Rapid Extraction of Sperm from Sexual Assault Kits

Draft Final Summary Overview
Submitted to
National Institute of Justice
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Washington, DC 20001

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Grant No: 2016-DN-BX-0161
Period of Performance: January 1, 2017 through February 28, 2018
Total Funding:
DUNS:
EIN:

PURPOSE OF THE PROJECT. The most common method for separating sperm cells from an attacker and epithelial cells from a victim in a sexual assault kit (SAK) is to perform a differential extraction. In this process, both sperm cells and epithelial cells are exposed to a mixture of reagents that is designed to rupture the epithelial cells while leaving the sperm cells intact. The epithelial cell lysate is removed from the intact sperm cells using several centrifugation steps; the heavier, intact sperm cells are pelleted during centrifugation from the reagent/lysate mixture and rinsed several times. After the epithelial cell lysate has been rinsed away from the sperm cell pellet, the sperm cells are lysed used using harsher reagents. After lysis, the DNA from the sperm cells is extracted, then quantified and identified using a series of downstream analyses, including quantitative polymerase chain reaction (qPCR) analysis and short tandem repeat (STR) analysis.

The most crucial step in SAK analysis is the isolation of the sperm cells from the attacker from the epithelial cells from the victim. The differential extraction process is long and complicated—it requires a significant amount of time, highly trained personnel, and substantial laboratory infrastructure. It is difficult to remove the female DNA from the victim from the sperm cell fraction; contamination with female DNA sometimes leads to identification data that is difficult to analyze and pinpoint to a specific individual. A *fast*, *simple*, *low-cost*, method for extracting sperm cells from mixtures of sperm and epithelial cells that results in a *pure* sperm cell extract is a necessity for analyzing SAKs. This would significantly decrease the time, complexity, and cost to process the kits, reducing the SAK backlog.

Our project highlights gradient elution isotachophoresis (GEITP) as a means for extracting sperm cells from mixtures of sperm cells and epithelial cells. GEITP marries isotachophoresis

and pressure-based flow to remove charged materials from solution. The goal of the present project is to develop a GEITP method for sperm extraction. The successful GEITP protocol is one in which only sperm cells are extracted from a mixture of both sperm and epithelial cells (i.e., no epithelial cells are present in the extract). Because SAKs contain an overwhelming majority of epithelial cells with respect to sperm cells, the process of extracting only sperm cells from this mixture is a challenging one. Our goal in this basic research project is to show that sperm cells can be extracted from a mixture of sperm cells and epithelial cells. We will assess different electrolyte systems to determine the one that allows sperm focusing but no epithelial cell focusing. We will use an optimized electrolyte system and GEITP protocol to assess the feasibility of GEITP-based sperm cell extractions.

PROJECT DESIGN AND METHODS. The primary method used in this work is GEITP, which uses a combination of pressure-based flow and isotachophoresis to focus charged molecules or particles on the basis of electrophoretic mobility, or their mobility in an electric field. GEITP uses two different aqueous buffers with different electrophoretic mobilities. The electrophoretically fast buffer is the leading electrolyte (LE) and the electrophoretically slow buffer, into which the sample is slurried, is the trailing electrolyte (TE). ***Before extraction***, the LE is in contact with the sample by means of a capillary, which is threaded through a capacitively-coupled contactless conductivity detector (C^4D) as well as a laser-induced fluorescence (LIF) detector. During the ***focusing*** step, a slight pressure is applied to the LE reservoir, thus expelling a small portion of the leading electrolyte into the sample solution. Concurrently, an electric field is applied. At this point, charged molecules or particles that possess an electrophoretic mobility between that of the LE and TE focus at the interface between the LE and TE buffers.

After focusing, a negative pressure is applied to the LE reservoir, thus removing the focused plug of analytes from the sample solution and allowing for ***detection*** of the analytes using LIF or conductivity detection. After detection, the capillary is moved to a clean, fresh vessel and a positive pressure is applied to the LE reservoir. The positive pressure results in ***delivery*** of the purified analytes into a clean sample tube for further downstream analysis.

We used two different GEITP setups, referred to as the horizontal apparatus and the vertical apparatus. In the horizontal apparatus, the capillary that connects the LE reservoir to the sample cup is parallel to the work surface. The two reasons for using this apparatus is to visualize the plug of extracted material during the GEITP process and use a high quality, sensitive C^4D. The horizontal apparatus consists of a capillary in fluid connection to an LE reservoir and a sample cup filled with TE. The outer diameter (OD) of the capillary is 360 μm and the inner diameter (ID) is experiment-dependent. The OD dimension of the capillary allows for facile incorporation into the LE reservoir with off-the-shelf capillary connectors machined into the reservoir. The LE reservoir, fabricated from poly(sulfone), is fitted with a lid that includes a Pt electrode connected to a Stanford Research Systems (Sunnyvale, CA) high voltage power supply (+/- 5000 V). The LE lid also contains poly(ether ether ketone) (PEEK) tubing that is plumbed to a Mensor (Mensor LP, part of WIKA Group, San Marcos, TX) pressure controller (+/- 30000 Pa) to afford pressure control during a GEITP run. The capillary passes through a C^4D (TraceDec, Innovative Sensor Technologies, Austria) and into a delrin sample cup; the ground electrode (Pt wire) is machined into a plastic cap that fits over the sample cup and is wired to the power supply. A multimeter (Agilent Technologies, Santa Clara, CA) is wired into the

apparatus to detect current through the capillary. The instrument is computer controlled via LabView software; C^4D data is collected using TraceDec software and analyzed using Origin.

In contrast to the horizontal apparatus, the capillary in the vertical apparatus is positioned perpendicular to the work surface. The LE reservoir, capillary, and detectors are mounted on an XYZ translational stage for robotic control during the GEITP protocol. The pressure supply is a lab-built syringe pump capable of pressure control from -5000-5000 Pa. The power supply is capable of 2000 V output (XP-EMCO, CA); we used both positive- and negative-polarity power supplies for this work. The capillary that we used for this work in the vertical apparatus is a *multibore* capillary that contains 16 bores (ID 75 μm). We used a multibore capillary to increase the extraction efficiency of sperm from the sample solution (as compared to a single capillary). The OD of the multibore capillary is 1.6 mm; we place the capillary into a PEEK sleeve and seal it with epoxy prior to fitting it into the LE reservoir. The LE reservoir was fabricated from poly(sulfone) and contains a machined hole with which ferrules and short nuts (LabSmith) are compatible. The multibore capillary is fitted through the ferrule and short nut and tightened into the LE reservoir. The capillary passes through an LIF detector, consisting of a 488-nm laser (Coherent, Santa Clara, CA), a liquid light guide, associated optics, and a photomultiplier tube (ThorLabs, Newton, NJ). The capillary also passed through a lab-built C^4D to monitor the presence of the LE and TE within the channel at any point during an extraction. The TE reservoir is a 200-μL tube; once the DNA is removed from the TE reservoir during the extraction process, the capillary is moved to a different location using the stage and pressure dispenses the DNA into a clean qPCR tube. All instruments were controlled with LabView software.

To detect the presence of sperm and/or epithelial cells in sample extracts, we used light microscopy. Each extract obtained from the vertical GEITP apparatus was approximately 16 mL in volume. We removed 8 μL of the extract and, in some cases, exposed it to 2 μL Bio-Rad nucleic acid dye. The liquid was then dropped on a microscope slide and covered with a coverslip. In making determinations of the presence or absence of cells, we counted every cell within this 8 μL sample (400X magnification. In some cases, the second 8 μL aliquot was used in an additional analysis or subjected to light microscopy.

We used quantitative polymerase chain reaction (qPCR) analysis to determine the amount of DNA in a sample as well as the amount of DNA in a sample extract. Because we are extracting cells, specifically sperm cells, rather than DNA, it was necessary to first perform a lysis step on the extracted cellular material. The lysis buffer contained 5 mM dithiothreitol, 1 mg/mL Proteinase K, 0.1% triton X, and 70 mM Tris HEPES. We incubated all extracts at 56 ^{o}C for 2 h, followed by Proteinase K destabilization at 100 ^{o}C for 10 min. Real-time qPCR data was collected on a Bio-Rad CFX96 and analyzed using Bio-Rad software (Hercules, CA). We first worked with a qPCR protocol that highlighted the THO1 locus (see Timken, M.D. et. al, *J Forensic Sci*., **2005**, *50(5)*, 1-17 for primer and probe sequences) to determine the presence and quantify human genomic DNA (25 ng/μL to 0.001 ng/μL). TaqMan Universal Master Mix 2X, no UNG (ThermoFisher Scientific, Waltham, MA) was used in addition to the primers, probes, and sample in the concentrations noted in the reference. The qPCR temperature program included one 10 min polymerase activation step (95 ^{o}C) and 45 cycles of denaturation followed by annealing/extension (15 sec, 95 ^{o}C; 60 sec, 60 ^{o}C). The Quantifiler Trio DNA Quantification Kit was used to quantify the total amount of human DNA and human male DNA in samples using a

TaqMan probe assay. The kit uses human-specific target loci on various autosomal chromosomes and the Y-chromosome to quantify 50 ng/μL to 0.005 ng/μL DNA. The qPCR program included of a holding (10 min) and denaturation (9 sec) step at 95 ^{o}C and 45 cycles of denaturation and annealing (9 sec, 95 ^{o}C; 30 sec, 60 ^{o}C). All samples were analyzed in triplicate.

DATA ANALYSIS

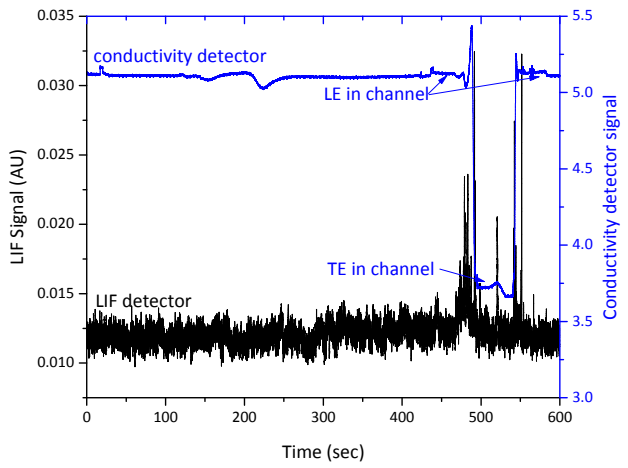

Figure 1: GEITP electropherograms highlighting the data collected from the LIF detector (black trace) and the conductivity detector (blue trace).

GEITP. GEITP electropherograms from both the LIF and the C^4D detector are collected during every extraction. With respect to the LIF data, a cell-permeable DNA intercalating dye is required to obtain LIF data. When we perform qPCR of sample extracts, the presence of such intercalating dye molecules interfere with the analysis. Thus, much of our GEITP data does not include electropherograms collected using the LIF detector. The electropherograms collected using the conductivity detector are used to monitor the extraction process. Figure 1 shows GEITP electropherograms of sperm extracted from a sperm cell slurry. In this case, SYTOX was added to the sample prior to extraction. The peaks corresponding to the sperm cells occur at ~480 sec and 540 sec; the first set of peaks is due to the plug of sperm cells passing the detector during the extraction step, the second set of peaks is due to the sperm cells passing the detector during the delivery step. The peak in the middle, at 510 sec, is sometimes present on the data and could be due to the sperm cells lingering in front of the

detector as the apparatus moves via the XYZ translational stage. Quantification of this data can be performed by integrating the area under the peaks and corresponding that data with quantification data such as that determined with qPCR analysis. We have not performed any quantification calibrations using sperm. With respect to the conductivity detector data, the rtace is somewhat stable until approximately 480 sec because the LE is in the channel. When the focused plug of sperm cells, followed by the TE, passes the detector at approximately 480 sec, the data decreases sharply, indicating the focused plug of sperm cells, and then the lower-conductivity TE, are in the channel. It is not until pressure is used to deliver the sperm cell plug, at approximately 540 sec, that the LE fills the channel again. This conductivity detector data is used for the express purpose of monitoring the extraction process. It is possible, however, to identify and quantify analytes using a similar, but more sensitive, detector. We are able to do this with the C^4D on the horizontal apparatus, and the different zones of analyte are present as "steps" within the large step indicating LE and TE.

Microscopy. Light microscopy with and without nucleic acid staining dye (Bio-Rad, Hercules, CA) was used to observe sperm cells and epithelial cells from samples as well as sample extracts. This data is present as the number of cell counted in 8 μL of sample or extract. These experiments were performed on each extract when we were determining the buffer system.

qPCR. Standard curve quantification was used for all qPCR analyses to determine the quantity of target DNA in all samples and sample extracts. Bio-Rad software compared the fluorescence signal from the standards to the unknown samples and calculate DNA concentration.

PROJECT FINDINGS. Our first experiments were designed to allow us to visualize sperm focusing during a GEITP extraction. The sperm cells were purchased from Fairfax Cryobank

(Fairfax, VA) and were washed with the TE buffer 3X before experimentation. The cells were exposed to SYTOX for fluorescent visualization. Using 50 mM Tris 25 mM tartrate as the LE and 12.5 mM Tris HEPES as the TE, we were able to visualize sperm cells after several minutes of focusing within the capillary. The voltage applied was +1000 V and the pressure was held at 500 Pa prior to focusing. This indicates that the sperm cells can focus and be extracted under these conditions. We also performed GEITP focusing studies using vaginal epithelial cells under the same experimental conditions that were used for sperm cell focusing. The cells were purchased in cell growth media (Lifeline Cell Technologies); the cells were washed 3X using 12.5 mM Tris HEPES TE before being subjected to GEITP focusing. We used SYTOX as the visualization dye for these experiments. The focusing experiments with epithelial cells were performed independently from sperm focusing experiments. The epithelial cells also focus within the capillary after several minutes of focusing time, indicating that the sperm and epithelials cells behave similarly in an electric field under these conditions.

Concurrent to the experiments in which the cells were visualized during focusing, we also performed GEITP experiments to determine the relative electrophoretic mobility of the sperm to various weak acids. In GEITP, analytes with an electrophoretic mobility between the leading and trailing electrolyte will focus. Our hypothesis is that we can determine which acids have similar electrophoretic mobilities to the sperm and use those acids as leading and trailing electrolytes. We determined that malic acid would be suitable as an LE and oxaloacetic acid and acetic acid would be appropriate as TE materials for sperm at pH 7.

We performed extractions of sperm cells using 50 mM Tris 25 mM tartrate as the LE and 12.5 mM Tris HEPES as the TE and visualized sperm cells in the extracted sample. We also

performed extractions of sperm and epithelial cell mixtures using 50 mM Tris 25 mM tartrate as the LE and 12.5 mM Tris HEPES as the TE as well as 50 mM Tris 25 mM malate as the LE and 12.5 mM Tris HEPES as the TE. In all cases, epithelial cells were extracted in addition to sperm cells. We hypothesized that the electrophoretic mobility of the sperm cells is very similar to the epithelial cells at pH 6-7. We thus experimented with other ways, to extract sperm cells from sperm/epithelial cell mixtures, including adding an antibody specific to epithelial cells to the sperm/epithelial cell mixture. We theorized that the presence of the antibody would slow the epithelial cells and thus afford focusing of the sperm cells and not the epithelial cells. Our data indicates that the epithelial cells were extracted from sperm/epithelial cell mixtures both with and without the antibody. We experimented with lysing the epithelial cells within the sperm/epithelial cell mixture and exposing the mixture to DNAse, an enzyme that catalyzes the degradation of DNA, to remove the DNA from the resultant solution. Our data shows that there is trace DNA in sample extracts that contained only epithelial cells, indicating that the DNA requires longer lysis times with DNAse. However, we also showed that we could extract sperm from this mixture. While this method could lead to clean sperm extracts, performing the lysis prior to extraction is not much different from the current differential extraction procedure.

We focused our efforts on GEITP extraction in acidic buffers. While we expected the electrophoretic mobility of both the sperm cells and the epithelial cells to decrease, we expected a more significant difference in mobility. We experimented with roughly 25 LE/TE pairs of different pH and composition. Our data indicated that either the sperm cells were not present in the extracts or that sperm cells and epithelial cells were both present in the extracts. We hypothesize that the sperm cells and the epithelial cells are very similar in electrophoretic

mobility, and we further postulate that the epithelial cells are actually slightly faster than the sperm cells at pH values pH 4.8-7 when the cells are exposed to a positive applied field. We thus modified our instrumentation and software to accept a negative power supply.

We have performed GEITP extractions using pH 4.6—pH 5.1 buffers; the LE is composed of acetic acid/NaOH and the TE is acetic acid/pyridine. At these pH values, our data (qPCR) indicate very little overall cell extraction and subsequent DNA detection.

IMPLICATIONS FOR CRIMINAL JUSTICE POLICY AND PRACTICE IN THE UNITED STATES

To date, there has been no impact on the scientific or forensic communities as a result of this research. However, we are still in the very basic research phase of this methodology and we feel confident that when the GEITP technique is optimized it will lower the burden on crime lab resources by reducing manpower, time, and cost. Using inexpensive instrumentation and reagents, this method will rapidly provide pure fractions of spermatozoa, free from contamination by victim cellular material or DNA, for STR analysis. The potential impact will be faster identification of perpetrators for ultimately faster prosecution. Alternatively, it may mean the faster exoneration of an innocent man. Once validated on our current laboratory platform, future development of the GEITP instrumentation will include multiplexing for even greater sample throughput. By reducing extraction time from a day-long differential extraction process to a minutes-long GEITP extraction, this method can revolutionize the handling and analysis of sexual assault kits.