FINAL TECHNICAL REPORT

QuantAssure Cassette Development Research

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This Final Technical Report represents the revision of the Draft Final Technical Report, as needed to respond to and incorporate all changes suggested by the NIJ reviewers.

Abstract

This project sought to produce the "QuantAssure Cassette," as a lateral flow immunochromatographic cassette assay for detecting sperm and semen proteins in sexual assault evidence samples.

Three target proteins were selected for obtaining antibodies to detect sperm and semen. Sperm specific thioredoxin3 (SPTRX3) detection indicates the presence of sperm cells. Semenogelin1 (SEM1), and prostate specific antigen (PSA/p30) both establish that seminal fluids are present. Positive results from all three target proteins using this assay would establish the presence of semen in the sample. Positive results with just SEM1 and PSA and not SPTRX3 indicates semen without sperm cells.

The assay works based on the sandwich-type of immunological detection where two antibodies are required for each target protein. One antibody is conjugated to colloidal gold and is mixed with the evidence sample so that it can form a complex with any target protein. This sample is added to the cassette, where chromatographic separation occurs. A capture antibody bound as a stripe on the assay membrane captures any target protein-gold antibody complexes. When the gold becomes concentrated at the stripe a band becomes visible indicating a positive antibody reaction and detection of the target protein.

The SPTRX3 gene is expressed in sperm cells and not in other tissues. A rabbit polyclonal anti-human SPTRX3 antibody immunized by two peptides unique to SPTRX3 was tested using western blots to determine the capacity of detecting SPTRX3 protein from dead, dry sperm cells in mock forensic samples. Our results demonstrate that the detection of a 17k Mr protein in samples on SDS-PAGE western blots indicates the presence of sperm cells. False positive cross reaction between standard reagent secondary antibodies and endogenous

antibodies found in semen and vaginal secretions interfered with the ability to test for SPTRX3 protein. Controlled experiments and a new specific secondary reagent allowed us to overcome this and establish an assay for developing the cassette.

Semenogelin1 and PSA protein are found in human semen even when sperm cells are absent. Antibodies for these were obtained commercially and tested with western blots to verify their ability to detect the target proteins in mock forensic samples. All of the antibodies were conjugated with colloidal gold and tested in sandwich assays. The PSA antibody pair performed as expected. Semenogelin1 antibodies will also require further development. Each semenogelin1 antibody selected individually detected semenogelin protein, but gold labeled antibodies did not function in sandwich assays. Future development of semenogelin detection will require a new antibody designed to encompass a stable peptide fragment.

A sandwich assay using the original polyclonal SPTRX3 antibody with the human TRX1 antibody did not give positive results. Because a second SPTRX3 antibody was needed to complete an assay, we decided to make two new antibodies specific for each of the two unique peptides of SPTRX3. The peptide from the first exon of SPTRX3 was used to immunize mice for monoclonal antibodies to be conjugated with gold. The peptide from the fifth exon of SPTRX3 was used to immunize rabbits for polyclonal antibodies to be used as capture antibodies in the cassestte. Although a good quality polyclonal antibody was produced using the two unique peptides together, our SPTRX3 antibody attempts subsequently failed, perhaps due to limited antigenicity of the peptides on their own.

Further development of the QuantAssure Cassette will require improved SPTRX3 and semenogelin antibodies. At this writing, a new SPTRX3 antibody has been developed at the University of Missouri and will be tested in sandwich assays in the future.

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Executive Summary

This Executive Summary provides an overview of the research project goals and design, the entities separately participating in segments of the research, and their respective roles, and the result of the research.

This grant research makes significant contributions to the body of knowledge needed in order to develop a lateral flow cassette to identify the presence of sperm and semen in evidence samples. The Executive Summary reviews the research steps that were taken, the issues that were faced, the successes along the way, the ultimate roadblock to success, and plans for ongoing research and development.

The grant was awarded to Paternity Testing Corporation (PTC) in Columbia, Missouri. The Principal Investigator is Dr. Christian Carson, who is on staff at PTC. The award period started on November 1, 2008 and the original award period ended on October 31, 2010.

Research was carried on throughout the original grant period in PTC's laboratory. Research was carried on simultaneously at the laboratory of Dr. Peter Sutovsky (the co-PI on this grant), through a research contract between PTC and the University of Missouri, which is reflected as the subcontract award in the budget for the grant. Later in the grant period, research for cassette and antibody development was also carried on at BioAssay Works and at BluePoint Bioscience, as discussed further in this summary.

The concept of this research project was to develop a lateral flow cassette that would allow crime laboratories to much more quickly and easily identify the presence of sperm and semen in an evidence sample. In turn, this would help to reduce the backlog of unprocessed rape kits.

The research goal was to ultimately allow crime lab staff to place the evidence sample in solution, and to pipette the sample onto a lateral flow cassette. Capillary action would pull the sample along the cassette. Bands of attached secondary antibody would attach to the primary antibodies in solution, leaving a visible signal on the cassette to indicate the presence of sperm and semen in the sample, and the relative amount of sperm, if present.

Three proteins were to be detected in this process. These included two proteins already in use in the forensic community for the detection of semen. Those are Prostate Specific Antigen (PSA/p30) and semenogelin. Detecting these proteins will successfully indicate the presence of semen in the absence of sperm. While currently in use in the industry for this purpose, both proteins are known to have false positive reactions to a number of substances. Based on current literature, to be confirmed by further testing, the expectation is that the false positive reactions are not mutual for any substance, and that therefore if both protein indicators show positive, the possibility of a false positive will have been eliminated.

The cassette design requires that, for each protein, there be a capture antibody fixed to the cassette strip, and a labeling antibody that binds to the protein when placed in solution with the evidence sample. For the PSA/p30 protein, the necessary antibodies were already available commercially. Because semenogelin is well documented and well understood, it was anticipated that development of the necessary antibodies for this protein under the research grant would be fairly straightforward.

The third protein is really the core of this project, and the discovery that made the QuantAssure Cassette concept possible. It was the discovery, in the field of human fertility diagnostics, of the sperm specific thioredoxin 3 protein (SPTRX3). One of the co-discoverers is Dr. Peter Sutovsky, at the University of Missouri, who is a co-investigator on this research grant. Dr. Sutovsky is a world renowned and well respected scientist in the fields of human and animal fertility.

Kim Gorman of PTC, in early discussions with Dr. Sutovsky, determined that there could also be a forensic application for SPTRX3.

SPTRX3 is found only in sperm. Therefore, positive indications for the presence of SPTRX3 will have no false positive issues (to also be confirmed by further testing under the grant). Thus, the conceived cassette would allow detection of sperm and semen in evidence samples, with a high degree of certainty, in much less time than under current practice.

Research was performed in pursuit of this technology, both in Dr. Sutovsky's laboratory and in the PTC laboratory, prior to the beginning of this research grant. Research to develop a cassette continues post-grant as well.

General Counsel for PTC negotiated an exclusive license from the University of Missouri for the use of SPTRX3 in forensic applications, and also negotiated a very detailed contract with BioAssay Works for the commercial production of cassettes, because of our firm belief in the viability of this technology.

Dr. Sutovsky's laboratory already possessed a polyclonal antibody to SPTRX3, which we hoped to use as the labeling antibody, and we believed that developing the necessary additional antibodies would be routine (because the original antibody was in short supply, once the cassette was developed and working we anticipated also developing a new monoclonal labeling antibody).

We began by performing cross-reactivity experiments with SPTRX3. It was especially important that any SPTRX3 antibody not cross react with any substance present in vaginal secretions. The cross reactivity experiments led to much additional work because of the cross-reactivity of various commercial secondary antibodies. Once that problem was finally corrected, and the lack of cross-reactivity was confirmed, the antibody was sent to BioAssay Works for colloidal gold labeling and development of the lateral flow cassette.

PTC also acquired commercially available antibodies for the PSA/p30 and semenogelin proteins, and forwarded those to BioAssay Works.

During the entire time that BioAssay Works and BluePoint Bioscience worked on cassette and antibody development, the PTC and Sutovsky laboratories continued to perform research especially in conjunction with providing support to the cassette and antibody development effort.

BioAssay Works, LLC (BAW) (located in Ijamsville, Maryland www.bioassayworks.com) was not the cassette development firm that PTC originally contemplated using for this project. However, upon continued research into the best company to use, we learned that, unlike the originally intended company, BAW had much experience and success in developing such cassettes. PTC also thoroughly grilled four references for which BAW had successfully

completed large projects. Based on the references, credentials, and our discussions with BAW, they were clearly our partner of choice for lateral flow cassette development.

When BAW received the antibodies for the three proteins from PTC, they established a test assay and proceeded to determine whether these antibodies could function properly as pairs in sandwich assays using colloidal gold as a label. BAW performed the assays and found that the PSA antibodies were sufficient for a functioning assay, but the semenogelin and SPTRX3 antibodies were not.

Because the SPTRX3 antibody wasn't sufficient for the assay, we proceeded to construct new SPTRX3 antibodies through BAW. For the monoclonal mouse antibodies, BAW used BluePoint Bioscience. BluePoint Bioscience is a company specializing in monoclonal antibody development. They are located in the same building as BAW, and have a close working relationship with BAW.

Also, because the project would not be able to succeed unless the SPTRX3 antibody issues were resolved, we decided that no new semenogelin antibodies would be planned and developed until the SPTRX3 antibodies were functioning correctly. We did not want to waste NIJ money on semenogelin antibody development if the project was not going to come to a successful conclusion. As it turned out, we were able to forego spending approximately $65,000 of the authorized grant award.

Two new SPTRX3 antibodies were planned. A polyclonal antibody was instigated by immunizing rabbits with the SPTRX3 exon5 peptide. A monoclonal antibody was started at BluePoint Bioscience by immunizing mice with the SPTRX3 exon1 peptide. None of the products from these immunizations produced a viable SPTRX3 antibody, even after a second set of mice were immunized and processed at BluePoint Bioscience.

As we approached the end of the initial grant period, we wanted to obtain a no-cost extension in order to continue the research, and we even initiated a request in GMS for the extension. However, as we continued to contemplate future research possibilities, we realized that we did not have a good handle on why SPTRX3 antibody development had failed. As a result, we had no confidence that spending down the remainder of the grant funds made sense or was likely to produce a positive result. So we decided that it was best to simply go ahead and end the grant.

Regarding possible reasons for the failure of SPTRX3 antibody development, Dale Dembrow at BluePoint Bioscience is unsure, but speculates that possibly the SPTRX3 protein is not stable. Chris Carson of PTC noted certain stability issues during research, and found that once the sperm protein was in solution it appeared to remain stable for only the remainder of that work day.

However, Dr. Sutovsky at the University of Missouri is certain that SPTRX3 is stable. His laboratory has continued to perform research following the close of the grant period (not funded by NIJ). Dr. Sutovsky states that in this interval his laboratory has identified a usable SPTRX3 antibody, and is in a position to proceed with cassette development.

PTC plans to continue research in an effort to complete cassette development. We have not yet had an opportunity to test Dr. Sutovsky's new antibody, or reproduce his results. But we will do that. If we determine that the new antibody is viable, then we will proceed with cassette research and development using the SPTRX3 protein.

If we determine that SPTRX3 is not useful for the cassette, then we hope to identify another sperm specific protein and attempt to develop antibodies for it.

We are truly sorry that this technology development effort has not been successful. In addition to the NIJ funds used in this grant research, both PTC and Dr. Sutovsky's laboratory at the University of Missouri have spent their own funds to work on this technology, and we continue to do so.

We want to thank NIJ very much for the vote of confidence, and the opportunity to pursue this technology.

I. Introduction

1. Statement of the Problem

Sexual assault crimes account for a significant portion of violent crime throughout the world. Fortunately, because of the nature of the crime, semen evidence left by rapists can provide the DNA identity of the rapist which can greatly enable his capture. Because the number of backlogged sexual assault cases to be processed is large and because the time currently needed to process them is so long, improved processing of sexual assault evidence will accomplish several goals. Reducing the time taken to confirm that the evidence contains spermatozoa or semen will shorten the overall processing time and increase the rate of cases processed. This will help eliminate the backlog of sexual assault cases waiting to be processed. It will also help individual rape victims to bring their cases to completion, by incarcerating the rapist more quickly. Most importantly, incarcerating the rapist more quickly will also reduce the number of repeat offenses.

Rape kit evidence from sexual assault crimes may carry semen from the perpetrator of the crime. Currently, the process of testing for spermatozoa and seminal fluid involves many steps. Seminal fluid proteins such as the PSA/p30 protein and the semenogelin protein from semen can be detected using lateral

flow cassette assays that employ antibodies that specifically bind to them. Detection of these proteins indicates the presence of semen even when spermatozoa are not present, as in the case of vasectomies and other azoospermia. However, some false positive results have been found with these assays. Regardless, currently, detection of the PSA/p30 protein in sexual assault evidence provides confirmation of semen.

Ultimately, the evidence must be examined for spermatozoa. DNA from spermatozoa can be isolated and analyzed in the crime laboratory to establish the identity of the perpetrator. Knowing whether or not spermatozoa are present in the evidence establishes the protocol for subsequent DNA extraction and analysis. If no spermatozoa are present, the analysts must still determine this before deciding to forego standard autosomal PCR. When sufficient numbers of intact spermatozoa are present, the evidence can be divided, using some for DNA extraction and saving some for later. When only a few spermatozoa are available, greater care must be taken with the evidence for DNA preparation.

Under current practice, the presence and relative amount of spermatozoa in sexual assault evidence is determined by removing some of the evidence, fixing it onto a slide and staining the cells for examination under a microscope. Microscopy does provide confirmation of spermatozoa in sexual assault evidence, but also consumes part of the evidence. Although it may be possible to recapture these cells from the slides for extraction or with laser micro-dissection, this only increases the level of complication for the evidence. When only limited, small amounts of sexual assault evidence are available, the crime laboratory analyst must proceed decisively. Most laboratories successfully and routinely carry out microscopy for sexual assault cases. However, as case loads increase the time spent on microscopy becomes enormous, and is a significant bottleneck in processing the backlog of such cases.

A speedier alternative method that could detect spermatozoa, without using up a portion of the sperm evidence, would be a valuable tool in reducing the backlog of sexual assault cases.

2. Literature Citations and Review

Out of the many proteins found in semen, some are found almost exclusively in semen. Thus, antibody reagents that are able to bind semen specific proteins can be used to establish the presence of semen in an evidence sample. Semen detection has been performed using the prostate gland secreted PSA/p30 protein as a target of antibody detection. Similarly, the seminal vesicle secreted semenogelin proteins have also been used for semen markers. Cassettes for the semenogelin and PSA/p30 semen markers can be obtained commercially and are used in current forensic applications.

Currently, forensics analysts use sperm cytology to establish and confirm the presence of spermatozoa in semen. A protein marker produced only by spermatozoa, the SPTRX3 protein, offers an opportunity for detecting spermatozoa with antibodies. Combined on a single test cassette, these three markers would provide assurance of whether semen is in the evidence or not. These will be used to produce a lateral flow assay for establishing the presence of semen including the specific detection and quantification of spermatozoa in an evidence sample.

The molecular reagents necessary to achieve this goal involve antibodies that specifically detect semen proteins. All three proteins are soluble and easily separated from spermatozoa by centrifugation. Human semen consists of a complex mixture of secreted materials including spermatozoa. The seminal fluid in semen carries secretions from the testicles, the seminal vesicles and the prostate gland. Further, spermatozoa are also able to secrete materials that are included in the seminal fluid. Much of the secreted material in seminal fluid is protein. While many proteins found in semen can be found in other human tissues, some are either significantly more abundant in semen or are exclusively found in semen. Proteins found mainly or only in semen provide good targets to enable semen and spermatozoa detection.

The *kallikrein-related peptidase 3* (KLK3), otherwise known as PSA (prostate specific antigen) or p30, is a major protein component found in semen and has a validated history of use in forensics for identifying semen stains (2, 12, 16, 31). PSA/p30 is a serine protease found in semen expressed mainly by the prostate gland in males (2, 18). Monoclonal antibody pairs for PSA/p30 are available commercially and can detect this protein at nanogram levels (14, 16). While PSA/p30 is found mainly in semen, it is expressed in other tissues as well (8). For example, PSA/p30 can be detected in amniotic fluid, endometrium, breast milk, breast tissues and tumors, and periurethral glands (8). It has been found in the blood and urine of women with polycystic ovaries (25). Thus, PSA/p30 has potential to indicate falsely the presence of semen in some evidence with this type of contamination. Regardless, PSA/p30 is used widely for forensic sexual assault cases and provides an accurate test for semen detection in most cases (12, 31).

Another semen protein marker will target the semenogelin protein, which is the predominant protein found in semen. It is secreted by the seminal vesicles and has multifunctional roles (15, 18). This abundant protein in semen is expressed by the *Semenogelin1* and *Semenogelin2* genes, and is produced mainly by secretory epithelium of the seminal vesicles and is the major protein in semen (21, 22, 32). Semenogelin proteins form the structural gel coagulum in semen and fragments of the protein have additional functions (26). Immunochemical tests for semenogelin using anti-semenogelin antibodies have been conducted successfully by many other laboratories (29, 30). A semenogelin test is available commercially. While semenogelin is mainly

expressed in semen, there are examples of semenogelin expression found in other tissues (4). Semenogelin protein was found in trachea and bronchi, skeletal muscle cells and a few scattered cells in the central nervous system. It has also been found expressed in association with lung cancer (28). Additionally semenogelin is expressed in the human retina (3).

Thus, semenogelin and PSA/p30 are expressed in tissues other than semen. However, based on the studies performed thus far, PSA/p30 and semenogelins are expressed together in semen, but are not found together in any other tissues. Detection of seminal fluid proteins when no spermatozoa are found indicates that the donor has had a vasectomy or is otherwise azoospermic.

The *Spermatocyte/Spermatid-specific Thioredoxin-3* (SPTRX3) protein gene is expressed only by spermatozoa (13). We have found that SPTRX3 protein is released from spermatozoa when semen is hydrated from desiccated samples (unpublished results). After testing for SPTRX3 expression in 15 tissue sources with Northern blots and 72 more tissue sources with mRNA arrays SPTRX3 expression was found only in testis (13, 23), see Figure 1.

Thus far, no expression of SPTRX3 has been found in other tissues of the male and female human body. The expression pattern is limited in the testis to spermatozoa. Antibodies to SPTRX3 protein detect this protein at nanogram levels and were used to detect its accumulation in spermatozoa at the subcellular level using microscopy. SPTRX3 protein is expressed by normal and abnormal spermatozoa. SPTRX3 accumulates in the Golgi complex and in cytoplasmic droplets of normal spermatozoa and in nuclear vacuoles and the superfluous cytoplasm of abnormal spermatozoa.

For spermatozoa detection, SPTRX3 is ideal as it is not present in other male or female somatic cells, tissues or tissue fluids, or in the female germ cells and reproductive tissues tested so far. Most importantly, our preliminary work shows that SPTRX3 protein remains stable in dried semen samples for long periods of time, and when the sample is hydrated the protein is released readily from the spermatozoa. Testing on samples stored at room temperature for days, months and years have yielded reliable results. These studies strongly indicate that detection of the SPTRX3 protein will provide an excellent indicator of the presence of spermatozoa in a sample. This causes SPTRX3 to be a good choice for the cassette assay this grant research is designed to develop, for the detection of sperm in evidence samples.

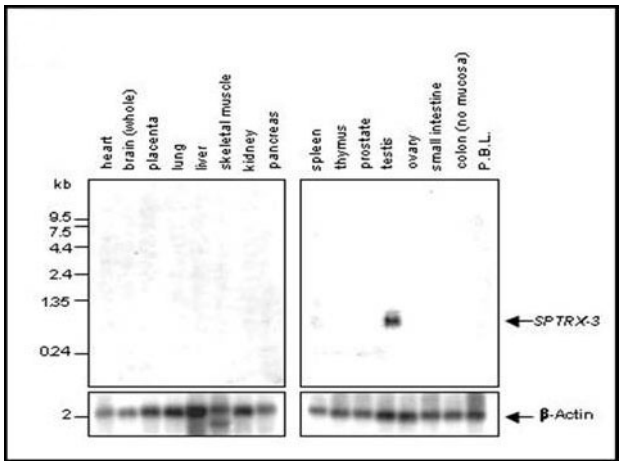


Figure 1. Detection of SPTRX3 mRNA in human testis. RNA samples from a variety of human tissues were characterized on northern blots using an SPTRX3 specific DNA probe. Expression of the SPTRX3 mRNA was detected only in testis tissue. ß-actin serves as an internal control for each lane.

Lateral flow assays provide a quick and relatively inexpensive method for immunochemical chromatographic detection of proteins in small samples (9, 5, 6). With suitable antibody reagents, and a lateral flow assay that incorporates these antibodies, detection of SPTRX3, PSA/p30, and semenogelin together will establish the serological status of sexual assault forensic evidence. The immunochemical methodology for ELISA tests and lateral flow cassettes is well documented (10, 11).

3. Hypothesis/Rationale for Research

Due to the enormous number of sexual assault cases with evidence ready for processing, efforts to improve this process are crucial. One of the steps in processing sexual assault evidence involves identifying and confirming the presence of seminal fluid and spermatozoa. Further, a determination of the amount of spermatozoa in the evidence is helpful for deciding the next steps in the process. Thus, a single cassette designed to detect seminal fluid and spermatozoa proteins together, and to identify the approximate quantity of spermatozoa, will be useful.

PSA/p30 and semenogelin assays are currently available. However, because each gives false positive results in some cases, we proposed combining these on the same cassette so that together a more reliable seminal fluid assay would be available. Because the SPTRX3 gene has spermatozoa specific gene expression, the SPTRX3 protein is expected to be detected only when spermatozoa are present. Thus, instead of using a microscope for visual examination for spermatozoa, we propose an assay for SPTRX3 protein to establish whether spermatozoa are present. Further, combining a SPTRX3 assay along with the PSA/p30 and semenogelin assays on a single cassette

allows the analyst to make a much quicker decision concerning the next steps in processing the case. In this fashion a single cassette will have the capacity to detect all three semen proteins.

Three target proteins were selected for obtaining antibodies to detect sperm and semen. Sperm specific thioredoxin3 (SPTRX3) detection indicates the presence of sperm cells. Semenogelin1 (SEM1), and prostate specific antigen (PSA/p30) both establish that seminal fluids are present. Positive results from all three target proteins using this assay would establish the presence of semen in the sample. Positive results with just SEM1 and PSA and not SPTRX3 indicates semen without sperm cells.

The assay works based on the sandwich-type of immunological detection where two antibodies are required for each target protein. One antibody is conjugated to colloidal gold and is mixed with the evidence sample so that it can form a complex with any target proteins in the sample. This sample is added to the cassette, where chromatographic separation occurs. A capture antibody bound as a stripe on the assay membrane captures any target protein-gold antibody complexes. When the gold becomes concentrated at the stripe a band becomes visible indicating a positive antibody reaction and detection of the target protein.

II. Methods

This Methods section of the Final Technical Report is presented in four parts, written by the four scientists who oversaw four different aspects of the research. Dr. Carson, the Principal Investigator on the grant, discusses the research conducted at PTC. Dr. Sutovsky, the Co-Principal Investigator and the co-discoverer of the SPTRX3 sperm specific protein, discusses the research conducted at his laboratory at the University of Missouri. Glen Ford discusses the initial efforts at lateral flow cassette implementation conducted at BioAssay Works in Maryland, leading to the discovery that initial antibodies for two of the three target proteins were not viable for use in a lateral flow cassette. Dale Dembrow discusses the work performed in attempting to generate monoclonal antibodies to SPTRX3 in his laboratory at BluePoint Bioscience in Maryland.

1. PTC Research Report

Dr. Christian Carson
Principal Investigator
Paternity Testing Corporation

This funding was used to develop the QuantAssure cassette for detecting sperm cells and semen. The goal to produce the

QuantAssure cassette has not yet been reached, but the essential components that were explored to reach this goal will be presented here.

The QuantAssure cassette was designed for the immunological detection of three target proteins found in human semen. Two of these proteins are found in the seminal fluid of males whether sperm cells are present or not (semenogelin and prostate-specific antigen). The third protein, sperm-specific thioredoxin3 (SPTRX3), is expressed and secreted only by sperm cells.
For immunological detection of a target using a lateral flow chromatographic assay, two antibodies for each target protein are required, a gold-conjugated labeling antibody and a capture antibody. A functioning assay indicates positive detection when both antibodies are bound to the target protein. This type of immunological assay is called a sandwich assay because the target protein becomes "sandwiched" by the two antibodies. One of these antibodies is the labeling antibody which is conjugated to gold particles and binds to one portion of the protein in the sample to be tested. The capture antibody binds the target protein in a different region than the labeling antibody. It is bound on the cassette membrane forming a stripe of immobilized antibody to bind target protein that flows past during the assay. Because the labeling antibody is also bound to the target protein when it passes over the stripe of capture antibody, the gold label becomes concentrated at the labeling antibody stripe and a band can be seen indicating the presence of the target protein in the sample.

While the human thioredoxin1 (TRX1) is expressed throughout the human body, expression of the SPTRX3 gene was shown to be specific for sperm cells (Jimenez *et al*., 2004, Miranda-Vizuete *et al*., 2004). SPTRX3 protein was detected in sperm cells specifically when using a rabbit anti-human SPTRX3 antibody (see Figure 2 for amino acid sequence comparison and the peptide used for this antibody). The SPTRX3 gene has sequence similarity to the TRX1 gene. They are both located on human chromosome 9 and their evolution probably involved duplication. However, differences between the two genes and in their resulting proteins provide opportunities to detect SPTRX3 expression specifically.

We sought to use this rabbit anti-human SPTRX3 antibody in an immunological assay to detect SPTRX3 protein from dried forensic samples. The first step required determining whether this antibody could detect SPTRX3 protein in forensic samples that had been stored dry for periods of time. Dried semen samples on tissue paper were placed into a buffered saline solution and vortexed vigorously. The tissue paper was then placed into a microfuge tube spin basket (Axygen Scientific), placed back into the microfuge tube and centrifuged for 10 minutes at 10,000 x g. The supernatant was transferred to another tube and the pellet was reserved for cell lysis and DNA purification. The supernatant was then prepared for SDS-PAGE electrophoresis using Laemmli buffer. After electrophoresis the gel was blotted onto PVDF membrane using the Hoefer miniVE electrophoresis and blot system. The subsequent blotted membrane was blocked with 10% nonfat milk in TBS and 0.01% tween20. The primary antibody, rabbit anti-human SPTRX3, was applied in 1% nonfat milk in TBS and 0.01% tween20 overnight. The secondary antibody, goat anti-rabbit horseraddish peroxidase conjugate, was applied in 1% nonfat milk in TBS and 0.01% tween20 for 1 hour. The substrate for the horseradish peroxidase enzyme conjugate on the secondary antibody was Immobilon Western Chemiluminescent HRP Substrate from Millipore.

Our results showed that we are able to detect the SPTRX3 protein from forensic samples. We found that by adding buffered saline to elute the sample from the substrate and by separating the sample from the substrate with a centrifuge, the resulting pellet contains intact cells and the supernatant carries detectable levels of SPTRX3 protein. This result demonstrates that SPTRX3 protein levels can be measured from the supernatant and the cells in the pellet can be entirely dedicated for DNA analysis. In our hands, the SPTRX3 protein is stable enough for detection, but once in solution, the protein is not stable for more than a day.

Consistently, SPTRX3 protein is found only when sperm cells are present, see figure 3. Samples were prepared and analyzed as described above. Although cross reactivity between the secondary antibody and endogenous human antibodies gave bands other than the SPTRX3 specific band, only samples with sperm cells result with the 17k Mr band indicative of SPTRX3. Vaginal swabs without sperm

cells do not have the 17k band in the supernatant fraction, while postcoital swabs do. Pellet fractions from sperm (not shown) and vaginal secretions do not have SPTRX3 protein.

As seen in figure 3, western blot data indicates non-specific cross reactivity in the assay. Because the SPTRX3 antibody has no label for detection itself, a secondary antibody, goat anti-rabbit, conjugated to horseradish peroxidase, was used to detect positive SPTRX3 antibody binding. We determined that the non-specific bands were coming from direct cross reactivity between the secondary goat anti-rabbit antibody and proteins in our samples, see figure 4. Goat anti-rabbit antibody from Invitrogen alone showed cross reactivity with human antibodies that are abundantly expressed in vaginal secretions and also to some extent in semen secretions. Thus, specific reactions between SPTRX3 antibody and SPTRX3 protein in semen secretions were obscured by cross reactivity between the secondary antibody and some human antibodies that were present. Further, attempts to show no reaction between SPTRX3 antibody and vaginal secretion proteins were obscured by cross reactivity between the secondary antibody and human vaginal secretion antibodies.

We then tried secondary antibodies from different sources. One possibility for cross reactivity between the goat anti-rabbit secondary antibody and human antibodies might have been due to binding between complement proteins from vaginal secretions and the Fc portion of the antibody. Thus, FAB fragment goat anti-rabbit secondary antibody from Jackson ImmunoResearch Laboratories was tested. However, these secondary antibodies also failed to not cross react with human immunoglobulins in the samples (data not shown).

To avoid using secondary antibodies and obtaining false positive signals due to cross reactivity between the secondary antibody and the abundant endogenous human antibodies we find, especially in vaginal secretion samples, we conjugated the SPTRX3 antibody directly with horseradish peroxidase using a SureLINK HRP Conjugation Kit obtained from KPL, Inc. The resultant conjugate SPTRX3 antibody detected a band of protein at ~44k on western blots, figure 5. When extracts from vaginal secretions and saliva were compared to sperm secretion preparations, this protein band

was only detected from sperm extracts. When greater concentrations of the conjugated SPTRX3 antibody were used, lower molecular weight bands were detected from vaginal secretion samples. Vaginal extracts gave three major bands at ~30k, 27k, and 24k M_r along with several other minor bands. The intensity of these bands was less than the intensity of the 44k band detected in sperm samples and they were nearly invisible when using 1:4000 dilution of the antibody. Because the 44k band was not the correct size for SPTRX3 protein, we were skeptical of the results. Further, the antibody may cross react with small molecular weight material found at the dye front found in both sperm and vaginal proteins. Because of these uncertainties, this portion of the project was discontinued.

Ultimately the problem of secondary cross reactivity was alleviated with a secondary antibody reagent obtained from BioAssay Works (see figure 4). They provided a goat anti-rabbit secondary antibody conjugated with horseradish peroxidase that is specific for rabbit antibodies and does not cross react with human antibodies. Western blot results using this secondary antibody gave a clean 17k Mr SPTRX3 band and no cross reacting bands in vaginal samples. With these results we were finally fully assured of no false positive signals in vaginal secretion proteins with the SPTRX3 antibody.

The rabbit anti-human SPTRX3 antibody was sent to BioAssay Works for labeling with colloidal gold. We also supplied BioAssay Works with frozen semen and sperm cell preparations for a source of target protein. To establish an assay for SPTRX3 using the rabbit anti-human SPTRX3 on hand, we had BioAssay Works label our antibody with colloidal gold. As a preliminary test we attempted to use a thioredoxin1 antibody from Santa Cruz Biotechnology and another from AbD Serotec to capture the SPTRX3 protein when bound to the SPTRX3 antibody. Both TRX1 antibodies failed to bind to the SPTRX3 protein. At this point we decided to produce new SPTRX3 antibodies to complement the original antibody, or to obtain an entire new set.

Two other target proteins were chosen for use in combination with SPTRX3 to obtain more complete information from a potential sexual assault sample with QuantAssure. The semenogelin1 protein is abundant in semen and it can be detected with semenogelin

antibodies (Lilja *et al.*,1989, Malm *et al.*,1996). However, full length semenogelin1 protein is degraded into fragments by the prostate specific antigen protein and this phenomenon must be taken into account when planning a sandwich assay for semenogelin1 see figure 6 (Robert *et al.*, 1997). Five semenogelin1 antibodies were obtained from Santa Cruz Biotechnology (see table below).

Semenogelin antibody	Semenogelin peptide region
H300	1-300
S17	50-100
E15	390-440
G16	370-420
C15	412-462

All were tested using dot blots to determine if semenogelin could be immunologically detected in the supernatant portion of a forensic sample, as described above. All five individually were able to detect semenogelin1. These were sent to BioAssay Works to label them with colloidal gold and to test them pairwise for their capacity to detect semenogelin1 protein using sandwich assay technology. None of these antibody combinations gave positive activity in the sandwich assay. While more work is needed to find a suitable antibody pair for one we already have, at this point we stopped working on semenogelin1 because of problems encountered with SPTRX3.

The third target protein we chose to include on the QuantAssure cassette is the prostate specific antigen (PSA, p30 protein) (Armbruster 1993, Johnson and Kotowski 1993). A known sandwich assay pair of PSA antibodies were purchased from AbD Serotec. These were sent to BioAssay Works where they labeled them with colloidal gold and found that they performed correctly in a test sandwich assay for PSA using our extracts.

BioAssay Works was recruited to help us create the QuantAssure cassette. Reagents and extraction supplies were sent to them for testing. BAW conjugated the antibodies with colloidal gold and tested each antibody for function in an immunological sandwich assay, as described above. Once we had determined which antibodies would

function in the QuantAssure assay, we set out to produce new SPTRX3 antibodies to enable this portion of the assay. Because the current SPTRX3 antibody provided the specificity needed, we focused on making two antibodies for SPTRX3, one from the first exon and one from the fifth exon to obtain a pair of antibodies with great specificity. We decided to make a mouse monoclonal antibody for gold conjugate from the first exon peptide and a rabbit polyclonal capture antibody from the fifth exon peptide. We were most interested in creating a prototype functioning QuantAssure assay to enable future consideration and development. Bluepoint Bioscience was recruited for the mouse monoclonal SPTRX3 antibodies and Southern Biotech recruited for rabbit polyclonal antibody production.

Serum from rabbits immunized with the first exon peptide of SPTRX3 was analyzed for immunological activity with semen extracts. The complexity of bands obtained proved to not allow progress with these antibodies (data not shown). Perhaps this peptide alone wasn't sufficient to stimulate a good antibody for SPTRX3.

Serum from mice immunized with the first exon peptide of SPTRX3 was analyzed for SPTRX3 detection. Many attempts were made to obtain the 17k Mr band with these sera, but we were not able to identify clones that were specific for the SPTRX3 protein.

Because the antibodies required for detection of SPTRX3 were not obtained by this process, an alternative approach to producing these antibodies will be necessary. One approach would be to try different unique peptides than the ones already tested. Alternatively, the whole protein or part of it can be expressed *in vivo* to obtain a larger peptide for immunization. In addition, semenogelin peptides will need to be constructed to produce a second antibody for semenogelin1 detection.

Armbruster DA (1993) Prostate-specific antigen - Biochemistry, analytical methods, and clinical application. Clinical Chemistry 39 (2): 181-195

Jiménez A, Zu W, Rawe VY, Pelto-Huikko M, Flickinger CJ, Sutovsky P, Gustafsson J-A, Oko R and Antonio Miranda-Vizuete A. (2004)

Spermatocyte/spermatid-specific thioredoxin-3, a novel Golgi apparatus-associated thioredoxin, is a specific marker of aberrant spermatogenesis. J. Biol. Chem. 279:34971-34982.

Johnson ED, Kotowski TM (1993) Detection of prostate specific antigen by ELISA. Journal of Forensic Sciences 38 (2):250-258

Lilja H, Abrahamsson PA, and Lundwall A (1989) Semenogelin, the predominant protein in human semen. Primary structure and identification of closely related proteins in the male accessory sex glands and on the spermatozoa. J. Biol. Chem. 264:1894–1900

Malm J, Hellman J, Magnusson H, Laurell CB, Lilja H (1996) Isolation and characterization of the major gel proteins in human semen, semenogelin I and semenogelin II. European Journal of Biochemistry 238(1):48-53

Miranda-Vizuete A, Sadek CM, Jiménez A, Krause WJ, Sutovsky P and Oko R (2004) The mammalian testis-specific thioredoxin system. Antioxid Redox Signal. 6:25-40

Robert, M., Gibbs, B.F., Jacobson, E. & Gagnon, C. (1997) Characterization of prostate-specific antigen proteolytic activity on its major physiological substrate, the sperm motility inhibitor precursor/semenogelin I. Biochemistry 36:3811-3819

Figure 2
Choosing unique peptides from SPTRX3 for specific antibodies.

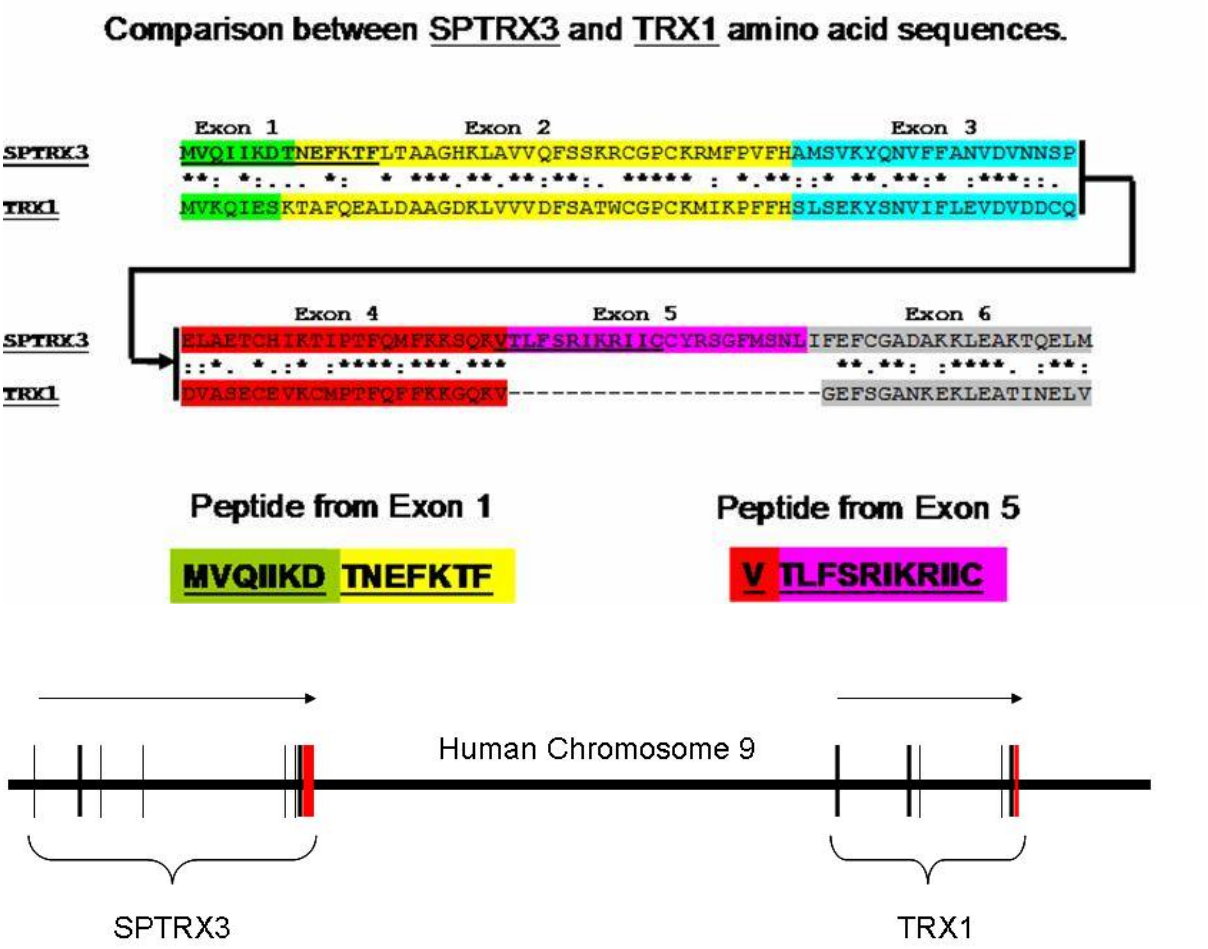


Figure 3
Detection of SPTRX3 protein using rabbit anti-human SPTRX3 polyclonal antibody
and horseradish peroxidase conjugated goat anti-rabbit secondary antibodies.

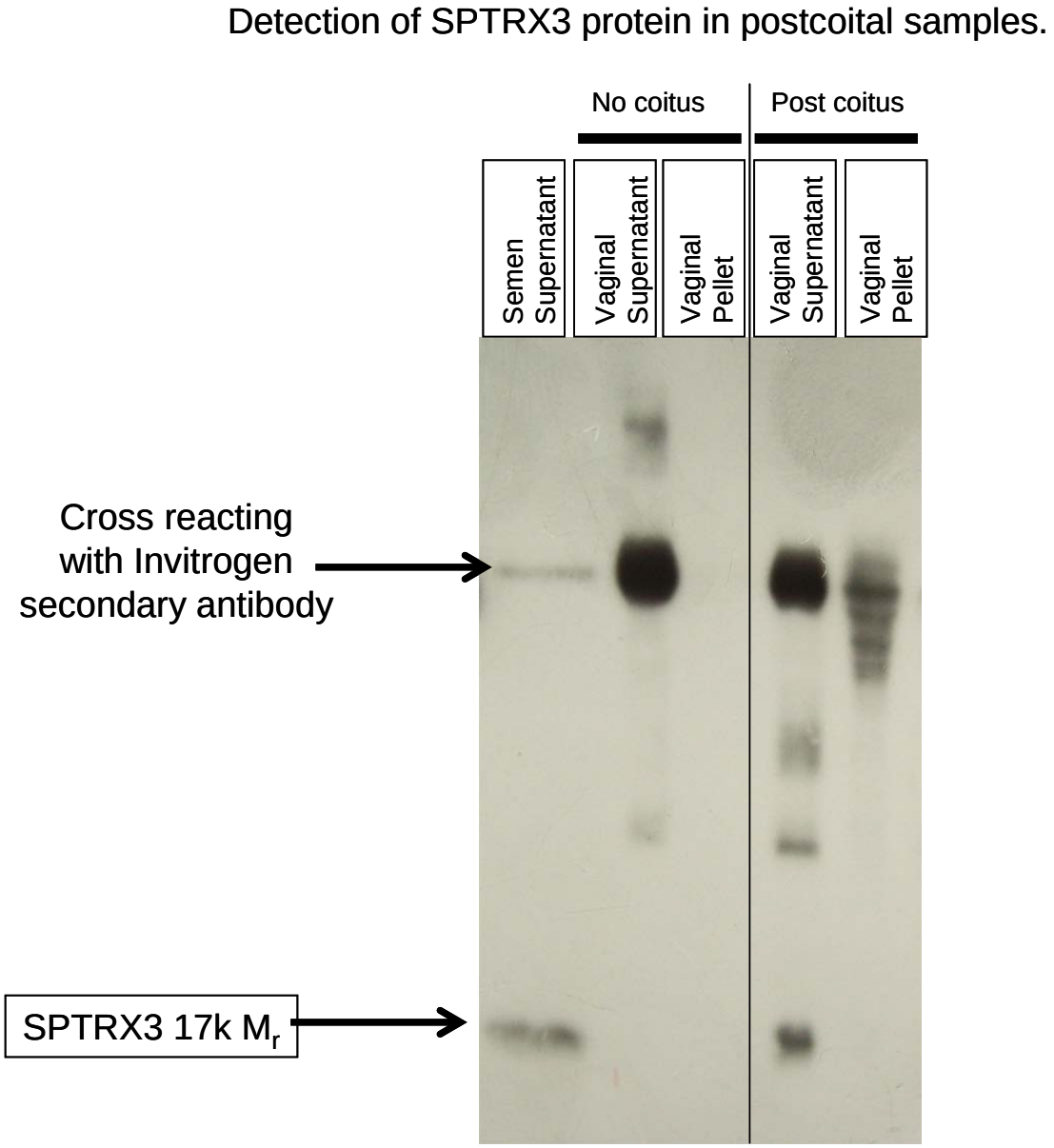


Figure 4
Detection of SPTRX3 protein in supernatants from dried semen. Complications and resolution with the secondary antibody.

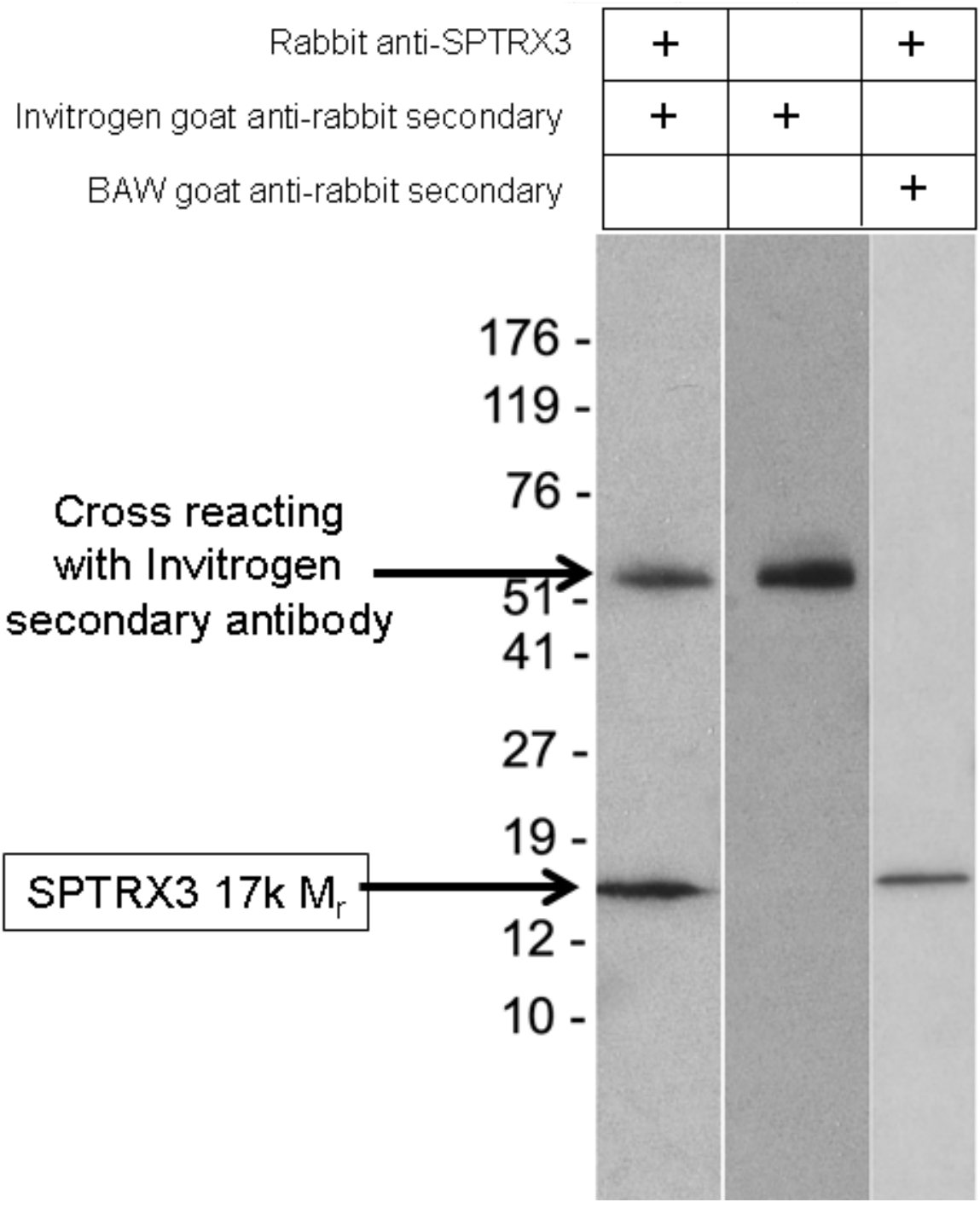


Figure 5
Rabbit anti-human SPTRX3 antibody conjugated directly to horseradish peroxidase
detects a 44k Mr band instead of the 17k Mr band.

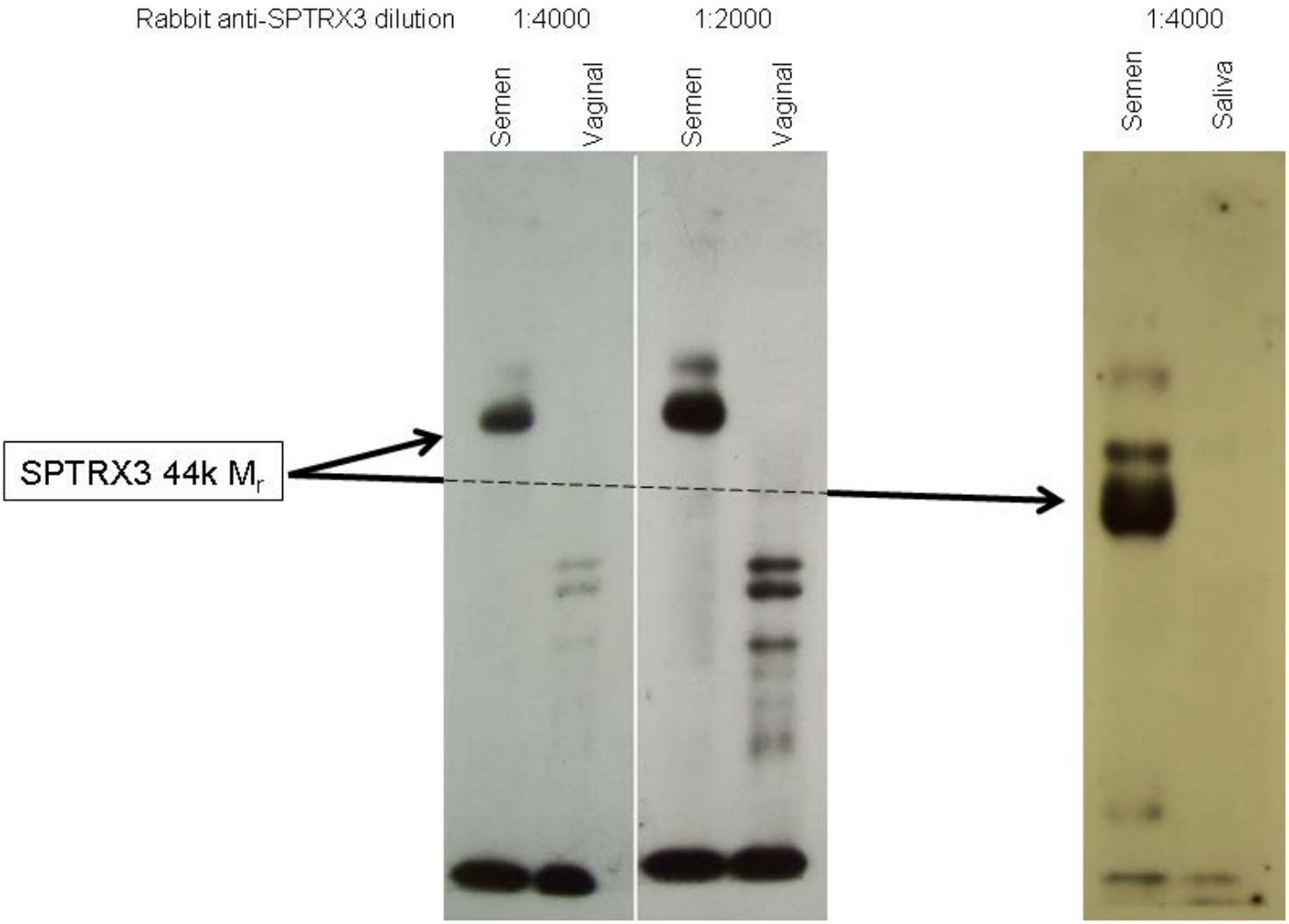
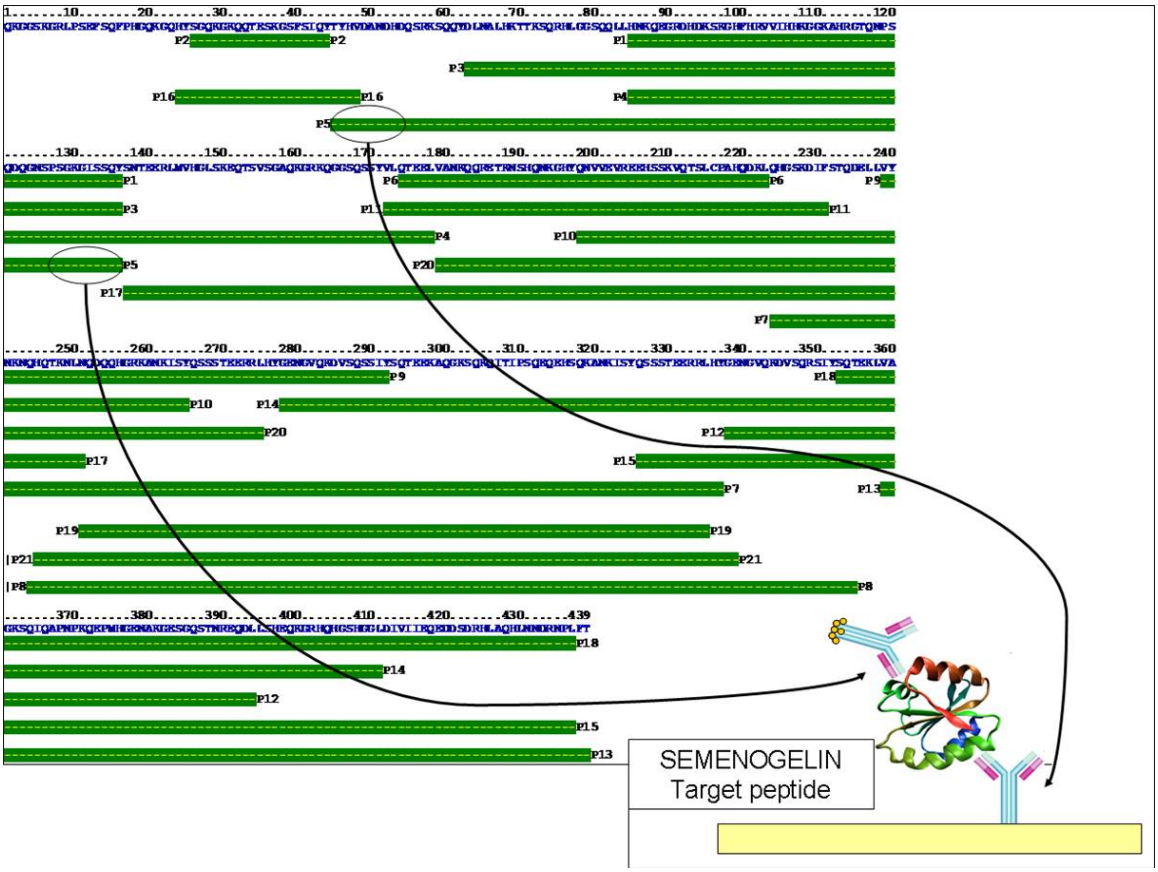


Figure 6
Semenogelin1 amino acid sequence showing proteolytic fragmentation and design of antibodies from the peptide sequences.

The figure shows the full length sequence of semenogelin1. Below the sequence are bars to indicate the peptide fragments produced by PSA digestion based on Roberts *et al*., 1997. The cartoon in the lower right hand corner of the figure shows a semenogelin peptide (peptide P5 in the figure) sandwiched between the lower capture antibody and the upper gold labeled antibody. The figure demonstrates the necessity of choosing a pair of antibodies that bind to the same peptide for semenogelin detection.



2. University of Missouri Research Report

Dr. Peter Sutovsky
Co-Principal Investigator
University of Missouri

1. Project Goal and Technical Principle

The goal of this project is to construct and validate a lateral flow device for detection of sperm cells in forensic rape case evidence kit. Such a kit is expected to contain victim's vaginal secretions and may also contain perpetrator's sperm or semen-derived proteins not present in the female bodily fluids. This kit will detect sperm-specific protein SPTRX3 using a paired antibody approach. For this, two anti-SPTRX3 antibodies, detecting two different domains of the SPTRX3 protein have to be produced. The first antibody, termed the labeling antibody is conjugated with 40 nm particles. This antibody is released from a wetting strip and binds to molecules of SPTRX3 present in the soluble sample. The second antibody, termed capture antibody binds to a domain of SPTRX3 different from the domain recognized by the labeling antibody. The capture antibody is immobilized on a test strip along which the soluble sample with colloidal gold tagged SPTRX3 molecules moves inside the lateral flow device. Capture of gold-tagged SPTRX3 molecules on the capture strip causes the strip to turn red because of high optical density of 40 nm gold particles. This will not happen unless there are SPTRX3 molecules, and therefore sperm cells or fragments present in the tested forensic evidence sample.

2. Production and Validation of Labeling Antibody

This antibody was produced by immunization of rabbits with a mixture of two peptides, corresponding to the following amino acid (AA) residues of the full length human SPTRX3 protein:

Peptide #1: **MVQIIKDTNEFKTFC** (AA residues 1-14)
Peptide #2: **VTLFSRIKRIIC** (AA residues86-97)

The resultant antibody was affinity purified and tested extensively by Western blotting of human sperm and vaginal secretion extracts.

Further testing was performed by immunofluorescence and flow cytometry. By western blotting, it was found that this antibody recognizes SPTRX3 from human sperm extract alone, or mixed with vaginal secretion extracts, but not with vaginal secretion extracts alone. Other human cell types and bodily fluids, used as negative control, also tested negative by Western blotting. By using ImageStreamtechnology that combines flow cytometry with epifluoresence

microscopy, this antibody was found to label SPTRX3 deposits typical of human spermatozoa, and not found in spermatozoa of other species. *We are therefore confident that this labeling antibody is specific to SPTRX3 derived from human sperm cells and does not cross-react with cells and bodily fluids from other male and female tissue organs in humans or animals.*

The above antibody was subsequently conjugated to 20 nm colloidal gold particles, which lend the molecules of antibody desired optical density without hindering its binding to target protein, SPTRX3. The resultant gold-dressed antibody was first verified by light microscopic indirect immunofluorescence technique. As anticipated, the gold-dressed antibody retained its ability to bind to human sperm-borne SPTRX3 aggregates, both in live and fixed spermatozoa (**Fig. 7 A**). The specificity of this binding was verified by transmission electron microscopy, which allowed us to directly visualize the antibody-coated colloidal gold particles bound to human sperm-borne SPTRX3 aggregates (**Fig. 7 B, C**). Therefore, we produced a functional labeling antibody for the SPTRX3-detecting lateral flow device.

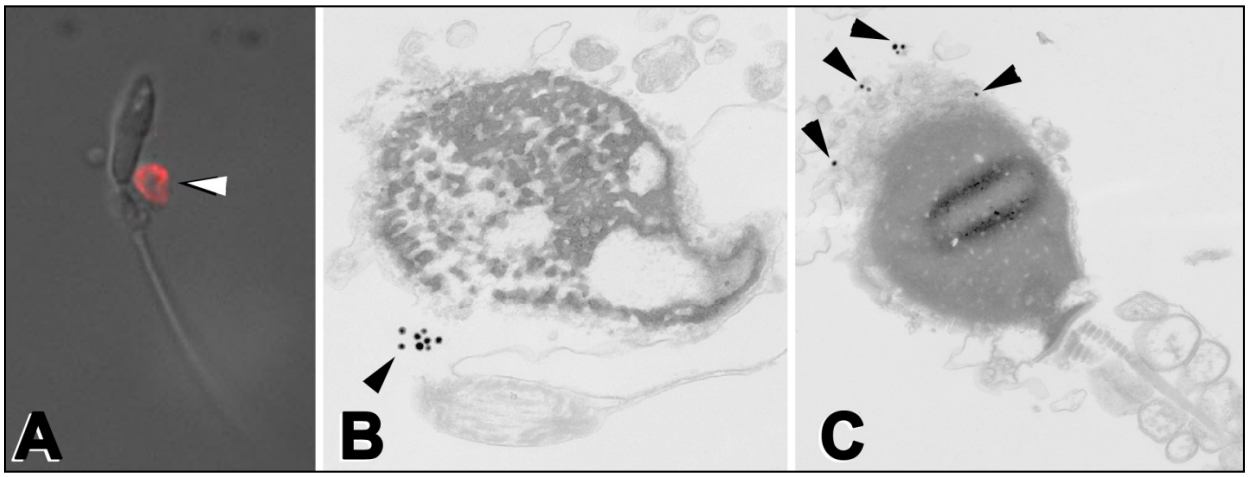


Fig. 7: Validation of the gold-dressed labeling antibody by immunofluorescence (**A**) and transmission electron microscopy (**B**) of human sperm cells. **A:** Red fluorescen anti-rabbit secondary antibody was used to detect the gold-dressed antibody bound to SPTRX3 aggregates (arrowhead) carried by whole-mounted human sperm cells. **B, C:** Gold particles coated with anti-SPTRX3 antibody (arrowheads), bound to SPTRX3 aggregates visualized on ultrathin sections of human sperm cells by electron microscopy.

3. Production and Validation of Capture Antibody

Initially, we attempted to produce this antibody by immunization of mice with a mixture of two peptides, corresponding to following amino acid (AA) residues of the full length human SPTRX3 protein:

Peptide #1: **NNSPELAETCHIKTI** (AA residues 60-74)
Peptide #2: **CYRSGFMSNLI** (AA residues 98-108)

Two rounds of immunization and serum testing have been completed. In the first round, the immunized mice failed to respond to immunization. Regardless, control sera were tested by ELISA, Western blotting and immunofluorescence in an extensive trial (Data not shown). In the second trial, a positive response to immunization was detected in five mice. Sera from all five animals were initially tested by immunofluorescence of human sperm cells, and find to react with some structures similar to SPTRX3 aggregates detected by the above labeling antibody. However, a high level of non-specific background fluorescence was detected, and some of the sperm cells with typical appearance of SPTRX3-aggreate bearing cells were not labeled specifically. All sera were therefore carefully analyzed by Western blotting of human sperm extracts. While several sera showed a protein band on western that was consistent with the anticipated SPTRX3 band, this was band often overshadowed by non-specific bands. Furthermore, a similar band, albeit weaker was also detected with the said sera in samples of human vaginal secretions.*Therefore, after a repeated immunization and validation trial spanning a period of one year, it was concluded that the immunized mice failed to produce antisera specific to human sperm-borne SPTRX3 protein.*

The developments described above necessitated a change of strategy. Therefore, two new capture antibodies were designed, with specificity to the following AA sequences/domains of human SPTRX3:

Peptide #1: **QMFKKSQKVTLFS**(AA residues78-90)
Peptide #2: **QFSSKRCGPCKRMFP** (AA residues26-40)

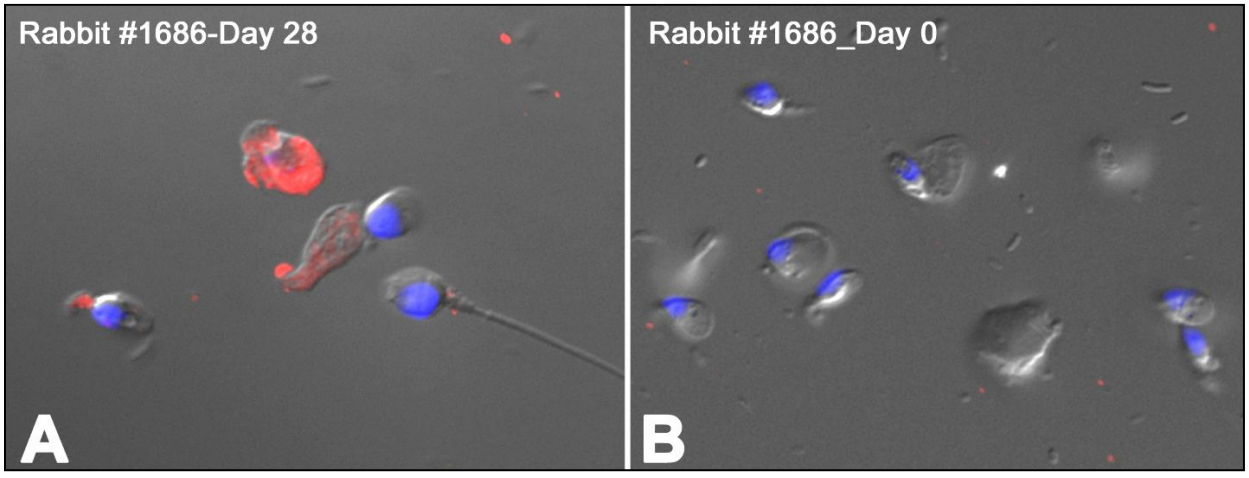


Fig. 8: Initial testing of new rabbit serum to be used as capture antibody. **A:** Immunofluorescence shows SPTRX3 labeling (red) of human sperm cells with a test pre-bleed serum collected from rabbit #1686 on day 28 after immunization with SPTRX3-peptide QMFKKSQKVTLFS. **B:** Serum from the same rabbit, taken one day prior to immunization shows no immunoreactivity reminiscent of SPTRX3 labeling. Both sera were tested at equal concentration, using the same human sperm cell preparation and identical labeling condition. DNA inside the sperm nuclei was counterstained with a blue fluorescent probe DAPI.

Two peptides corresponding to the above sequences were synthesized, conjugated to keyhole limpet hemocyanin (KLH) to increase its immunogenic properties, and used individually (as opposed to in mixture) to immunize two rabbits each. At the time of this report completion, the test pre-bleed performed on Day 28 after immunization yielded four sera, one of which (Peptide #2) shows strong and specific immunoreactivity with SPTRX3 aggregates in human spermatozoa by immunofluorescence (**Fig. 8**). No such immunoreactivity was observed by using a preimmune serum collected from the same rabbit one day before the initial immunization. Strong immunoreactivity with some background fluorescence was observed at the dilution of 1:100, which is typically used as starting dilution for immunofluorescence antibody testing in our laboratory. The antibody was retested using a progressive dilution approach. While the non-specific background fluorescence was reduced, the anticipated labeling of SPTRX3 aggregates was still observed at a dilution of 1:2,000. Next, this serum was tested by Western blotting and found to recognize a band in the 13-19 kDa range, consistent with SPTRX3 (**Fig. 9**). Additional bands were detected above this range, which came as no surprise since this was a crude serum containing a variety of immunoglobulin species. Meanwhile, booster immunization was given to all four rabbits and the next set of sera was collected on Day 56. Testing of these sera by immunofluorescence and Western blotting confirmed previously found SPTRX3-immunoreactivity.

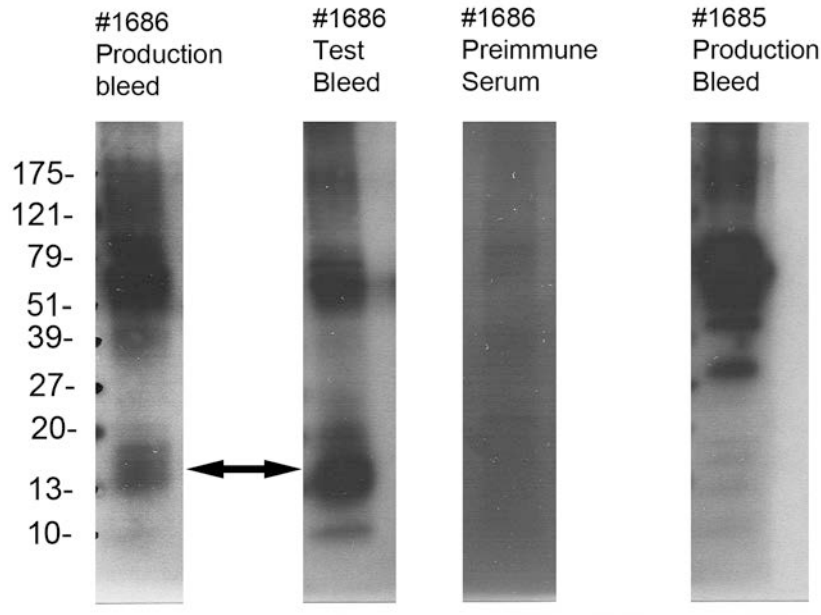


Fig. 9. Sera from two of the four rabbits immunized to produce capture antibody. Note the prominent band in 13-19 kDa range (arrow) in rabbit number 1686, consistent with SPTRX3. Additional bands above said range are non-specific IgGs that were subsequently removed by affinity purification

Terminal bleeding of rabbit #1868 yielded a crude serum which was affinity purified against the immunization peptide. A total of 8 mL were recovered and tested by immunofluorescence and Western blotting. Immunofluorescence (**Fig. 10 A**) showed human sperm labeling consistent with that observed by using our original serum (labeling antibody). Most importantly, Western blotting detected a single band consistent with mass of SPTRX3 in human sperm extracts (**Fig. 10**

B). This AP-antibody is currently being tested for cross-reactivity with proteins found in forensic rape samples. Since then, one more rabbit also produced a crude serum recognizing SPTRX3, and was also terminally bled. The resultant serum is currently being affinity purified.

In conclusion, despite a delay due to a failed mouse immunization trial, we have produced and affinity purified an SPTRX3 antiserum suitable for capture antibody to be used for the completion of the construction our lateral flow device.

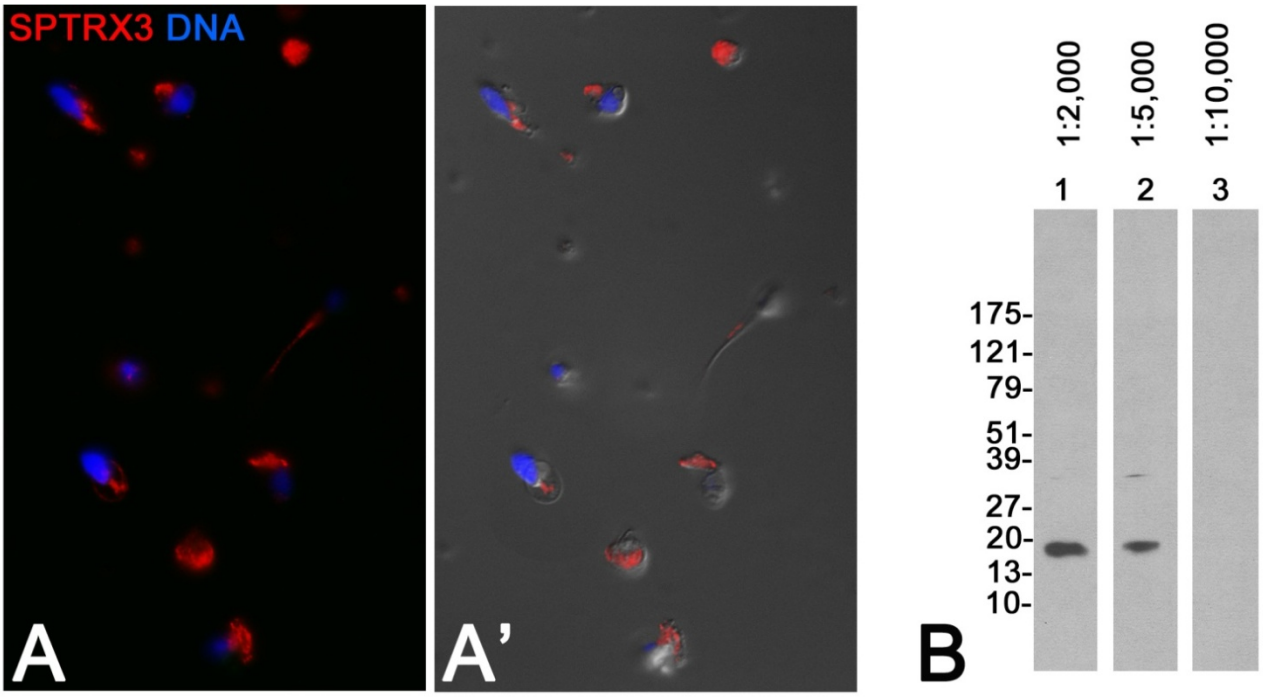


Fig 10. Validation of affinity purified capture antibody. (**A**) Immunofluorescence; red SPTRX3 signal is seen in typical sites of SPTRX3 accumulation in the redundant cytoplasm of defective human spermatozoa. Panel A' shows overlay of fluorescence with a parfocal image acquired using differential interference contrast (DIC) optics. (**B**) Western blotting; a single band consistent with SPTRX3 is detected in human sperm extract probed with AP-antibody diluted 1:2,000 or 1:5,000. Further supporting the specificity of this antibody, high dilution (1:10,000) eliminated the band.

project. Funding for this additional work has been secured by Dr. Sutovsky. No additional funding is being requested from NIJ.

- Testing of capture antibody by ELISA, immunofluorescence and Western blotting of forensic samples (ongoing)
- Construction and calibration of a test strip
- Lateral flow cassette assembly
- Validation of lateral flow device by using solubilized mixtures of human sperm cells and vaginal secretions

5. Materials & Methods

Production of rabbit polyclonal antibodies: The KLH-conjugated peptides (se amino acid sequences identified above) were dissolved in 1 ml of Freund's complete adjuvant (FCA; Sigma, St. Louis, MO, #F5881) and injected into ten separate subcutaneous sites in each of two New Zealand White rabbits per peptide. The following immunization schedule was used:

Day 0: Pre-Immune Bleed
Day 1: Initial Immunization
Day 14: 1st Boost
Day 21: 2nd Boost
Day 28: Test Bleed (5-7ml)
Day 35: 3rd Boost
Day 49: 4th Boost
Day 57: Production Bleed (20-25ml) and boost
Day 78: Terminal exsanguination (55-65 mL)

The test bleed sera were obtained by bleeding of the marginal ear vein and rabbits were reimmunized. With the exception of currently immunized rabbits in the ongoing trial, the rabbits were sacrificed on day 57, blood was collected and immune sera were prepared using standard procedures (Harlow and Lane, 1988). Immune sera referred to as p64 Lillie and p64 Fran, as well as pre-immune sera collected on the day of first immunization, were screened by Western blotting of human sperm extracts and immunofluorescence on formaldehyde-fixed human spermatozoa.

All animal procedures, as well as the health and well-being of all rabbits, were carefully monitored by veterinary staff and all procedures were performed under the guidance of approved ACUC protocols in a humane manner and with the use of anesthesia.

Production of mouse monoclonal antibodies: Performed by BioAssay Works using standard laboratory and live animal procedures.

Human sperm samples: Cryopreserved human sperm samples for serum testing were purchased from Fairfax Cryobank, Fairfax, VA, and used under strictly followed guidelines of a protocol approved by the Internal Review Board (IRB) of the University of Missouri. All samples were collected from pre-screened healthy, fertile donors who signed appropriate consent form. All samples were coded by Fairfax Cryobank and kept anonymous to researchers and staff involved with the present project.

Western blotting. Proteins from human sperm samples were extracted by adding an extraction buffer (pH 6.0), consisting of 50 mMTris HCL, 1 M NaCl, 20mM imidazole, 1 mMethylenediaminetetraaceteic acid (**EDTA)** and 5 mMbenzamidine. Protease Inhibitor Cocktail (Sigma, St. Louis, MO, #P-8340), PMSFand 10% Triton X-100 were added at 10 μl/1 ml of extraction buffer. The

extraction buffer was then added to the sperm sample at 1:1 and ground for 5 minutes using a pestle attached to a 9.6 volt electric drill (Black and Decker®, model no. CD9600). Samples were centrifuged in a refrigerated centrifuge (Sorvall®, Biofuge Fresco, #75005516) at 4ºC at 16,000 x g for 1 hour. The protein loads were standardized by applying the Bradford protein estimation assay (Bio-Rad Protein Assay kit; Bio-Rad Laboratories, Hercules, CA) measured at 595 nm wavelength absorbance. All samples were brought to an equal final protein concentration by dilution using a 2X loading buffer consisting of 2% SDS, 10% glycerin, 0.0125% brom phenol blue, 5% beta mercaptoethanol, 62.5 mMTrisHCl pH 6.8 and distilled water. Samples were boiled in a hot water bath for 3 minutes. Electrophoresis on a 10% Tris-glycine polyacrylamide gel (Cambrex Bio Science Rockland, Inc, Rockland, ME, #59508) was run using 6 µg of protein in each lane. Ten microliters of Prosieve Color Protein Marker (Cambrex Bio Science Rockland, Inc, Rockland, ME) were used as the molecular weight markers. After 1 hour of electrophoresis, the proteins were transferred to a polyvinylidenedifluoride(PVDF)membrane (Immunobilin™-P Transfer Membranes, Millipor Corp., Bedford, MA) using a wet transfer system (Owl Scientific, Inc., Woburn, MA, Model #VEP-2). The membranes were blocked for 1 hour in 5% non-fat dried milk then washed in 0.25% TBS-Tween 20 5 times 5 minutes each. The membranes were then incubated overnight at +4°C with immune or preimmune mouse or rabbit serum. Serial dilutions were made ranging from 1:500 to 1:5,000 to test individual mouse and rabbit sera.After overnight incubation, membranes were washed again in 0.25% TBS-Tween 20 5 times 5 minutes each then incubated in secondary antibody (Goat Aanti-MouseIgG-HRP for mouse sera; Goat Anti-Rabbit IgG-HRP for rabbit sera) for 1 hour (diluted at 1:10,000). Membranes were washed one last time in 0.25% TBS-Tween. Membranes were developed using the SuperSignal® West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc., Erembodegem, Belgium, #34077) for 4 minutes then developed for 1-10 minutes on Kodak BioMax Light Film (Kodak, Cedex, France) using the Kodak M35A X-OMAT processor

Immunocytochemistry and epifluorescence microscopy on whole mounts of human spermatozoa. Detailed procedures are described in (Sutovsky, 2004). Briefly, whole sperm samples were mounted to poly-L-lysine coated cover slips and fixed in 2% formaldehyde in 0.1% phosphate buffered saline (PBS)for 40 minutes. Samples were washed, permeabilized for 40 min with 0.1% Triton-X-100 and blocked in 5% normal goat serum (NGS) in PBS for 25 minutes. To detect the binding of mouse/rabbit sera to sperm-borne PSTRX3 molecules, samples were incubated for 40 minutes with the said serum diluted in PBS containing 1% NGS and 0.1 % TX-100. Serial dilutions ranging from 1:50 to 1:2,000 were tested. Incubation I with immune/preimmune serum was, followed by a brief wash in PBS then incubation for 40 minutes with a mixture containing either a goat anti-mouse IgG-FITC (Zymed, San Francisco, CA, #62-6511; diluted 1:100) or goat-anti rabbit IgG-TRITC (Zymed, San Francisco, CA, #81-6114; diluted 1:100), depending on which animal species' serum was being

tested. To counterstain sperm head, a DNA-stain DAPI (Molecular Probes, Eugene, OR, #D1306) was added to second antibody solution at a concentration of 5 ug/ml. After secondary antibody incubation, coverslips were washed again in PBS and mounted on microscopy slides in VectaShield medium (Vector Laboratories, Inc., Burlingame, CA, #H-1000). Multi-channel acquisition (visible light, ultraviolet-blue, green, red and far-red spectra) was performed by using a Nikon Eclipse E800 microscope equipped with differential interference contrast optics (DIC) and a CoolSnap HQ CCD camera operated by the MetaMorph software.

Preparation of colloidal gold-dressed anti-SPTRX3 antibody . Rabbit serum raised against a mixture of peptides composed of equal amounts of peptide #1 (MVQIIKDTNEFKTFC; AA residues 1-14) and peptide #2 (VTLFSRIKRIIC; AA residues 86-97) was provided to BioAssay Works LLC, (BAW) Ijamsville, MD, for conjugation to 40 nm colloidal gold. This conjugation was performed b using a proprietary procedure developed by BAW.

Ultrastructural colloidal gold immunocytochemistry and transmission electron microscopy (TEM). Gold dressed anti-SPTRX3 antibody was used in a procedure adapted from immunofluorescence protocol described above. Human spermatozoa were fixed in 2% formaldehyde, washed and pelleted by centrifugation, and then resuspended for 40 min in PBS containing 0.1% NGS. Following this permeabilization step, spermatozoa pelleted by centrifugation were resuspended for 25 min in PBS with 0.1% TX-100 and 5% NGS, to block non-specific serum-sperm binding. After blocking and centrifugation, supernatant was removed and sperm pellet resuspended in PBS containing 0.1% TX-100, 1 % NGS and either a 1:200 dilution of 40 nm colloidal gold dressed with rabbit anti-SPTRX3 antiserum or the same dilution of control colloidal gold particles, provided by BioAssay Works, Ijamsville, MD. Samples were incubated overnight at +4C, then washed by centrifugation, pelleted and processed for TEM as follows. Briefly, gold-labeled sperm pellets were fixed in 0.6% glutaraldehyde/2% paraformaldehyde fixative, postfixed in 1% osmium tetroxide, dehydrated, and embedded in PolyBed 812. Ultrathin sections were cut on a Leica Ultracut UCT ultramicrotome, stained by uranylacetate and lead citrated, and photographed in a Jeol1200 EX electron microscope. Photographs were scanned by UmaxMagic Scan flat-bed scanner (Umax Technologies Inc., Dallas, TX) and edited by Adobe Photoshop CS3 (Adobe, Mountain View, CA).

3. BioAssay Works Research Report

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BioAssay Works (BAW) received antibody from Paternity Testing Corporation (PTC) for three proteins, in order to develop a lateral flow cassette to detect the presence of sperm and semen in solution.

We tested the antibodies for the PSA/p30 protein, and found them to work acceptably for use in a cassette.

We tested five antibodies provided by PTC for the semenogelin protein. We attempted to determine an acceptable pair of antibodies (to act as labeling antibody and capture antibody). No combination of the five antibodies gave positive activity in the sandwich assay. Further development of antibodies for the semenogelin protein was put on hold by PTC, pending the outcome of attempts to resolve difficulties regarding the SPTRX3 antibodies.

With regard to the polyclonal rabbit antibody received from PTC for the SPTRX3 protein, using the cassette construct described below, semen samples from two different sources were solublized by low-level sonication and tested at three different log-dilutions. No activity was observed, leading to the conclusion that the Rabbit anti-SPTRX3 polyclonal antibody does not react with native SPTRX3 antigen. Since the SPTRX3 antigen has multiple repeat-epitopes on the sperm cells, a reaction was expected.

Furthermore, Western Blot analysis of the rabbit anti-SPTRX3 polyclonal showed no activity with the expected molecular weight banding patterns of SPTRX3 antigen.

Based on these findings, PTC decided to pursue generating antibodies to the different Exon transcribed regions of the SPTRX3 molecule that, based on data-base information, might be immunogenic.

Lateral Flow Cassette Design

Specific details of the Anti SPTRX3 - LFI strip assembly

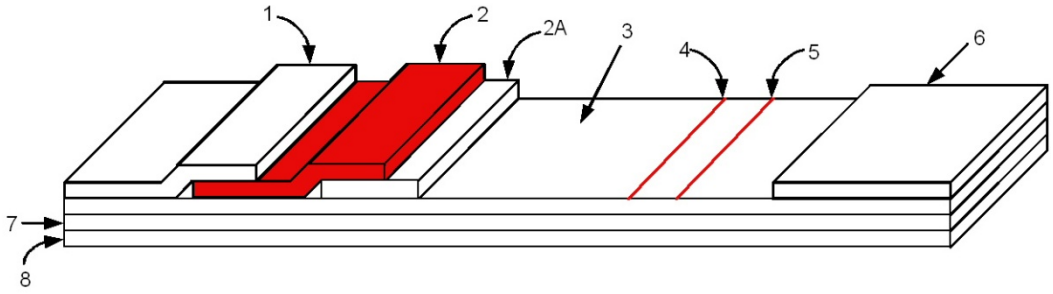

Diagram Key

1. Sample Pad A sample pad containing fibrous mesh material to absorb the sample-and–gold-conjugate immune complexes to allow gradual migration through the membrane.

2. Conjugate Pad A rabbit polyclonal antibody to SPTRX-3 was conjugated to BAW colloidal gold at or slightly above the isoelectric point of the antibody, it was blocked with BSA, and mixed with proprietary solubilizing and releasing compounds, and dried on polyester ribbon, together with a semi-permeable polyester spacer ribbon (membrane–2A), and overlaid on the striped nitrocellulose membrane (3) and attached to a plastic laminate (the laminate-film backing).

3. Nitrocellulose Membrane Test-zone membrane region onto which rabbit polyclonal antibody to SPTRX-3 and capture reagents are striped.

4. First antibody test line rabbit antibody reactive with SPTRX-3 analyte if present in the sample tested.

5. Second antibody control line A control-zone, indicating to the user whether or not the test was successfully run. The control-line is an antibody to the gold-coated species antibody.

6. Absorbent medium A reservoir wick or sink pad at the distal end of the device that absorbs the liquid sample and conjugate material to facilitate capillary migration through the membrane.

7. Polyester film backing attached to analytical membrane.

8. Laminate-film backing Pressure-sensitive acrylic laminated to vinyl or polypropylene.

4. BluePoint Bioscience Research Report

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Project Summary: SPTRX3 Peptide Monoclonal Antibody Development

19 March 2009 Hybridoma development project was initiated. Project goal was to develop monoclonal antibodies to SPTRX3, Exon 1 peptide, a spermatozoa specific protein, for use in rapid test platforms. Antibody producing hybridoma were to be screened by ELISA for peptide activity and confirmed by Western Blot to ensure activity to endogenous protein. Additional negative screening and confirmation/optimization to be determined.

03 April 2009 Peptide was conjugated to Keyhole limpet hemocyanin (KLH) to ensure peptide hyperimmunity.

17 April 2009 Balb/c mice were immunized 3 times at ~21 day intervals to optimize immune response.

15 June 2009 Serum samples were evaluated by ELISA for activity to peptide. All animals evinced high titre response (1/64,000-1/256,000) to peptide. Optimal window of opportunity for successful fusion 30-60 days

19 June-16 October 2009 Polyacrylamide gel electrophoresis (PAGE) and subsequent protein to membrane transfer and Western Blot analysis was performed. Nine endogenus protein preparations were attempted for optimizing the western blot confirmation assay. No activity was evinced at the specified protein molecular weight of ~16kDa and it was surmised that a larger protein, ~68 and 48kDa, may be precursor forms of the smaller SPTRX3.

30 October 2009 Cell fusions were performed for hybridoma development. Nine hundred and forty-three (943) fusion outgrowths were tested by ELISA; no peptide positive wells were identified.

20 November 2009 Cell fusions were performed for hybridoma development. Eighteen hundred and eighty-six (1886) fusion outgrowths were tested by ELISA; one peptide positive well was isolated and tested for western blot confirmation and found to be non-reactive to endogenous protein.

04 December 2009 Project was reviewed by BioAssay Works with PTC. It was determined that a continued effort hybridoma development project should be initiated. It was also determined that confirmation protein for western blot would be provided by P. Sutovsky lab and that the fusions would be performed in a timely manner following western blot confirmation of activity.

06 January 2010 Balb/c mice were immunized 3 times at ~21 day intervals to optimize immune response.

03 March 2010 Serum samples were evaluated by ELISA for activity to peptide. All animals evinced high titre response (>1/25,600) to peptide.

09 March-26 July 2010 Polyacrylamide gel electrophoresis (PAGE) and subsequent protein to membrane transfer and Western Blot analysis was performed. Fourteen endogenous protein preparations were attempted for optimizing the western blot confirmation assay. Only one sample, numbered "170," provided by P. Sutovsky evinced activity at the targeted protein molecular weight of ~16kDa with additional bands at higher molecular weights. Repeated analysis of "170" was not reproducible (possible protease denaturation) and additional samples provided by P. Sutovsky yielded no specific activity.

26 July 2010 Normal mouse serum was provided to P. Sutovsky to ensure assay results were not artifact or non-specific; no responses were provided.

Project was terminated due to inability to provide a consistent endogenous protein which could be used to develop a reliable confirmatory assay.

The Table of Contents to the lab notebook used for this research is attached as an exhibit for additional reference.

III. Results

1. Statement of Results

The result of the initial experiments, regarding cross-reactivity between SPTRX3 antibody and any substances found in vaginal secretions, were ultimately successful. Because we encountered reactions between various commercial secondary antibodies and other proteins found in our sample, this phase of the research became much larger than anticipated. Ultimately we were able to find antibodies that did not cross-react with other proteins in our sample, and also confirmed that no products of vaginal secretions cross-reacted.

After labeling the antibodies furnished to them by PTC with colloidal gold, BioAssay Works determined that the commercially available antibodies for the PSA/p30 protein do function properly as pairs in a sandwich assay. Thus, one of

the three target proteins are ready to proceed to the next phase of testing and optimizing the lateral flow cassette.

BioAssay Works experiments determined that the antibodies provided for SPTRX3, including the antibody previously developed by Dr. Sutovsky, did not function properly as pairs in a sandwich assay. As a result, additional antibodies would need to be developed.

Likewise, the commercial antibodies acquired for the semenogelin protein did not function properly as pairs in a sandwich assay. As a result, additional semenogelin antibodies would also need to be acquired. However, further effort regarding semenogelin antibodies awaited the outcome of the development of effective SPTRX3 antibodies.

The effort to develop a polyclonal antibody by immunizing rabbits with the SPTRX3 exon5 peptide failed.

BluePoint Bioscience immunized mice with the SPTRX3 exon1 peptide in order to develop a monoclonal antibody. This effort at SPTRX3 antibody development also failed. A second round of mouse immunizations met with the same fate.

The inability to develop SPTRX3 antibodies brought the research project to a close.

2. Tables

The failure of this project to develop a lateral flow cassette, because of the inability to develop antibodies to the SPTRX3 protein, prevented us from getting to the point of running forensic samples through a cassette and recording the cassette's performance. Therefore there are no tables showing results.

3. Figures

Likewise, although there are figures included in the discussion of specific research methods, above, the inability to develop the intended cassette prevented the generation of results that could be demonstrated in figures.

IV. Conclusions

1. Discussion of Findings

Regarding possible reasons for the failure of SPTRX3 antibody development, Dale Dembrow at BluePoint Bioscience is unsure, but speculates that possibly the SPTRX3 protein is not stable. Dr. Chris Carson of PTC noted certain stability issues during research, and found that once the sperm protein was in solution it appeared to remain stable for only the remainder of that work day. In order to

determine the reasons for the failure, whether protein instability or otherwise, additional experiments will need to be performed.

However, Dr. Sutovsky at the University of Missouri is certain that SPTRX3 is stable. His laboratory has continued to perform research following the close of the grant period (not funded by NIJ). Dr. Sutovsky states that in this interval his laboratory has identified a usable SPTRX3 antibody, and is in a position to proceed with cassette development. PTC has not had an opportunity to run experiments with Dr. Sutovsky's most recent antibody at this point in time.

2. Implications for Policy and Practice

The inability to develop SPTRX3 antibodies during the course of this project has prevented the intended effect of making the processing of rape kits in the forensic laboratory faster and more effective.

3. Implications for Further Research

We believe that the concept of developing a lateral flow cassette to determine the presence of spermatozoa and semen in evidence samples remains viable.

Of the three proteins targeted for use in this cassette, we have antibodies for the PSA/p30 protein that will work in the cassette.

While we did not continue to pursue additional antibodies for the semenogelin protein, pending development of SPTRX3 antibodies, we continue to anticipate that it will be possible to find or develop effective semenogelin antibodies for this purpose.

We are not confident that antibodies can be developed for the SPTRX3 protein. Our experience during the conduct of this grant research causes us to seriously doubt that it is possible. If it is not, then we believe that the originally conceived cassette design may still be viable if a different sperm specific protein can be identified.

PTC plans to continue research in an effort to complete cassette development. We have not yet had an opportunity to test Dr. Sutovsky's new antibody, or reproduce his results. We will do that. If we determine that Dr. Sutovsky's new antibody is viable, then we will proceed with cassette research and development using the SPTRX3 protein.

If we determine that SPTRX3 is not useful for the cassette, then we hope to identify another sperm specific protein and attempt to develop antibodies for it.

V. References

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VI. Dissemination of Research Findings

The findings from this research grant have been disseminated to NIJ by virtue of this Final Technical Report. They have not been disseminated elsewhere.