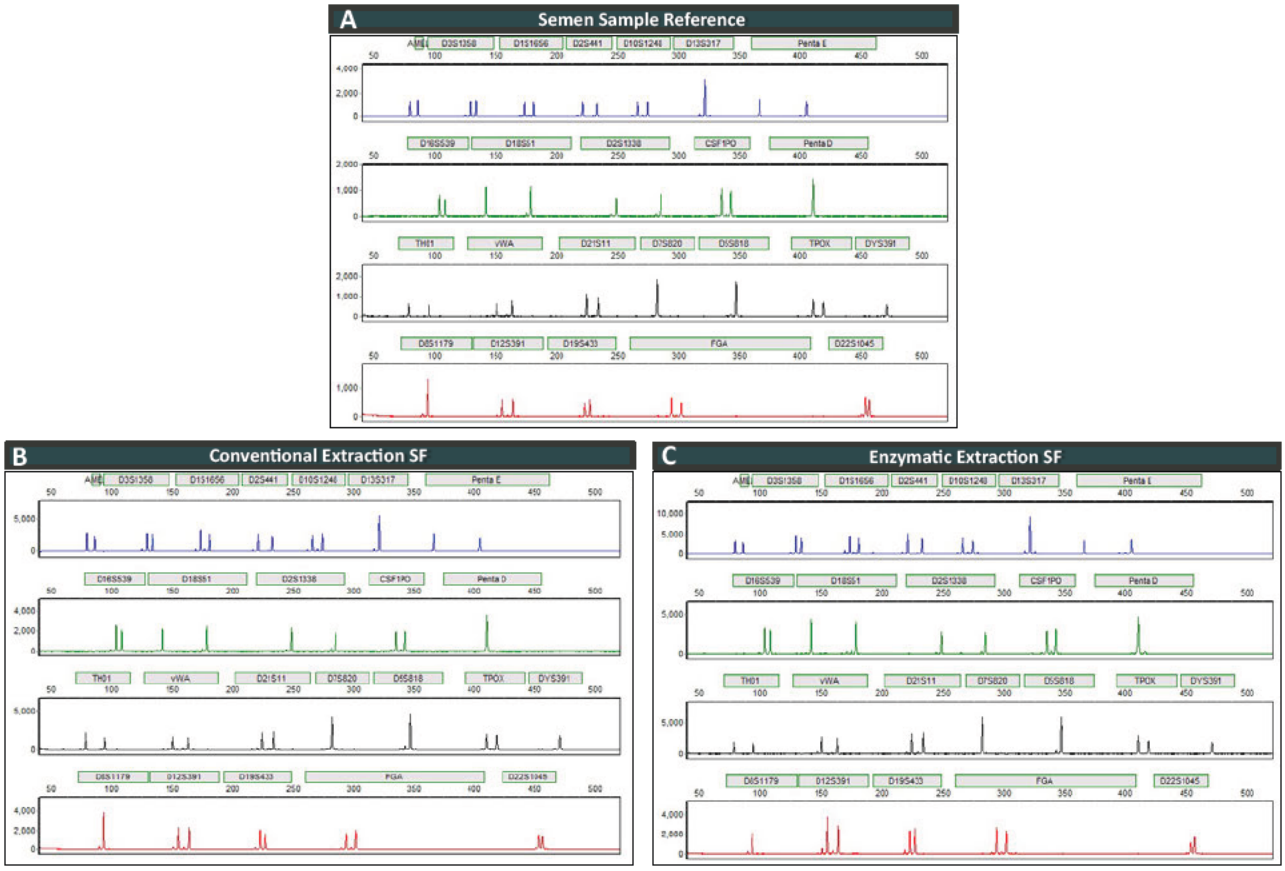

Figure 13. STR profiles. (A) Reference STR profile obtained from the seminal fluid used to make all mock SAECK samples. **(B)** Profile obtained from the SF of a mock SAECK extracted with a ~20h. 'conventional' method. **(C)** Profile obtained from the SF of an identical mock SAECK extracted with the 'short' enzymatic DE method.

9. Monitoring DNA Recovery on the Microfluidic Architecture

a. Non-sperm DNA

Colorimetric evaluation of fluidic control within the microdevice suggests the DNA originating from non-sperm cells is gradually removed from the architecture prior to ingress in the SF. Here, true enzyme-assisted extraction was performed on-disc from buccal swabs with the non-sperm lysis (NSL) mix only to verify whether this behavior would persist with cellular extracts. Stock hgDNA was diluted in 1X TE buffer to create a calibration curve with concentrations ranging from 10, 1, 0.1, 0.01, and 0.001 ng/µL for relative DNA quantification and showed excellent linearity, with an R2=0.995 and 90.3% efficiency. The extracted fractions were recovered from the microdevice and probed for the presence of TPOX for total human-specific DNA detection using a 2X SensiFAST Probe qPCR mix. As shown in **Figure 14-A**, results agree with dye testing studies and show the larger amount of female DNA present in the first isolate (NSF; 2.45 ± 0.45 ng total DNA) gradually decreases in the ensuing wash fractions and only a minimal amount is detected in the SF (0.24 ± 0.11 ng total DNA).

b. Sperm DNA

To track the behavior of sperm cellular extracts in the microfluidic architecture, extraction was performed using both the NSL and SL mixtures. Swabs containing a dried 10 µL aliquot of neat seminal fluid were sealed into the 4-plex microdevice and the on-disc workflow was completed for three domains, at least. A calibration curve of known concentrations (10, 1, 0.1, 0.01, and 0.001 ng/µL) of male hgDNA in 1X TE buffer was generated to extrapolate DNA amounts in the recovered fractions. The curve presented excellent linearity, with an R2=0.993 and 98.7% efficiency. The SRY locus was probed for the detection of male-specific human DNA with the 2X SensiFAST Probe qPCR mix and female hgDNA standard at 1 ng/µL was used as a negative control. The initial lysate (NSF) contains 0.27 ± 0.08 ng of Y-DNA on average (**Figure 14-B**), which gradually decreases in the wash fractions until the SL mixture is delivered to the sample reservoir. In this final step, lysis of spermatozoa is initiated in the substrate and chamber trough per the introduction of sperm cell lysing reagents to the workflow for the first time. An approximate 8X Y-specific DNA increase is detected in the SF (2.1 ± 0.88 ng) when compared to the NSF, indicating the coveted DE goal of SF enrichment with sperm cell DNA is attainable with enzyme-assisted extraction on a microfluidic platform.

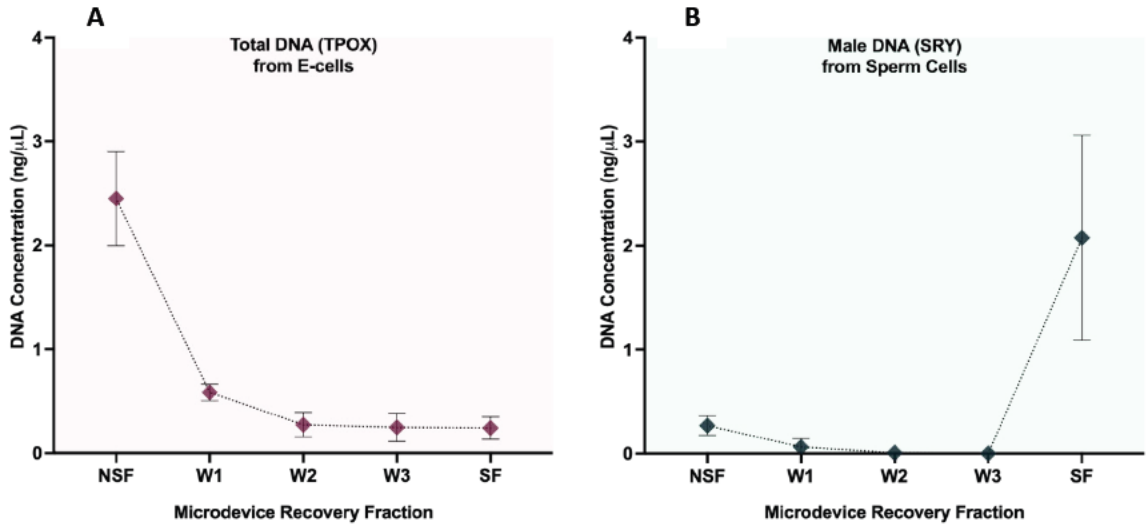

Figure 14. DNA Recovery on the Microdevice. Extraction from e-cells (left) shows gradual decrease of non-sperm DNA from the workflow prior to ingress in the SF. Conversely, extraction from sperm cells (right) shows increase of Y-DNA in the SF.

10. On-Disc DE

The microfluidic device was fabricated according to the *print-cut-laminate* (PCL) method: the architectural features that emulate a traditional DE workflow were designed on AutoCAD and laser-ablated using a CO_2 laser cutter; the layers and accessory pieces were vortexed in deionized H_2O for 20 minutes and left to dry on the benchtop prior to fabrication. The 5 main layers (**Figure 1**) were wiped with 70% IPA, aligned and laminated, then incubated at 40°C for 45-60 minutes under 20 lbs. PMMA and accessory pieces are also wiped with 70% IPA prior to being placed on the 5-layer disc. Mock samples, previously prepared and dried onto swabs, are embedded onto the sample chamber at this time. The completed disc (containing PMMA piece and sample swab(s)) was left under 20 lbs. weights at room temperature for approximately 2 days prior to running a full on-disc assay. The test microdevice was prepared by adding a dry, sterile swab to

the sample chamber of domain 1 (D1) to serve as a negative control, while domains 2, 3, and 4 (D2, D3, D4) contained an identical mock sample preparation dried on the same type of swab (**Figure 15**). On-disc DE was performed according to the parameters in **Table 3**. NSLC refers to the non-sperm lysis cocktail, whereas SLC refers to the sperm lysis cocktail. These reagents are mixed outside of the disc, on a PCR tube, spin vortexed, and then pipetted into the appropriate reagent chamber. 100 µL of NSLC goes into the leftmost reagent chamber, 100 µL of PCR grade water goes into each of the middle reagent chambers, and 100 µL of SLC goes into the rightmost reagent chamber.

Table 3. Reagent volumes for on-disc enzymatic DE.

NSLC	SLC
89 µL Water	56 µL Water
10 µL 10X Blue Buffer	20 µL 10X Blue Buffer
1 µL *prep*GEM	20 µL Acrosolv
-	2 µL *prep*GEM

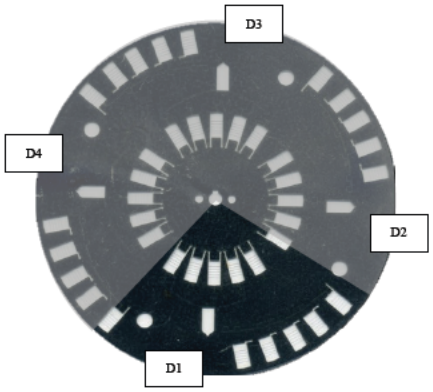

Figure 15. The four identical disc domains. Each domain is capable of single piece of evidence. D1 was run with a dry swab for a negative control. D2, D3, and D4 processed the exact same mock sample for replicates.

The on-disc workflow was performed in a way to mimic the in-tube enzymatic process. After the workflow was completed for each individual domain, the fractions were collected for downstream analysis. The PeT in each individual recovery chamber was punctured with a sterile needle (wiped with 70% EtOH between recoveries) and a 200 µL pipette was used to recover the volumes contained within. All 5 fractions (NSF, SF, and the intermediate washed, termed W1, W2, and W3 here) were collected into different 0.2 mL PCR tubes and stored in a -80°C freezer until use. All extracted fractions were amplified and analyzed for Y-DNA with the Quantifiler Trio Kit (ThermoFisher) using a QuantStudio-5 Real-Time PCR System for Human Identification. Reactions were carried out at half-volume, with a 2 µL sample input, and cycling conditions followed the manufacturer's instructions. A calibration curve was created with the Quantifiler Trio DNA Standard and Diluent with concentrations of 50, 5, 0.5, 0.05, and 0.005 ng/µL for quantification of the DNA extracted from the mock samples run on-disc. **Figure 16** contains the results of the quantification of the extracts retrieved from the CDx disc.

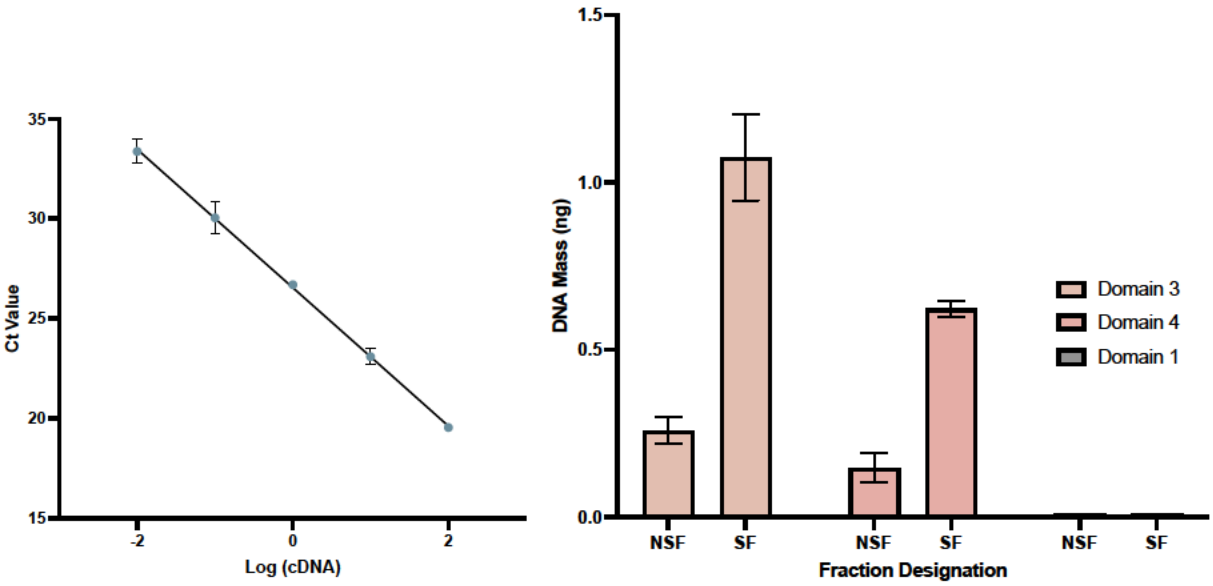

Figure 16. On-disc DE of dried mock sample. Left: calibration curve with known amounts of DNA used to quantify the DNA on the samples extracted on-disc. Right: Y-DNA in the NSF and SF of samples extracted on-disc, showing enrichment of the SF with Y-DNA. There was a failure mode on D2 of the disc, so it is not accounted for above. Further, D1 (which contained a dry, sample-free swab) shows no detection of DNA, showing there is no contamination on the disc.

11. Comparing the Standard and Short Enzymatic Process On-Disc

On-disc extraction of genetic material from the discrete cell types found in SAECK evidence shows concordance with gold-standard DE processes, whereby most of the non-sperm or 'victim' DNA is diverted into wash fractions prior to ingress in the critical sperm fraction, which, in turn, is enriched with Y-DNA originating from the spermatozoa of the perpetrator. The goal was then to investigate whether analogous conclusions could be drawn of true on-disc differential extraction from samples of mixed cellular types. Full on-disc DE was performed with both a 'standard' and 'short' enzymatic protocol(s). Recall that the standard enzymatic process recommends a 10-min heating step for non-sperm cell lysis (NSL), followed by an additional 13-min incubation step for sperm cell lysis (SL). The final stages in both NSL and SL steps require holding the sample at 95°C for 5 min to deactivate the lysing enzyme. To avoid incompatibility with our polymeric devices owning to prolonged exposure to direct heat at high temperatures, we devised a 'short' enzymatic DE protocol that reduces the 95°C heating steps to 1 min each. No loss in sensitivity was observed from this time reduction and both 'standard' and 'short' processes show concordance with a conventional, SDS-ProK-DTT-type DE process. A single domain in at least four independent microdevices (n=4) was processed as a 'blank' with sterile, cell-free swabs enclosed in the sample chamber. No DNA was detected in these recovered fractions, negating the presence of nucleic acid contamination on the microdevice. Total and Y-DNA quantification results from Quantifiler™ Trio (**Figures 17 and 18**) show clear enrichment of the SF with the male target for both enzymatic processes (standard and short) with a workflow that is completed in > 1h per device domain. Significant discrimination between the average carryover non-sperm DNA (standard: 0.18 ng/µL, short: 0.54 ng/µL) and Y-specific DNA (standard: 1.70 ng/µL, short: 2.63 ng/µL) in the SF is demonstrated with unpaired t-tests (p-values < 0.0001, $\alpha = 0.05$). This suggested enrichment of the forensically-relevant SF with sperm DNA is encouraging evidence that attaining single-source male profile(s) through STR profiling is possible and was investigated next.

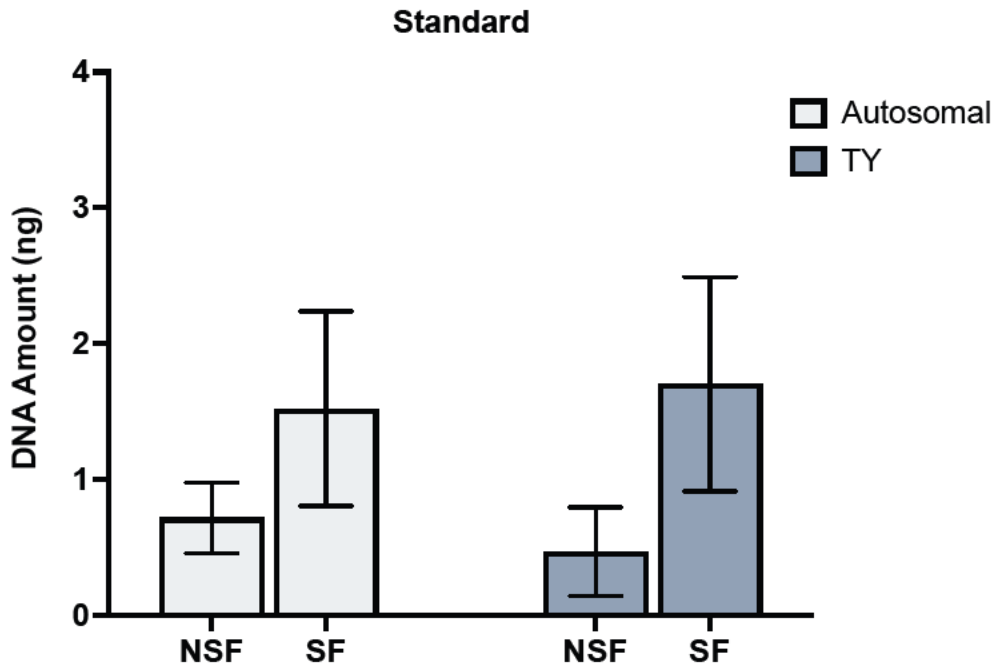

Figure 17. Autosomal and Y-DNA quantification on NSF and SF with the 'standard' enzymatic extraction. DNA quantification shows enrichment of the SF with Y-DNA relative to its NSF.

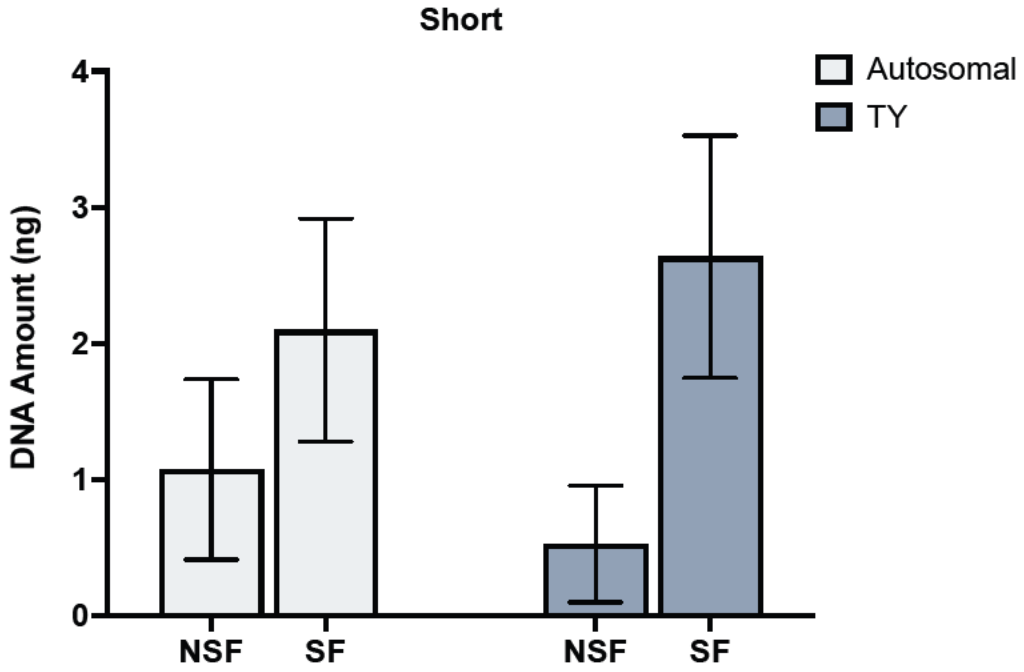

Figure 18. Autosomal and Y-DNA quantification on NSF and SF with the 'standard' enzymatic extraction. DNA quantification shows enrichment of the SF with Y-DNA relative to its NSF.

12. Post-Microfluidic DE STR Analysis by CE

Separation of amplified targets by CE is ubiquitously used in forensic human identification to inspect STRs for human identification. The sizes of these repeated sequences differ from individual to individual and allow for subjects to be distinguished with a high degree of statistical certainty. Following quantitation of the microfluidic differentially-extracted samples, STR fingerprinting was employed to investigate whether obtaining single source profiles from the male-DNA enriched sperm fractions was possible. Eluates recovered from on-disc workflow(s) were processed for comprehensive interrogation of the core CODIS and ESS loci with the PowerPlex® Fusion kit. Reaction input volumes were calculated based on the previous quantification results and ranged from 1 µL to the entire recommended 15 µL for samples with low DNA quantitation. Full STR profiles can be seen in Figure 19. Both standard and short protocols produce STR profiles comparable to the male reference profile. Similarly, the residual NSF DNA that carries over into the SFs does not seem to mask the male peaks. These data show that our simple, rapid, and hazard-free DE approach enriches the SF with sperm DNA to such a magnitude that generation of single-source profiles from Y-DNA is possible. This is of the utmost importance to STR profiling as it obviates the need for interpretation and deconvolution of complex mixtures.

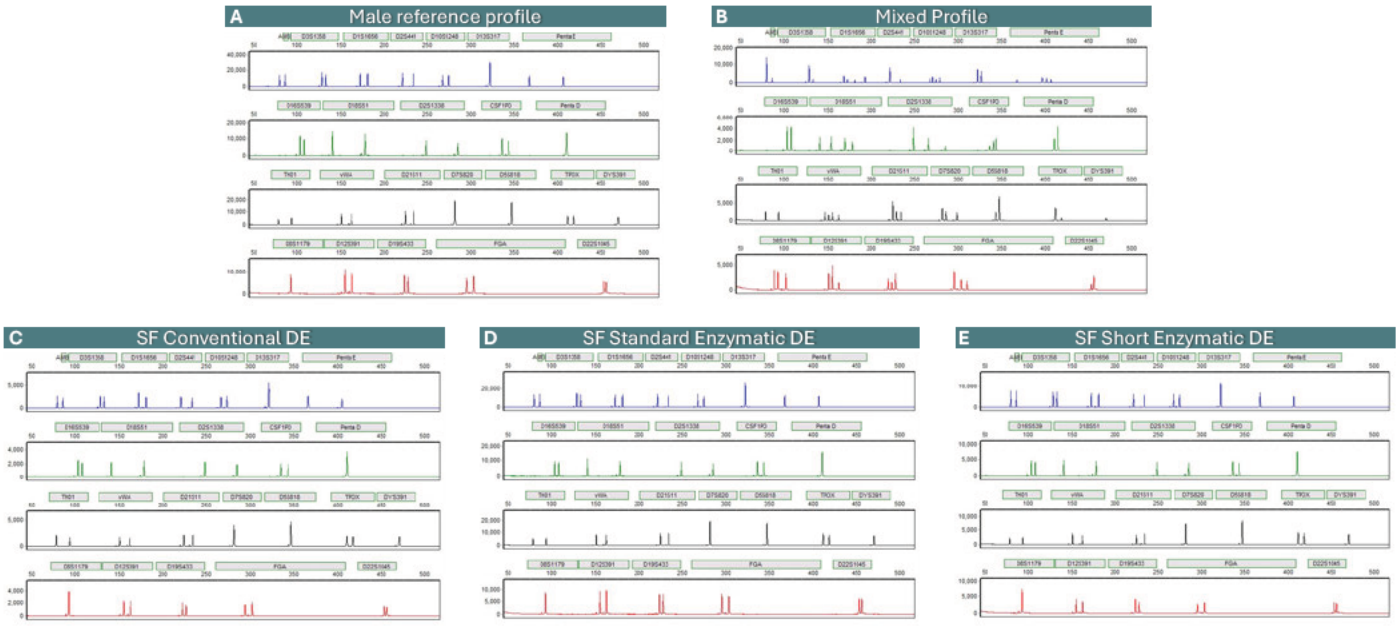

Figure 19. Full STR profiles from enzymatically differentially-extracted samples. (A) Male reference profile was obtained from the SL extraction of the seminal fluid sample used to create mock mixed samples. (B) Mixed profile from mock sample extracted with a non-DE protocol. (C) SF from a mock sample extracted with a conventional, column-based DE protocol. (D) SF from a mock sample extracted on-disc with the standard enzymatic DE process. (E) SF from a mock sample extracted on-disc with the short enzymatic DE process.

13. Optimization of the TCDE Protocol

The Temperature Controlled Differential Extraction (TCDE) chemistry was optimized off-disc for microfluidic implementation. Mock sexual assault samples were dried on cotton swabs and extraction was performed on whole swabs. The objective was to verify whether the TCDE process is compatible with our microdevice or if fine-tuning of the protocol is necessary. The main difference between the TCDE and standard/short enzymatic DE methods is the addition of a nuclease treatment following lysis and release of DNA from non-sperm cells. Three variations of the TCDE method were tested in-tube, each containing five different lytic mixtures. The five master mixes consisted of the following, A: 47 µL water, 50 µL 10X Blue buffer, and 3 µL *prep*GEM; B: 2.5 µL 1:10 *prep*GEM in 1X Blue buffer, 10 µL 2.5U Benzonase in 2X Benzonase buffer, and 20 µL 2X Benzonase buffer (which was prepared by mixing 4 mL of 1M Tris HCl pH 8, 0.0381g of $MgCl_2$, 0.2338g of NaCl brought up to 100 mL with DI water); C: 2.5 µL 1:10 *prep*GEM, and 10 µL 2.5U Benzonase; D: 38.5 µL water, 5 µL 10X Blue buffer, 20 µL Acrosolv, and 4 µL *prep*GEM; E: 47.5 µL water, 5 µL 10X Blue buffer, 25 µL Acrosolv, and 10 µL *prep*GEM. The full TCDE protocol followed these steps: master mix A was added to the tube containing the whole swab, vortexed, and incubated at 75 °C for 15 min. The liquid was removed from the swab by placing it in a spin basket and centrifuging at 15,000 rpm for 5 minutes. The supernatant was removed and heated for 5 min at 95 °C, the resulting fraction was labeled ‘EF’ or ‘NSF’ for epithelial/non-sperm fraction. Master mix B was added to the swab, while master mix C was added to the sperm pellet. Both fractions were vortexed and incubated at 37 °C for 15 min, followed by 15 min at 75 °C. The liquid in the swab was removed by centrifuging at 15,000 rpm for 5 min. The fraction from the swab was labeled ‘MF’ for material fraction, and the one from the sperm pellet was labeled ‘SF’ for sperm fraction. Master mixes D and E were added to the MF and SF, respectively, and incubated at 52 °C for 5 min, 75 °C for 3 min, and 95 °C for another 3 min. Total and Y-DNA was quantified on the EF/NSF, MF, and SF using the Quantifiler™ Trio Kit. **Figure 20** shows the Y-DNA and autosomal quantification results. The results suggest a substantial amount of both autosomal and Y-DNA is present in the MF, suggesting a significant amount of sperm cells stick to the swab. Hence, the TCDE was adapted to reflect this finding. This adapted protocol used the same master mixes, with the same input reagent volumes and incubation times, but a Benzonase treatment was not done on the swab, only on the sperm pellet. Master mix B was used to resuspend the sperm pellet, incubations followed the times and temperatures described previously, and the sperm was re-pelleted at 15,000 rpm for 5 min. The supernatant was recovered and labeled as BF, or Benzonase fraction. Master mixes D and E were added to both the swab and sperm pellet, and the remaining of the process followed the instructions above.

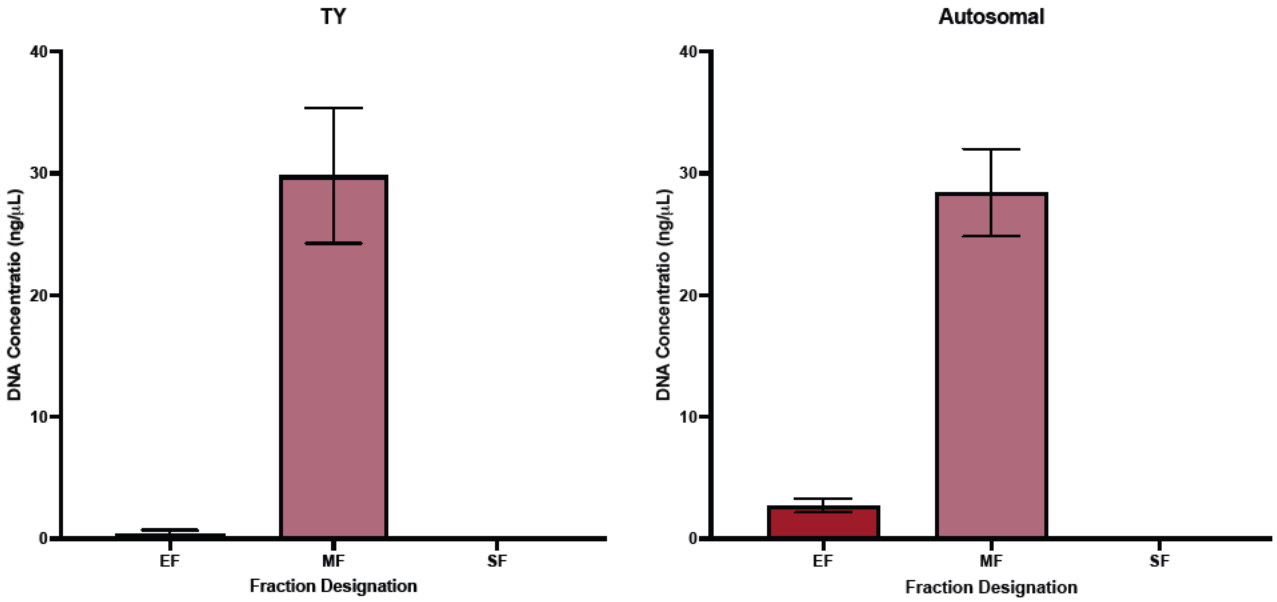

Figure 20. Y and autosomal DNA quantification from the TCDE process. The protocol utilizes the sperm lysis mixture on both the material fraction (swab) and sperm pellet. The large amount of DNA in the MF indicates that the cells stick to the substrate, and the protocol was modified accordingly.

The results in **Figure 21** agree with the previous, showing the largest amount of DNA is present in the 'MF', which is the resulting fraction from lysis performed directly on the swab. These findings are promising towards our goal of implementation of the TCDE protocol on the microdevice, as the CDx disc employs a swab-in approach intended to exploit this natural tendency to maximize sperm cell DNA recovery by retaining unbound and loosely bound sperm cells and by lysing them directly from the cutting during the last enzymatic treatment step of the CDx workflow. Simply stated, unlike conventional DE, the swab-in approach retains and treats the cutting throughout the CDx workflow to improve yield. Studies show that DNA yields indeed increase when the extraction of sperm cells is done directly from the swab as opposed to the sperm pellet.

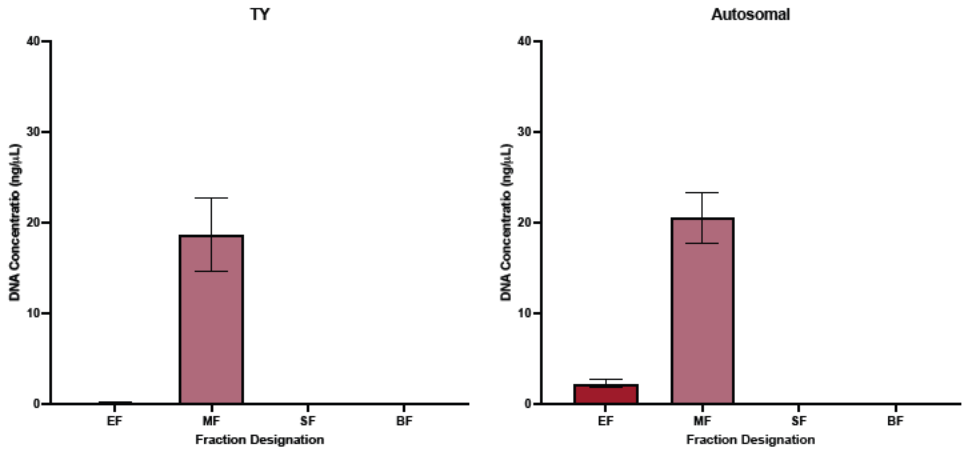

Figure 21. Y and autosomal DNA quantification from the modified TCDE process. The modified protocol agrees with the previous, and shows most of the DNA is extracted from the substrate/swab, which shows promise towards microfluidic implementation given the swab-in approach of the CDx disc.

14. On-Disc TCDE

The TCDE protocol was performed on the CDx disc. As before, the device was fabricated according to the *print-cut-laminate* (PCL) method. The test microdevice was prepared by adding dried mock samples into the swab chambers. On-disc TCDE was performed according to the parameters that follow. The NSLC consisted of 47 µL of water, 50 µL of 10X Blue Buffer, and 3 µL of *prep*GEM. This reaction mix was centrifugally-driven to the swab chamber by spinning the disc at 3,000 rpm for 60 seconds in the clockwise direction, followed by a 3,000rpm/60s spin in the counterclockwise direction, followed by another spin in the clockwise direction (3,000rpm/60s). The 'Benzonase' mixture consisted of 67.5 µL of water, 20 µL of 20X Benzonase Buffer, and 2.5 µL of 1:10 *prep*GEM. The SLC mixture consisted of 56 µL of water, 20 µL of Acrosolv, 20 µL of 10X Blue Buffer, and 4 µL *prep*GEM. The Benzonase as well as the SLC mixtures were spun into the swab chamber at 3,000 rpm for 60s, with no additional spin events. Similarly, all recoveries into the downstream chambers also featured a single spin event of 3,000 rpm for 60s. The incubation profiles were the following: NSL (76°C/900s and 96°C/60s), Benzonase (37°C/900s, 76°C/900s and 96°C/60s), SL (52°C/300s, 76°C/180s and 96°C/60s). **Table 4** depicts the mean Y-DNA amounts from samples extracted with the TCDE method on the microdevice, showing enrichment of the SF with male DNA. Data is shown from each of the four individual domains on a single microdevice.

Table 4. Y-DNA Quantification on NSF and its SF counterpart. Quantification from mock samples extracted on the microdevice using the TCDE method.

Domain 1		Domain 2		Domain 3		Domain 4	
NSF	SF	NSF	SF	NSF	SF	NSF	SF
0.15 ± 0.02	10.76 ± 1.42	0.16 ± 0.03	4.08 ± 0.55	0.14 ± 0.03	4.71 ± 1.65	0.17 ± 0.02	2.82 ± 0.35

Conclusions, Project Aims, and Future Work

We propose a proof-of-principle microfluidic approach to DE and further characterize it to demonstrate it promotes successful fractionation of nucleic acids of different origins (e.g., victim vs. perpetrator) and that on-disc enzymatic differential extraction of mock sexual assault evidence containing mixtures of female epithelial cells and seminal fluid is feasible. The microfluidic device is the size of a standard compact disc, and fluid is driven away from the center of rotation via centrifugal forces. The microfluidic system semi-automates the DE process by replacing the numerous pipetting steps with a laser-valving approach that promotes fully-enclosed cell lysis and lysate fractionation. The 'standard' enzymatic DE protocol can be completed in about 30 minutes and demonstrated analogous sensitivity to conventional protocols that rely on long incubation times and PCR-inhibiting reagents. When compared to a 6-hour conventional DE process (1.5h incubation of NSF, 4h incubation of SF, and 0.5h automated extract purification), an approximate 12X time gain is acquired. Further, the on-disc enzymatic approach produces discrete forensic fractions with significant statistical differences in male-DNA when SAECK-like samples are used. Though carryover of female DNA to the SF still occurs, the amounts do not mask male peaks in the STR electropherograms. These studies highlight the fractionation capabilities proffered by integrated laser valve opening/closing strategies and the plasticity of the PCL disc fabrication method for addressing fluid handling, unit operation integration, and other fluidics-related issues. It appears that a modest rotational frequency (≤ 3,000 rpm) generates sufficient rotational forces to facilitate reasonable on-disc fluid recovery from sample cuttings (≥ 90% with cotton swab cuttings). This is ideal as PCL-fabricated microdevices often struggle with higher spin speeds. Dye and buccal swab extraction studies established that small molecules (red dye) and non-sperm cell DNA

are removed from the sample chamber prior to extraction and elution of the critical sperm fraction. On-disc extraction studies reaffirmed that *prep*GEM does not to appear to adversely impact sperm cell integrity, i.e., the enzyme lacks the substrate specificity needed to simultaneously rupture non-sperm and sperm cells.

As part of the project's aims, we have: 1) optimized the TCDE chemistry for samples dried on common substrates such as swabs (**Figures 7-12)**, 2) refined the existing TCDE protocol for use with both common laboratory equipment and the low-volume rotational CDx disc (**Figures 20 and 21**), 3) developed a novel, centrifugally-driven microfluidic device that can be inexpensively prototyped and fabricated (**Figures 1-5**), and 4) adapted the TCDE protocol to the CDx disc (**Figures 20-21, Table 4**). For future work, we aim to design, build, and test a prototype instrument that integrates and leverages the in-house engineering expertise garnered during the development of existing Landers Lab spin systems. The instrument will achieve supreme flow control and step-wise, on-disc TCDE reagent delivery by employing the laser-based, 'closable valve' methods developed in the Landers Lab. Briefly, the CDx system exploits foundational rotational microfluidic principles that drive fluid flow through the architecture and allow the disc to function as a centrifuge (which seems somewhat apropos given that most current DE protocols incorporate lengthy, repeated centrifugation steps), a pipettor, and a mixing/metering device. The instrument will minimally provide for: timed reagent release, temperature control for novel, sequential enzymatic reactions, and fractionation that yields discrete sperm (SF) and non-sperm (NSF), and waste fraction from SAECKs. Further, laser-based valving stands to effectively reduce device footprint and cost by eliminating the need for external pumps and valves to control fluid movement.

What individuals have worked on the project?

Name: James Landers
Project Role: Principal Investigator
Nearest person month worked: 1.0
Collaborated with individual in foreign country: Yes
Travelled to foreign country: Yes

Name: Larissa Cunha
Project Role: Graduate Student
Nearest person month worked: 24
Collaborated with individual in foreign country: No
Travelled to foreign country: No

Name: Miracle Enwere
Project Role: Graduate Student
Nearest person month worked: 4
Collaborated with individual in foreign country: Yes
Travelled to foreign country: No

Name: Junyi Yao
Project Role: Graduate Student
Nearest person month worked: 10
Collaborated with individual in foreign country: No
Travelled to foreign country: No

Name: Sadie Kiendzior
Project Role: Graduate Student
Nearest person month worked: 11
Collaborated with individual in foreign country: No
Travelled to foreign country: No

Name: Jayne Francis
Project Role: Undergraduate Student
Nearest person month worked: 1
Collaborated with individual in foreign country: No
Travelled to foreign country: No

Name: Zoey Golabek
Project Role: Technician
Nearest person month worked: 2
Collaborated with individual in foreign country: No
Travelled to foreign country: No

What other organizations have been involved as partners?

Boston University (Dr. Robin Cotton)

Describe any other special reporting requirements specified in the award terms and conditions not covered by the questions above.

Nothing to Report.

Products

- AAFS 2024 Abstract (B86).
- MSB 2023 Program (this one doesn't contain the abstract of the talk).
- MSB 2023 3-Minute Research Showcase.
- Posters presented at the 2022 Gordon Research Conference and UVA's 3rd Year Poster Session are located in the physical space of the lab.
- Cunha, L. L.; Roberts, A.; Cotton, R. W.; and Landers, J. P. A Rapid, Fully-Enzymatic Method for Forensic Differential Extraction of Biological Sexual Assault Evidence. Forensic Science International: Genetics. In preparation.
- Cunha, L. L.; Woolf, M. S.; O'Neill, K.; Momand, E.; and Landers, J. P. Implementation of a Fast, Fully-Enzymatic Forensic Differential Extraction Method on a Centrifugal Microfluidic Device for Sexual Assault Sample Preparation. Analytical Chemistry. In preparation.

Dissemination: (presentations at conferences)

A Microfluidic Approach Towards Automated Forensic Differential Extraction of DNA-Based Sexual Assault Evidence. Larissa L. Cunha, M. Shane Woolf, S. Kiendzior, K. N. O'Neill, E. Momand, James P. Landers. **Oral Presentation.**
2024 76th American Academy of Forensic Sciences (AAFS) Annual Scientific Meeting. Denver, CO, USA.

A Microfluidic Tool for Automated Forensic Differential Extraction (DE) of DNA-Based Sexual Assault Evidence. Larissa L. Cunha, M. Shane Woolf, K. N. O'Neill, James P. Landers. **Oral Presentation.**
2023 39th International Symposium on Microscale Separations and Bioanalysis (MSB). Tallahassee, FL, USA.

Seeking Justice: Microfluidic Approaches to Improve Forensic Sample Extraction and Preparation. Larissa L. Cunha. **3-Minute Research Showcase.**
2023 39th International Symposium on Microscale Separations and Bioanalysis (MSB). Tallahassee, FL, USA.

Toward the Development of an Automatable Differential Extraction (DE) Process on a Centrifugal Microfluidic Disc for Sexual Assault Evidence Analysis. Larissa L. Cunha, M. S. Woolf, J. P. Landers. **Poster Presentation.**
2022 Gordon Research Conferences – Forensic Analysis of Human DNA. Mount Snow, VT, USA.

Development of a Microdevice for Automated Differential Cell Lysis from Sexual Assault Evidence. Larissa L. Cunha, M. S. Woolf, J. P. Landers. **Poster Presentation.**
2022 University of Virginia Chemistry Department Graduate Student 3rd Year Poster Session. Charlottesville, VA, USA.