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Extended development of RSID-Semen and SERATEC PSA Semiquant assays with diluted semen samples

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Thesis

EXTENDED DEVELOPMENT OF RSID-SEMEN AND SERATEC PSA SEMIQUANT ASSAYS WITH DILUTED SEMEN SAMPLES

by

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ABSTRACT

In sexual assault cases, forensic scientists routinely employ assays to detect cellular material and proteins, such as spermatozoa and acid phosphatase (AP), to identify biological material, notably semen, on items of evidence. Due to the limit of detection (LOD) for all chemical presumptive tests and confirmatory immunochromatographic assays, more diluted samples may not be detected when testing for the presence of proteins such as prostate-specific antigen (PSA) and semenogelin (Sg). The concentrations of the various elements in semen can differ significantly from person to person due to many factors including lifestyle and genetic conditions. The variable concentrations of different seminal components between individuals result in a wide range of protein and cellular material detection in semen stains. Negative immunochromatographic assay results, in conjunction with azoospermia, or the absence of spermatozoa, during microscopic evaluations, can falsely lead forensic serologists to conclude the absence of semen on evidentiary items.

By testing different sample dilutions bordering the LOD of both RSID™-Semen (Independent Forensics, Hillside, IL) and SERATEC® PSA Semiquant (SERATEC GmbH, Göttingen, Germany), this study demonstrates the potential to increase the detection rate of Sg when extending the development period beyond the suggested 10

minutes. Although PSA detection did not improve with extended testing time, Sg detection increased, resulting in positive results developing between 10 and 45 minutes. The 45-minute time limit of this study takes into consideration the large backlog of cases at laboratories and attempts to mediate the balance between increased time expenditure and improved detection rates.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AP	Acid phosphatase
cm	Centimeter
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
kDa	Kilodaltons
KPIC	Kernechtrot-picroindigocarmine
mg	Milligrams
mL	Milliliters
mM	Millimolar
ng	Nanogram
nL	Nanoliter
LOD	Limit of detection
pH	Potential of hydrogen
PSA	Prostate-specific antigen
Sg	Semenogelin
SgI	Semenogelin I
SgII	Semenogelin II
SVSA	Seminal vesicle-specific antigen
µg	Microgram
µL	Microliter

1. INTRODUCTION

When a forensic scientist processes a sexual assault kit or analyzes an item from a crime scene associated with a sexual assault, the primary objective is often to identify the biological fluids present prior to conducting deoxyribonucleic acid (DNA) testing and to subsequently determine the likely source of DNA. The process typically begins with a visual examination of the crime scene or collected evidence items, which may include the use of an alternate light source to enhance latent stains. Preliminary testing of potential semen stains for acid phosphatase (AP) is useful in determining the stain's probative value for DNA testing to the case. If the preliminary test is positive for AP, an enzyme present in high concentrations in semen, then further testing can be performed to attempt to confirm the biological fluid as semen (1). Confirmatory testing for semen routinely employs microscopic examination of spermatozoa., however, this method cannot always be relied upon because not all semen samples contain spermatozoa.

1.1 Semen Components

The composition of semen significantly varies between individuals, and even within individuals over the course of their life due to lifestyle, genetic conditions, injury, and other factors that impact the production of seminal fluid (2). As a result, forensic biologists cannot rely solely on the presence of one component to confirm the presence of semen in all cases. Males without spermatozoa in their ejaculate, termed azoospermia, will produce samples that cannot be confirmed as semen during microscopic analysis due to the absence of sperm cells. This also holds true for men who have undergone a vasectomy

procedure to seal the vas deferens tubes, which transports sperm cells from the testes to the urethra, and therefore inhibit sperm cells from combining with the seminal fluid (3). Conversely, older or aged semen samples may generate negative results for protein activity or sperm cell detection due to degradation or poor release from the substrate during extraction. To circumvent these drawbacks, there are various methods to aid in the identification of semen, including the use of immunochromatographic assays to detect semenogelin (Sg) or prostate-specific antigen (PSA).

The understanding of the composition of semen, or any biological fluid, is crucial for forensic scientists to appropriately analyze items of evidence. Semen is a mixture of organic and inorganic compounds produced by the male reproductive system. Semen is generally comprised of two components, spermatozoa as the cellular portion produced in the testes and seminal plasma as the fluidic fraction (4). The sperm cell is delineated into three morphologically distinct structures: head, midpiece, and tail. A typical sperm cell head contains a condensed haploid nucleus and an acrosomal cap, or vesicle, located on the anterior end of the head. This cap contains hydrolytic enzymes to facilitate penetration through the egg's membrane during fertilization (4). Most spermatozoa have a flagellum, or tail, to mobilize sperm cells during fertilization. A plasma membrane surrounds both the flagellum and the haploid nucleus to protect the sperm from the extracellular environment, otherwise known as the extracellular matrix (ECM). Since sperm cells are required to travel through the female reproductive system and break through the egg's outer coat to initiate fertilization, mobility is an important aspect of a healthy intact sperm cell (4). The midpiece of the sperm sits between the flagellum and the head. It contains mitochondria to support

energy production necessary to facilitate the propulsion of the flagellum. Understanding the structure and composition of spermatozoa is important in microscopic techniques, including cell staining, during forensic analysis.

Spermatozoa make up approximately 2-5% of semen. The remainder is the seminal plasma containing a mixture of organic and inorganic components, such as hormones, proteins and enzymes, antioxidants, amines, vitamins, and inorganic ions (5). The plasma provides nutrition for the spermatozoa during fertilization because it contains fructose, citric acid, amino acids, and lactic acids in addition to other precursors for important metabolic pathways. It also provides the sperm with protection from infection-causing toxins and shields the sperm's genetic material against the acidic conditions of the vaginal and cervical cavities.

1.1.1 Proteins in Semen

Semenogelin I (SgI) and semenogelin II (SgII) are the predominant proteins found in human seminal fluid. Production of Sg mostly occurs in the seminal vesicle, with a low concentration of SgII that is produced in the epididymis. The epididymis connects the testis to the vas deferens and matures and stores sperm cells during spermatogenesis (6). SgI and SgII are about 78% similar in their primary structures, contributing to their different atomic weights (6). SgI has an atomic mass of 52 kDa and SgII has an atomic mass of 71 kDa (7). They bind to each other through disulfide bridges to form high molecular weight complexes of crosslinked Sg proteins to aid in the coagulation of semen (7). During ejaculation, the sperm cells from the epididymal fluid is mixed with seminal

vesicle secretions containing Sg, as well as prostatic fluid containing zinc and PSA. The presence of the Sg in the seminal vesicle secretions causes the epididymal fluid to coagulate and entrap the sperm cells with its thick consistency (8). The Sg and zinc ions have a high binding affinity for one another and bind to the sperm. As more Sg proteins enclose around the sperm cells causing immobilization, the sperm cells experience strain and tension, causing immediate coagulation. This transforms the once-liquified seminal fluid into a viscous gel-like structure known as the semen coagulum.

This coagulum is also impacted by fibronectin, which is a glycoprotein residing in the ECM of human tissue. Hundreds of immobilized proteins make up the ECM to fill the space between the cells and facilitate cellular communication between the intracellular and extracellular environment. Like many proteins in the ECM, fibronectin is critical for wound healing because it has an important role in cellular adhesion. It binds to the integrins, or receptors, on the outside of a cell, linking it to the actin filaments inside the cell (9). This occurs in human tissue, including the connective tissue surrounding the testis. It is suggested that the fibronectin on the sperm head is required for the sperm cell to adhere to the egg, and ultimately induce fertilization.

Like the interaction between the sperm and the egg, fibronectin also plays a role in the interaction of sperm and seminal plasma (10, 11). When fibronectin binds to the $\alpha 5\beta 1$ receptor on the sperm, cell-to-cell adhesion causes further coagulation of semen (12). This entraps the spermatozoa, inhibiting sperm motility and fertilization. While it has been well documented how fibronectin impacts overall interactions, the ways in which fibronectin impacts an individual spermatozoon and its motility are not widely understood (13). A

study using enzyme-linked immunosorbent assays to quantify seminal fluid and cell-bound fibronectin, showed an inverse correlation between the concentration of fibronectin in the plasma and sperm motility (14). Using a computer assisted sperm analysis, a video camera tracked the movement of the sperm through the microscope. The results showed that increasing amounts of fibronectin decreased sperm motility. When the concentration reached 0.18 to 0.5 μg of fibronectin per ejaculation, sperm motility stopped completely. Moreover, the addition of fibronectin antibodies to decrease the concentration of bound fibronectin demonstrated an increase in sperm motility as expected.

PSA is a glycoprotein produced in the prostate gland and secreted into the seminal fluid at concentrations of 0.5-3.0 mg/mL (15). It is responsible for hydrolyzing Sg during ejaculation. PSA can also be found in blood serum, apocrine sweat glands, urine, fecal material, and breast milk. Women produce PSA at levels that are significantly lower and typically undetectable by forensic PSA immunochromatographic assays when testing semen-free vaginal samples. For comparison, an average healthy male has approximately 1-2 ng of PSA in a serum sample taken during a blood test, whereas an average healthy female has approximately 0.002 ng (16). Elevated levels of PSA in males could indicate a prostatic condition such as prostate cancer or inflammation (17), or may also indicate a man's level of fertility. A study found that the average PSA concentration in infertile men was higher, while average testosterone concentrations were lower (18). If the liquefaction time of the semen is normal, this may suggest that a different factor other than PSA is causing fertility issues. Another study with similar results specified that increased PSA correlates with decreased sperm motility, however, sperm count was not affected (19).

Overall, the literature on this subject suggests scientific disagreement, leaving the effects of PSA on fertility unclear.

After Sg and fibronectin cause the semen to coagulate into a viscous gel-like substance, PSA then immediately facilitates the liquefaction of the seminal fluid to reverse the coagulation process and allow successful transport of the sperm cells to the fallopian tubes (20). PSA does this through the degradation of the coagulate-forming proteins, Sg and fibronectin, to release the sperm (21). During this liquefaction process, zinc ions are released from PSA and begin binding with Sg to facilitate the hydrolysis of Sg (22, 23). Zinc, while present in various body fluids, has the highest concentration in the prostate gland. Due to zinc's ability to bind and inactivate PSA, most PSA activity is ceased when zinc concentration approaches 7 mM, allowing Sg to induce coagulation before zinc is released from PSA (19). The coagulation and liquification processes are therefore controlled by PSA and Sg's dependence of zinc. Following ejaculation, the presence of Sg in the released seminal fluid causes zinc to unbind from and activate PSA because Sg has a higher binding affinity to zinc than PSA (22). This allows full liquefaction to occur in approximately 5-20 minutes (22). Unusual liquefaction times outside this range may be caused by an individual's concentration of Sg or PSA in the seminal fluid. Research suggests the amount of zinc in the seminal plasma is important for male reproductive health, however, one study suggests that having an overabundance of zinc may cause sperm abnormalities (24).

1.2 Immunochromatographic Assays

An immunochromatographic assay, or lateral flow immunoassay, is a simple, sensitive, quick, and relatively inexpensive method to detect a target analyte present in a substance. For semen, commercially available lateral flow immunochromatographic assays detect either Sg or PSA (Figure 1). Generally, immunochromatographic assays have three main components in each test cartridge that include the sample application pad, conjugate pad, and nitrocellulose membrane (25). These assays only test for one target analyte at a time, however, different tests can be performed to identify the fluids that make up a potential mixture. Since these tests are generally very sensitive, only a small portion of the test sample is required. This decreases sample consumption and allows increased samplings for multiple tests, if necessary. The sample preparation procedure is relatively simple, requiring only ten minutes to two hours for extraction in an optimized buffer solution. Older or less concentrated stains may require longer extraction periods compared to fresh, highly concentrated samples. A portion of the sample can be extracted using sterile water or buffer; however, the manufacturer supplied buffer is recommended for use because it is better able to separate the antibodies from the conjugate pad (25). In addition, the buffer is designed to mediate the sample's pH which in turn helps avoid unexpected issues such as reduced sample migration or non-specific binding.

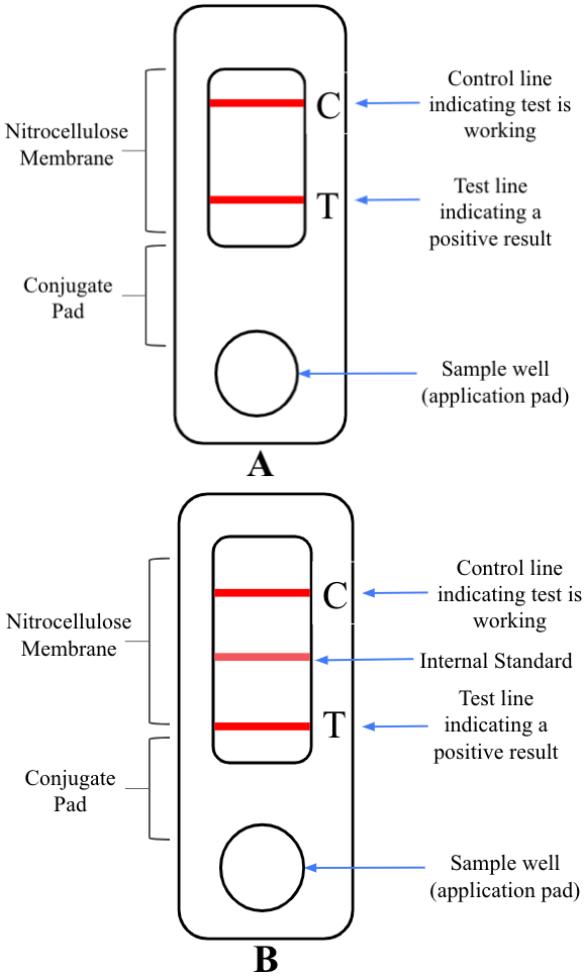

Figure 1. General layout of lateral flow immunochromatographic assay. (A) positive RSID™-Semen result; (B) positive SERATEC® PSA Semiquant result.

1.2.1 High-Dose Hook Effect

When using immunochromatographic assays in the testing of semen, sample preparation is crucial to avoid the "high-dose hook effect". Semen samples with a high analyte concentration may generate a false negative result when excess free target antigens migrate toward the test line before the labeled antibody-antigen complexes. The saturation of antigens prevents the complexes from binding to the test line and allowing the red band to be visualized. Fortunately, the high-dose hook effect can be avoided by diluting the

sample extract prior to testing (26). Validation study data provided by each assay manufacturer provides the range of concentrations that each test can adequately detect. In casework, the concentration of the sample is unknown, however the sample can be diluted and re-run in situations where the high dose hook effect is suspected.

1.2.2 Immunochromatographic Assay for Sg

Two commonly used forensic test kits for semen are RSID™-Semen (Independent Forensics, Hillside, IL) and SERATEC® PSA Semiquant (SERATEC GmbH, Göttingen, Germany). RSID™-Semen was the first available immunochromatographic assay for the detection of Sg in human semen (26). It is a qualitative analysis and gives no indication of concentration for the analyte of interest. Instead of testing for PSA like other semen immunochromatographic assays, it detects the presence of seminal vesicle-specific antigen (SVSA), including the SgI and SgII proteins. The test utilizes two monoclonal mouse antibodies specific for human Sg, in the typical lateral flow format, to detect the presence of the target analyte. Monoclonal antibodies, as opposed to polyclonal antibodies, bind to only one specific site on the target antigen.

The prepared liquid sample extract is deposited onto the sample pad where it comes into contact with and binds to mobile monoclonal mouse antihuman semenogelin antibodies conjugated to colloidal gold when Sg is present. The colloidal gold becomes dissolved into the sample. The complex migrates and binds to the test line where additional mouse anti-human Sg antibodies are immobilized. As the antigen bound with the gold-conjugated antibody accumulates, the red test line appears. If no Sg is present or does not

exceed the test's limit of detection, the complexes do not form, and no line will be visible at the test zone. Lastly, the immobilized anti-mouse immunoglobulin at the control line will capture anti-mouse antibodies moving through, yielding a red line at the control region to signify that the test is working as expected. According to most manufacturing companies, the test is complete 10 minutes after the sample is applied to the cartridge, yielding a positive or negative result (Figure 2).

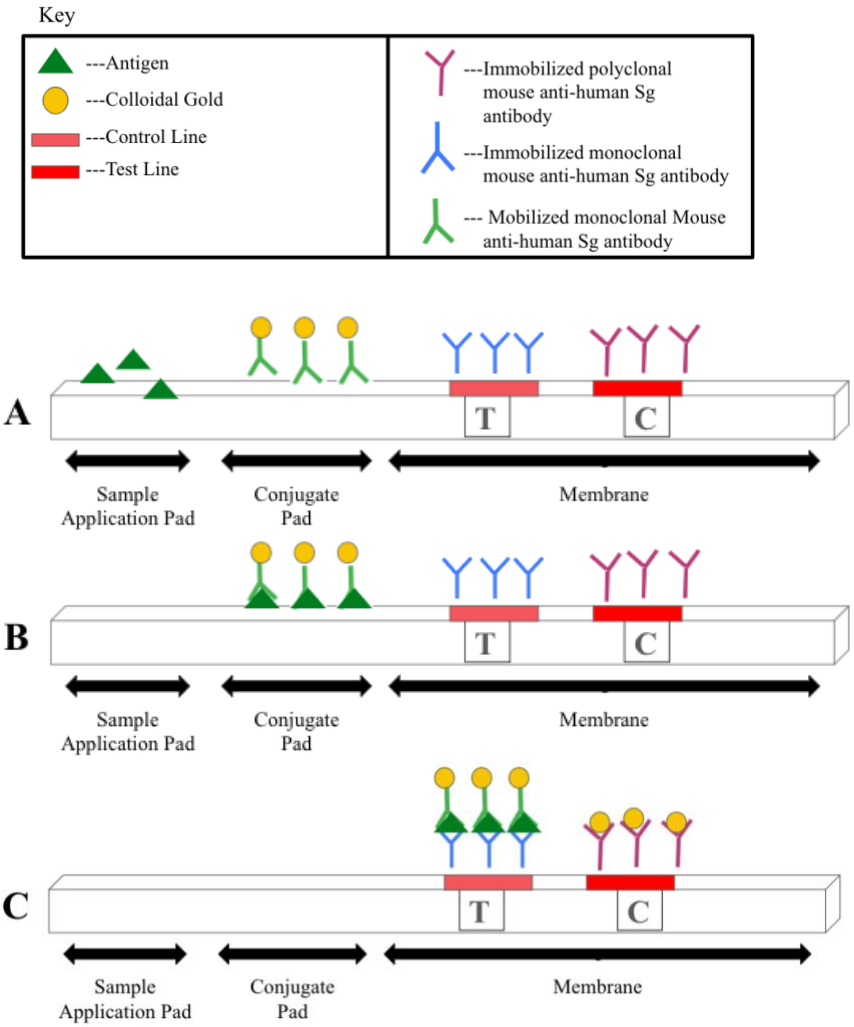

Figure 2. Antibody-antigen interaction in RSID™-Semen. This figure shows the initial position of all components involved (A), the interaction of antigens and monoclonal gold-labeled antibodies at the conjugate pad (B), and final interactions at the control and test strip (C). The diagram reflects the process for developing a positive result for Sg.

1.2.3 Immunochromatographic Assay for PSA

The SERATEC® PSA Semiquant assay detects the presence of PSA. This test is semi-quantitative because the intensity of the test line color can be compared to the assay's internal standard band, which represents the intensity of a sample with a PSA concentration of 4 ng PSA/mL (27). The internal standard region sits between the test and control line for relative comparisons. The inclusion of the internal standard means a positive result will display three lines instead of two. The principle of the test is similar to RSID™-Semen, with one immobilized antibody at the test line, however, SERATEC® PSA Semiquant uses two monoclonal mouse anti-PSA antibodies. The control region and the internal standard use immobilized polyclonal goat anti-mouse antibodies. Once the sample is loaded, the solution travels to the conjugate pad with a gold-labeled second monoclonal mouse anti-PSA antibody. Any PSA in the sample will bind to the gold-labeled antibodies to form a PSA-gold-conjugated anti-PSA complex that will migrate up the membrane with the buffer. The sample then continues up the membrane, reaching the test region. If PSA is present, the PSA-gold-conjugated anti-PSA antibody complex will be captured by the immobilized monoclonal antibody and a red line will appear. When the sample reaches the control line and the internal standard line, the gold-labeled anti-PSA antibody binds to the anti-mouse antibody, revealing two red lines (Figure 3).

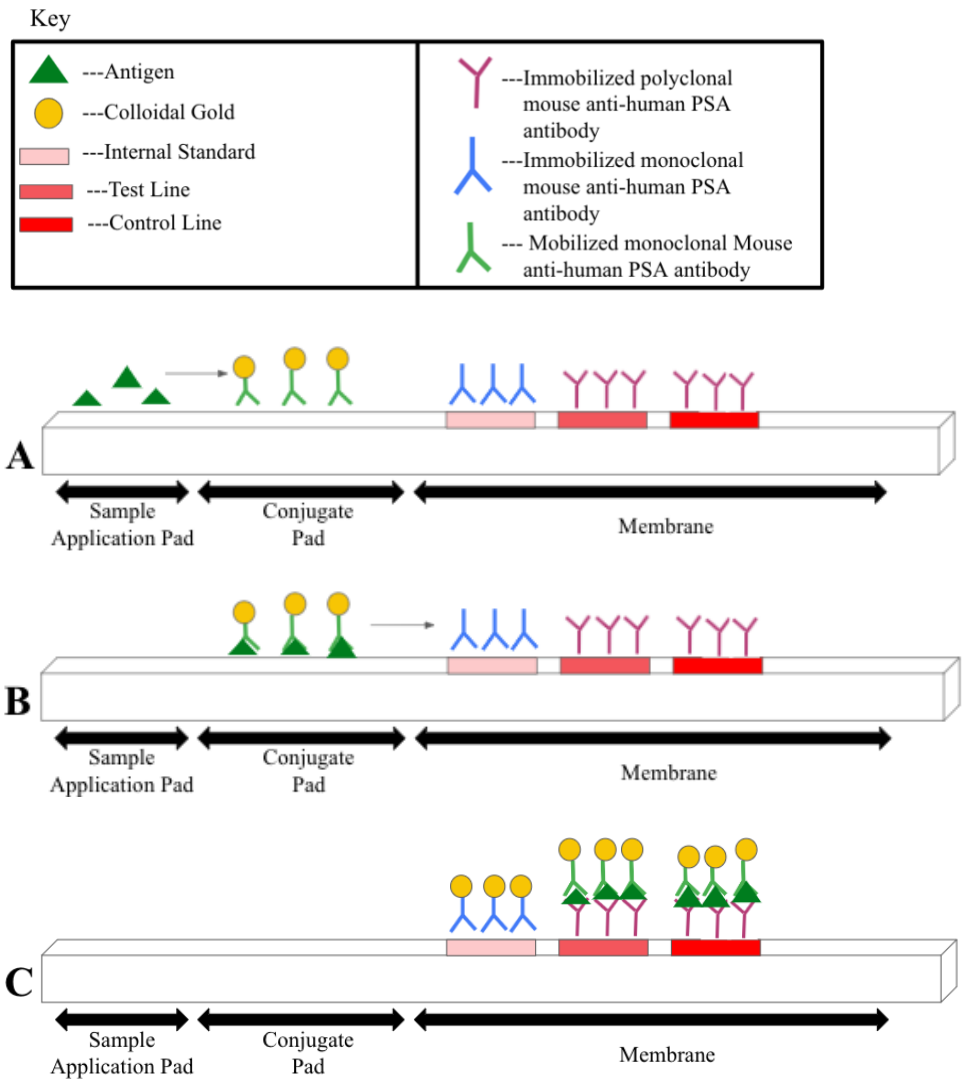

Figure 3. Antibody-antigen interaction in SERATEC® PSA Semiquant. This figure shows the initial position of all components involved (A), the interaction of antigens and monoclonal gold-labeled antibodies at the conjugate pad (B), and final interactions at the control, internal standard, and test strip (C). The diagram reflects the process for developing a positive result for PSA.

Literature exists regarding the comparison of RSID™-Semen and SERATEC® PSA Semiquant (28, 29). Each kit has advantages and disadvantages based on sensitivity, specificity, ability to analyze old or degraded samples, and cost. Both detect seminal proteins, making them suitable for all human semen samples, not just those containing

spermatozoa. Immunochromatographic assays are more sensitive and specific than the presumptive AP colorimetric test, which recognizes an enzyme that is not unique to semen. In the field of forensics, it is common to have cases with older degraded stains in which AP activity is no longer detectable. The interaction between antigens and antibodies can result in non-specific binding (false positives) if the membrane's pH is not within the preferred range. Buffer included with the assays helps to maintain the optimized pH, reducing false positive occurrences from pH changes. Since PSA is not prostate-specific, false positives may also occur from the presence of PSA in other biological fluids including blood serum, apocrine sweat glands, urine, fecal material, and breast milk (30). SERATEC® PSA is reported to be more sensitive than RSID™-Semen. The limit of detection is 200 ng/mL, compared to 8,000 ng/mL for RSID™-Semen and it is less expensive (29). The average cost of a SERATEC® PSA test is $4.12 as of 2024. Cost is one of the biggest disadvantages of using RSID™-Semen. It is one of the most expensive semen assays, costing approximately $7.80 per test as of 2024.

One of the advantages of testing for Sg instead of PSA is that seminal fluid has been shown to have higher concentrations of Sg compared to PSA (28). Also, one study showed SERATEC® PSA had a false-positive rate of 2.9%, while RSID™-Semen had no false positives; however, these results are not consistent across all studies with some reporting different false-positive rates (29, 31, 32). This is because Sg is less likely to be detected in urine or vaginal fluid compared to PSA, even though Sg is found in other tissues in the body besides prostate and penile tissue. Other biological samples that may contain semenogelin include urine and vaginal fluid. Semenogelin can also be found in skeletal

muscle, colon, kidney, and lung tissues, however, these are not common sources of evidence and will likely not interfere with forensic testing. Another benefit of testing for Sg instead of PSA is its detection on post-coital vaginal swabs. In one study that examined assay sensitivity, RSID™-Semen had a higher rate of detection, making it more ideal for sexual assault kits after the window for optimal time of collection has passed, and the seminal components begin to degrade (29).

1.3 Research Objectives

This research focuses on the detection of Sg and PSA, particularly in low-level concentrations. A primary goal is to identify if there is an increase in positive Sg or PSA results when extending the manufacturer recommended 10-minute sample migration and development to 45 minutes. The samples used in the study were microscopically analyzed to document the sperm cell count. Following the data collection of the immunochromatographic tests, the results were analyzed to determine a potential correlation between the concentration of Sg and PSA and sperm cell count. The study will also determine if Sg or PSA has a more sensitive detection range for semen samples with low sperm concentrations.

2. MATERIALS AND METHODS

2.1 Microscopic Examination

Semen samples were acquired from 22 anonymous donors. All human samples were collected in compliance with a protocol approved by the Boston University School of Medicine Institutional Review Board. An additional 2 semen samples were purchased from a commercial vendor (Lee BioSolutions, Maryland Heights, MO), for a total of 24 semen samples. Of these samples, 12 were frozen upon submission in liquid form to preserve sperm cells and prevent PSA and Sg degradation. The remaining 12 samples were stored in the freezer as neat stains that had previously been prepared on white cotton swatches. Since most biological casework involves extraction and testing of dried stains, both liquid specimens and dried stains were examined. The dried samples and the liquid samples were evaluated separately as they could not be compared with each other because they were from separate donors. Donors 1-12 were the liquid samples and donors 13-24 were the dried samples. All samples were stored in a freezer, between -25 and -10 degrees Celsius, to preserve the protein concentration and avoid the degradation of intact sperm. Seminal proteins do not thrive outside the environment of the reproductive organs, unlike sperm, and will therefore deteriorate unless frozen (33). Kernechtrot-Picroindigocarmine (KPIC) staining was utilized for improved visualization during microscopy.

For all liquid samples, 1 µL of semen was directly pipetted onto the microscope slide and then diluted with 1 µL of distilled water. Since the liquid semen samples were more viscous than the prepared extractions of the stain samples, a 1:1 dilution of semen to water was used when making the slides to decrease the overall viscosity of the sample and

avoid large clusters of spermatozoa overlapping with each other. For consistent comparison of sperm count, it was important to use the same amount of semen for each slide. The process of making slides for the dried samples required an extraction preparation step prior to deposition on a microscope slide. A cutting of each stain measuring approximately 0.5 cm^2 was excised and placed in 250 µL of RSID™-Universal Buffer, where it remained on the orbital shaker for a 1-hour extraction period at room temperature. Once the extractions were complete, the tubes were placed in a centrifuge at 14,000 rpm for 2 minutes. The supernatant of each sample was pipetted into a separate clean tube, carefully labeled, and saved for Sg and PSA testing. The remaining pellet in the original tube was vortexed for homogeneity and then 1 µL of the pellet was distributed onto a microscope slide. Each liquid or dried semen sample was prepared in duplicate to account for sperm cells that are not distributed uniformly or the possibility that the extraction efficiency is not consistent in releasing the sperm cells from the substrate.

Following the extraction of the stains, the procedures for the dried stains and liquid samples were identical. Once the pellet or liquid sample was deposited on the slide, the slides were heat fixed using a hot plate until the sample appeared dry. Then 1-2 drops of nuclear fast red, or Kernechtrot stain, were applied to each specimen and allowed to absorb for 15 minutes. Following the absorption process, the stain was gently rinsed with distilled water and allowed to dry. Then 1-2 drops of picroindigocarmine solution were applied to each specimen and allowed to absorb for 1 minute. Each slide was then rinsed with a gentle stream of ethanol and given time to dry. Lastly, 1 drop of Cytoseal™ mounting media was placed at the center of a glass cover slip, which was then placed over the stain with enough

pressure to allow the Cytoseal™ to spread out and cover the entire stain. Following staining, the slides were left to dry for several minutes before viewing them under the microscope at 400x magnification.

As a result of KPIC staining, the sperm cells heads appear red with a light pink acrosomal cap, while the sperm's flagellum and midpiece, if present, appear green when viewed microscopically. In the presence of epithelium, the cell cytoplasm present in post-coital vaginal swabs appear green and the epithelial nuclei are red. An example of a slide with KPIC staining is shown in Figure 4.

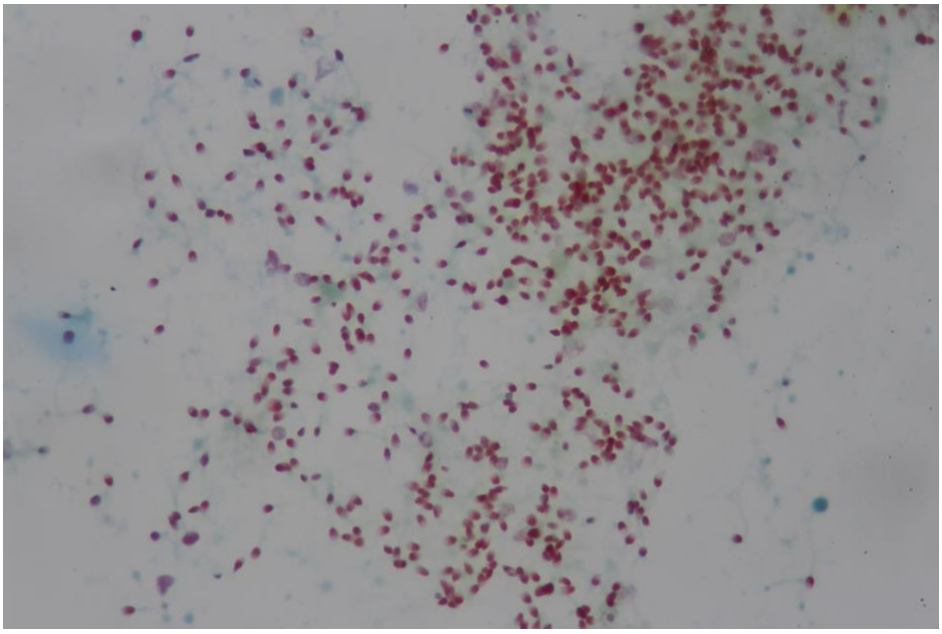

Figure 4. KPIC Staining. One field of view, as seen through the microscope at 400x magnification.

A sperm count classification system was utilized to designate a score to each microscope slide for comparison of sperm concentration with protein detection (Table 1). Except for slides displaying azoospermia, the number of sperm cells per slide was estimated by counting the number of sperm cells observed in 10 representative fields and

determining the average, rounding up. For each slide assigned a 1+, the total amount of located sperm was also specified. Based on this estimation, one of the values shown in Table 1 was assigned to each slide. The presence of tails was not recorded, as it was not necessary for this research.

Table 1. Sperm Count Classification System

Sperm Score	Description
0	No sperm present (azoospermia)
1+	Few sperm on the entire slide; difficult to locate
2+	At least one sperm in most fields
3+	Several sperm in most fields; easy to locate
4+	Many sperm in most fields

2.2 Preliminary Testing

Samples were diluted to levels approaching the limit of detection to determine if an immunoassay could yield positive results past the recommended 10-minute timeframe. Two samples were selected to undergo a preliminary testing phase to determine a baseline level of sensitivity. The two selected samples, from donors 18 and 23, were used to create a series of serial dilutions. One donated sample and one purchased sample were arbitrarily selected to account for potential differences in protein concentrations due to storage conditions. Each dilution was tested for both PSA and Sg until a positive result was reported or the 45-minute proposed time limit was reached.

The initial sample of each serial dilution started with 0.5 μL of seminal fluid mixed with RSID™-Universal Buffer. Distilled water can be used in place of buffer when making dilutions; however, buffer was used because it helps the sample maintain an appropriate pH. RSID™-Universal Buffer was selected because it works well for both RSID™-Semen

and SERATEC® PSA Semiquant assays (26). The buffer is classified as an aqueous solution containing a mixture of acids, varying in strength, that help reduce deviation from the optimal pH and serve as a diluent. It contains salts (e.g., sodium chloride and potassium chloride) for physiological stability, and proteins such as bovine serum albumin to improve the sensitivity and specificity of the test. This protein serves as a carrier protein to dilute antibodies and reduce non-specific binding of other antibodies. The buffer also contains preservatives and surfactants (e.g., Tween-20) to create self-assembled molecular clusters that allow for better solubility (34, 35).

2.3 Immunochromatographic Testing

2.3.1 Preparation

The positive control used a known semen sample, and the negative control used a clean cotton swab. Following the same serial dilution procedure as described in the preliminary testing, the 24 semen samples were tested for PSA and Sg using the 1:10,000, 1:20,000, and 1:30,000 dilutions. For the items stored as dried stains, an additional step was required for sample preparation. A portion of each stain, approximately 0.5 cm^2 as suggested by the RSID™-Semen kit instructions, was cut and extracted in approximately 300 μL of RSID™-Universal Buffer for 1 hour as instructed by RSID™-Semen. Sample tubes were placed on an orbital shaker at room temperature during this process.

2.3.2 Data Collection

As the immunochromatographic assays for PSA and Sg were developing, the results were simultaneously documented. If the result was positive within the first 10 minutes, the test was concluded and documented as a positive result for PSA or Sg. If the test was negative at 10 minutes, the test was allowed to continue developing for up to 45 minutes, while the results were documented every 5 minutes. If the test line appeared at the test region at any point up to the 45-minute mark, the test was concluded and documented as a positive result. If the test line was not apparent by 45 minutes, then the test was concluded and documented as a negative result. As the assays developed beyond 10 minutes, a few drops of buffer were added to the sample well approximately every 10 or 15 minutes to keep the sample membrane from drying out. Within the initial 10-minute development period, drying of the membrane is not a concern because the solvent will remain wet throughout the duration of the test. If the sample becomes dry, then it will stop migrating up the test strip, leaving no opportunity for the target analyte to bind at the test line.

2.3.3 Replicate Testing

Once all 3 dilutions for each sample were tested, the process was repeated once more to yield two sets of reproducible data. The extraction methods, dilutions, and run time all remained the same. The purpose of replicate testing was to see if the results could be duplicated with consistency. Variability between sample duplicates could be expected due to the stochastic nature of these low-level samples.

3. RESULTS AND DISCUSSION

3.1 Microscopic Analysis

Each sample was evaluated microscopically to categorize the amount of sperm present (Table 2 and 3). During the microscopic analysis, duplicate slides from the same donor were not reviewed at the same time to reduce confirmation bias, or the tendency to make conclusions based on preconceived beliefs or known information. Since the sperm score classification system is subjective, this is especially important for designating the most appropriate classification score.

Table 2. Microscopic Examination of Dry Samples. This table shows the sperm score designated for each slide examined.

	Sperm Score: Slide 1	**Sperm Score: Slide 2**
Donor 1	3+	3+
Donor 2	2+	3+
Donor 3	1+ (6 sperm)	1+ (7 sperm)
Donor 4	1+ (7 sperm)	1+ (4 sperm)
Donor 5	2+	2+
Donor 6	1+ (11 sperm)	2+
Donor 7	4+	4+
Donor 8	3+	4+
Donor 9	3+	3+
Donor 10	4+	4+
Donor 11	2+	2+
Donor 12	3+	4+

Table 3. Microscopic Examination of Liquid Samples. This table shows the sperm score for each slide examined.

	Sperm Score: Slide 1	**Sperm Score: Slide 2**
Donor 13	3+	4+
Donor 14	2+	3+
Donor 15	4+	4+
Donor 16	4+	4+
Donor 17	0	0
Donor 18	3+	2+
Donor 19	0	0
Donor 20	3+	4+
Donor 21	1+ (3 sperm)	0
Donor 22	4+	4+
Donor 23	4+	4+
Donor 24	2+	2+

A comparison was made using the test results and the sperm scores to determine if there may be a correlation between sperm count and PSA or Sg concentrations. Although immunochromatographic assays are qualitative, samples that develop a positive test line faster than others may suggest that the concentration of the tested proteins are also higher. All tests, including the microscopic examination for spermatozoa, were performed in duplicate to consider reproducibility as well as variability within seminal fluid samples. The objective of this comparative analysis is to determine if a protocol change by extending the time limit of the test would be beneficial for both laboratory and field testing. The sperm score for slide 1 and slide 2 for each sample appeared to be relatively consistent in all the samples, with minor exceptions. Most notably, no sperm were found on slide 2 of donor 21, however, 3 sperm were located on slide 1. At low level such as these demonstrated, it is not unexpected to attain differing sperm counts. Tables 2 and 3 show the persistency and stability of sperm, as most dry and liquid samples scored a relatively

high score. Although the sperm count was not evaluated on fresh samples, it is unlikely that the sperm count drastically changed while frozen in storage. The strength of disulfide bonds in sperm allows stabilization of the DNA so the sperm do not degrade once ejaculated (36). If the sperm count on the slide is not representative of the sample's initial sperm count, it is likely due to the difficulties of releasing cells from fabric, instead of the degradation of the sperm (36).

3.2 Preliminary Testing Results

Table 4. Preliminary Testing Results. Results were recorded within 10 minutes for a series of serial dilutions, to decide what 3 dilutions should be used for the rest of the study.
+ Indicates a positive result.
Neg Indicates a negative result.

Donor Number	Dilution	RSID™ - Semen	SERATEC® PSA Semiquant
Donor 18	1:1,000	+	+
	1:5,000	+	+
	1:10,000	+	+
	1:15,000	Neg	+
	1:30,000	Neg	Neg
Donor 23	1:5,000	+	+
	1:10,000	Neg	+
	1:15,000	Neg	+
	1:30,000	Neg	+

For both donor 18 and donor 23 samples, the sensitivity of the SERATEC® PSA Semiquant was higher, as predicted based on previous literature (28, 29) This made it difficult in selecting the appropriate dilutions to test for the remainder of the experiment. To adequately demonstrate if the SERATEC® PSA Semiquant and the RSID™-Semen assays would continue to develop following a negative result after 10 minutes, the dilutions

included should be on the precipice of each test's sensitivity. Since SERATEC® PSA was more sensitive, higher dilutions were included, while also performing lower dilutions to accommodate the RSID™-Semen sensitivity. To meet both requirements, the dilutions selected had a larger range than initially anticipated. Based on the preliminary testing the selected dilutions were 1:10,000, 1:20,000, and 1:30,000. The number of dilutions tested was limited by cost. Determining the dilutions that would be ideal for this study proved to be difficult due to the amount of variation in the semen samples. Not all samples had similar concentrations of PSA and Sg, resulting in a significant range of sensitivity levels.

3.3 Immunochromatographic Results

3.3.1 RSID™-Semen Results

All 24 semen samples, each with three dilutions, were tested in duplicate for the presence of Sg and PSA using RSID™-Semen and SERATEC® PSA Semiquant, respectively. There was a clear correlation with dilution and time required for development. As the samples were more diluted, they required additional time to develop. The tests that required more than 10 minutes to develop showed very faint test lines, suggesting a low concentration of Sg present. For each progression to the next dilution, some tests failed to detect at all, while other were able to detect Sg with time extensions. While all samples were diluted the same, they did not have the same starting concentrations of proteins, since this is presumed to vary among individuals. Therefore, the tests reached their limit of detection (LOD) at different dilutions for each sample donor. The inclusion of less disparate dilutions could help determine the approximate LOD for each sample. It would

also likely result in more samples that continue to develop beyond the 10-minute mark. The jump from the 1:10,000 to the 1:20,000 to the 1:30,000 dilution is large, forcing some samples to reach their limit of detection before exhibiting their ability to develop beyond the recommended 10 minutes.

The results presented in Tables 5 and 6 exhibit slight variability in sensitivity and development time. There were 7 samples in which the replicate testing did not provide the same result; however, all of them, except donor 9, eventually reached the same result (positive) at different time increments. If left longer than 45 minutes to develop, donor 9 may have also turned into a positive result during trial 2 since trial 1 showed a positive result at 45 minutes. From this data, certain assays may take longer to develop depending on the sample being tested. Semen composition varies in protein concentration and sperm count from person to person, which may influence the time an assay takes to develop a result. Considering that 9 out of the 24 samples took more than the allotted 10 minutes to develop for at least one dilution, forensic laboratories could improve the rate of detection for Sg in seminal samples by extending the time limit of the assay or possibly by extending the extraction time.

One potential issue with extending the assay development time is that a positive result might occur in the absence of the target protein if nonspecific binding occurs due to pH changes. Immunochromatographic assays are sensitive to low pH values, the high dose hook effect, and certain non-target fluids such as urine, breast milk, vaginal fluid (31, 33). The high dose hook effect was not an issue in this experiment since all samples were diluted at very low concentrations. It is not known if a change in pH may have occurred or some

reaction with another seminal component may have taken place during the extended time to 45 minutes. However, all negative controls yielded negative results throughout the duration of the experiment, indicating that the delayed positive results observed for some samples did not occur when semen was absent.

Table 5. Immunochromatographic Assay Results for Sg in Dry Samples. The table shows the duration of time, in minutes, required for the immunochromatographic assay to detect the presence of Sg in the dried samples. It documents the test results in 5-minute increments for all three dilutions for each sample. Replicate testing is only included when the results differ.
+ Indicates a positive result.
Neg Indicates a negative result.
▭ Indicates trial 2 results when they were different than trial 1 results for the same dilution. Trial 2 results are not shown if the response was the same as trial 1.

Donor Number	Dilution	Time (minutes)								
		5	10	15	20	25	30	35	40	45
1	1:10,000	+								
	1:20,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	1:30,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
2	1:10,000	+								
	1:20,000	Neg	Neg	Neg	Neg	+				
	1:20,000	Neg	Neg	Neg	+					
	1:30,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
3	1:10,000	Neg	+							
	1:20,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	1:30,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
4	1:10,000	+								
	1:20,000	Neg	+							
	1:30,000	Neg	Neg	Neg	Neg	Neg	Neg	+		
5	1:10,000	+								
	1:20,000	+								
	1:30,000	+								
6	1:10,000	+								
	1:20,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	1:30,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
7	1:10,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	1:20,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	1:30,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg

Donor Number	Dilution	Time (minutes)								
		5	10	15	20	25	30	35	40	45
8	1:10,000	+								
	1:20,000	Neg	Neg	Neg	+					
	1:30,000	+								
9	1:10,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	1:20,000	Neg	Neg	Neg	+					
	1:30,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	1:30,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	+
10	1:10,000	+								
	1:20,000	Neg	Neg	Neg	Neg	Neg	Neg	+		
	1:20,000	Neg	Neg	Neg	+					
	1:30,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
11	1:10,000	Neg	+							
	1:20,000	Neg	+							
	1:30,000	Neg	Neg	Neg	Neg	Neg	+			
12	1:10,000	+								
	1:20,000	+								
	1:20,000	Neg	+							
	1:30,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg

The change in test results during the extended testing time for the liquid samples showed a similar pattern to the dried samples (Table 6). If given more time to develop, some of the diluted liquid samples eventually yielded a positive result. While not all samples demonstrated this, 6 of the 12 liquid samples did follow this trend. Additional testing with more samples is required to draw conclusions regarding the different development patterns after 10 minutes.

Table 6. Immunochromatographic Assay Results for Sg in Liquid Samples. The table shows the duration of time, in minutes, required for the immunochromatographic assay to detect the presence of Sg in the liquid samples. It documents the test results in 5-minute increments for all three dilutions for each sample. Replicate testing is only included when the results differ.
+ Indicates a positive result.
Neg Indicates a negative result.
▭ Indicates trial 2 results when they were different than trial 1 results for the same dilution. Trial 2 results are not shown if the response was the same as trial 1.

Donor Number	Dilution	Time (minutes)								
		5	10	15	20	25	30	35	40	45
13	1:10,000	+								
	1:20,000	Neg	+							
	1:30,000	Neg	Neg	+						
14	1:10,000	+								
	1:20,000	+								
	1:30,000	Neg	Neg	Neg	+					
	1:30,000	Neg	Neg	+						
15	1:10,000	+								
	1:20,000	+								
	1:30,000	+								
16	1:10,000	+								
	1:20,000	+								
	1:20,000	Neg	Neg	+						
	1:30,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
17	1:10,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	1:20,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	1:30,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
18	1:10,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	1:20,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	1:30,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
19	1:10,000	+								
	1:20,000	Neg	+							
	1:30,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
20	1:10,000	+								
	1:20,000	Neg	Neg	+						
	1:20,000	Neg	+							
	1:30,000	Neg	Neg	Neg	+					
21	1:10,000	+								
	1:20,000	+								
	1:30,000	Neg	Neg	Neg	+					

Donor Number	Dilution	Time (minutes)								
		5	**10**	**15**	**20**	**25**	**30**	**35**	**40**	**45**
22	1:10,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	1:20,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	1:30,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
23	1:10,000	+								
	1:20,000	Neg	Neg	+						
	1:30,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
24	1:10,000	Neg	+							
	1:20,000	Neg	Neg	Neg	Neg	+				
	1:30,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg

3.3.2 SERATEC® PSA Semiquant Results

All samples testing positive using SERATEC® PSA Semiquant demonstrated a positive result within the first 5 minutes. Contrary to the results using RSID™-Semen, none of the immunochromatographic assays continued to develop beyond 10 minutes. Therefore, all tests with a negative result at the 5-minute mark remained negative for the total 45-minute duration of the experiment. For the 1:10,000 dilutions, all immunochromatographic assays were able to detect the presence of PSA within 5 minutes. All 1:10,000 dilutions demonstrated a test band with a more intense color than the internal standard. The internal standard contains a specified amount of antibodies to represent a color intensity for a sample with a PSA concentration of 4 ng/mL, therefore demonstrating that the PSA concentrations of the 1:10,000 diluted samples have a concentration greater than 4 ng/mL.

The 1:20,000 dilutions testing positive for PSA also all developed within 5 minutes, similar to the 1:10,000 dilution. There were two samples that tested negative when diluted

to 1:20,000 and these negative tests did not turn positive within the 45-minute window, suggesting that increasing the time allowed for detection of PSA may not be beneficial when using SERATEC® PSA Semiquant. Whenever PSA was detected, the test line appeared positive within 5 minutes, regardless of the concentration. When the 1:30,000 diluted samples were tested, 14 of the total samples had a negative result with the other 10 samples yielding a positive result within 5 minutes. While some samples were still more concentrated than the internal standard, as shown by the intensity of the test line, most tests showed a fainter test line. The vibrancy of the test band was reproducible in the duplicate testing. Regardless of the concentration, all positive results appeared quickly with no significant difference in time development between fainter and brighter lines. This further demonstrates that the time necessary for detection does not appear to be dependent on the concentration of PSA when using SERATEC® PSA Semiquant.

Table 7. Immunochromatographic Assay Results for PSA in Dry Samples. The table shows the duration of time, in minutes, required for the immunochromatographic assay to detect the presence of PSA in the dry samples. It documents the test results in 5-minute increments for all three dilutions for each sample. Replicate testing is not shown because results were the same.
+ Indicates a positive result.
Neg Indicates a negative result.

Donor Number	**Dilution**	**Time (minutes)**								
		5	**10**	**15**	**20**	**25**	**30**	**35**	**40**	**45**
1	1:10,000	+								
	1:20,000	+								
	1:30,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
2	1:10,000	+								
	1:20,000	+								
	1:30,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg

Donor Number	Dilution	Time (minutes)								
		5	10	15	20	25	30	35	40	45
3	1:10,000	+								
	1:20,000	+								
	1:30,000	+								
4	1:10,000	+								
	1:20,000	+								
	1:30,000	+								
5	1:10,000	+								
	1:20,000	+								
	1:30,000	+								
6	1:10,000	+								
	1:20,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	1:30,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
7	1:10,000	+								
	1:20,000	+								
	1:30,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
8	1:10,000	+								
	1:20,000	+								
	1:30,000	+								
9	1:10,000	+								
	1:20,000	+								
	1:30,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
10	1:10,000	+								
	1:20,000	+								
	1:30,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
11	1:10,000	+								
	1:20,000	+								
	1:30,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
12	1:10,000	+								
	1:20,000	+								
	1:30,000	+								

Table 8. Immunochromatographic Assay Results for PSA in Liquid Samples. The table shows the duration of time, in minutes, required for the immunochromatographic assay to detect the presence of PSA in the liquid samples. It documents the test results in 5-minute increments for all three dilutions for each sample. Replicate testing is not shown because results were the same.
+ Indicates a positive result.
Neg Indicates a negative result.

Donor Number	Dilution	Time (minutes)								
		5	10	15	20	25	30	35	40	45
13	1:10,000	+								
	1:20,000	+								
	1:30,000	+								
14	1:10,000	+								
	1:20,000	+								
	1:30,000	+								
15	1:10,000	+								
	1:20,000	+								
	1:30,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
16	1:10,000	+								
	1:20,000	+								
	1:30,000	+								
17	1:10,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	1:20,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	1:30,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
18	1:10,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	1:20,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	1:30,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
19	1:10,000	+								
	1:20,000	+								
	1:30,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
20	1:10,000	+								
	1:20,000	+								
	1:30,000	+								
21	1:10,000	+								
	1:20,000	+								
	1:30,000	+								
22	1:10,000	+								
	1:20,000	+								
	1:30,000	+								

Donor Number	Dilution	Time (minutes)								
		5	10	15	20	25	30	35	40	45
23	1:10,000	+								
	1:20,000	+								
	1:30,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
24	1:10,000	+								
	1:20,000	+								
	1:30,000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg

3.4 Comparing RSID™ - Semen and SERATEC® PSA Semiquant

The preliminary testing showed that SERATEC® PSA Semiquant is more sensitive than RSID™-Semen, as expected based on previous research, and the experimental data further demonstrated this (29). There were no samples in which RSID™-Semen outperformed SERATEC® PSA Semiquant in terms of sensitivity. This suggests that SERATEC® PSA Semiquant is more equipped to handle dilute, older, or environmentally impacted samples. Furthermore, SERATEC® PSA Semiquant was more reliable for yielding reproducible results between duplicates compared to RSID™-Semen. There was less variation in the time required to produce positive results for the detection of PSA, with all yielding a positive band within in the first 5 minutes, whereas the detection of Sg was detected between 10-45 minutes for some samples. Therefore, PSA testing may be more suitable for most preliminary crime scene testing, requiring faster and more sensitive results. Depending on the concentration of the sample, RSID™-Semen assays may produce a false negative on less concentrated semen samples if the test is concluded at 10 minutes as instructed. Furthermore, an extended testing period is not ideal for crime scene work when efficiency is critical.

Overall, the detection of PSA was more suitable than Sg in terms of speed, reproducibility, and sensitivity; however, there is potential to increase the rate of detection for Sg by extending the test duration, unlike for PSA. The data demonstrated that some samples required more time than the suggested 10 minutes for Sg detection. By extending the window of time allowed for assay development, laboratories can more accurately determine the presence of Sg when semen concentration is relatively low. If the experiment had recorded the results for more than 45 minutes, it is possible that more samples could have yielded a positive result. If this continued experimentation is performed, it is important that the negative controls be monitored closely to ensure all changes are true positives. While no false positives occurred with the negative control during this experiment, allowing the tests even more time may change the outcome of the negative controls, indicating whether there is a limit to the time a test can develop without incurring a false positive. According to the manufacturer's developmental validation, it is not expected that the buffer alone would yield a positive result over an extended period of time. Immunochromatographic assays are designed to be quick with results available in under ten minutes, thus there is a trade-off between the efficiency and detection limits with RSID™-Semen. Since laboratories often have a backlog of cases, it is understandable that they would not employ a protocol for RSID™-Semen using increased development time, although detection rates would likely improve. Finding a balance between time expended and test accuracy would allow laboratories to potentially increase the efficiency of results.

Another factor for consideration between the two tests is the ability to approximate semen concentration. While these are qualitative tests since the exact concentration is not

determined, SERATEC® PSA Semiquant gives some indication of concentration due to the inclusion of an internal standard. Since the actual PSA concentrations of the samples were unknown in this research, the accuracy of the internal standard could not be tested, but it is important to note that the test line appeared less bright as the samples became more diluted, suggesting that it is possible to compare the concentration of PSA in different samples based on their relative intensities. While RSID™-Semen does not have an internal standard for concentration, it is possible that the intensity of the test band may be correlated with sample concentration. As samples were further diluted, the bands became fainter. In addition, the increased time required for the detection of Sg may be indicative of relatively lower concentrations. The more diluted samples often took more time to develop compared to the less diluted samples. All 1:10,000 dilutions showed a positive result for Sg within the first 10 minutes, and the 1:30,000 dilutions took the longest to develop if they ended in a positive result. Being able to estimate the relative concentration of samples may be helpful in forensic science because it could help scientists decide which samples are most suitable for testing.

Based on visual observations, RSID™-Semen assays developed more slowly. The speed at which the buffer moved up the membrane was noticeably slower and for some tests, the buffer had yet to reach the test line by the 5-minute mark. Adding additional buffer did not seem to change the pace at which it moved up the membrane. The buffer in the SERATEC® PSA Semiquant cartridge traveled faster, which contributed to the quicker development of the tests.

3.5 Correlation Between Sperm Count and Concentrations of Sg and PSA

Based on the RSID™-Semen and SERATEC® PSA Semiquant results and sperm scores obtained in this study, there is no definitive correlation between Sg or PSA protein concentration and sperm count. However, this lack of correlation cannot be reported with confidence since the original concentrations of the proteins when the samples were fresh are unknown. In comparison to sperm cells, these proteins are not as persistent over time (33). This especially applies to Sg because it is actively degraded by PSA in the body (22). The results of the liquid and dried samples were evaluated separately since they did not originate from the same specimens. There are mixed opinions in the literature about the relationship between sperm concentration and PSA levels (21, 34). A study designed to compare sperm count with hormonal parameters, PSA levels, semen volume, and other factors in middle-aged men reported a negative correlation between PSA levels and sperm count, as well as semen volume. This suggests that PSA concentration is related to other reproductive parameters (37). Another study reported higher PSA levels in infertile men (18). In contrast to these studies, the data presented here shows no correlation between the two. However, not all samples were evaluated when they were fresh, and it has been well established that sperm elution from stains on cotton is highly inefficient (38, 39). It is also possible that the protein concentrations during testing had since decreased from the time the samples were first deposited.

Similarly to PSA, it is recognized that Sg plays a significant role in fertility, but more research is necessary to understand the relationship between these proteins and sperm count (37). Most of the existing literature focuses on the impacts of PSA quantities in the

body since high PSA levels are often associated with cancer in both men and women (16, 37). Sperm count is a significant factor in fertility, but it is not the only one. With such a wide variety of elements involved in fertility, it is difficult to accurately determine the cause and effect of any patterns.

4. CONCLUSIONS

While the components of semen are well documented, the concentrations of its constituents differ significantly from person to person and change within a person over time due to many factors including lifestyle and genetic conditions. As a result, forensic serologists cannot always rely on a single detection method to determine if semen is present. The three methods of detection discussed in this study - microscopic examination and the detection of PSA or Sg - are used in conjunction with other chemical presumptive tests in forensic laboratories or at crime scenes.

Validation studies providing information on a test's sensitivity and specificity are pertinent to understanding their limits and disadvantages. The findings of this study suggest that a change in protocol for RSID™-Semen may expand the limits of detection for Sg. Diluted semen samples developed a positive test line beyond the instructed time limit of 10 minutes, demonstrating that this may not be enough time for less concentrated samples to accumulate enough target analyte at the test line for a visible pink band to appear signifying the presence of Sg. Allowing additional time for RSID™-Semen results to develop may lead to better detection limits since the tests developed more slowly than SERATEC® PSA Semiquant. Unlike RSID™-Semen, the PSA assays showed no evidence that a negative result at 10 minutes will change to a positive result if given additional time to develop. All positive results for PSA occurred within the first 5 minutes, making it a much quicker assay than RSID™-Semen. While it could be beneficial for RSID™-Semen tests to continue to develop past 10 minutes, based on the results of this study, it would not

be an efficient use of analysts' time to extend the period of waiting for SERATEC® PSA Semiquant.

4.1 Future Directions

For future studies, the dried stains should be made from the same samples used for the liquid semen testing to demonstrate any differences are sperm or protein concentration over time using different preservation methods. Semen varies significantly in protein concentration and sperm count from person to person, and therefore, it is not useful to compare the dried stain and liquid samples originating from different donors. As a result, it was not possible to directly compare sperm count and assay test results for all 24 samples. To fairly evaluate the relationship between Sg or PSA concentrations and sperm count, future studies should not only compare liquid and dried stain samples originating from the same source, but they should also take note of the initial sperm count of the sample before it is potentially impacted by the natural degradation that occurs during storage. An accurate comparison or correlation could not be determined in this study because the results of the microscopic examination may not have reflected the donor's actual sperm count, despite the persistence of sperm. It would also be beneficial to perform this experiment using different storage temperatures. According to the National Institute of Justice, storage of dried stains is optimal at 15-24 degrees Celsius (40).

Furthermore, future research should evaluate if extended development for RSID™-Semen beyond 45 minutes can lead to false positive results or additional true positive results. In this study, all negative controls remained negative throughout the 45 minutes,

suggesting that the changes in the test results were caused by the delayed detection of Sg and were not false positives. It is possible that other factors impacted the test after the 10-minute mark, but the results of the negative control show that this is unlikely. Another future experiment could also be done with more dilutions between 1:10,000, 1:20,000, and 1:30,000, and/or more dilutions extending from 1:30,000. Increasing the dilutions would help determine the limit at which the tests will not continue to develop.

There is an abundant amount research attempting to better explore the relationship between seminal proteins and sperm, as well as the relationship between specific seminal proteins and fertility (18, 21, 23, 24, 37). Even though much of this ongoing research is garnered towards understanding the causes of infertility, or learning what causes dangerously high PSA levels in men and women, there are also forensic applications to consider. By expanding forensic knowledge of the components and interactions within the seminal fluid, forensic serologists may find new ways to increase detection rates. There may even be other proteins in seminal fluid with the potential for high detection rates and/or limits in diluted semen. Although Sg and PSA are the predominant proteins involved in liquefaction and coagulation, therefore making them important for reproductive health, there are certainly other components in semen that also have relatively high concentrations such as zinc, calcium, lipids, and sugars (41). These all play a role in sperm function. Using this information, new methods or improvements in current methods could change the way serologists approach cases involving potential seminal fluid evidence, and possibly make forensic laboratories more efficient.

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