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Signed _____

Greggory S. LaBerge, Principal Investigator

Final Technical Report

"Sperm Capture Using Aptamer Based Technology"

NIJ Award #2007-DN-BX-K143

SomaLogic Inc., Boulder, Colorado and Denver Police Crime Laboratory, Denver, Colorado

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Abstract

The Denver Police Department Crime Laboratory Bureau in cooperation with SomaLogic, Inc., in Boulder, Colorado, have undertaken a research project to investigate if DNA aptamers can be developed to bind sperm heads, and used to both identify and immobilize the sperm heads for purification and later DNA analysis. Over the last two years, work has been conducted with commercially available sperm samples to develop a set of specific aptamer molecules, which are short strands of DNA that bind specifically to sperm heads. Research, to date, has identified several candidates with the ability to bind sperm under very stringent and selective conditions. Recently, these aptamers were tested in a magnetic bead system with Biotin and Streptavidin linkers and found to selectively cluster sperm cells. This is the first aptamer based system used in whole cell sperm capture. Further research is ongoing to fully characterize and optimize aptamers under various stringent and forensically relevant conditions, with the goal of developing an inexpensive and rapid system for sperm cell capture. The first phase of the project involved the isolation of pure sperm heads used as templates for the development of aptamer sequences to bind preferentially to both the outer protein membrane and the stripped perinuclear calyx of sperm cells. Both conditions yielded aptamers with the ability to bind the sperm heads specifically after counter selection experiments with HeLa cells to mimic non-sperm epithelial cells. Work is continuing to characterize the best candidate aptamers and establish manufacturing conditions for these sequences. As the project develops, a full forensic validation study will be conducted that addresses reproducibility, sensitivity, reliability, and both mock and adjudicated forensic case samples. This system has the potential to enable very high throughput systems for sperm capture and lysis of sperm cells based on magnetic beads, 96 well plastic plates, or other isolation techniques.

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Executive Summary

Evidence from sexual assault cases routinely consists of swabs taken during the examination of victims in the hospital in addition to evidence collected from crime scenes. This evidence is commonly comprised of a mixture of body fluids containing both male and female cells with DNA. The separation of spermatozoa containing the putative perpetrator's DNA from the victim's DNA is crucial in the analysis of sexual assault case evidence. Improved automation of sperm head detection and separation will enable crime laboratories to process more sexual assault cases in a timely manner and populate the CODIS database with more evidentiary samples from sexual assaults.

The goal of this research project was to use aptamer based DNA capture technology, in partnership with SomaLogic, to specifically capture sperm cells for forensic applications. We focused our work on the development of a system to enable the separation of spermatozoa from female epithelial cells in mixed sexual assault evidence, and result in the amplification of male DNA from the putative perpetrator in a rapid, cost effective, and high throughput format, amenable to robotic workstations that are becoming more common in forensic laboratories. Aptamers are short DNA molecules that have the molecular specificity and affinity recognition properties of antibodies; however aptamer DNA molecules are more stable than antibodies for uses involving "affinity" chromatography since their complex, three-dimensional structures are capable of reforming after denaturation. Aptamers are reusable molecules that can routinely be chemically modified to increase their stability. They are currently used as diagnostic reagents in various applications, such as in the detection of environmental contaminants in biosensors, as well as monitoring carcinogen or drug levels in the blood of patients. Additionally, they can be used in therapeutics for the treatment of disease. Aptamer targets can include both intracellular proteins, such as transcription factors; extracellular proteins (such as growth factors or coagulation factors) or more complex targets such as viruses or bacteria.

The specific objective of the project was to develop aptamers with the highest possible affinity and specificity to capture and immobilize intact sperm cells in the presence of female epithelial cells and other non-sperm semen components.

The longer term vision for this research is directed toward the development of a cost-effective aptamer based assay suitable for high throughput analysis that can easily be implemented in forensic laboratories. A suitable matrix for aptamer binding will be selected (for example streptavidin bead systems or coated 96 well plates) and the optimal procedure to isolate DNA for subsequent STR analysis will be developed. Later stages of this research are focusing on development of the sperm-capture assay as a commercial product at SomaLogic.

Results, Findings and Conclusions:

Development of aptamers specific for protein components of sperm cells:

The timeframe for the original project was 18 months, which was extended to meet the goals of the project. Commercially available spermatozoa from different donors were isolated and used in several selective rounds to achieve aptamer specificity for human sperm cells. Strategies to avoid possible individual genetic variations in the sperm surface structure were used, such as stripping the outer sperm cell membrane with Triton X100, as well as reducing any non-specific binding to epithelial cells and non-sperm semen components by using counter selection procedures.

Several rounds of aptamer selection were run according to a custom protocol against pooled sperm samples. This protocol was designed to select aptamers that retain specific sperm-binding properties in the presence of any contaminating materials that might be found in forensic samples. Such contaminating materials would be expected to include human epithelia, but may also include proteolytic and nucleolytic activities contributed by contaminating bacteria. In addition, the selection procedure was designed to generate aptamers that target common epitopes, also called apatopes (target structures bound by aptamers), on all sperm, independent of donor age or ethnicity. This was accomplished by using a selection medium that contained a high concentration of Triton X100 non-ionic detergent. This buffer stripped away the plasma

membrane of the sperm cells, exposing the highly conserved proteins of sperm perinuclear calyx. To reduce or eliminate any cross reactivity with epithelial cells, the selection was designed to selectively subtract aptamers that bind to these cell types. HeLa cells were used for this step as they were readily available and were a reasonable substitute for human epithelial cells. As a safeguard against nucelolytic activity, magnesium (a necessary cofactor for nearly all known deoxyribonucleases), was omitted from the selection medium. High sodium chloride concentrations, inhibitory to nucleases, were also included to enforce specificity and to minimize nuclease activity. A heavy burden of nucleic acid competitors was included to ensure performance in the presence of a heavy burden of released DNA from lysed epithelia. Future refinements could include epithelial cells from donors as the system becomes more established.

Seven rounds of selection were performed, with selection monitored by Cot curves, which provide a measure of the complexity of a given nucleic acid population and are commonly used to assess the degree of completion of a SELEX experiment. Figure 1 depicts the results of a large-scale screen of cloned aptamers from the seventh round of selection against sperm cells. Aptamers chosen for immediate characterization are shown in green.

Single-point (1 x10^5 cells/uL) Affinity Assay

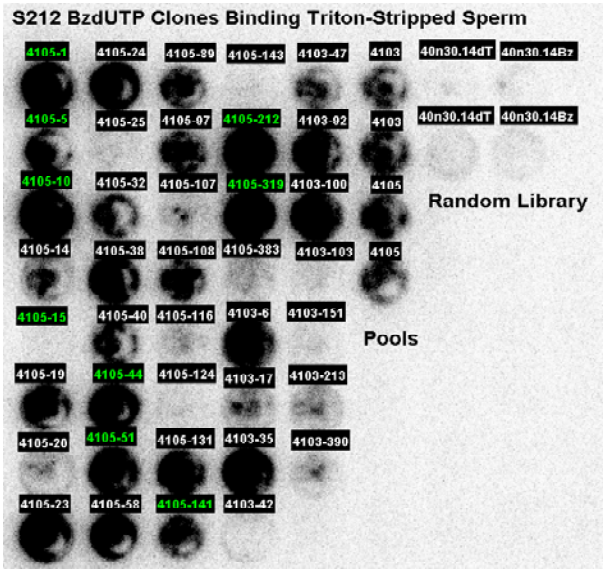


Figure 1 *Affinity assay showing binding to human sperm cells indicating results of the seventh round of aptamer selection against human sperm cells. Green highlights represent aptamers selected for further molecular characterization.*

A control experiment in which HeLa cells were substituted for sperm cells showed no significant binding of any screened aptamers to HeLa cells (data not shown).

Formal binding curves of selected aptamers from the large-scale screen were generated. Sperm cells (heads with intact tails and heads alone) (Figure 2 left panel) or HeLa cells (Figure 2 right panel) were incubated with the indicated radiolabeled aptamer, in the presence of tRNA and herring sperm DNA competitors (1 mg/ml each) for 60 minutes at 37° C. Mixtures were then filtered through DuraPore filters (0.45 uM pore size) and washed. Retained radioactivity was quantified by autoradiography on phosphor screens and analyzed using a Fuji BioAnlalytic scanner. The fraction bound was defined as the total radioactivity retained on the washed DuraPore filter divided by total radioactivity contained within the reaction. Total radioactivity was determined by spotting a portion of reaction mixtures onto Durapore filters and subsequent autoradiography without further processing. This experiment clearly demonstrates that the aptamers selected have bound to sperm cells (heads with intact tails and heads alone) and not to HeLa cells that were used as a surrogate for human epithelial cells.

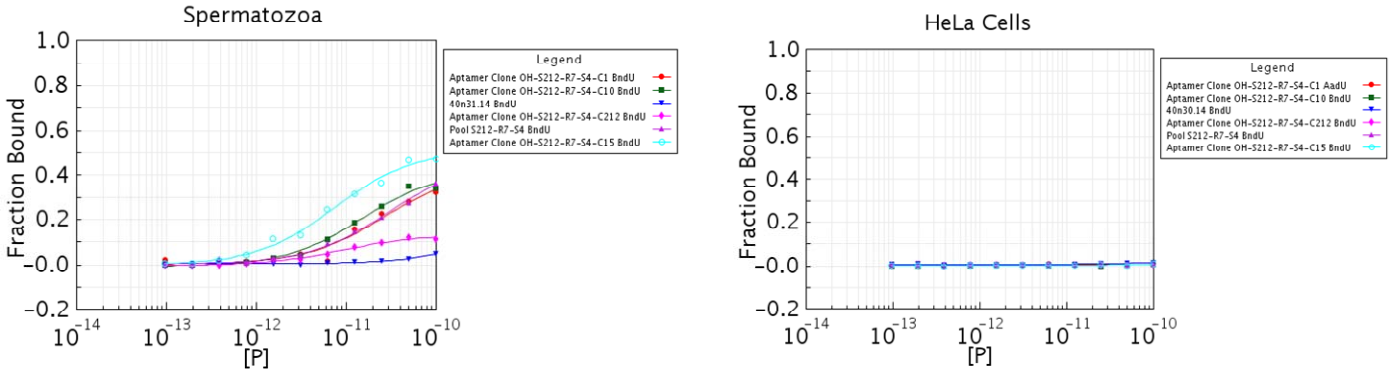


Figure 2 *Binding curves of selected radiolabeled aptamers from previous rounds of selection to both human sperm cells and HeLa cells. Total radioactivity retained is plotted as fraction of cells bound to filters versus concentration of cells.*

As this work continues, we will also test dried sperm samples that mimic forensic samples and continue selection with more forensically relevant materials.

<u>Elution of Sperm from Cotton swabs as targets for capture:</u>

While a formal dissociation constant cannot be determined owing to the unknown nature and quantity of specific protein targets, we have documented that binding of selected

aptamers to sperm cells is strong and specific, with little or no binding to control HeLa cells observed. Future experiments will address directly assess the capability of individual aptamers to bind sperm in the presence large quantities of contaminating human epithelial cells.

Elution of Sperm from Cotton swabs as targets for capture:

There are currently two media commonly used for sperm elution from cotton swabs to collect forensic samples. The first is phosphate buffered saline, which is inefficient (recoveries typically less than 10%). The second is a 1% solution of sodium lauryl sulfate (sodium dodecyl sulfate, SDS), used in the differential extraction method. SDS is an efficient medium for recovery; however, it is incompatible with aptamer binding and is normally used with centrifugation-based wash steps, which are low-yielding, require large amounts of sperm cells at the outset of the operation, and is time consuming. Our C2 selection buffer was designed to support efficient elution of sperm from forensic samples as well as aptamer binding.

We compared the efficiency of elution of sperm from cotton swabs to which semen had been absorbed and dried in C2 selection buffers at various concentrations with a solution containing 1% SDS (intended to mimic differential extraction elution buffer). In these pilot experiments, we found that C2 selection buffer supported recoveries that compared favorably with the recoveries supported by SDS. Recoveries ranged from 44% of the SDS control (C2 selection buffer) to 78% of the SDS control (2-fold concentrated C2 selection buffer. More concentrated C2 buffer (4-fold concentrated) yielded no obvious improvement, with 54% recovery relative to the SDS control.

Aptamer target identification:

One consideration for selection of the proper aptamer for use in sperm capture is the protein target. Desirable protein targets for sperm capture aptamers are sperm structural proteins present in all, or nearly all, of the sperm-producing population. Hence, we have commenced efforts to identify the targets of aptamers isolated in our whole-cell selection.

Preliminary data suggests that we can detect specific aptamer-binding activity in urea extracts. The fact that such a binding activity can be detected in soluble extracts suggests that aptamer targets can be purified by affinity chromatography. We are currently developing an affinity chromatography strategy and hope to have enough relatively pure protein to identify the protein targets of these aptamers by mass spectroscopy.

Capture of sperm by biotinylated aptamers and magnetic streptavidin beads:

As proof-of-principle, we tested the ability of one of the aptamers we generated to mediate capture of sperm cells onto magnetic streptavidin beads. Biotinylated aptamer (Figure 1, upper left, 4105-1) was immobilized on streptavidin-coated magnetic beads. The aptamer-substituted beads were incubated with sperm in the presence of a competitor cocktail (herring sperm DNA, tRNA, and dextran sulfate). The mixture was pipetted onto a microscope slide and a small magnet placed near the droplet for a short period. Figure 3 depicts light micrographs of bead-and-sperm mixtures, with and without added aptamer. Clearly sperm cells can be isolated and magnetically clustered as in panels B and C.

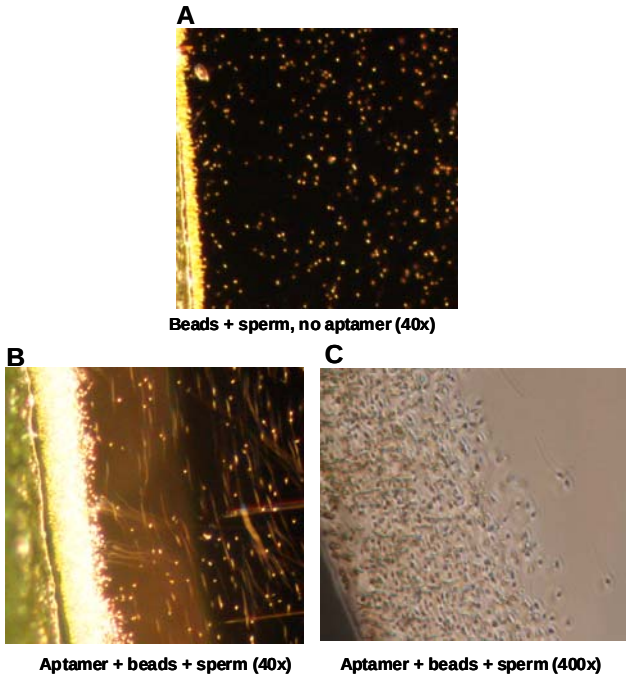


Figure 3 *Light micrographs of human sperm cell capture by biotinylated aptamer 4105-1 and magnetic streptavidin beads.*

Further research beyond the scope of this project and future development:

Following aptamer identification, the focus of the work will be on the selection of a suitable matrix for the development of a sperm-capture assay. The goal is to covalently link the synthesized aptamers to a solid matrix by coating the surface of a tube, a 96-well plate, slide, column/beads, or other suitable matrix. Swabs prepared to mimic sexual assault evidence will be eluted in sample buffers, providing sperm cells. Dilute sperm cells can be captured using magnetic beads (as presented in Figure 3), 96-well plates, or lateral flow devices. We are particularly interested in lateral flow devices, in which isolated spermatozoa are observed and quantified using secondary aptamers labeled with visible reporters in a manner similar to sandwich immuno-assays with antibodies used for clinical diagnostics [19]. We anticipate that one or more of our sperm-specific aptamers will be labeled with a visible dye and used to quantify the sperm attached to the "band" of capture aptamers.

Further experiments will concentrate on optimizing the specific covalent aptamer binding and sperm immobilization to the selected matrix while recovering epithelial cells and non-sperm semen components. This will also test aptamers for their ability to bind to varying small numbers of sperm in mixtures with female epithelial cells. Wash steps or cellular digestion will allow for the complete removal of non-sperm cells while retaining immobilized sperm cells. The development of a procedure to isolate DNA from the immobilized spermatozoa will follow, potentially involving either strong detergent lysis or an enzymatic digestion step to lyse the sperm cell wall. Also, the potential use of DNA isolation using magnetic beads has been tested with streptavidin beads and the results are presented in Figure 3.

During the development of these protocols, the suitability for high throughput processing has been evaluated as well as the potential for automation of this sperm-capture system. The expected affinity of isolated aptamers to spermatozoa should enable the binding of a single sperm cell. This sensitivity could allow for an increase of the post-coital interval in which sperm can be detected beyond the traditional 24-72 hour time interval [31]. Additionally, the coating of a potential matrix could be manipulated to enable the binding of sperm cells at a specific density. This would allow for a constant

amount of DNA being isolated in the sperm-capture assay and would potentially make DNA quantitation steps obsolete, thereby further increasing the processing speed of sexual assault evidence.

Implications for Policy and Practice:

The potential impact of this proposed project could be very significant for the forensic community in terms of sexual assault case backlog reduction. The National Forensic DNA Study Report from December 2003 [34] estimated a backlog of 169,000 rape cases in the United States; therefore, DNA laboratories could very strongly benefit from a cost effective assay that allows for fast, easy and reliable processing of sexual assault evidence.

This sperm capture assay will allow for faster processing of evidence from sexual assault cases and can be used in all forensic laboratories. By using the developed assay, will decrease the amount of time required to obtain a DNA profile from a putative perpetrator will decrease and may result in a faster identification and prosecution of suspects, possibly preventing later crimes. Additionally, more profiles will be created for entry in the CODIS database, which in turn can help link and potentially solve further crimes. In cases without a suspect and no immediate identification of the putative perpetrator, a DNA profile can lead to a John Doe warrant, which halts the Statute of Limitations.

Final Technical Report

I. Introduction

1. Statement of the problem:

According to the Federal Bureau of Investigation's Uniform Crime Reports, 93,934 forcible rapes were reported in the United States in 2005 [1]. Evidence from sexual assault cases routinely consist of swabs taken during the examination of victims in the hospital and evidence collected from crime scenes. This evidence is commonly comprised of a mixture of body fluids containing both male and female cells with DNA. The separation of spermatozoa containing the putative perpetrator's DNA from the victim's DNA is crucial in the analysis of sexual assault case evidence. Improved automation of sperm head detection and separation will enable crime laboratories to process more sexual assault cases in a timely manner and populate the CODIS database with more evidentiary samples from sexual assaults.

2. Literature citations and review:

The most widely used protocols in the analysis of sexual assault evidence are based on the differential extraction procedure originally described by Gill *et al*., 1985 [2]. This method is used to enrich sperm cells in the presence of an excess of epithelial cells, creating a separation of DNA profiles by differential lysis of both types of cells. The process is time consuming, labor intensive, and results in possible DNA mixtures due to incomplete separation of the different cell types, especially when sperm head counts are low. Commercially available kits, like the Differex™ System by the Promega Corporation [3] are improvements on traditional methods through the addition of a Separation Solution that is meant to enable an easier and more efficient separation of epithelial and sperm fractions followed by using a paramagnetic resin for DNA isolation [4], but these protocols still require a significant amount of handling by scientific personnel.

The same argument can be made for other recent improvements to the traditional differential extraction procedure. The addition of cellulose-digesting enzymes can

enhance the release of sperm and epithelial cells from a cotton swab, especially for older swabs with decreased sperm cell recovery due to longer drying times [5]. The application of sperm and epithelial fractions to FTA cards allows for direct amplification without an extraction step [6]. Both methods, however, still require a great deal of sample handling.

Several recent publications deal with possible improvements and partial automation of existing protocols. The use of the paramagnetic resin for DNA isolation in the DNA IQ™ system, together with the BioMek 2000 Laboratory Automation Workstation, has been described as suitable for forensic casework samples [7]. Additionally, new technologies are described in the literature which involve separation of sperm and epithelial cells before DNA extraction. Microfabricated devices exploit the differential physical properties of sperm cells versus other cells by allowing for the sedimentation and adsorption of epithelial cells to the bottom of an inlet reservoir on a glass microfluidic device. Subsequent buffer flow through the system causes spermatozoa to be washed out into the outlet reservoir resulting in the separation of the different cell types [8]. Microchip-Based Cell Lysis [9] allows for a silica-based DNA extraction method in a micro-chip based format. However, it requires the application of a solution to wash sperms cells. Multiple publications describe the uses of Laser Microdissection [10, 11, 12] to selectively capture and isolate sperm cells. This method uses a laser beam fitted with an optical microscope to cut cells of interest from cell smears. This results in high specificity and allows the use of limited numbers of spermatozoa on microscope slides for DNA extraction and Forensic Short Tandem Repeat (STR) analysis. This system is time-consuming, labor-intensive and not easily amenable to automation.

Efforts directed towards improving the differential extraction process and sperm separation are also featured on the website of "The President's DNA Initiative - Advancing Justice Through DNA Technology" [13] with the physical separation of cells identified as a high priority need by the DNA Forensics Technical Working Group. A total of seven research projects on this topic were sponsored by the National Institute of Justice (NIJ) in 2005, with an additional two projects in 2006 [13].

3. Statement of rationale for this research and background information:

Since its first description in 1990 [14], a process called the 'systematic evolution of ligands by exponential enrichment' (SELEX) has been used to identify single-stranded nucleic-acid molecules called aptamers. Aptamers have the molecular specificity and affinity recognition properties of antibodies; however aptamer DNA molecules are more stable than antibodies for uses involving "affinity" chromatography since their complex, three-dimensional structures are capable of reforming after denaturation [15, 16, 17]. Numerous applications of aptamers are reviewed in Bunka and Stockley, 2006 [15] including their use as affinity purification media in biotechnology due to the high specificity and high affinity to target proteins. Aptamers are reusable molecules that can routinely be chemically modified to increase their stability. They are currently used as diagnostic reagents in various applications, such as in the detection of environmental contaminants in biosensors, as well as monitoring carcinogen or drug levels in the blood of patients. Additionally, they can be used in therapeutics for the treatment of disease [15]. Aptamer targets can include both intracellular proteins, such as transcription factors; extracellular proteins, such as growth or coagulation factors; or more complex targets like viruses or bacteria. In December 2004, the Food and Drug Administration approved the first aptamer-based therapeutic agent for the treatment of age-related macular degeneration [18]. This therapeutic agent, Pegaptanib sodium (Macugen, Eyetech Pharmaceuticals/Pfizer) is an RNA aptamer directed against vascular endothelial growth factor (VEGF)-165, the VEGF isoform primarily responsible for pathological ocular neovascularization and vascular permeability. Through binding of this growth factor, the drug was shown in clinical trials to be effective in treating choroidal neovascularization associated with age-related macular degeneration. This is the first aptamer therapeutic approved for use in humans, paving the way for future aptamer applications.

In another example of aptamer applications, arrays of aptamers are used to discover protein signatures of disease. Many diseases involve multiple, often overlapping, physiologic and pathophysiologic processes. Rarely does one biomarker provide sufficient information in medical diagnostics. Therefore, patterns of biomarkers or disease signatures reflect the true complexity of the disease. By using aptamer arrays,

multiple protein markers can be measured with high specificity and low background noise [19].

The SELEX process has been successfully used to create aptamers that are specific to human osteoblasts and are capable of capturing target cells out of a cell solution [20]. Furthermore, when these aptamers were immobilized onto titanium alloy surfaces, they worked as capture molecules and allowed the attachment of osteoblasts and their subsequent growth on the alloy surface [21]. This improves cell seeding efficiency and cytocompatibility, allowing for the development of optimized bone-implants. This principle for the application of surface-coated aptamers as capture molecules facilitating cell adhesion offers the possibility of further optimizing and developing the surface properties of medical implants and scaffolds for various therapeutic applications and opens new perspectives towards clinical applications of stem cell and tissue engineering strategies.

The SELEX process allows for the identification of RNA or DNA aptamers that bind to a variety of defined molecular targets with high affinity and specificity. In a modification of the process, called cell-SELEX, the aptamer targets are located on the surface of entire cells, such as glioblastoma cells [22], leukemia cells [23] or tumor microvessels [24]. To increase the specificity of the aptamer pool, a counter-selection strategy is used ensuring that the targets bound by the aptamers are specific for the desired target cells but not for the control cells. The entire process, as previously described in [23], involves the incubation of target cells with a library consisting of a large pool of random single-stranded (ss) DNA oligonucleotides. The cells are washed, and the ssDNA oligonucleotides, potentially aptamers, bound to the cell surfaces are eluted by heating to 95° C. The collected aptamers are then allowed to interact with excess negative control cells and only DNA sequences remaining free in the supernatant will be collected and amplified for a next-round selection, thereby removing aptamers that bind to the control cells. This process is repeated between 5 and 15 times. After multiple rounds of selection, the process will result in a small population of ssDNA aptamers that bind to the target cell but do not bind to the control cell. These aptamers can then be cloned, sequenced and further characterized to identify a single aptamer with the best

overall binding properties. This cell-based selection process can be done without prior knowledge of target molecules on the cell surface and, through the effective counter-SELEX process, result in the isolation of highly specific aptamers.

Human semen consists of approximately 200 to 500 million spermatozoa per ejaculate and seminal plasma, which is composed of a complex range of organic and inorganic constituents. These constituents include various proteins and enzymes, such as prostate acid phosphate, prostate specific antigen, proteolytic enzymes, amino acids, citrate, flavins, fructose, phosphorylcholine, prostaglandins, vitamin C, zinc, galactose, sialic acid, and various alkaline bases [25]. A normal human sperm head ranges between 2.5 and 3.5 microns in width and 5 to 7 microns in length and includes several sperm-specific surface proteins in its basic structure. These proteins are unique, cell-specific, immunogenic, and are involved in the ability of sperm to bind to ovulated eggs [26, 27]. Sperm cells have a plasma membrane that plays a very active role in sperm fertilization capacity and its biochemical composition remains a field of interest for current research. A network of disulfide bonds leaves the sperm membrane more resistant to digestion than other membranes [28], an attribute that is utilized in current differential extraction protocols. This stability, together with the number of sperm-specific proteins as possible targets, makes sperm cells good candidates for an aptamer-based sperm capture system.

The goal of this research project was to use aptamer based DNA capture technology, in partnership with SomaLogic, to specifically capture sperm cells for forensic applications. We focused our work on the development of a system to enable the separation of spermatozoa from female epithelial cells in mixed sexual assault evidence and result in the amplification of male DNA from the putative perpetrator in a rapid, cost effective, and high throughput format, amenable to robotic workstations that are becoming more common in forensic laboratories.

The specific objective of the project was to develop aptamers with the highest possible affinity and specificity to capture and immobilize intact sperm cells in the presence of female epithelial cells and other non-sperm semen components.

The longer term vision for this research is directed toward the development of a cost-effective aptamer based assay suitable for high throughput analysis that can easily be implemented in forensic laboratories. A suitable matrix for aptamer binding will be selected (for example streptavidin bead systems or coated 96 well plates) and the optimal procedure to isolate DNA for subsequent STR analysis will be developed. Later stages of this research are focusing on development of the sperm-capture assay as a commercial product at SomaLogic.

II. Methods

Research Design:

Development of aptamers specific for protein components of sperm cells The timeframe for the original project was18 months and extended to meet the goals of the project. Commercially available spermatozoa from different donors were isolated and used in several selective rounds to achieve aptamer specificity for human sperm cells. Strategies to avoid possible individual genetic variations in the sperm surface structure were used like stripping the outer sperm cell membrane with Triton X100 as well as reducing any non-specific binding to epithelial cells and non-sperm semen components by using counter selection procedures.

Several rounds of the SELEX aptamer design and selection process were run according to standard SomaLogic protocols against pooled sperm samples. Aptamers were isolated that are capable of binding to common epitopes, also called apatopes (target structures bound by aptamers), on all sperm, independent of donor age or ethnicity. This was accomplished by using an elution buffer that contained a high concentration of Triton X100 non-ionic detergent. This buffer stripped away the plasma membrane of the sperm cells exposing the sperm perinuclear calyx. This cytoskeletal structure was used as a target for aptamer design. To reduce or eliminate any cross reactivity with non-sperm components such as epithelial cells, experiments were designed to selectively subtract aptamers that bind to these cell types. HeLa cells were used for this

step as they were readily available and were a reasonable substitute for human epithelial cells. Future refinement could include epithelial cells from donors as the system becomes more established. These counter-SELEX protocols [23, 29, 30] guarantee the kinds of specificity required for efficient spermatozoa capture.

After completion of counter selection experiments, we focused on development of specific aptamer pools by conducting convergence experiments. After about 6 rounds of aptamer selection to sperm cells a smaller number of aptamer sequences was isolated from millions of possibilities. These sequences formed the basis for a round 7 of selection for sperm binding presented below in the materials and methods section. 9 aptamers have been identified for characterization and are currently being sequenced. As this work continues we will also test dried sperm samples that mimic forensic samples and continue selection with more forensically relevant materials such as adjudicated case samples.

Following the aptamer identification, the focus of the work will be on the selection of a suitable matrix for the development of a sperm-capture assay. The goal is to covalently link the synthesized aptamers to a solid matrix by coating the surface of a tube, a 96-well plate, slide, column/beads, or other suitable matrices. Swabs prepared to mimic sexual assault evidence will be eluted in sample buffers, providing sperm cells. Dilute sperm cells can be captured using magnetic beads, 96-well plates, or lateral flow devices. We are particularly interested in lateral flow devices in which isolated spermatozoa are observed and quantified using secondary aptamers labeled with visible reporters in a manner similar to sandwich immuno-assays with antibodies used for clinical diagnostics [19]. We anticipate that one or more of our sperm-specific aptamers could be labeled with a visible dye and used to quantify the sperm attached to the “band” of capture aptamers.

Further experiments will concentrate on optimizing the specific covalent aptamer binding and sperm immobilization to the selected matrix while recovering epithelial cells and non-sperm semen components. This will also test aptamers for their ability to bind to varying small numbers of sperm in mixtures with female epithelial cells. Wash steps

or cellular digestion will allow for the complete removal of non-sperm cells while retaining immobilized sperm cells. The development of a procedure to isolate DNA from the immobilized spermatozoa will follow, potentially involving either strong detergent lysis or an enzymatic digestion step to lyse the sperm cell wall. Also, the potential use of DNA isolation using magnetic beads has been tested with streptavidin beads.

During the development of these protocols, the suitability for high throughput processing has been evaluated as well as the potential for automation of this sperm-capture system. The expected affinity of isolated aptamers to spermatozoa should enable the binding of a single sperm cell. This sensitivity could allow for an increase of the post-coital interval in which sperm can be detected beyond the traditional 24-72 hour time interval [31]. Additionally, the coating of a potential matrix could be manipulated to enable the binding of sperm cells at a specific density. This would allow for a constant amount of DNA being isolated in the sperm-capture assay and would potentially make DNA quantitation steps obsolete, thereby further increasing the processing speed of sexual assault evidence samples.

Methods, Materials, and Procedures:

Purchased materials: sperm cells (research vials, prepared by density gradient centrifugation and subsequent washing) were purchased from California Cryobank. Oligonucleotides were synthesized and purified in-house by standard methods. Bacterial plasmids encoding sperm membrane proteins were purchased from Blue Heron Biotechnology. Radiolabeled nucleoside triphosphates and deoxynucleoside triphosphates were purchased from Perkin-Elmer. Magnetic streptavidin beads (MyOne C1) were purchased from Invitrogen. KOD DNA polymerase was purchased from EMD Biosciences.

Buffers: C1 selection buffer consisted of 40 mM Hepes, 350 mM NaCl, 1 mM EDTA, and 0.5 mM EGTA. C2 selection buffer consisted of 40 mM Hepes, 350 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, and 0.1 % w/v Triton X100 detergent. Elution buffer consisted of 40 mM Hepes pH 7.5, 0.025% sodium dodecyl sulfate, and 5 mM EDTA. SQ20 buffer is a proprietary formulation that has been found to support DNA

polymerase activity somewhat more effectively than conventional amplification and primer extension buffers.

Amplification reactions: Aptamer pool DNA was amplified by an initial step of primer extension at 76 C (30 minutes), followed by 30 rounds of standard amplification. The initial primer extension step is necessitated by the relative inefficiency of replication of benzyl dU-containing templates.

Aptamer libraries: A biotinylated oligonucleotide library consisting of 40 random deoxynucleotides flanked by constant regions (ggcagtccgtccgtc at the 5' end; gccagaagcagaaggacg at the 3' end) was synthesized and purified via standard methods. Aptamer libraries were prepared by annealing deoxynucleotide primers complementary to the 3' constant region of the oligonucleotide library, then immobilizing the primed library on MyOne magnetic streptavidin beads. A mixture consisting of deoxynucleoside triphosphates (0.8 mM each) and KOD DNA polymerase (0.1 unit/ul) in SQ20 buffer was then added, and the mix was incubated for two hours at 68 C. The newly synthesized DNA's, comprising the aptamer libraries, were eluted with 20 mM NaOH, and neutralized. Aptamer libraries were concentrated on YM10 centrifugal ultrafiltration units, quantified, and analyzed by gel electrophoresis. Two aptamer libraries were prepared: one bearing the four canonical deoxynucleoside triphosphates, the other substituting benzylated deoxyuridine triphosphate for deoxythymidine triphosphate. Aptamer selections that employ benzyl-substituted deoxynucleotide residues have been found to yield higher affinity aptamers and have a higher success rate than selections performed with conventional dT-bearing libraries.

Enzymatically prepared aptamer ("eDNA"): eDNA was generated by amplification of aptamer pools with a 5' unsubstituted primer and a biotinylated 3' primer. The amplified DNA was adsorbed to magnetic streptavidin beads, and the non-template strand of DNA stripped from the immobilized DNA with 20 mM NaOH, generating an immobilized template DNA. Primer complementary to the 5' end of the immobilized template, KOD DNA polymerase, and deoxynucleoside triphosphates (0.8 mM) were then added. The mixture was incubated at 75 C for 1 hour with shaking. The extended products were

recovered by elution with 20 mM NaOH and neutralized. The products were evaluated by gel electrophoresis.

Sperm cell preparation: Washed sperm cells were prepared either by three washes and suspension in C1 buffer, or by three washes in C2 buffer supplemented with Triton X100 detergent and NaCl to final concentrations of 1% v/v and 600 mM, respectively, followed by suspension in C2 buffer.

Aptamer selection: Approximately 150 ul of 16 uM dT-containing oligonucleotide library or 150 ul of 16 uM of benzyl-dU library (2.4 nMol, or 1.5 x 10 E15 molecules) in C1 or C2 buffer were heated to 95 C, then slow-cooled to 37 C. Aptamer libraries were then combined with an equal volume of a competitor solution composed of 2 mg/ml herring sperm DNA, 2 mg/ml yeast tRNA, 200 uM dextran sulfate, 2 uM prothrombin, 2 uM casein, and 2 mg/ml human serum albumin in 1X C1 or C2 buffer. Note that the high-salt conditions and high concentrations of competitor were found to be necessary to diminish non-specific binding of aptamer library to sperm cells. 10 E7 HeLa cells were added and the mixtures were incubated for ten minutes. HeLa cells and debris were removed by centrifugation. Sperm cells (10 E7) were then added to the preadsorbed libraries. The mixtures were incubated for 1 hour. The mixtures were centrifuged at 6000 x *g* for 5 minutes, and the supernatants removed. Sperm cells were suspended in C1 or C2 buffer, mixed, then centrifuged and the supernatants removed. This wash step was repeated 8 times. Adsorbed aptamer was eluted from sperm cells by addition of a solution consisting of 40 mM Hepes (pH 7.5), 0.025% SDS, 5 mM EDTA, and 50 ug/ml proteinase K, incubation at 55 C for 15 minutes, and removal of sperm cells by centrifugation. Eluted aptamer DNA was purified by adsorption to “primer beads”, which are magnetic streptavidin beads to which biotinylated oligonucleotide complementary to the 3’ constant primer regions of the aptamer library had been adsorbed. Beads were washed and aptamer recovered by elution with 20 mM NaOH. Eluted aptamer solutions were neutralized, amplified, and eDNA made as described above. Subsequent rounds of were performed in a similar fashion, albeit using eDNA from the previous round in lieu of aptamer library. Seven rounds of selection were performed. The progress of the selection was monitored at the end of each round via measurement of the rate and

extent of reassociation of thermally denatured aptamer pools. These so-called C0t curves reflect the complexity of a DNA population and thus serve as a measure of the of Rounds 3-7 included incubations for increasing periods of time in a solution of 10 mM dextran sulfate in C1 or C2 buffer. Incubations in dextran sulfate comprise a "kinetic challenge" intended to remove aptamers with high off-rates. After round 7, eluted aptamer was primer-extended, amplified, and cloned.

Binding assays: aptamer preparations were radiolabeled with □-^{32}P adenosine triphosphate and T4 polynucleotide kinase according to standard methods. Radiolabeled aptamers were separated from free label by gel filtration on small spin colums (ProbeQuant G50). Radiolabeled aptamer (about 10,000 cpm/reaction) was combined with sperm cells in C1 or C2 buffer supplemented with 1 mg/ml herring sperm DNA, 1 mg/ml tRNA, 100 uM dextran sulfate, 100 ug/ml human serum albumin, and 1uM casein. Reactions were incubated for 1 hour at 37 C, and filtered through filter plates under vacuum. Plates were washed with 200 ul C1 or C2 buffer, dried, and exposed to phosphor screens for several hours. Images were analyzed and quantified on a Fuji Bioanalytic scanner.

III. Results

1. Statement of Results:

We have generated aptamers that bind specifically to sperm cells under stringent conditions. The buffer used for selection and binding of aptamers to sperm supports efficient recovery of sperm from cotton swabs. We have demonstrated that one of our biotin-substituted aptamers can cause sperm to bind to streptavidin beads, supporting the notion that immobilized aptamers can be used to purify sperm from forensic samples

Optimization of selection conditions: Pilot experiments with sperm cells revealed a powerful non-specific DNA-binding activity that would have precluded a successful aptamer selection. We thought to suppress non-specific DNA-binding with elevated

sodium chloride concentration. Shown below is a plot of sodium chloride concentration versus non-specific benzylated DNA binding, radiolabeled aptamer library DNA as a probe. As shown below, a binding minimum occurs at about 350 mM NaCl.

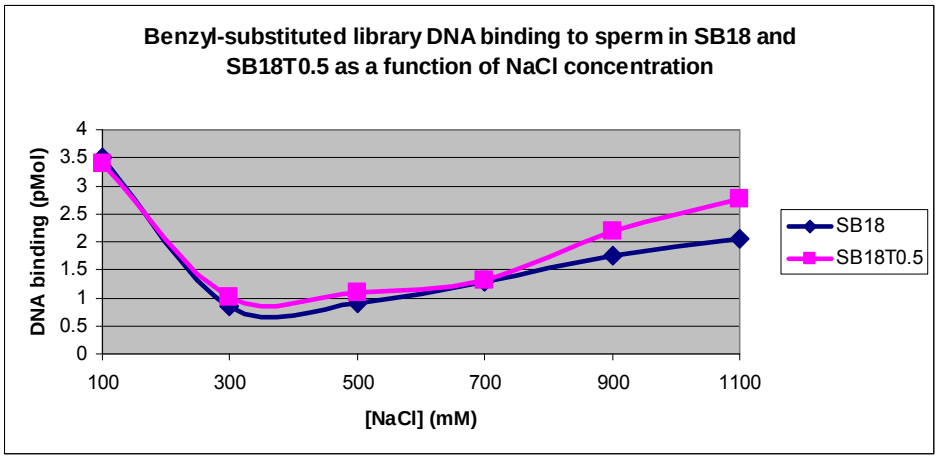


Figure 1. *Benzyl-substituted DNA library binding to sperm cells as a function of NaCl concentrations.*

Reasoning that such an elevated salt concentration was likely to support efficient elution of sperm from cotton swabs as well as diminish non-specific DNA binding to sperm, we selected 350 mM NaCl as a condition for further optimization.

We measured non-specific DNA-binding to sperm as a function of competitor nucleic acid concentration at 350 mM NaCl, again using radiolabeled aptamer DNA as a probe. We found that non-specific binding of benzyl-substituted library DNA was resistant to high (>1 mg/ml herring sperm DNA and >1mg/ml tRNA) concentrations of nucleic acid competitor (shown below):

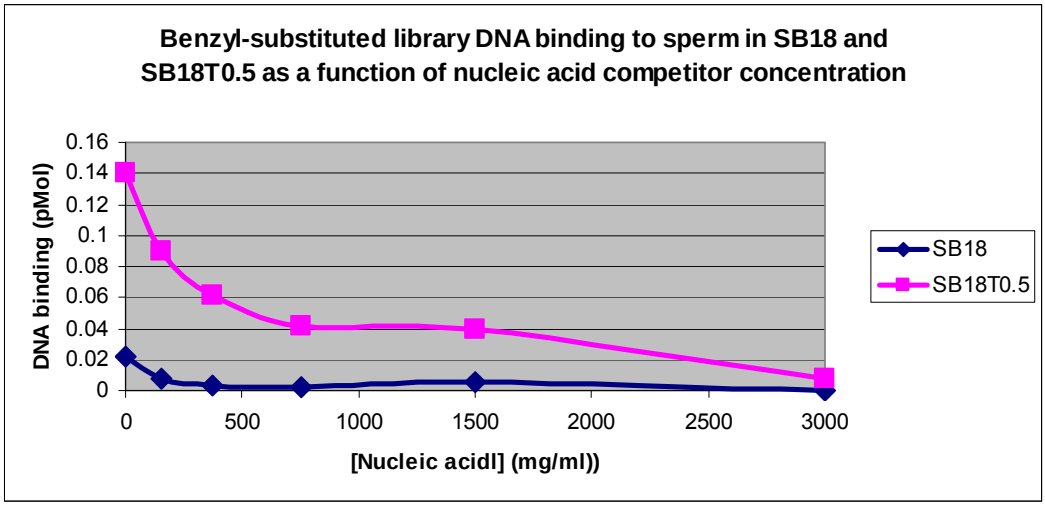


Figure 2. *Benzyl-substituted DNA library binding to sperm cells as a function of competitor nucleic acid concentrations.*

Even at 1 mg/ml herring sperm DNA and 1 mg/ml tRNA, the amount of non-specific DNA-binding to sperm is one to three logs too high to support a successful aptamer selection. While it was clear that an additional competitor would be required to diminish non-specific binding to acceptable levels, we chose to include 1 mg/ml tRNA and 1 mg/ml herring sperm DNA in the next round of optimization, as forensic samples would be expected to contain large quantities of contaminating DNA from epithelial cells

We next measured non-specific benzylated DNA library binding as a function of dextran sulfate concentration at 350 mM NaCl and 1 mg/ml herring sperm DNA and 1 mg/ml tRNA. We found that roughly 100 uM dextran sulfate was sufficient to diminish non-specific library binding to levels that would likely support a successful aptamer selection. One such titration is shown below:

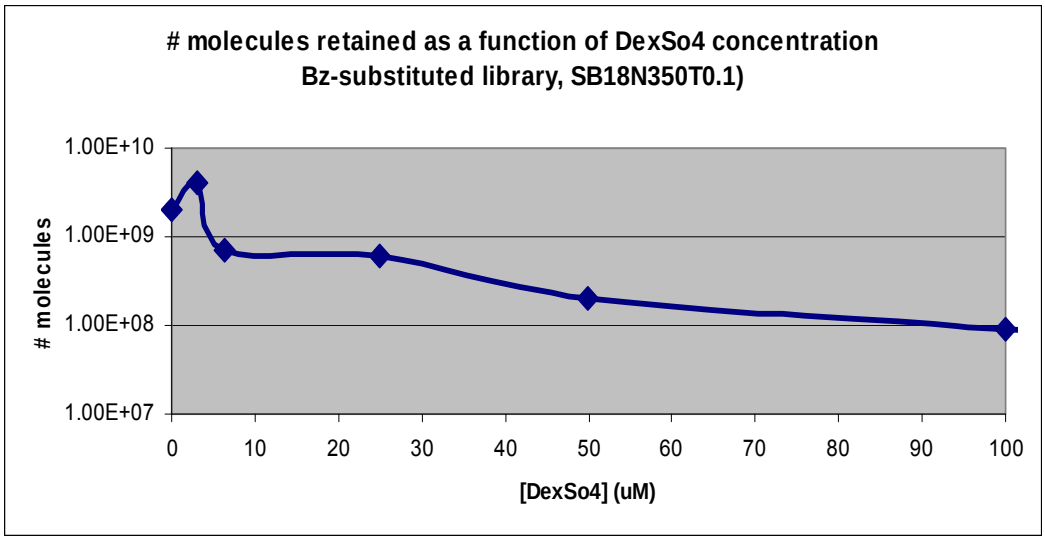


Figure 3. *Benzyl-substituted DNA library binding to sperm cells as a function of dextran sulfate concentrations.*

Rationale for selection buffer design: A primary consideration in our selection buffer design was the notion that the buffer used to elute sperm from cotton swabs and the buffer used for binding of sperm to aptamers for capture should be similar, if not identical. This was intended to facilitate the development of a simple and robot-compatible scheme for forensic sample processing. We chose to retain high NaCl concentrations in our selection buffer, facilitate elution from swabs, and omit and chelate remaining divalent cations from the selection buffer to suppress proteolytic and deoxynucleolytic activities that might degrade samples over a prolonged elution period. Large quantities of competitor DNA and tRNA were included, both to minimize non-specific library binding and to ensure that selected aptamers would perform in the presence of large amounts of contaminating epithelial DNA that might be expected in forensic samples. Two buffer conditions were chosen: one without added detergent, and one containing a relatively high concentration of non-ionic detergent. The first condition was intended to select for aptamers that bind to membrane proteins of intact sperm; the second condition, which strips away the plasma membrane of sperm, was intended to select for aptamers that bind to proteins of the so-called “perinuclear calyx”, the cytoskeletal structure that remains after conventional differential extraction techniques.

Rationale for selection scheme: We chose to use a "subtractive" selection procedure to ensure that selected aptamers would have little or no affinity for contaminating epithelial cells from forensic samples. In a subtractive selection procedure, aptamer libraries are pre-adsorbed to a particular non-target ligand to remove binders of that ligand from the library prior to selection against the desired target. We used HeLa cells for this subtractive step, reasoning that their origin (from a human cervical cancer cell line) made them a reasonable facsimile of the epithelial cells that we expect to encounter in forensic samples. We chose to use centrifugation as a so-called partition (separation of target and bound aptamer from solution), reasoning that while laborious, it involves no carrier species (e.g., streptavidin beads) and therefore carries no inherent tendency to select for aptamers to extraneous targets.

Convergence of aptamer pools: A standard measure of the progress of a selection is the loss of sequence complexity from a selected aptamer pool as it advances through selection rounds. The loss of sequence complexity represents selection of a few binding sequences from the enormous sequence variety contained within the original library. This so-called "convergence" can be measured as a function of the rate of re-association of heat-denatured amplification products from the selected aptamer pool. Plots describing such reassociation kinetics are termed "C0t" curves. C0t curves from four successive rounds of aptamer selection against sperm cells are shown below:

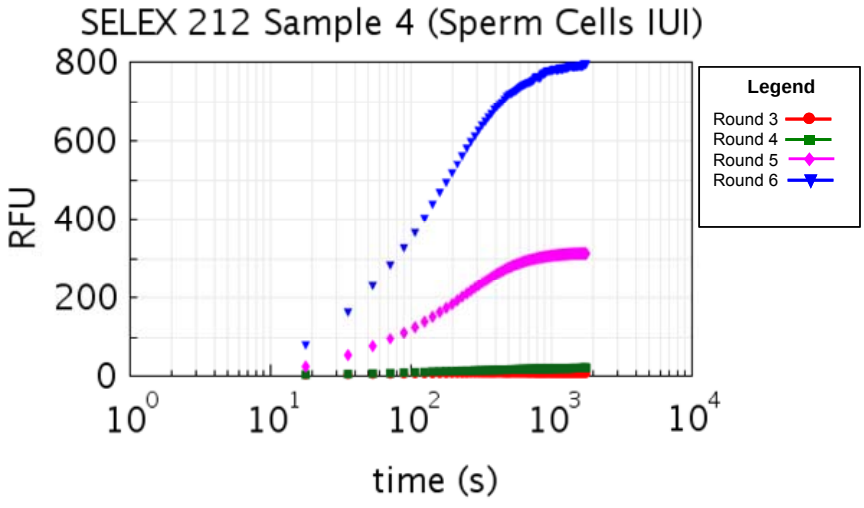
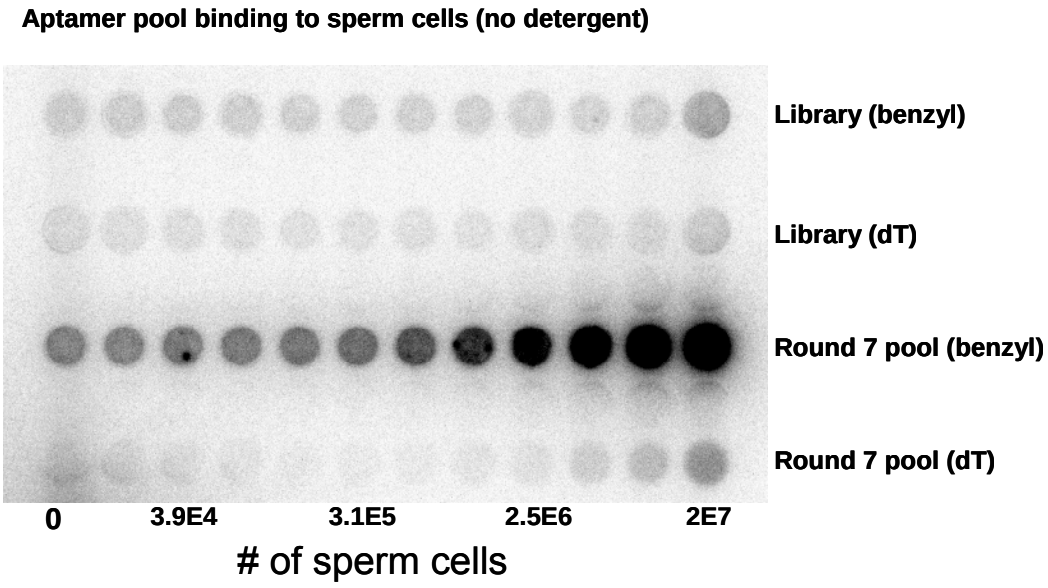
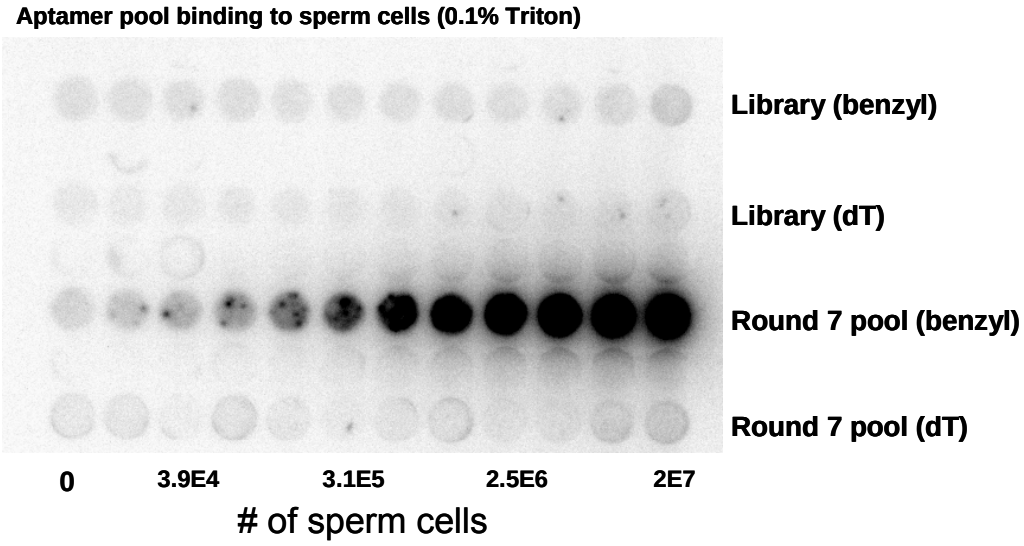


Figure 4. *C0t curves reflecting four rounds of aptamer selection against sperm cells.*

The extent and rapid rise of Rounds 5 and 6 are indicative of a relatively small population of sequence species indicating a successful selection of a small number aptamers from the vast number of sequences represented in the original library.

Aptamer pools bind to sperm: We directly tested aptamer pools from Round 7 of the selection for binding to sperm cells, using radiolabeled aptamer pools as probes and the very stringent conditions used for selection. The results are shown below:





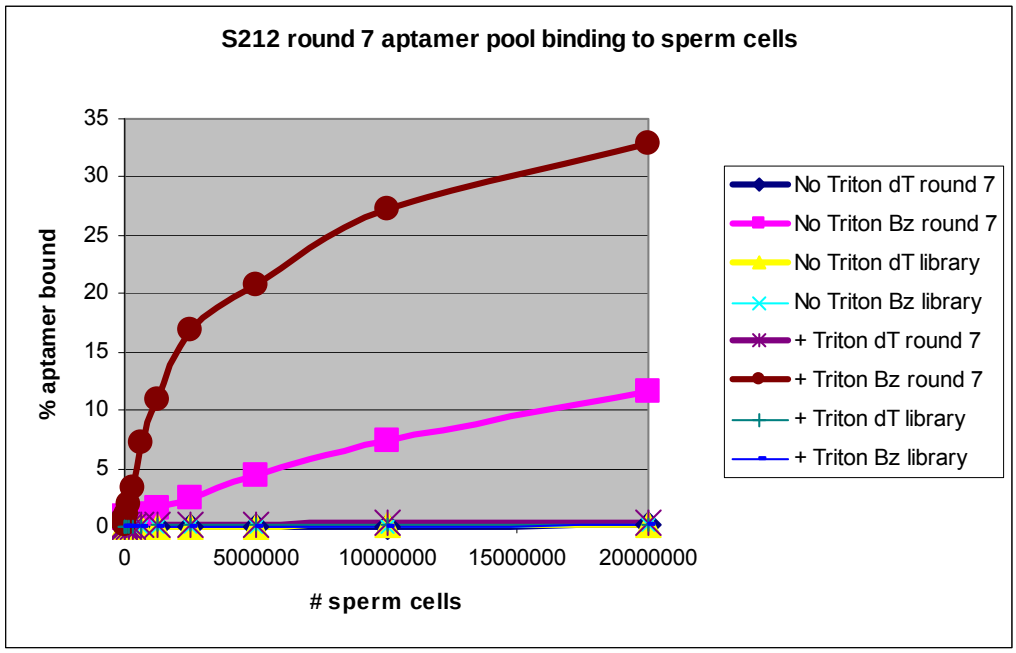


Figure 5. Selected *aptamers from round 7 selection experiments binding to sperm targets as compared to cDNA library binding to sperm cells either with no detergent treatment of sperm cells or 0.1% Triton 100 treatment of sperm cells to expose perinuclear calyx.*

Benzylated aptamer pools from Round 7, but not the original library, bind vigorously to sperm cells, in both the presence and absence of the non-ionic detergent Triton X100 used to strip the plasma membranes from sperm.

Individual aptamers bind to sperm: Aptamer pools were cloned and individual aptamer sequences determined. Individual aptamers were generated from isolated plasmid DNA, and tested for binding to sperm cells and for binding to competitor HeLa cells, which we have used as an epithelial cell substitute. Sequence alignments were performed and aptamers were selected for their ability to bind sperm. Shown below is the large-scale screen against sperm cells that we used to identify aptamers for further study. Aptamers chosen for immediate characterization are shown in green.

Single-point (1 x10^5 cells/uL) Affinity Assay

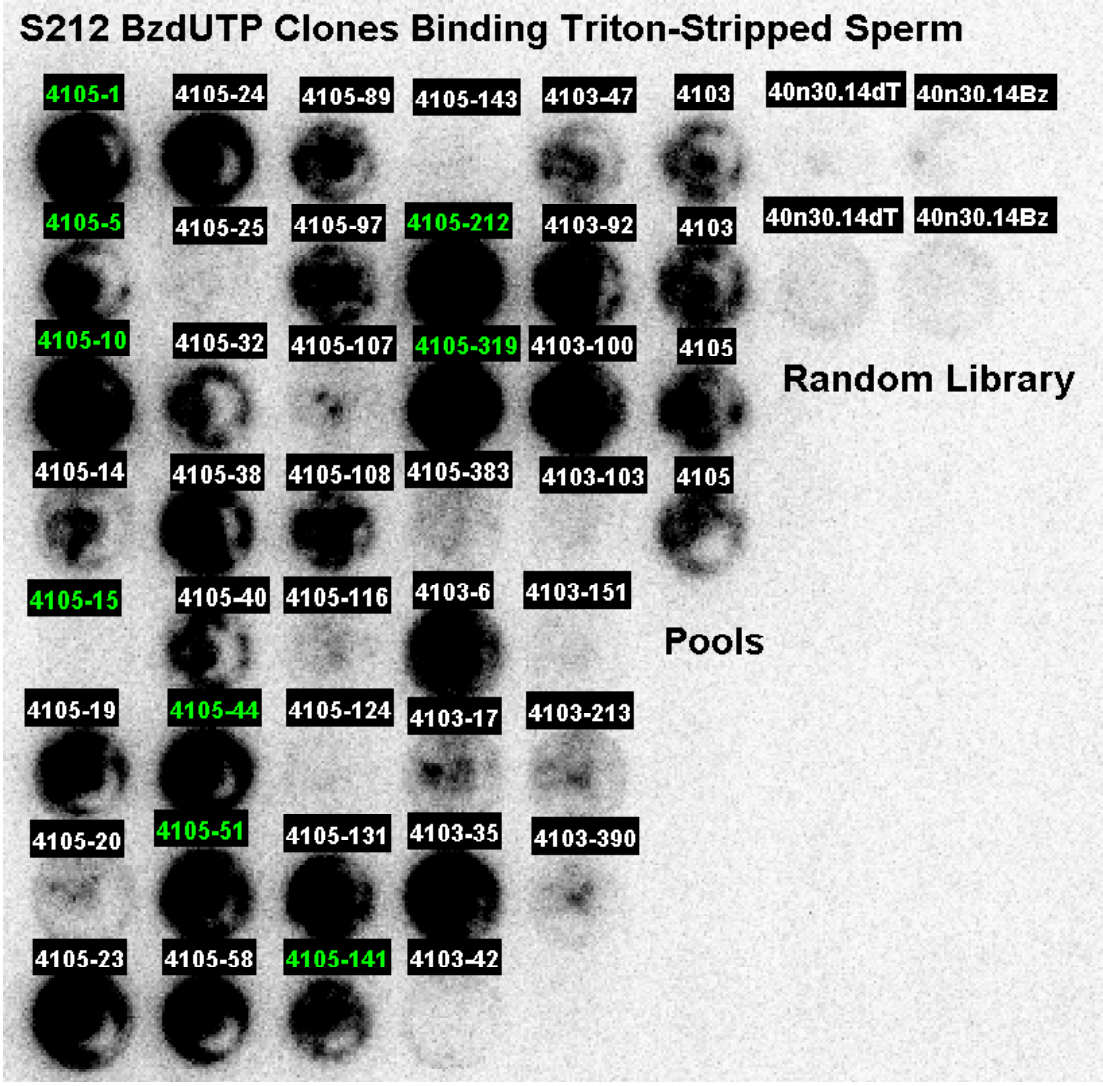


Figure 6. *Aptamer pool tests for binding to sperm cells and characterization of results. Results highlighted in green font indicate the best binding candidates for further characterization experiments.*

Shown below are the sequences of the random regions of tested aptamers. Note that deoxynucleotide residues designated “T” are not thymidine, but benzylated deoxyuridine residues. Again, aptamers selected for immediate characterization bear green identification numbers.

Clone	Mean % CPM Bound	Mean Rank	Sequence	Family
4105-212	69	1	GACACTGTATTATTCAGGCGTGGCGGAGTATGAATCGATC	Orphan
4105-10	53	2	TGCCAAGCTCTGCACA GGTTTTTGCGTGATCTC TGTGCAG	Pattern 3
4105-319	52	3	GATCTCATTATTAATTGGGTCACGCTTCGGCATGTTAGTG	Orphan
4105-1	39	7	AT CATGCCCATTCCAGAGCTC GGTTTTTGCGTGATCTC GA	Pattern 3
4105-15	34		TGATCAGCCGAGTGTTAGTAGAGGCCTGCGGTGTTAGTGC	Orphan
4105-44	27	7	AGGGTCCTGTCAGATGCTGCCG GGTTTTTGCGTGATCTC C	Pattern 2
4105-38	27	7.5	AACTTCCGAGTCCAG GGTTTTTGGGTGATCTC CTGGCTGA	
4103-6	26	9	ACTTCTG TCCAGC AT GCA GGTTTTTGTGTGATCTCTG CCA	
4103-92	26	9.5	AAACTTGCGCAC GTA GGGTTTTTGTGTGATCTCC TGTCGC	
4105-23	26	7.5	CAGCTGACGCCTGACGC GGTTTTTGGGTGATCTC GCGATA	
4105-58	22	9.5	ACTGCATATCCGAG TTTTGT GCTGTGATTTTCAACTCGGT	
4103-100	21	11	AGCGAAGCTATGCCGTA GCC GGGTTTTTGTGTGATCTCCG	
4105-24	20	12.5	TCTGACACCGAAAGCTGGCCG GGTTTTTGTGTGATCTC CG	
4103-35	20	12.5	AGATCAGCCGAGTGTTAGTAGAGGCCTGCGGTGTTAGTGC	
4105-51	18	11.5	GGCGAGGCCAAGCA AGTTTTAAT CCGGC ATCCC TTTTCG C	Pattern 1
4105-131	13	15.5	AGGGTCCTGTCAGATGCTGCCG GGTTTTTGCGTGATCTC C	
4105-97	12	17	TGGCCCCCGG AGTTTTAAT ACCT CACC TGTTTT CCCGCAT	
4105-19	11	19.5	CCAGTCGCTGAG AGTTTTAAT CCCGC ACCCC TTTTCCTCA	
4105-89	10	20.5	GCACCCCATCATAGAGCTGTC TCTTGT CCATCGTCATAA	
4105-108	10	21.5	AGAATACCAGGCGC AGTTTTAAT CAGGC AACCC TTTTCG C	
4105-40	10	21.5	TGCAAAACCAGC AGTTTTAAT ACGTC ACCCC TTTTCG GTG	
4105-5	10	21.5	GGCGAGCACTGC AGTTTTAAT ACACC CACCGC TTTTCG CG	
4105 Pool	9	22.5	4105 Pool	
4105-32	9	22.5	GAAGTAGCAGAC AGTTTTAAT ATGC ACCGG TTTTCG TCGC	
4105-141	7	27	AGAGCGTTGCGATTATCGGTGAGTTTCGAACGCATCCAG	Orphan

Figure 7. *DNA sequences for the selected aptamers from figure 6 used in subsequent characterization experiments.*

Binding curves for four of the selected aptamers are shown below:

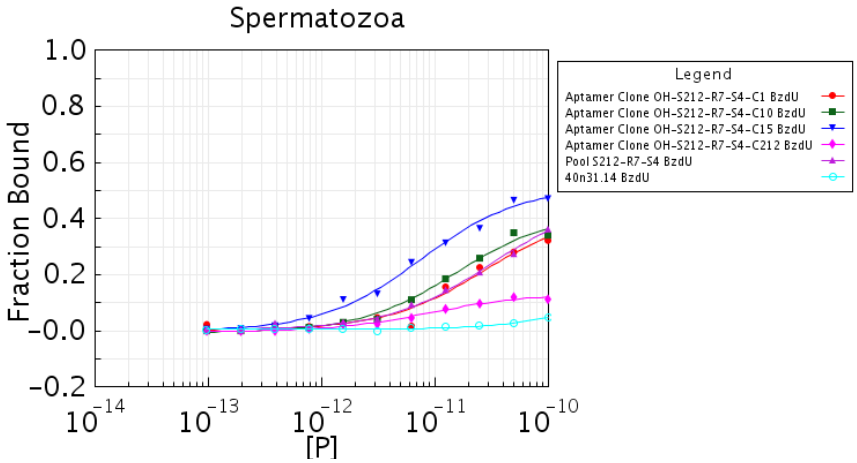


Figure 8. *Binding curves for four selected aptamer clones against sperm cells.*

All four tested clones specifically bind to sperm cells (compare library, turquoise circles, with pink, purple, red, green and blue curves). Three of the four tested clones bind with a higher apparent affinity than the original pool (compare purple triangles with red circles, green squares, and blue triangles). More than twenty clones remain to be tested.

C2 selection buffer supports efficient elution of sperm from cotton swabs:
There are currently two media commonly used for sperm elution from the cotton swabs commonly used to collect forensic samples. The first is essentially phosphate buffered saline, which is inefficient (recoveries typically less than 10%). The second is a 1% solution of sodium lauryl sulfate (sodium dodecyl sulfate, SDS), used in the differential extraction method. SDS is an efficient medium for recovery, but is incompatible with aptamer binding, and is normally coupled with centrifugation-based wash steps which are both low-yielding and require large amounts of sperm cells at the outset of the operation. Our C2 selection buffer was designed to support efficient elution of sperm from forensic samples as well as aptamer binding.

We compared the efficiency of elution of sperm from cotton swabs to which semen had been adsorbed and dried in C2 selection buffers at various concentrations with a solution containing 1% SDS (intended to mimic differential elution buffer). In these pilot experiments, we found that C2 selection buffer supported recoveries that compared favorably with the recoveries supported by SDS. Recoveries ranged from 44% of the SDS control (C2 selection buffer) to 78% of the SDS control (2-fold concentrated C2 selection buffer. More concentrated C2 (4-fold concentrated) yielded no obvious improvement, with 54% recovery relative to the SDS control.

Aptamer target identification:
One consideration for selection of the proper aptamer for use in sperm capture is its protein target. Desirable protein targets for sperm capture aptamers are sperm structural proteins present in all, or nearly all, of the sperm-producing population. Hence, we have commenced efforts to identify the targets of aptamers isolated in our whole-cell selection.

Preliminary data suggests that we can detect specific aptamer-binding activity in urea extracts. Shown below is a binding curve, using radiolabeled aptamers and a 2-fold dilution series (from left to right) of a 4M urea extract from sperm calices.

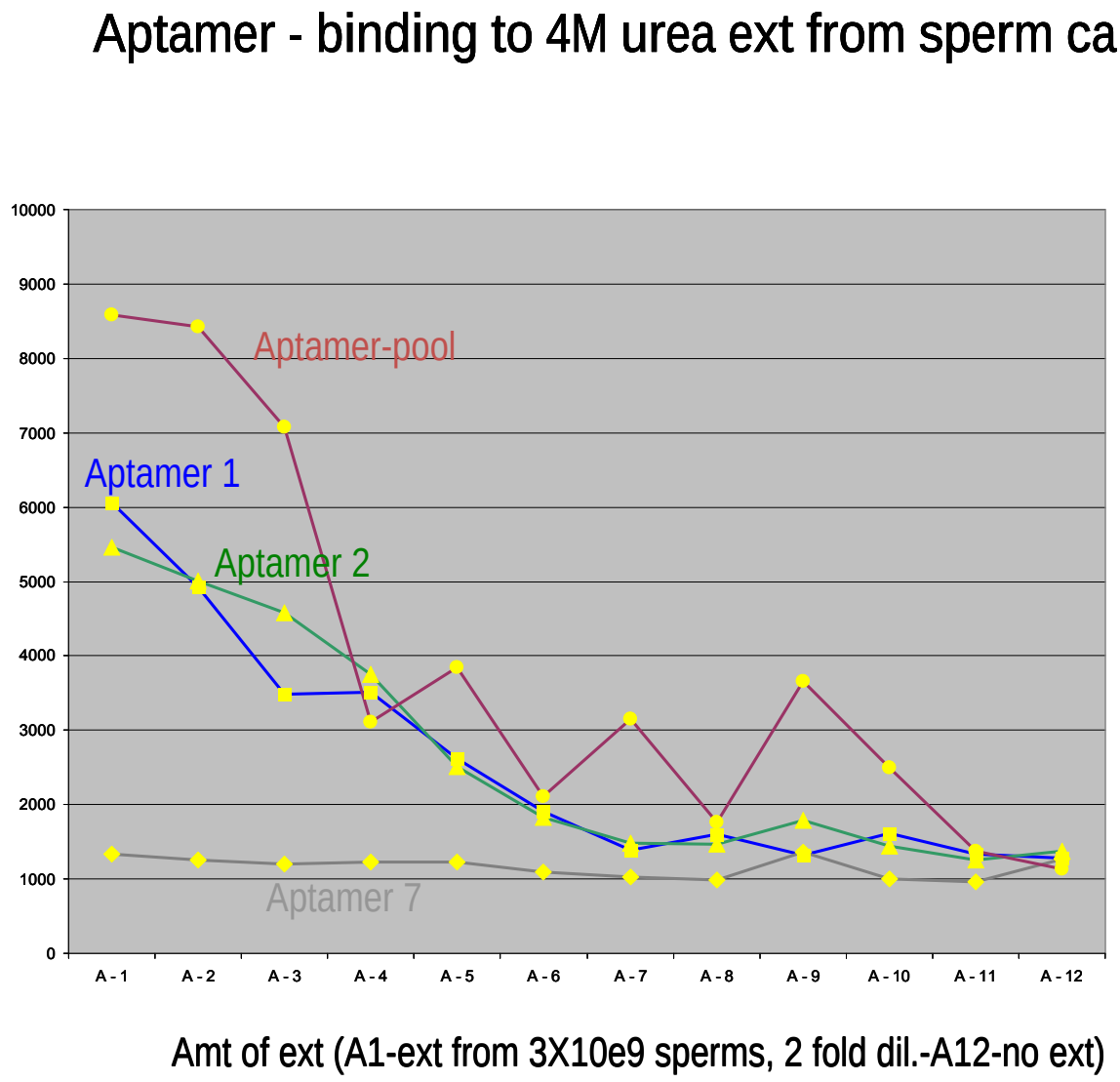


Figure 9. *Sperm proteins isolated from sperm pericalyx in 4M Urea extracts and aptamer binding of three selected aptamers versus a pool of several aptamer types. Extent of binding is represented in radioactive counts on the Y-axis and a 2-fold dilution series of 4M Urea protein extracts presented on X-axis from left to right.*

The fact that such a binding activity can be detected in a soluble extract suggests that aptamer targets can be purified by affinity chromatography. We are currently developing an affinity chromatography strategy and hope to have enough relatively pure protein to identify the protein targets of these aptamers by mass spectroscopy.

Capture of sperm by biotinylated aptamers and magnetic streptavidin beads:

As proof-of-principle, we tested the ability of one of the aptamers we generated to mediate capture of sperm onto magnetic streptavidin beads. Biotinylated aptamer (4105-1) was immobilized on streptavidin-coated magnetic beads. The aptamer-substituted beads were incubated with sperm in the presence of a competitor cocktail (herring sperm DNA, tRNA, and dextran sulfate). The mixture was pipetted onto a microscope slide, and a small magnet placed near the droplet for a short period. Shown below are light micrographs of bead-and-sperm mixtures, with and without added aptamer. Sperm cells are clearly isolated and magnetically clustered as in panels B and C.

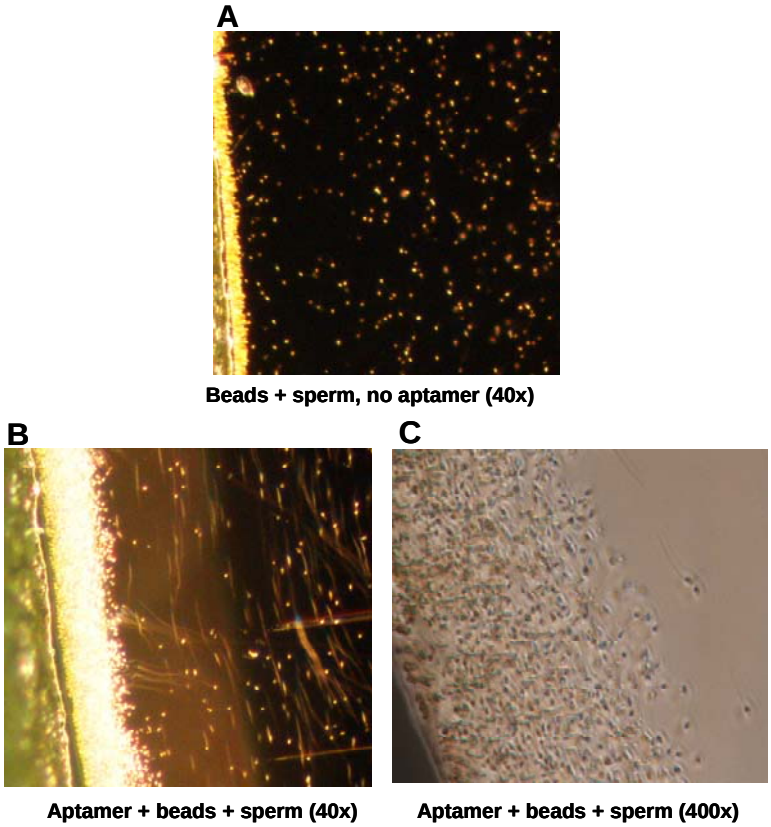


Figure 10. *Binding of one of the seven selected aptamers from experiments above to sperm cells on a microscope slide. Panel A-40x magnification of sperm cells in presence of magnetic streptavidin coated beads without aptamer. Panel B-40X magnification of sperm cells in presence of magnetic streptavidin coated beads with biotinylated aptamer present and magnet present on left side of panel. Panel C-400x magnification of isolated sperm cells to left side of slide under magnetic attraction of bead-aptamer complexes.*

IV. Conclusions

1. Discussion of findings:

This project has demonstrated that specific aptamers can be designed to bind specifically to sperm cells and subsequently used to design a sperm capture assay with neutral media such as magnetic beads. This work opens the door toward a very high throughput assay based on engineered aptamers that will specifically capture sperm cells under highly stringent conditions. The work over the last two years has been successful in developing an elution buffer for cotton swabs widely used in sexual assault evidence collection. This buffer enabled recovery of 44% to 78% of sperm cells bound to the cotton swabs. Used in conjunction with our 9 selected aptamers, we anticipate a system to be developed soon for sperm binding initially based on strepavidn beads.

High-affinity aptamers that preferentially bind to sperm heads will be synthesized and immobilized via appended biotin tags on 96-well streptavidin plates. Immobilized aptamers will be evaluated for their ability to capture sperm cells (in various conditions including samples from adjudicated forensic cases), to capture sperm in the presence of large amounts of epithelial cells and cell debris, to retain sperm cells under stringent washing conditions without retention of contaminating epithelial cells or debris, and finally, their ability to release sperm cells or sperm DNA in the presence of a chaotropic salt (Triton X100) and/or a reducing agent such as dithiothreitol (DTT). These steps will be monitored by quantitative assessment of human DNA or human male DNA through use of commercially available DNA quantification kits (Quantifiler® Human DNA Quantification and Quantifiler® Y Human Male DNA Quantification Kits, Applied Biosystems), using standard curves generated from sperm cell stocks. Ultimately, selected aptamers will be subject to truncation and binding analysis to identify the functional region of each aptamer and to establish a minimal functional sequence length. This step will ensure manufacturing efficiency, final aptamer purity, and minimize expense.

Following aptamer identification, the focus of continuing work will be on the selection of an ideal matrix for the development of a sperm-capture assay. The goal is to covalently link the synthesized aptamers to a solid matrix by coating the surface of a tube, a 96-well plate, slide, column/beads, or other suitable matrices. Swabs prepared to mimic sexual assault evidence and evidence from adjudicated cases will be eluted in sample buffers, providing sperm cells. Dilute sperm cells can be captured using beads, 96-well plates, or lateral flow devices. We are particularly interested in lateral flow devices in which isolated spermatozoa are observed and quantified using secondary aptamers labeled with visible reporters in a manner similar to sandwich immuno-assays with antibodies used for clinical diagnostics. We anticipate that one or more of our sperm-specific aptamers will be labeled with a visible dye and used to quantify the sperm attached to the "band" of capture aptamers.

Further experiments will concentrate on optimizing the specific covalent aptamer binding and sperm immobilization to the selected matrix while recovering epithelial cells and non-sperm semen components. This will also further test aptamers for their ability to bind to varying small numbers of sperm in mixtures with female epithelial cells. Wash steps or cellular digestion will allow for the complete removal of non-sperm cells while retaining immobilized sperm cells. The development of a formal procedure to isolate DNA from the immobilized spermatozoa will follow, potentially involving either lysis with a chaotropic salt (e.g. guanidine isothiocyanate) and a reducing agent, or, conceivably, an enzymatic digestion step to lyse the sperm cell wall or with addition of DTT as with current protocols. Also, the potential use of DNA isolation using magnetic beads will be tested further to the work presented in this report. PCR amplification and STR analysis on the separated male DNA will be performed at the Denver Police Department Crime Laboratory to ensure that the recovered DNA is viable for DNA profiling.

During the development of these protocols, the suitability for high throughput processing was evaluated as well as the potential for automation of this sperm-capture system. Additionally, the coating of a potential matrix could be manipulated to enable the binding of sperm cells at a specific density. This would allow for a constant amount of DNA being isolated in the sperm-capture assay, and would potentially make DNA quantitation

steps unnecessary, thereby further increasing the processing speed of sexual assault evidence samples.

2. Implications for policy and practice:

The potential impact of this proposed project could be very significant for the forensic community in terms of sexual assault case backlog reduction. The National Forensic DNA Study Report from December 2003 [34] estimated a backlog of 169,000 rape cases in the United States; therefore, DNA laboratories could very strongly benefit from a cost effective assay that allows for fast, easy, and reliable processing of sexual assault evidence.

This sperm capture assay will allow for faster processing of evidence from sexual assault cases and can be used in all forensic laboratories. By using the developed assay, the amount of time required to obtain a DNA profile from a putative perpetrator will decrease and may result in a faster identification and prosecution of suspects, possibly preventing later crimes. Additionally, more profiles will be created for entry in the CODIS database, which in turn can help to link and potentially solve further crimes. In cases without a suspect and no immediate identification of the putative perpetrator, a DNA profile can lead to a John Doe warrant which halts the Statute of Limitations.

This project proposes to develop a sperm capture assay that will be amenable to automation using robotic workstations that are becoming more common in forensic casework. Automation can further decrease the amount of time required to process sexual assault cases and decrease the backlog in forensic laboratories. This would improve capacities for other casework that do not involve mixed evidence samples and will also allow laboratories to revisit cold sexual assault cases and attempt to identify perpetrators.

To make the product commercially available, a partnership with a supplier of forensic products will be sought and their existing distribution and marketing network will be used. Therefore, we expect the finished sperm-capture assay to be very cost-effective and affordable for forensic laboratories.

Considering that the majority of evidentiary samples in crime laboratories arise from sexual assault cases, this project can have a significant impact on the forensic community by decreasing the amount of time required to handle this evidence in a cost-effective way.

3. Implications for further research:

The continuing goal of this research is the development of a cost-effective assay suitable for high throughput analysis that can easily be implemented in forensic laboratories. Upon successful development of the sperm-capture assay, a developmental validation must be conducted to prepare for the use in forensic casework. According to the Revised Validation Guidelines issued by the Scientific Working Group on DNA Analysis Methods (SWGDAM) in 2004, the developmental validation must demonstrate the accuracy, sensitivity, precision and reproducibility of a novel methodology and provide the forensic community with the necessary information to assess the abilities, required conditions and limitations of this method.

Various spermatozoa concentrations and different ratios of sperm to epithelial cells will be used to test the affinity of sperm binding. The samples will be extracted using the developed sperm capture assay and lysis protocol, the resulting DNA concentration of the extracts determined by real-time PCR analysis and STR profiles will be developed using the major STR analysis kits used in the forensic community (AmpFℓSTR® Profiler Plus®, COfiler® and Identifiler® PCR Amplification Kits from Applied Biosystems as well as the PowerPlex® 16 System from the Promega Corporation). This will evaluate the feasibility of using the sperm-capture assay in the context of forensic casework samples.

Later research will focus on evaluation of known inhibitors, non-human sperm samples, mixed samples, as well as non-probative casework samples. Additionally it will be used to demonstrate the accuracy, precision, and reproducibility, as well as the sensitivity and specificity of the assay and identify possible limitations of the procedure.

Following the successful completion of these developmental validation studies, the product will be useful for forensic DNA casework and be made available to the forensic community.

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VI. Dissemination of Research Findings

July 2008-National Institute of Justice Annual Meeting: Oral Presentation: Sperm Capture using Aptamer Based Technology, Washington D.C.

March 2009-Presentation “Sperm Capture using Aptamer Based Technology” presented at the DNA technical working group-future of DNA-National Institute of Justice, Arlington VA.

June 2009-National Institute of Justice Annual Meeting: Poster Presentation: Sperm Capture using Aptamer Based Technology, Washington D.C.