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Brief Synopsis of Goals:

The primary aims of our program focus on the adaptation of conventional chromatin immunoprecipitation (ChIP) methodology to an optimized, sample specific method for use on mixed samples from sexual assault casework. Our Sperm DNA Capture method takes advantage of the uniqueness of sperm chromatin wherein the majority of histone proteins have been replaced by a class of proteins called protamines. Protamines facilitate the packaging of the sperm genome into a highly condensed state, roughly 85% of the sperm genome is protamine bound. We target the DNA/protamine complex using monoclonal antibodies in order to completely separate the sperm DNA from other DNAs in the mixture. We previously reported development of the methodology, screening of antibodies, and use of a variety of samples including initial testing on self-collected post-coital swab material. Throughout the program the key performance evaluations have been DNA yield, clean separation of the DNAs in mixed samples, and the method's sample-to-purified-DNA time commitment benchmarked against current differential extraction methods.

Purpose

There is an average of 89,000 reported rape cases in the United States annually (Statistic Brain Research Institute 2015, and data from National Sexual Violence Resource Center 2015 and references therein). In the United States, roughly16% of the female population, and 3% of the male population have experienced an attempted or completed rape. Other sources put these numbers much higher (National Sexual Violence Resource Center 2015). Despite the severity of this crime, there has become a national backlog of possibly 500,000 or more unprocessed samples obtained from forensic sexual assault exams (Petersen et al. 2012). This backlog is caused by limitations in the current methods for processing and analyzing the samples. Federal legislation was recently passed to increase funding on the order of $36 million to address the backlog, but the fact remains that there are a limited number of trained examiners who can process the samples and the manual workflows used in processing these samples are rate limiting.

While many victims report the event within a short period of time, the evidence in the form of analyzable DNA can be compromised by degradation of the sperm over time or washing of the body or materials (Janisch, Meyer, et al. 2010; Vuichard 2011). In practice, this limits the time frame for the forensic exam to be performed for the collection of evidence to a span of 72-120 hours after the assault (Boston Area Rape Crisis Center 2015). For analysis of post-coital vaginal swabs from consenting donors, intact sperm could be detected in 38% of the samples after 36 hours, while only 18% of anal samples contained sperm after 24 hours. Other studies have shown through Y-chromosome detection, however, that the sperm DNA can be detected as far out as three weeks (Mayntz-Press 2008). Thus, methods to isolate sperm DNA that are not dependent on intact sperm bodies are desirable and could impact the time in which exams can be conducted and still produce reliable full STR profiles.

Current State of Research

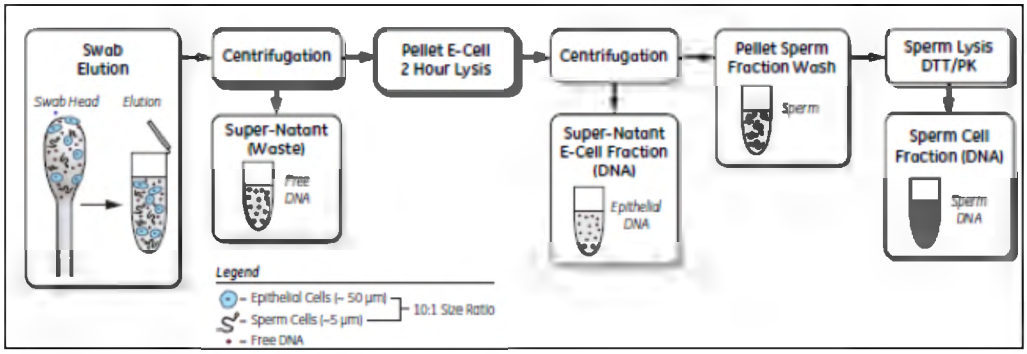

Figure 1: Process of differential extraction. Intact cells eluted from swab material followed by a differential centrifugation and cell lysis to produce 2 fractions containing the victim and the sperm DNAs.

The analysis of sexual assault samples, consisting of a mixture of epithelial and sperm cells, is conventionally started using a differential extraction process (Figure 1), and is followed by STR-typing. This process requires many manual handling steps (25 to 30), takes about six hours to complete, and requires a skilled technician.

Differential extraction, whether done manually or partially automated, entails elution of intake cells from the collection swab. Then differential lysis and centrifugation steps produce fractions of epithelial DNA, sperm cells, and free DNA from lysed sperm cells. A recent study (Vuichard et al. 2011) comparing differential extraction protocols used by forensic laboratories found that >90% of the male DNA initially present in the simulated sexual-assault samples was lost after differential DNA extraction due to inefficient recovery of the cells from the swabs, highlighting the need for the development and comparative testing of alternative cell-separation techniques. Studies by Ballantyne in his NIJ funded studies, however, have shown that male DNA can be detected after several weeks when Y-STR is performed to detect sperm DNA.

DNA and associated proteins are fundamentally different in mature spermatozoa from other somatic cells (Balhorn 2007, Johnson et al. 2011, Boskovic and Torres-Padilla 2013, Ward 2010, Steger et al. 2000, Yoshii et al. 2005, Zini et al. 2007). A key element of this difference is the packaging of the DNA into chromatin through the use of protamine proteins in lieu of histones typically found in somatic cells where as much as 80-90% of the histones are replaced by protamines. This results in altered structure of the DNA, allowing compaction into the small volume of the sperm head. Protamines have an extremely high affinity for DNA and protect it from nuclease cleavage. Further, while intact sperm rapidly degrade in the vaginal environment (Vuichard et al. 2011), we believe protamine bound DNA is still largely protected in this environment and can be isolated for subsequent analysis. This has been shown as shown by the presence of Y-chromosome DNA several weeks post-coitus (Mayntz-Press et al. 2008). Recognizing that protamines held both high specificity and high affinity (possible off rates on the order of years) (Brewer, Corzett, and Balhorn 1999), we have now developed a sperm DNA capture approach based on antibodies raised against protamine proteins. We initially started with a traditional chromatin precipitation (ChIP) approach (Mercer 2013, Han 2013) and modified each step to be optimized for the capture of sperm DNA that is bound by protamines. In all other cell types (male and female), naked chromosomal DNA is nucleosomes composed of histone proteins.

Design and Methods:

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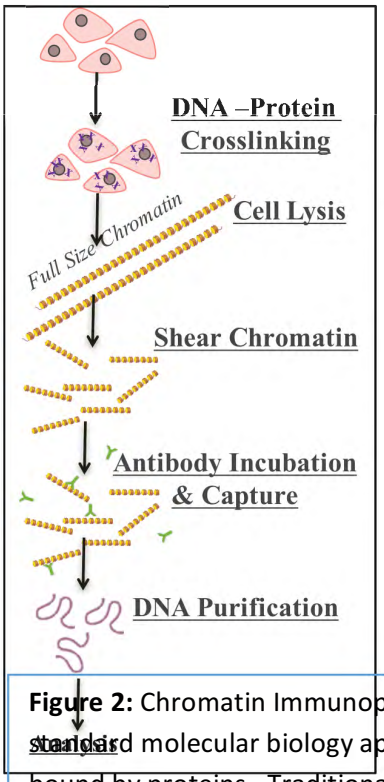

Figure 2: Chromatin Immunoprecipitation overview: ChIP is a standard molecular biology approach for isolating DNA that is bound by proteins. Traditionally, these interactions are weak and thus fixation is performed to stabilize the interactions allowing for

The approach taken to isolate DNA from sperm in mixed samples using the premises of Chromatin Immunoprecipitation (ChIP) as a method for capturing sperm specific DNA through antibody capture of the protamines associated with the sperm DNA and not other somatic DNA. ChIP is a well-established method for isolating specific DNA through affinity capture of associated proteins. ChIP has been used for isolation of protamine DNA complexes, though it has never been used for purifying sperm specific DNA for HID applications. A key advantage to this method is that it should be independent of the sample cells "intactness" which currently limits the time window from which suitable sample material can be obtained. The general method entails crosslinking of DNA binding proteins to the DNA with formaldehyde, lysing the cells, shearing the chromatin, capturing specific proteins bound to the DNA using antibodies, and finally eluting the DNA for downstream analysis such as sequencing.

Mock Forensic samples

All biological mock forensics sample were sourced from Lee Biosolutions℠ under internal IRB approved protocols. Both pre and post-coital vaginal swabs were self-collected by the donor Reference buccal swabs were generated by inserting a sterile cotton swab into the oral cavity. Fresh never frozen semen was self-collected by donor into sterile container and shipped overnight on wet ice. Upon receipt of fresh never frozen semen the spermatozoa count was assessed using C-Chip™ Disposable Hemacytometer (INCYTO™). To apply semen to sterile cotton swab, 50 µl of semen was diluted in 450 µl PBS buffer in a 1.7 ml micro centrifuge tube. Sterile cotton swab is inserted into tube completely submerging the swab head. Twist swab 180° back and forth five times. Remove swab and place in location to dry completely overnight. Alternatively, 15 µl of semen was diluted in 135 µl PBS buffer in a 1.7 ml micro centrifuge tube. Sterile cotton swab is inserted into tube and used to completely soak up semen dilution. Remove swab from tube and place in location to dry completely overnight.

Biacore Antibody Screen

To accurately identify promising antibodies screening work required they be screened independent the other method processes under development. Three separate assays were developed for use on a Biacore® 3000 (GE Healthcare) to assess antibody candidates' ability to bind mock presentations of sperm chromatin. To confirm binding of the antibody candidates' human protamine one and protamine two were loaded onto a CM5 flow cell (GE Healthcare) and presented with antibody candidates. To assess antibody/protamine binding sensor chips were prepared by first activating the surface by flowing 100 µl of 10 µg/ml EDC/NHS at a flow rate of 5 µl/min. Followed by human protamine binding flowing 100 µl of 50 µg/ml at a flow rate of 5 µl/min. Unbound amine chemistry was quenched by flowing 50 µl of 1 M ethanolamine at a flow rate of 10 µl/min. Sensor Chip was equilibrated using 1X PBS pH 7.4 buffer (Gibco™) flowing 100 µl at a flow rate of 10 µl/min. Antibodies were diluted to 0.2 mg/ml in 1X PBS pH7.4 prior to flowing 100 µl at 5 µl/min to assess

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binding. Background binding was quantified by flowing 100 µl of 0.2 mg/ml antibody flowing at 5 µl/min over a CM5 Sensor Chip that had been blocked by first surface activation and quenching as described above. Dissociation of antibody from protamine target was done using 40 µl of 10 µg/ml Glycine pH 2.0 at a flow rate of 10 µl/min. Sensor Chip were then re-equilibrated using 1X PBS pH 7.4 as described above prior to evaluation of the next antibody.

To assess antibody binding to protamine that has bound human genomic DNA CM5 Sensor Chips were also used. Surface activation was done by flowing 100 µl of 10 µg/ml EDC/NHS at a flow rate of 5 µl/min. Followed by human protamine binding flowing 100 µl of 50 µg/ml at a flow rate of 5 µl/min. Unbound amine chemistry was quenched by flowing 50 µl of 1 M ethanolamine at a flow rate of 10 µl/min. Human genomic DNA was loaded onto the bound protamine by flowing 100 µl of 10 µg/ml DNA at a flow rate of 5 µl/min. Sensor Chip was equilibrated using 1X PBS pH 7.4 buffer (Gibco™) flowing 100 µl at a flow rate of 10 µl/min. Antibodies were diluted to 0.2 mg/ml in 1X PBS pH7.4 prior to flowing 100 µl at 5 µl/min to assess binding. Background binding was quantified by flowing 100 µl of 0.2 mg/ml antibody flowing at 5 µl/min over a CM5 Sensor Chip that had been blocked by first surface activation and quenching as described above. Dissociation of antibody from protamine target was done using 40 µl of 10 µg/ml Glycine pH 2.0 at a flow rate of 10 µl/min. Human DNA was bound to protamines as previous prior to next antibody trail. Sensor Chip were then re-equilibrated using 1X PBS pH 7.4 as described above prior to evaluation of the next antibody.

A third scenario was generated using SA Sensor Chips to assemble a more realistic surrogate target for the antibodies to bind. Human genomic DNA was biotinylated using Nick Translation Kit (Amersham) and biotinylated dCTP (Thermo Fisher) per manufacturer's instructions. DNA was bound to the SA Sensor Chip surface by flowing 100 µl of 10 µg/ml biotinylated DNA at a flow rate of 5 µl/min., followed by human protamine binding flowing 100 µl of 50 µg/ml at a flow rate of 5 µl/min. Fragmented human genomic DNA was loaded onto the biotinylated DNA/protamine complex by flowing 100 µl of 10 µg/ml DNA at a flow rate of 5 µl/min. Sensor Chip was equilibrated using 1X PBS pH 7.4 buffer (Gibco™) flowing 100 µl at a flow rate of 10 µl/min. Antibodies were diluted to 0.2 mg/ml in 1X PBS pH7.4 prior to flowing 100 µl at 5 µl/min to assess binding. Background binding was quantified by generating a biotinylated DNA only Sensor Chip by flowing 100 µl of 10 µg/ml biotinylated DNA at a flow rate of 5 µl/min. Antibody candidates background binding was quantified by flowing 100 µl of 0.2 mg/ml antibody flowing at 5 µl/min over the DNA only Sensor Chip Dissociation of antibody from protamine target was done using 40 µl of 10 µg/ml Glycine pH 2.0 at a flow rate of 10 µl/min. Both human protamine and fragments human DNA was bound to the biotinylated DNA as previously described prior to next antibody trail. Sensor Chip were then re-equilibrated using 1X PBS pH 7.4 as described above prior to evaluation of the next antibody.

Conditioned Media MAb purification

Screening of monoclonal antibody clones from conditioned media was carrying out using HiTrap Protein A HP (GE Healthcare) prepacked columns on ÄKTA Avant (GE Healthcare) system per manufacturer's instructions. Eluted antibody collection fractions were concentrated and buffer exchanged into 1X PBS pH7.4 (Gibco) using Amicon® Untral-4 Centrifugal Filer Units (Millipore) per manufacturers instruction. Purified antibodies were quantified using a NanoDrop 2000 (Thermo Scientific) spectrophotometer per manufactures instruction.

Sperm DNA Capture Method

Lysis is performed directly on swab based or material cutting samples. Typical 1/3 swab cutting samples are placed in 2 ml Costar® Spin-X® Plastic Centrifuge Tube (Corning). To sample add 150 µl lysis buffer composed of 10 mM Tris pH8 (Life Technologies), 0.1 mM EDTA(Life Technologies), 0.5% SDS (w/v) (Life Technologies), and 20 mM DTT (Sigma) added just prior to lysis incubation. Incubate samples at 45°C in a Thermomixer (Eppendorf) mixing at 1000 RPM for 30 minutes. Following lysis incubation use forceps to remove sample substrate from lysis tube and insert it into spin basket before inserting the spin basket back into the 2 ml lysis tube. Centrifuge the Spin-X® tube at top speed (>20,000 RCF) for 1 minute. Remove and discard spin basket and sample substrate and discard to waste. In order to prepare the lysis solution for antibody incubation it needs to be cleaned up using the pre-packed mini spin trap consumable as detailed in the Sperm DNA Capture Protocol. Following lysis clean-up add 20 µl of 10X antibody incubation buffer composed of 17 mM Tris pH8(Life Technologies), 1.5% Triton X 100 (Sigma), 1.2 mM EDTA (Life Technologies), 167 mM NaCl (Sigma), and 0.01% SDS (w/v) (Life Technologies) to the cleared lysate. Antibody can either be added to sample alone or pre-conjugated to magnetic beads as described for each experiment. Following capture by magnetic beads, us a magnetic stand to pull the beads to the wall of the tube and using a pipet collect the supernatant fraction containing unbound DNA. To prepare supernatant fraction for quantification use the corresponding pre-packed mini spin trap consumable as detailed in the Sperm DNA Capture protocol. Wash the capture sperm DNA by adding 500 µl 1X PBS pH7.4 (Gibco) w/ 0.02% (w/v) TWEEN 20 (Sigma) and re-suspending the beads into solution. Use the magnetic stand to pull beads to the tube wall and aspirate wash buffer. Repeat PBS wash once more, followed by a TE buffer wash and subsequent resuspension in TE as per the Sperm DNA Capture Protocol. Sample elution was carried out using either Chelating Sepharose Fast Flow (GE Healthcare) media or heat based elution as described for each experiment.

Data Analysis and Key Findings:

Each step of the standard Chromatin precipitation (ChIP) protocol was evaluated as pertained to the successful capture of sperm DNA specifically. Key findings from the evaluation of each step are described below. Comprehensive data files and results will be included in an upcoming publication the team is preparing.

Cross-linking with formaldehyde is an initial step typically done to stabilize the DNA protein interactions. We found that the protamine DNA binding affinity is sufficient that this step is unnecessary. As a result, this allows us to also eliminate a cross-linking reversal step downstream.

Lysis of the sample is the next step in ChIP. Several key findings came about from this step during the optimization of lysis reagents used which included different lysis buffers and a time course evaluation. The first is that the conditions needed to be altered after we moved to swab material, and this was not altogether surprising, but does emphasize the value in working with swabs over liquid samples. The second finding was that significantly more DNA was recovered when lysis is done directly on the swab as opposed to releasing the cells from the swab prior to lysis, as is done with traditional differential extraction. This ultimately gives higher amounts of DNA for downstream analysis to be performed on, and strongly demonstrates that cell integrity is not required for the ChIP assay as it is for standard differential extraction. Finally, following lysis, we found we could simply add Captocore beads to the lysate to sequester out the SDS and DTT, which would be inhibitory to subsequent antibody capture.

Following lysis, typically either a DNAse treatment or sonication are done to fragment the DNA. We found this step completely unnecessary and the protamines seem to render the DNA itself resistant to DNAse digestion.

After sample lysis, an antibody specific to the target protein is added. The antibody used must be specific to the target, with sufficient affinity to allow down-steam capture. In the first year of the project, we evaluated 10 different antibodies that were a mix of monoclonal and polyclonal species from either mice or rabbits. A monoclonal antibody is ultimately desired for dissemination of the assay, as the generation of a polyclonal requires a new immunization for each lot. In addition to DNA capture, we used a Biacore assay to determine relative affinities. We identified a lead candidate from Briarpatch, but subsequently learned that the antibody was under license for other forensic applications. We then worked with Briarpatch for the generation of a new antibody, and over 24 clones were evaluated with preferential affinities to either P1 or P2 protamines. DNA capture amounts using a standard sample were compared to ELISAs run to determine affinity. One clone of each lot (P1 and P2) was expanded and are being used for final commercial development. Interestingly, the P2 antibody has shown higher pull down, and making a cocktail of the 2 antibodies did not have any significant advantage. These new antibodies are mouse monoclonals, thus ensuring long term supply of this critical

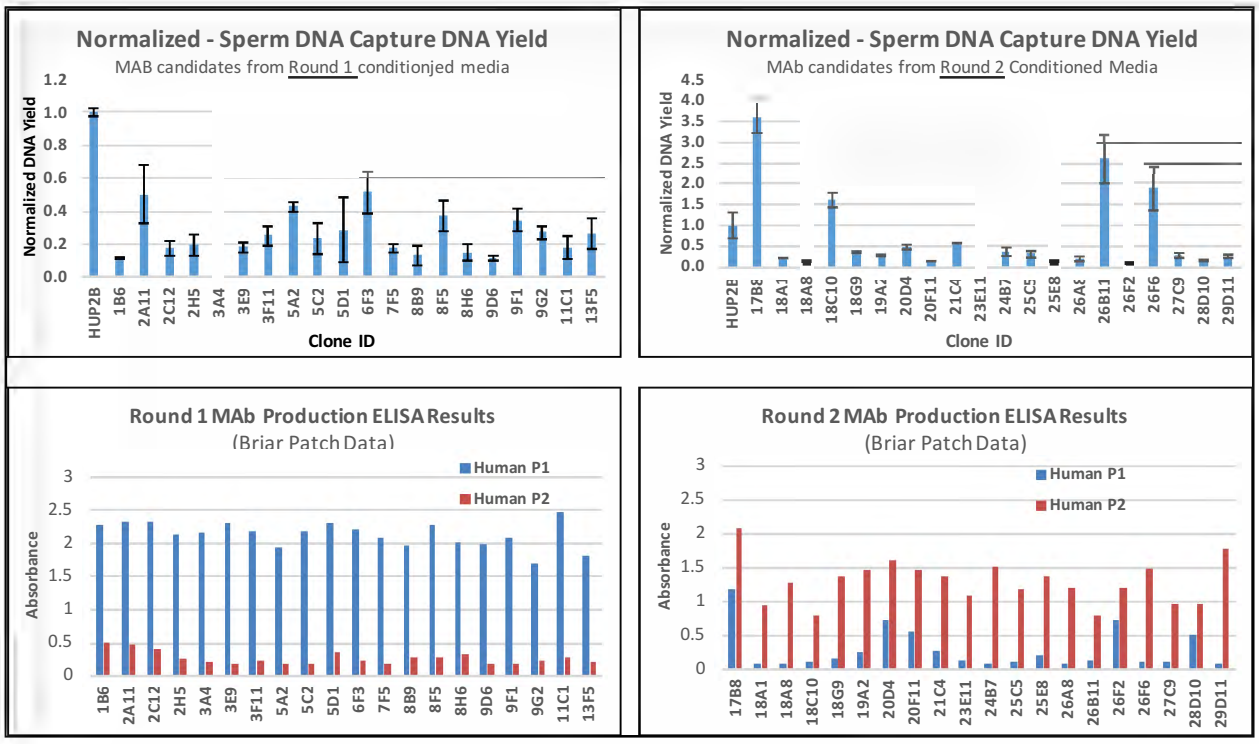

Figure 4: Testing of multiple new antibody clones for optimal performance with DNA capture from mixed samples. Antibodies were specific to either P1 or P2 or cross reacted with both as determined by ELISA (bottom panels) .

reagent. Dextran was tested as a crowding agent and showed limited improvement on sensitivity of the assay, though this will be re-evaluated in the preparation of the commercial assay as it has the potential to decrease amount of antibody needed or increase the relative affinity.

Antibody:protamine:DNA capture was evaluated using 2 different types of protein A/G conjugated beads. The first type are relatively large beads (Dynabeads) or smaller beads (Seramag), both of which

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can be pulled out of the sample with magnets. While the Seramag beads took some optimization, they ultimately gave equivalent performance to the Dynabeads,

and ultimately will cost less to include in a commercial kit for the sperm DNA capture. We evaluated a range of Bead:antibody ratios relative to total volume for the optimization.
Elution of DNA from the beads is the final step in the sperm DNA isolation. For this step, we tested washing conditions, elution buffer, time, and heat to derive optimal yields in a minimal volume and thus avoiding a concentration step prior to final analysis. One unexpected finding was that higher yields were obtained by having the final wash be in TE rather than the standard wash buffer. This is thought to possibly chelated out a cation and weakened the protamine DNA interactions, though this will require more extensive investigation to verify.
Throughout the studies the team relied on DNA quantifications for total human DNA and Y-chromosome specific DNA. These assays rely on PCR for quantification using standard curves, and thus we knew the end samples were PCR compatible. For selected experiments, STR analysis was performed to verify that the end sample was suitable for final assays and performed as well or better when compared to replicate samples processed using standard differential extractions.

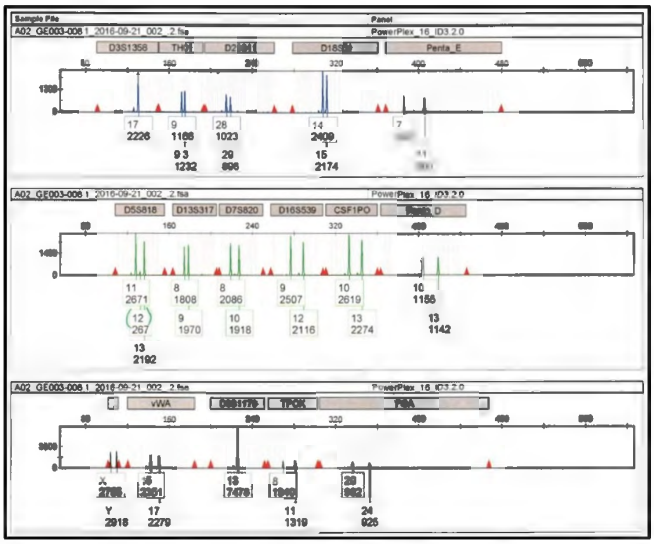

Figure 14: Example STR profiles show specific peaks for male profiles.

Implications to Forensic Community:

We successfully overcame technical risk to deliver a novel streamlined method with many benefits over differential extraction. Sperm's unique chromatin structure proved to be a near ideal target for immunocapture using ChIP. Interest from the forensic community as a viable replacement for differential extraction as well as from GE's commercial avenues was immediate. The method has been de-risked of any technical bottlenecks, and this project will result in a new commercial offering for the forensics community. We've demonstrated the high specificity of the method in mixed samples, and the ease of use when compared to differential extraction. Our method will be able to isolate sperm DNA from samples that have up until now been unsuitable for differential extraction. Leading to a higher success generated full autosomal STR profiles, reduced backlogs, and compliance with sample processing regulations.

Evaluation of automation for the assay

From program on set we have been designing the proof of concept development work with a path to a fully automated format. The flow-through lysis clean-up, magnetic beads, elution conditions are all amenable to automation on existing liquid handling robots. Discussion began during the program with robotics companies to collaborate and get Sperm DNA Capture onto their platforms. Deck layouts and method scripting has begun with the goals of fully automating the method under the NIJ award 2016-DN-BX-0156.

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Progress to Assay Commercialization

As a direct result of this program's success an internal new product introduction project was granted for GE Healthcare's HID & Forensic business. Final formulation and development work is ongoing during 2017. Initial commercial offering scheduled for early 2018 release.

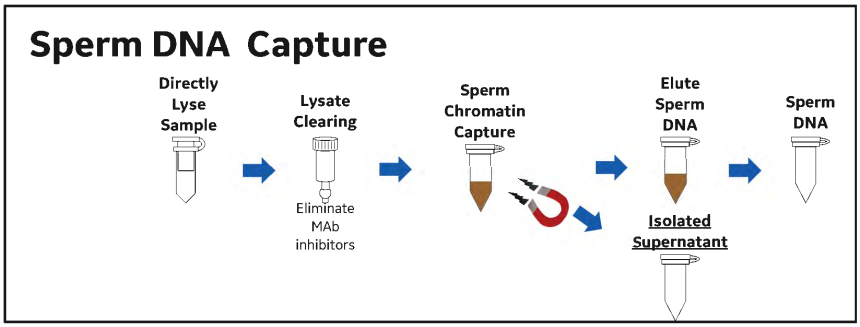

Figure 6: Schematic representation of final sperm DNA isolation by protamine affinity capture.

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