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# A COMPARISON OF RAPID STAIN IDENTIFICATION TEST FOR SEMEN (RSID™ – SEMEN), SERATEC® PSA SEMIQUANT, AND ABACARD® P30 TESTS FOR THE FORENSIC IDENTIFICATION OF SEMINAL FLUID

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By

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ABSTRACT

A Comparison of Rapid Stain Identification Test for Semen (RSID™ – Semen), Seratec® PSA Semiquant, and ABAcard® p30 Tests for the Forensic Identification of Seminal Fluid

By

Melanie E. Chang

Several methods exist to aid in the detection of semen or seminal fluid in forensic casework. Detection methods for seminal fluid rely on the presence of prostate specific antigen (PSA) or acid phosphatase. Studies have shown that both are not specific for seminal fluid and can be found in other body fluids, including vaginal secretions. The Rapid Stain Identification Test for Semen (RSID™ – Semen) targets semenogelin, a protein said to be specific to seminal fluid. In this study, RSID™ – Semen was compared to ABAcard® p30 and Seratec® Semiquant PSA, two immunological tests that target PSA. The effects of proprietary extraction buffers supplied by each manufacturer were also studied.

The sensitivity of all three tests with liquid semen diluted in the appropriate buffer was 1:100,000. The sensitivities with liquid seminal fluid were 1:10,000 (RSID™) and 1:100,000 (ABAcard® and Seratec®). The sensitivities of dried semen stains extracted in the appropriate buffers were 1:10,000 (RSID™), 1:1,000 (Seratec®), and 1:100 (ABAcard®). The sensitivities of dried seminal fluid stains extracted in the appropriate buffers were 1:10,000 (RSID™) and 1:1,000 (ABAcard® and Seratec®).

All three tests reacted positively with male urine. A 1:10 dilution of male urine did not react with ABAcard® or RSID™ and a 1:100 dilution did not react with Seratec®.

RSID™ also reacted positively with semen-free vaginal fluid and female urine. A negative result was observed with a 1:10 dilution of vaginal fluid and a 1:10 dilution of female urine. Each of the three cards did not react with blood, sweat, feces, saliva, or breast milk.

Post-coital samples and semen/saliva mixtures extracted in each of the buffers were tested with a one-step α-naphthyl acid phosphate solution, stained with nuclear fast red and picroindigo carmine for a microscopic examination, and evaluated with the Amylase Radial Diffusion Test. No interference was observed with the RSID™ and ABAcard® buffers for all three tests. No interference was observed on the Amylase Radial Diffusion Test with the Seratec® buffer. For Seratec® buffer extracts, a yellow color was observed after 30 seconds with the acid phosphate solution. Microscopically, epithelial cells appeared blue and sperm cells appeared purple.

Samples were extracted in both phosphate buffered saline and water, then diluted by 1:10 in each of the buffers. No interference was observed on the test cards. With PBS and water control, negative results were observed on both ABAcard® and Seratec®. PBS gave a positive result with RSID™. A failed test (white lines at the test and control positions) was observed with water on the RSID™ card.

RSID™ – Semen also has the advantage of detecting a different protein. RSID™ – Semen had greater sensitivity with stains compared to ABAcard® and Seratec®. However, RSID™ – Semen gave positive results with female urine and vaginal samples. The root cause of the false positive results has not been identified. Although the RSID™ – Semen protocols recommend dilution of all samples before testing, these cards are not suggested for forensic casework at this time.

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# CHAPTER 1

## Introduction

### *Background*

The ability to detect semen and seminal fluid in samples submitted to forensic laboratories for sexual assault crimes is crucial. Often, samples are collected in sexual assault kits, in which semen or seminal fluid must be detected to establish that sexual activity occurred. Once detected, samples may be forwarded for DNA analysis to determine contributors. Without the ability to properly identify semen or seminal fluid in a sample, it becomes more difficult to establish that a crime took place.

#### *Semen*

Ejaculated semen is typically a viscous, creamy, slightly yellowish or grayish fluid, containing spermatozoa suspended in a fluid medium called seminal fluid [1]. When semen is ejaculated, it begins to coagulate immediately. Three main gel-forming proteins, fibronectin, semenogelin I, and semenogelin II, are secreted from the seminal vesicles into the seminal fluid. Minutes after ejaculation, the coagulated semen begins to reliquify due to the p30 enzyme, also known as prostate-specific antigen (PSA), which cuts semenogelin into smaller fragments. [2] Semen is not homogeneous during the ejaculation; the first part of the emission contains more spermatozoa and prostatic fluid (PSA and acid phosphatase). Later in the ejaculate, there is a greater concentration of fluid from the seminal vesicles. [3]

#### *Spermatozoa*

The average human ejaculate is 3.5 mL, with a sperm density of approximately 100 million sperm/mL [3]. A confirmatory test for semen involves the microscopic

identification of spermatozoa. The human sperm cell is a flagellated cell with a tail length of approximately 50-55μm. The head is pear-shaped when viewing from the side and is approximately 4-5 μm long and 2-3 μm wide. An acrosomal cap surrounds the head and contains the enzymes necessary to fertilize a female ovum [3]. The head contains the male contributor's nuclear DNA. When it fertilizes the female egg, half of the resulting embryo's DNA comes from the sperm cell and the other half comes from the ovum. Additional components of the spermatozoa include the midpiece, which is roughly the same length as the head for a human sperm cell. In normal fertile males, there may be up to 40% abnormal sperm present, which includes irregular heads, small heads, elongated heads, thin heads, large heads, duplicate heads, coiled tails, bent tails, short tails, no tails, and duplicate tails. The percentage of coiled tails rises after the age of forty. [3]

Sperm are identified based on an oval or teardrop-shaped head. The Christmas tree staining technique is often used for the identification of sperm. Nuclear fast red stains the head of the sperm cell red, the acrosomal cap a lighter pink, and picroindigo carmine stains the tail green. The identification of human spermatozoa can be difficult, as sperm from other animals may appear very similar.

Spermatozoa are usually found in the human vagina up to 3 days after intercourse and occasionally up to 6 days later. Tails are frequently found attached to spermatozoa on swabs taken within one hour of intercourse. They are commonly found up to 16 hours and rarely up to 72 hours. [4]

*Seminal Fluid*

Spermatozoa are suspended in seminal fluid. Seminal fluid contains many enzymatic and non-enzymatic components, including acid phosphatase, alkaline phosphatase, nucleotidases, pyrophosphatases, and ATPases. It is the richest known source of acid phosphatase. Freshly ejaculated semen is also rich in phosphorylcholine, which is immediately dephosphorylated by acid phosphatase to choline and orthophosphate. [5]

*Acid Phosphatase*

Seminal acid phosphatase (SAP or AP) is an enzyme present in significant quantities in seminal fluid.

Seminal acid phosphatase is a dimeric glycoprotein with a molecular weight of 100,000 to 120,000. SAP is a dimer consisting of two subunits of approximately 50,000 to 55,000 dalton molecular weight. SAP is classified as a non-specific orthophosphoric monoester phosphohydrolase, which catalyzes the hydrolytic removal of an ester-linked phosphate group from a monophosphate substrate. [3] The optimal pH for enzyme activity is 4.9 [6].

SAP is produced in the epithelial cells in the prostate gland and is excreted into the seminal plasma when the gland contracts during ejaculation. SAP levels are not affected after a vasectomy is performed [3]. There is no evidence showing that the testes or seminal vesicles contribute acid phosphatase to semen [7].

The amount of SAP in seminal fluid is about 1 mg/mL and is independent of the number of spermatozoa produced by the individual [3]. Acid phosphatase synthesis and secretion by the prostate is under androgen control. SAP is first produced at puberty,

reaches its peak from the ages of approximately 15 to 40, and then declines thereafter. There is variation between individuals and variation within individuals over time based on hormone levels. Frequency of ejaculation has little effect on the acid phosphatase concentration in semen. However, semen volume decreases with shorter ejaculation intervals, thus the total amount of acid phosphatase decreases. [7]

SAP is normally found in very low concentrations in the blood of healthy males [3]. Serum levels are often elevated in men with prostate cancer and other diseases [7]. Acid phosphatase is also found in low concentrations in semen-free vaginal swabs [3]. Variation exists in the acid phosphatase level in vaginal samples between women and within the same individual. Although the time of cycle does not appear to contribute significantly to the acid phosphatase level, it has been seen to decline with age. Levels in women over 50 were found to be approximately one-third of levels in younger women. [7] Samples from pregnant women and samples containing a large amount of bacteria may give fast acid phosphatase reactions.

Acid phosphatase activity decreases in the vagina after sexual activity due to dilution by vaginal fluids, drainage from the vaginal vault, and degradation by vaginal and/or seminal hydrolases. The majority of acid phosphatase activity loss is due to drainage and dilution. [7]

SAP analysis is popular because the enzyme is present in large quantities in human semen, it is very stable, and relatively quick and easy to analyze. Semen stains maintained dry and frozen can retain SAP activity for years. It may be washed out or destroyed by mold, putrefaction, chemicals, or heat. [3] Seminal acid phosphatase is

rarely detected in the human vagina two days after intercourse, but may sometimes be detected up to 3 days. [4]

*Prostate-Specific Antigen*

Prostate-specific antigen (PSA), also known as p30, is another enzyme found in seminal fluid. The biological function of PSA is to break down the gel-forming proteins in semen, causing it to liquefy after ejaculation. PSA is a glycoprotein consisting of 237 amino acid residues and exists in five different forms. The molecular weight has been reported to be 33,000 to 34,000. [3]

Studies have shown that PSA is produced in the epithelial cells that line the ductal elements of the prostate. As with seminal acid phosphatase, PSA is excreted into the seminal plasma when the gland contracts during ejaculation. Thus, PSA levels are not affected by vasectomies. [3]

The mean concentration of PSA in semen has been reported at different levels (800 µg/mL, 1200 µg/mL, 1550 µg/mL, and 1900 µg/mL) by various sources. Male urine has been reported to contain PSA, but may be due to drainage from the prostatic ducts. The first 1-5 $cm^3$ of urine is richer in PSA than midstream urine. [3]

Studies show that low amounts of PSA are detectable in the urine of eleven year old boys. The amount of PSA in the urine of healthy men was found in some cases to be 800 ng/mL. [8] Amniotic fluid, nipple aspirate fluid, and breast milk have detectable levels of PSA [9]. Very low levels are also found in the peripheral blood of healthy males, vaginal secretions, and female urine [3]. The concentration of PSA in male blood serum is normally low (< 4 ng/mL) and is elevated up to 200 ng/mL in the presence of

prostatic disease. The range of PSA in female vaginal samples is typically 0.0-1.25 ng/mL [8].

PSA has also been detected in saliva. Manello et al. studied a group of 40 female volunteers, half of which were taking oral contraceptives (0.5 mg Gestoden and 0.035 mg Ethinylestradiol per pill). The group taking the contraceptive exhibited an average of 0.099 ng PSA/mL (range 0.04- 0.34 ng PSA/mL). The average value in the control group was 0.048 ng PSA/mL (range 0.02-0.15 ng PSA/mL). [10]

The PSA concentration in female saliva was measured throughout the menstrual cycle. The highest values were found on day 9 (follicular phase) and day 14 (midcycle) with values of 0.024 ng PSA/mL and 0.029 ng PSA /mL, respectively. The maximum concentration of PSA in this study was 0.06 ng/mL in saliva. [10]

PSA is typically a stable enzyme when stored at room temperature, and especially when stored frozen. Semen stains maintained dry and frozen can retain SAP activity for years. For post-coital samples in the vaginal cavity, PSA levels depend on exposure and time since intercourse. Vaginal levels typically return to normal in approximately 48 hours. PSA has been reported to be detectable in the vagina for approximately 14-47 hours after a sexual assault. The stability of PSA is generally higher than that of acid phosphatase. Sperm cells are detectable for the longest period (maximal several days). Kamenev et al. (1989) detected postcoital PSA for 10.5 –24 hours. Detection times by Graves *et al*. (1985) were 13 to 47 hrs (average 27 hrs). [10]

*Semenogelin*

Semenogelin (Sg) is a major component of human seminal fluid and gives rise to the gel-like coagulum of newly ejaculated semen. Following ejaculation, human semen

coagulates and appears as a dense network of narrow and long fibers of approximately 0.15 µm in length. The coagulum consist of complexes of the predominant protein called semenogelin I (Sg I) and semenogelin II (Sg II). [11] Sg I and Sg II interact non-covalently to instantly form a coagulum upon ejaculation via disulphide bridges. Semenogelin I consists of 439 amino acid residues and semenogelin II is comprised of 559 amino acid residues. [12]

Semenogelin concentrations range from 4–68 mg Sg/mL and are generally higher than PSA concentrations, which typically range from 0.2–5.5 mg PSA/mL in seminal fluid [13].

Semenogelin has been detected in several tissues including the seminal vesicles, vas deferens, prostate, epididymis, skeletal muscle, kidney, colon, trachea, and in lung tumors [2]. However, semenogelin has not been reported in any other body fluids besides semen.

The stability of semenogelin has not been reported.

*Identification of Semen and Seminal Fluid*

In forensic cases, the most desired scenario for the identification of semen is by the microscopic identification of spermatozoa. The detection of spermatozoa is not always simple, as a microscopic analysis may take hours. Even for trained and experienced analysts, some animal sperm are difficult to distinguish from human origin. Furthermore, not all ejaculates contain spermatozoa. Azoospermia is a condition in which there is no measurable level of sperm in seminal fluid. A vasectomized male is azoospermic due to the surgical severing of the vas deferens, thus preventing the ejaculation of sperm cells. Azoospermic males release seminal fluid, as any normal male

would do in an ejaculation. Seminal fluid contains various proteins and enzymes, including acid phosphatase, PSA, and semenogelin. Screening methods take advantage of the proteins that are found in high concentrations in seminal fluid when sperm cells are not observed microscopically. [3]

Seminal acid phosphatase and PSA have been established as components of seminal fluid, present in high quantities and fairly specific to semen. These proteins are known to be present in lower levels in other body fluids, including vaginal secretions. This can be challenging in sexual assault cases since evidentiary submissions commonly include vaginal samples. More recently, semenogelin has been suggested as an alternative protein to target in forensic analysis because of its specificity for seminal fluid.

*Relevance of Study to Forensic Science*

In the Bureau of Justice Statistics' National Crime Victimization Survey, the number of rape and sexual assaults of U.S. residents age 12 or older decreased by 38.7% from 203,830 in 2008 to 125,910 in 2009. From 2000-2009, the rate dropped by 57%. [14] However, according to police and prosecutors who spoke with Human Rights Watch, it can take as long as 12 months from the time rape kit testing is requested until test results are received by the requesting law enforcement officer [15]. More efficient methods at the laboratory can lead to a shorter turn around time. With more specific semen detection methods, false positive samples that get forwarded for DNA analysis may be eliminated. Seminal acid phosphatase and PSA have been well-established methods for seminal fluid detection in forensic casework. These proteins are known to be present in lower levels in other body fluids, including vaginal secretions. Positive

screening results for acid phosphate or PSA may give DNA results that only show a female profile. This can be especially costly and time-consuming to process DNA samples where false positive screening results were obtained.

Additionally, samples that test negative for acid phosphatase and p30 may in fact contain seminal fluid if semenogelin is detected since the concentration of semenogelin is greater. This may be helpful in cases where victim samples were not collected until several days after the assault or a very low amount of seminal fluid was ejaculated.

*Purpose of Study*

Independent Forensics has developed a commercially available kit for the detection of semen. Rapid Stain Identification of Human Semen (RSID™ – Semen) is a quick and easy tool to use in the forensic laboratory and is based on the detection of semenogelin, a protein said to be specific for seminal fluid.

The purpose of this study was to evaluate RSID™ – Semen for the identification of seminal fluid. This alternative method for semen detection was compared to ABAcard® p30 and Seratec® PSA Semiquant. These are two commonly utilized immunological tests for PSA used for seminal fluid testing in forensic laboratories.

*Research Objectives*

This study was designed to evaluate the ability of RSID™ – Semen to detect seminal fluid in comparison to the Seratec® PSA Semiquant and ABAcard® p30. The detection of semen by each method (dependent variable in the study) was measured as a function of the following independent variables: dilution factor (1:10, 1:100, 1:1,000, 1:10,000, 1:100,000, and 1:1,000,000), semen obtained from non-vasectomized versus vasectomized donors, liquid versus dried samples, non-semen samples, mixtures, and the

extractant used to isolate the semen sample. Samples extracted with the provided RSID™ Universal Buffer were assessed by testing for acid phosphatase, amylase, and microscopic staining then compared to the extraction buffers provided by ABAcard® and Seratec®. The following research questions were addressed in this study:

1. Can RSID™ – Semen detect both non-vasectomized and vasectomized ejaculates?
2. How sensitive is RSID™ – Semen compared to ABAcard® p30 and Seratec® PSA?
3. How specific is RSID™ – Semen compared to ABAcard® p30 and Seratec® PSA?
4. Does RSID™ – Semen react with common substrates encountered in forensic samples?
5. Can RSID™ – Semen detect semenogelin in samples containing mixtures of semen and other body fluids?
6. Can RSID™ – Semen detect semenogelin in post-coital vaginal samples?
7. Can samples extracted with the RSID™ Universal Buffer be used for acid phosphatase testing, amylase testing, and staining for a microscopic examination?
8. Can other extractants be used and tested on RSID™ – Semen?

*Hypotheses*

RSID™ – Semen is predicted to have the ability to detect semen and seminal fluid. Since semen typically contains higher levels of semenogelin than PSA or acid phosphatase, it is predicted that RSID™ – Semen will be able to detect more dilute

samples of semen or seminal fluid than other methods. RSID™ – Semen is expected to be more specific than the other tests since semenogelin is said to be specific to seminal fluid. In contrast, acid phosphatase and PSA are found in other body fluid samples including vaginal fluid. RSID™ – Semen is not expected to have a reaction with any substrates tested. RSID™ – Semen is expected to be able to detect semen in mixtures with other body fluids. It is unknown whether RSID™ – Semen can detect semenogelin in post-coital vaginal samples as the stability of the protein in the vagina is unknown. It is unknown whether samples extracted in RSID™ Universal Buffer will affect acid phosphatase testing, amylase testing, and staining for a microscopic examination. It is unknown if other extractant can be used on RSID™ – Semen.

# CHAPTER 2

## Review of Relevant Literature

### *Acid Phosphatase*

#### *Methods of Detection*

The Acid Phosphatase Test is a presumptive test that targets the performance of an enzymatic function. The test reagent consists of a monophosphate substrate, typically α-naphthyl phosphate, p-nitrophenyl phosphate, phenyl phosphate, or thymolthalein monophosphate and a diazo dye. When the reagent is added to a semen stain, the acid phosphatase removes the phosphate from the substrate and the dye causes a color change [3].

#### *False Positive Reactions*

There are no tests that are specific to seminal acid phosphatase, as the test will react with vaginal acid phosphatase. Since there are typically lower levels of acid phosphatase in semen-free vaginal samples, the time for a color change is typically longer than that of a semen sample. False positive results have also been observed with cauliflower, fungi, contraceptive creams containing hexyl resorcinol, and non-human semen [3, 7]. Some feminine hygiene products have been found to contain phenols or naphthols that react with the diazo dyes [7]. Anal and rectal swabs may also give a positive result.

### *Prostate-Specific Antigen*

#### *Methods of Detection*

PSA detection is based on the antigenicity of the molecule. Methods used to detect PSA include Ouchterlony double diffusion, crossover electrophoresis, rocket

immunoelectrophoresis, and ELISA testing. Commercially available immunoassay tests are also used for PSA detection. A sample is extracted from a stain and is added to the sample well of a test card. The sample diffuses horizontally across the membrane. The sample first encounters a dye-labeled mobile monoclonal antibody that binds to the PSA antigen in the sample, if present. The PSA antibody-antigen complex continues to diffuse across the membrane to the test area, where immobile polyclonal PSA antibodies reside. If PSA is present, the complex will bind to the immobilized antibody and the dye will cause a pink line to develop. The control area contains immobilized antihuman immunoglobulin antibodies. Excess dye-labeled mobile monoclonal antibodies bind to the immobilized antibodies, resulting in a colored band. A pink line at the control area must be present for a valid interpretation. The Seratec® Semiquant PSA contains an internal standard that contains 4ng PSA/mL and allows semiquantitation of results. A high dose hook effect can be observed with these cards. When a high concentration of PSA is added, there is more PSA than dye-labeled mobile monoclonal antibody. The unbound PSA binds to the mobilized antibody in the test region, but no colored line develops since there is no dye attached. This results in a false negative result.

The immunoassay cards have the benefit of being a quick and easy to interpret method for the identification of PSA. PSA testing has an advantage over acid phosphatase testing when an older stain has lost its enzymatic activity. The manufacturer of the ABAcard® p30 (Abacus Diagnostics) states that the test's sensitivity is 4 ng PSA/mL. In comparison, Seratec® states that the PSA Semiquant test has a sensitivity of 2 ng of PSA/mL. [16, 8]

*Seratec PSA Semiquant Validation*

Vaginal swabs from 70 abstinent females were collected throughout their menstrual cycle. 130 vaginal samples tested negative for PSA. 23 of these swabs tested positive for acid phosphatase, demonstrating that PSA is a more specific marker than acid phosphatase. In all swabs, no spermatozoa were detected. In 50 sexual assault case samples, 42 samples tested positive for PSA with Seratec®, while only 35 tested positive for acid phosphatase. Saliva samples have also been shown to contain low levels of PSA. [10]

## *Semenogelin*

*Methods of Detection*

For many years, semenogelin was detected using ELISA, but recently, dot-blot immunoassays and one-step immunochromatographic assays have been developed. Rapid Stain Identification of Human Semen (RSID™ – Semen) is a commercially available immunochromatographic strip test for the detection of semenogelin. These test strips are designed similarly to the PSA immunochromatographic tests. When the sample and supplied buffer are added to the sample well, they are deposited onto a mobile mouse monoclonal antibody conjugated to colloidal gold. If semenogelin is present, it will bind to the antibody forming an antigen-antibody-gold complex. This diffuses laterally to the test line, where the sample encounters an immobilized mouse monoclonal anti-semenogelin antibody. If 1 μL or greater of semenogelin is present, the antibody-antigen complex line will bind to the immobilized antibody and a red line will be observed. The control area contains an anti-mouse IgG antibody that captures any excess mobile mouse monoclonal antibody conjugated to colloidal gold. This results in a red line and

demonstrates that the components of the test are working properly. There is a wick at the end of the strip that absorbs the excess sample and running buffer, thus preventing back flow. As with the PSA immunochromatographic tests, RSID™ – Semen has the potential of false negatives due to the high dose hook effect. When a highly concentrated semen sample is added to the strip, there are not enough mobile mouse antibody-gold complexes to bind to the semenogelin. As a result, unbound semenogelin will bind at the test site, resulting in a weak or absent line. [17]

*Benefits of Semenogelin Detection*

When semenogelin coagulates semen upon ejaculation, it is soon cleaved by PSA to liquefy the sample. The semenogelin cleavage products are detected by the monoclonal antibodies in RSID™ – Semen and results in a positive test. Furthermore, the concentration of semenogelin (4-68 mg/mL) is typically significantly higher than the PSA concentration (0.2-5.5 mg/mL) in seminal fluid. Therefore, samples containing low amounts of semen should give a positive semenogelin and a negative PSA result. [13]

*RSID™ – Semen Validation*

Independent Forensics conducted a validation study for RSID™ – Semen. The sensitivity of the test was determined to detect as little as 2.5 nL of semen. A high dose hook effect was observed when greater than 3 µL of semen was added to the sample well. No other body fluids gave false positive results with RSID™ – Semen including breast milk, vaginal fluid, and urine. No false positives were observed with spermicides or lubricants. Vaginal samples were obtained from female volunteers post-coital without the use of a condom on days 0, 1, 2, 3, 4, 5, 6, 7, and 9. Days 0 and 1 gave strong positive results, day 2 was weak, and all other days were negative. For one volunteer's

post-coital samples, day 1, 2, and 3 contained menstrual blood. RSID™ – Semen showed positive results for day 0, 1, and 2. Menstrual blood did not interfere with RSID™ – Semen. [17]

*Comparison of Tests*

*RSID™ – Semen versus ABAcard® p30*

A study by Hobbs *et al.* was conducted to compare the results of IMx PSA, ABAcard® p30, and RSID™ – Semen. A total of 581 vaginal swabs were extracted in saline and were measured using the quantitative IMx assay by Abbott Laboratories. Vaginal swab extracts were also tested using ABAcard® p30. In samples containing ≤1 ng PSA/mL, semenogelin detection was greater than the detection of PSA by ABAcard®. PSA detection was significantly higher than semenogelin in samples containing >1.0 ng PSA/mL. Since there is generally a higher concentration of semenogelin than PSA in seminal fluid, some positive semenogelin results with negative PSA results were expected. In 10% of all positive vaginal swab samples, positive semenogelin with negative PSA results were obtained. However, in 25% of all positive vaginal samples, PSA results were positive and semenogelin results were negative. The authors noted that the manufacturer's recommended procedure for RSID™ – Semen testing was not followed. This may have resulted in false negative results due to the high dose hook effect because the samples were not diluted as recommended with the running buffer. Furthermore, the authors used saline to extract the vaginal swabs, which was not recommended by the manufacturer. Buffered saline alone produced false-positive results; however, buffered saline diluted 1:5 in running buffer was negative. Based on these results, the authors chose to test the vaginal samples extracted in saline and further

diluted in the running buffer. Overall, ABAcard® p30 detected 91% of semen samples compared to 74% detected by RSID™ – Semen [13].

Pang and Cheung conducted another study comparing RSID™ – Semen and ABAcard® p30. Human semen, a purchased semen standard, animal semen, human body fluids, and casework samples were tested. RSID™ – Semen could detect human semen and a seminal fluid standard up to a 1:100,000 dilution, while the ABAcard® could detect up to a 1:50,000 dilution. A 1:50,000 dilution was equivalent to 2 ng PSA/mL. Urine samples from 10 males and 10 females were tested. All ten of the male urine liquid samples tested positive with ABAcard® p30. Extracted dried stains tested negative. The same urine samples tested negative with RSID™ – Semen. All other body fluids (blood, saliva, sweat, and fecal material) gave negative results with both tests. There were no interferences observed when these body fluids were mixed with semen samples. Lubricants and spermicides did not interfere with either test. Semen samples from cock, pig, bull, stallion, cat, and dog were tested. Cock semen gave a weak positive color change for acid phosphatase activity. All animal semen tested negative for PSA and semenogelin. Of the 54 casework samples tested, three tested positive for semenogelin and acid phosphatase with observed spermatozoa, but tested negative for PSA. The authors concluded that the detection of semenogelin in semen stains was an appropriate and effective method for identifying semen.

*RSID™ – Semen versus Seratec® PSA Semiquant and Acid Phosphatase*

A post coital study was conducted to compare RSID™ – Semen and Seratec® PSA Semiquant. Vaginal swabs were collected on day 0, 1, 2, 3, 17, 19, and 21. Days 1, 2, and 3 contained menstrual blood. A positive result was seen with RSID™ – Semen on

days 0-3. Positive results were seen with Seratec® PSA Semiquant on days 0 and 1. Acid phosphatase tests were only positive for days 0 and 1. The authors concluded that RSID™ – Semen was more sensitive than acid phosphatase and Seratec® PSA testing methods. [17]

*ABAcard® p30 versus Seratec PSA Semiquant*

In a study conducted by Laux, negative results were obtained with the ABAcard® at a concentration of 6.25 ng/mL. Seratec® kits were positive below 1 ng/mL PSA. Samples were compared using each of the kits. In 15% of the cases, a positive result was observed with Seratec® and a negative result was observed with ABAcard®. There were no occurrences of a positive ABAcard® results and a negative Seratec® result. The study concluded that the Seratec® PSA Semiquant Kit was more sensitive than the ABAcard® p30 Test. [18]

# CHAPTER 3

## Materials and Methods

### *Subject Recruitment*

Approval was granted from the California Department of Justice, Riverside Laboratory prior to initiating this study. Samples used in this study were donated by employees at the Department of Justice, Riverside Laboratory. Semen, seminal fluid, and other non-semen samples used in this study were obtained from a collection of previously submitted reference samples at the Riverside Laboratory. Additional non-semen samples were collected from voluntary participants who are employees at the Riverside Laboratory. The donors of post-coital vaginal samples voluntarily participated and were asked to not engage in sexual intercourse for two days prior to the study. They were asked to self-collect vaginal swabs at specified times after having intercourse with a male partner and to not engage in sexual intercourse during the course of swab collections. Females were allowed to engage in all other normal activities such as showering and exercising. All donors were also informed that participation was voluntary and that all forms of personal identification were considered confidential. The donated semen, breast milk, and post-coital vaginal samples were stored frozen prior to sample preparation. The urine and blood samples were refrigerated prior to sample preparation and extraction. All other samples were collected on swabs and allowed to dry overnight before extraction the following day. Female urine and vaginal samples were considered semen-free since collection was taken at least 5 days post-coital.

## *Experiment Design*

The primary objective of this study was to evaluate the use of RSID™ – Semen as a seminal fluid identification tool in forensic laboratories. Several independent variables were explored, including sample dilution, liquid semen versus dried stains, different biological fluids, mixtures of semen with biological fluids, and extractant. Three different semen screening methods (RSID™ – Semen, Seratec® PSA Semiquant, and ABAcard® p30 tests) were compared to evaluate their abilities to detect semen.

## *Sample Preparation*

### *Sensitivity and Reproducibility Study*

A dilution series was prepared for a semen sample from a non-vasectomized and from a vasectomized male. Diluted samples were prepared using each of the buffers provided in the three test kits as follows: 1:10, 1:100, 1:1,000, 1:10,000, 1:100,000, and 1:1,000,000. The same dilutions were also prepared in autoclaved ultrapure water. The dilutions prepared in water were used to prepare dried stains by adding 100 µL to a sterile polyester swab or 50 µL to an approximately 1 $cm^2$ sized cotton substrate. The swabs and substrates were allowed to dry overnight.

### *Contamination Study*

A reagent blank (provided extraction buffer) and substrate controls (polyester swab and cotton substrate) were tested to assess any contamination issues. For the substrate controls, no fluids were added to a 1 $cm^2$ sized cotton fabric or sterile polyester swab.

*Specificity Study*

100 µL of urine and breast milk samples were added to sterile Puritan polyester swabs. 50 µL of blood was added to a sterile polyester swab. Vaginal fluid, sweat, saliva, and feces were collected directly onto a sterile polyester swab. All samples were allowed to dry overnight before extraction.

*Mixture Study*

The extracts for all body fluids that tested negative with each of the individual tests and a 1:10 semen dilution (in autoclaved ultrapure water) were added together in equal volumes. The total volume of the mixture was dependent on the volume required by each of the individual tests.

*Extraction Method for Immunochromatographic Tests*

Before extraction, frozen samples were brought to room temperature. Dried samples were extracted in the appropriate buffer specified by the manufacturer of the test strips. For Seratec® PSA samples, half of one swab or a 1 $cm^2$ cotton substrate was extracted in 120 µL of the provided buffer for a minimum of 2 hours. For ABAcard® p30 samples, half of one swab or a 1 $cm^2$ cotton substrate was extracted in 200 µL of the provided buffer for a minimum of 2 hours. For RSID™ – Semen samples, half of one swab or a 1 $cm^2$ cotton substrate was extracted in 100 µL of the provided Universal Buffer for a minimum of 2 hours. All substrates were transferred to spin baskets, placed back in the tube, and centrifuged for 5 minutes at 13,000 g.

*Seratec® PSA Semiquant Testing and Interpretation*

Approximately 120 µL of extract was added to the sample well on the test strip. The time for a positive test line to appear was noted. The results were read up to 10

minutes. A negative control using 120 µL of the provided buffer was tested to ensure the lot of cards was performing correctly and that contamination was not present.

A test was negative when only an internal standard and a control line appeared. A negative result meant that the PSA concentration was below the detection limit or was not present. If the PSA concentration was too high, false negative results may have been observed due to over concentration of PSA. When a test line, internal standard line, and a control line were present, the sample contained PSA. When the internal standard line and/or control line were not observed, the test was invalid. If the sample contained high amounts of PSA it was possible that the color intensity of the control line was weak.

*ABAcard® P30 Testing and Interpretation*

Approximately 200 µL of extract was added to the sample well on the test strip. The time for a positive test line to appear was noted. The results were read up to 10 minutes. A negative control using 200 µL of the provided buffer was tested to ensure the lot of cards was performing correctly and that contamination was not present.

Pink lines in the test and control areas resulted in a positive test and indicated that the PSA level was at or above 4 ng/mL. If there was only one pink line in the control area, the test result was negative. This indicated the absence of PSA or that there was less than 4 ng/mL of PSA present. A negative result may also have been due to the high dose hook effect due to over concentration of PSA on the test strip. The test was invalid if there was no pink line in the control area.

*RSID™ – Semen Testing and Interpretation*

Approximately 100 µL of the extract was added to the sample well on the test strip. The time for a positive test line to appear was noted. The results were read up to

10 minutes. A negative control using 100 µL of the Universal Buffer was tested to ensure the lot of cards was performing correctly and that contamination was not present.

Semenogelin was detected when red lines were visible at both the control and test positions. Semenogelin was not detected when a red line was observed only in the control region. The high dose hook effect may have produced false negative results. A test failure occurred when a red line was not observed in the control area.

### *Additional Testing*

#### *Acid Phosphatase Testing and Interpretation*

The following semen and seminal fluid dilutions were prepared in each of the supplied buffers: 1:10, 1:100, 1:1,000, 1:10,000. 5 µL of each dilution was added to a piece of autoclaved filter paper, followed by a drop of a one-step alpha-naphthyl acid phosphate reagent. A positive control (neat semen or seminal fluid) and a negative control (extraction buffer only) were also tested. A purple color change was noted up to 5 minutes.

#### *Radial Amylase Diffusion Test*

A gel containing 1% agarose and 0.1% starch from SERI amylase buffer and SERI EA agarose was prepared. Wells were punched in the gel. An alpha-amylase standard of an approximate concentration of 1 u/mL was diluted 1:10 and 1:100 in each of the provided buffers to serve as positive controls. Each buffer alone served as a negative control. 5 uL of sample was placed in each well. The plate was placed in a moisture chamber and incubated for 16-24 hrs in a 37 $^{o}$C oven. After incubation, the gel was stained with 1:10 iodine diluted in autoclaved ultrapure water. Each of the clearings were measured.

*Post-Coital Vaginal Samples Study*

Female donors were asked to not engage in sexual intercourse for at least two days prior to the study. One participant was asked to have sexual intercourse with a non-vasectomized male partner, with complete ejaculation occurring, and to not engage in sexual intercourse for the length of the study. Another female donor was asked to participate in the same study with a vasectomized male partner. Additionally, one of the donors conducted this study with a non-vasectomized male while beginning her menstrual cycle. Tampons were not worn at any time. Each of the participants self-swabbed the vaginal area after approximately 1 hour and 24 hours post-coital. They were allowed to shower, exercise, and complete all activity as normal.

Approximately one-fourth of a swab was extracted in 200 μL of each buffer for a minimum of 2 hours. All substrates were transferred to spin baskets, placed back in the tube, and centrifuged for 5 minutes at 13,000 g.

The extract was tested for acid phosphatase using the method described previously.

Slides were prepared from approximately 5 μL of each of the cell pellets of selected samples. Slides were stained for 3 minutes with nuclear fast red and 25 seconds with picroindigo carmine. Flotexx mounting medium and a coverslip was added. The slides were observed with a Nikon Eclipse 50i microscope under bright field and phase contrast. Slides were scanned at 200x and examined under bright field and phase contrast at 400x. Specimen staining and characteristics of sperm cells (if present) were noted. These characteristics included shape, size, and the presence of tails.

A 1:10 dilution of the extract in the appropriate buffer was prepared. The 1:10 sample dilution was tested on the appropriate semen test card using the procedures described previously.

*Semen and Saliva Mixture Study*

A buccal sample was collected with a polyester swab, then 50 µL of a 1:100 semen dilution (in autoclaved ultrapure water) was deposited on the swab. 200 µL of each buffer was used to extract approximately one-fourth of the swab for a minimum of 2 hours. All substrates were transferred to spin baskets, placed back in the tube, and centrifuged for 5 minutes at 13,000 g.

The extract was tested for acid phosphatase using the method described previously.

Slides were prepared in the same manner as described previously. Specimen staining and characteristics of sperm cells (if present) were noted.

A 1:10 dilution of the extract in the appropriate buffer was prepared. The 1:10 sample dilution was tested on the appropriate semen test card using the procedures described previously.

The samples were tested for amylase using the Amylase Radial Diffusion Test. The α-amylase standard was used to prepare dilutions of 1:10, 1:100, and 1:500 in the appropriate buffer as positive controls. The plate was incubated overnight, stained, and the clearings were measured.

*Alternative Extraction Methods*

Approximately one-fourth of a post-coital (1 hr) vaginal sample and a semen-free vaginal sample were each extracted in 200 uL of PBS and autoclaved ultrapure $H_2O$ for a

minimum of two hours. The substrates were transferred to spin baskets, placed back in the tube, and centrifuged for 5 minutes at 13,000 g.

A 1:10 dilution was prepared using the extract and the appropriate buffer. PBS and autoclaved ultrapure $H_2O$ were both run separately on each of the cards. Each of the buffers was run separately on the appropriate cards in a prior study.

The pH of each of the RSID™ Universal Buffer, ABAcard® extraction buffer, and PBS were tested using a digital pH meter. The Seratec® extraction buffer was stated to be 8.3. The pH of autoclaved ultrapure $H_2O$ was not measured.

# CHAPTER 4

## Results

### *Sensitivity, Reproducibility, Specificity, and Contamination Studies*

#### *Sensitivity and Reproducibility of Liquid Samples*

The sensitivities of RSID™ – Semen (card lot 080211S1, buffer lot 080811UB), ABAcard® p30 (card/buffer lot 23200702), and Seratec® PSA (card lot F11261, buffer lot 11263B) were 1:100,000 for liquid semen diluted in the provided buffers. For liquid seminal fluid, the sensitivities for the same lots of ABAcard® p30 and Seratec® PSA were 1:100,000. The sensitivity of the same RSID™ – Semen lot was 1:10,000.

The repeatability of the results was studied by testing the same semen and seminal fluid dilutions a second time. Seratec® did not provide a second lot. The tests were repeated on the same lot and the same results were obtained for the sensitivities of both semen and seminal fluid. The same results were also obtained for both semen and seminal fluid using RSID™ – Semen (card lot 01051151, buffer lot 080811UB) and ABAcard® p30 (card/buffer lot 23200921). (Table 1)

#### *Sensitivity of Dried Samples on Cotton Cloth*

Dried semen stains were extracted in the provided buffer. The sensitivity of RSID™ – Semen (card lot 080211S1, buffer lot 080811UB) for semen was 1:100,000. The sensitivities for ABAcard® p30 (card/buffer lot 23200702) and Seratec® PSA were 1:1,000. (Table 2)

The sensitivities of RSID™ – Semen and Seratec® PSA for dried seminal fluid stains were 1:1,000. The sensitivity for ABAcard® p30 was 1:100. The test was repeated by extracting a separate sample. (Table 2)

*Sensitivity of Dried Samples on Polyester Swabs*

Dried semen stains were extracted in the provided buffer. The same sensitivities obtained for dried semen stains on cotton cloth were observed for semen stains on polyester swabs for RSID™ – Semen (card lot 01051151, buffer lot 080811UB), ABAcard® p30 (card/buffer lot 23200921), and Seratec® PSA. (Table 2)

The same sensitivities for ABAcard® p30 and Seratec® PSA as the dried seminal fluid stains on cotton cloth were obtained for dried seminal fluid on polyester swabs. The sensitivity observed for RSID™ – Semen was 1:10,000. (Table 2)

*Contamination Study*

RSID™ – Semen (card lot 080211S1, buffer lot 080811UB), RSID™ – Semen (card lot 01051151, buffer lot 080811UB), ABAcard® p30 (card/buffer lot 23200702), ABAcard® p30 (card/buffer lot 23200921), and Seratec® PSA (card lot F11261, buffer lot 11263B) were each tested with the provided buffer. A portion of clean cotton cloth and a sterile polyester swab were each extracted in the provided buffer then tested on each of the cards listed above. Negative results were obtained for all samples.

*Specificity and Reactivity with Other Body Fluids*

A positive result for male urine was obtained for RSID™ – Semen (card lot 080211S1, buffer lot 080811UB), ABAcard® p30 (card/buffer lot 23200702) and Seratec® PSA ABAcard® p30 did not react with a 1:10 dilution of the same male urine. Seratec® PSA reacted positively with a 1:10 dilution, but did not react with a 1:100 dilution of the same male urine. RSID™ did not react with a 1:10 dilution of male urine.

ABAcard® p30 and Seratec® PSA did not react with the following body fluids:

vaginal fluid, female urine, female blood, male blood, sweat, feces, saliva, and breast milk.

RSID™ – Semen reacted with two different semen-free vaginal samples. A negative result was obtained when diluting one of the vaginal samples to 1:10. RSID™ – Semen also reacted with two different semen-free female urine samples. RSID™ did not react with two different 1:10 dilutions of female urine samples. RSID™ – Semen did not react with any of the remaining non-semen samples listed previously.

Positive results were observed with all three cards when testing an equal mixture of negative non-semen samples and a 1:10 dilution of semen. (Table 3)

*Use of Extraction Buffers on Other Biology Screening Tests*

*Acid Phosphatase*

Liquid samples of neat semen and dilutions in the provided extraction buffers were tested with a 1-step α-naphthyl acid phosphate reagent on filter paper. The samples were observed for 5 minutes. The sensitivity of the test for samples diluted in RSID™ Universal Buffer (lot 080811UB) and ABAcard® extraction buffer (lot 23200921) was 1:1,000.

The sensitivity of the AP test with the Seratec® extraction buffer (lot 11263B) was 1:100. It should also be noted that the negative control and dilutions of 1:100 and greater had a yellow color change after 30 seconds. The Seratec® buffer without any acid phosphatase reagent was added to filter paper and no color change was observed up to 5 minutes. The sensitivity study was repeated using the Seratec® extraction buffer on a second set of semen samples and the same results were obtained.

Liquid samples of neat seminal fluid and dilutions in the provided extraction buffers were also tested with a 1-step α-naphthyl acid phosphate reagent. A sensitivity of 1:1,000 was obtained with all three test card buffers. (Table 4)

*Radial Amylase Diffusion Test*

Positive and negative controls were tested. A positive α-amylase standard diluted 1:10 and 1:100 in each of the provided buffers had the same clearing sizes with each buffer for both dilutions. No clearings were observed for negative controls where only buffer was tested. (Table 7)

*Post-Coital Vaginal Samples*

Portions of swabs containing vaginal samples taken approximately 24 hrs post-coital were each extracted with RSID™ Universal Buffer (lot 080811UB), ABAcard® extraction buffer (lot 23200921), and Seratec® extraction buffer (lot 11263B). Each extract was tested with a 1-step α-naphthyl acid phosphate reagent. A 1:10 dilution of the extract was tested with RSID™ – Semen (lot 01051151), ABAcard® p30 (lot 23200921), and Seratec® PSA. The extracts were also tested for amylase using the Radial Amylase Diffusion Test. Slides were prepared from vaginal samples with semen donors and observed microscopically. (Table 5)

A post-coital vaginal sample with a semen donor and extracted with the ABAcard® buffer gave a positive acid phosphatase result. The same sample extracted in the two other buffers gave negative results. Negative results were obtained for all extractions for a post-coital vaginal sample with a semen donor while on her menstrual cycle and a post-coital vaginal sample with a vasectomized donor.

The post-coital vaginal sample with a semen donor extracted with the RSID™ Universal Buffer was the only sample to give a positive result with RSID™ – Semen. All other extracts tested negative with their respective test cards.

Low levels of amylase were obtained for all samples. The clearing sizes were all of similar size (Tables 8-11, Figures 1-4).

The staining characteristics of epithelial cells and sperm cells were observed on each slide. The presence of sperm cells was noted, but were not counted. Sperm cells were observed on all slides except the vaginal sample containing menstrual blood extracted in the Seratec® buffer. No sperm cells were observed on a second post-coital vaginal sample containing menstrual blood sample extracted in the Seratec® buffer. Typical staining colors were observed on slides prepared from samples extracted in RSID™ Universal Buffer and ABAcard® extraction buffer. When heat fixing the slides prepared from samples extracted in the Seratec® buffer, a shiny appearance was noted. This may have contributed to the staining characteristics for these slides as epithelial cells had a bluer color rather than a green color and sperm cells had a purple color rather than a red color.

*Semen and Saliva Mixture Study*

Portions of a buccal swab containing a 1:100 diluted semen sample were extracted in RSID™ Universal Buffer (lot 080811UB), ABAcard® extraction buffer (lot 23200921), and Seratec® extraction buffer (lot 11263B). The same tests were performed on these samples as the post-coital vaginal samples. (Table 5)

A positive acid phosphatase result was observed with the sample extracted in RSID™ Universal Buffer. Negative results were obtained for the two other extracts.

Positive results were observed on all extracts with their respective test cards.

The clearing sizes were all of similar size using the Amylase Radial Diffusion Test (Tables 9-11, Figures 2-4).

Sperm cells were observed on all slides. Similar staining characteristics were observed on these samples as with the post-coital vaginal samples. Typical colors were observed on the samples extracted in RSID™ Universal Buffer and ABAcard® extraction buffer. Blue epithelial cells and purple sperm cells were observed on the sample extracted with the Seratec® buffer.

*Alternative Extraction Methods*

A vaginal sample with a semen donor taken approximately 1 hr post-coital and a semen-free vaginal sample were each extracted in phosphate buffered saline and autoclaved ultrapure water. 1:10 extracts were prepared by diluting in the provided buffer then tested with RSID™ – Semen (card 01051151, buffer lot 080811UB), ABAcard® p30 (card/buffer lot 23200702) and Seratec® PSA. Positive results were obtained for all post-coital vaginal samples. Negative results were obtained for all semen-free vaginal samples. Phosphate buffered saline and autoclaved ultrapure water were each run on the test cards. Both gave negative results with ABAcard® p30 and Seratec® PSA. A positive result was observed with phosphate buffered saline on RSID™ – Semen. Two white lines at the control and test areas were observed with water on RSID™ – Semen. (Table 6)

The pH level of each of PBS were measured and compared to those of each of the provided extraction buffers (Table 11). It was noted that the pH of RSID™ Universal Buffer was higher than all other extractants.

Table 1

*Sensitivity and Reproducibility of Liquid Semen Samples*

| Sample | RSID™ | | ABAcard® | | Seratec® | |
|---|---|---|---|---|---|---|
| | Card 080211S1 Buffer 080811UB | Card 01051151 Buffer 080811UB | Lot 23200702 | Lot 23200921 | Card F11261 Buffer 11263B | |
| 1:10 Semen Liquid | + 1:24 | + 10:00 (faint) | + 1:00 | + 1:08 | + 0:30 | + 0:27 |
| 1:100 Semen Liquid | + 1:21 | + 0:40 | + 0:38 | + 0:35 | + 0:45 | + 0:17 |
| 1:1,000 Semen Liquid | + 1:47 | + 0:52 | + 0:47 | + 0:38 | + 0:48 | + 0:23 |
| 1:10,000 Semen Liquid | + 1:15 | + 0:52 | + 1:05 | + 1:01 | + 0:53 | + 1:15 |
| 1:100,000 Semen Liquid | + 4:20 | + 6:46 | + 3:39 | + 3:37 | + 3:53 | + 5:07 |
| 1:1,000,000 Semen Liquid | - | - | - | - | - | - |
| 1:10 Seminal Fluid Liquid | + 1:04 | + 0:53 | + 0:43 | + 0:59 | + 0:46 | + 0:25 |
| 1:100 Seminal Fluid Liquid | + 0:45 | + 0:28 | + 0:22 | + 0:58 | + 0:50 | + 0:24 |
| 1:1,000 Seminal Fluid Liquid | + 1:05 | + 0:26 | + 0:32 | + 0:22 | + 0:49 | + 0:40 |
| 1:10,000 Seminal Fluid Liquid | + 4:57 | + 2:12 | + 1:16 | + 0:59 | + 1:21 | + 1:17 |
| 1:100,000 Seminal Fluid Liquid | - | - | + 2:35 | + 4:33 | + 10:00 (faint) | + 3:40 (faint) |
| 1:1,000,000 Seminal Fluid Liquid | - | - | - | - | - | - |

Table 2

*Sensitivity of Extracted Semen Stains on Cotton Cloth and Polyester Swabs*

| Sample | RSID™ | | ABAcard® | | Seratec® | |
|---|---|---|---|---|---|---|
| | Card 080211S1 Buffer 080811UB | Card 01051151 Buffer 080811UB | Lot 23200702 | Lot 23200921 | Card F11261 Buffer 11263B | |
| | Cotton | Polyester | Cotton | Polyester | Cotton | Polyester |
| 1:10 Semen | + 1:09 | + 1:27 | + 0:48 | + 0:25 | + 0:27 | + 0:22 |
| 1:100 Semen | + 1:07 | + 0:57 | + 1:26 | + 0:35 | + 0:27 | + 0:26 |
| 1:1,000 Semen | + 1:32 | + 1:30 | + 4:07 | + 2:48 | + 0:58 | + 1:07 |
| 1:10,000 Semen | + 6:09 (faint) | + 4:47 | - | - | - | - |
| 1:100,000 Semen | - | - | - | - | - | - |
| 1:1,000,000 Semen | - | - | Not tested | Not tested | Not tested | Not tested |
| 1:10 Seminal Fluid | + 1:30 | + 0:59 | + 0:30 | + 0:34 | + 0:24 | + 0:27 |
| 1:100 Seminal Fluid | + 1:36 | + 0:54 | + 1:36 | + 0:34 | + 0:27 | + 0:32 |
| 1:1,000 Seminal Fluid | + 7:23 | + 1:09 | - | - | + 1:18 | + 2:50 |
| 1:10,000 Seminal Fluid | - | + 4:32 (faint) | - | - | - | - |
| 1:100,000 Seminal Fluid | - | - | Not tested | Not tested | - | - |
| 1:1,000,000 Seminal Fluid | Not tested | - | Not tested | Not tested | Not tested | Not tested |

Table 3

*Specificity & Reactivity with Other Body Fluids*

| Sample | RSID™ Card 080211S1 Buffer 080811UB | ABAcard® Lot 23200702 | Seratec® Card F11261 Buffer 11263B |
|---|---|---|---|
| Vaginal Fluid 1 | + 10:00 (faint) | - | - |
| Vaginal Fluid 2 | + 5:30 | Not tested | Not tested |
| 1:10 Vaginal Fluid 2 | - | Not tested | Not tested |
| Female Urine 1 | + 10:00 (faint) | - | - |
| 1:10 Female Urine 1 | - | | |
| Female Urine 2 | + 9:35 | Not tested | Not tested |
| Male Urine | + 10:00 (faint) | + 9:45 | + 1:20 |
| 1:10 Male Urine | - | - | + 10:00 (faint) |
| 1:100 Male Urine | Not tested | Not tested | - |
| Female Blood | - | - | - |
| Male Blood | - | - | - |
| Sweat | - | - | - |
| Feces | - | - | - |
| Saliva | - | - | - |
| Breast Milk | - | - | - |
| Fluid Mixture with Semen* | + 1:30 | + 0:30 | + 0:27 |

*Equal volumes of 1:10 Semen and all negative non-semen samples

Table 4

*Acid Phosphatase Test Sensitivity of Semen Dilutions in Supplied Buffers*

| **Sample** | **RSID™ Buffer Lot 080811UB** | **ABAcard® Buffer Lot 23200921** | **Seratec® Buffer Lot 11263B** | |
|---|---|---|---|---|
| Neat Semen Liquid | + immediate | + immediate | + immediate | + immediate |
| 1:10 Semen Liquid | + 0:05 | + 0:03 | + 0:04 | + 0:04 |
| 1:100 Semen Liquid | + 0:20 | + 0:15 | + 1:00* | + 1:09* |
| 1:1,000 Semen Liquid | + 1:50 | + 0:45 | -* | -* |
| 1:10,000 Semen Liquid | - | - | -* | -* |
| Negative Control | - | - | -* | -* |
| Buffer Only (No AP Added) | Not tested | Not tested | Remained clear | Remained clear |
| Neat Seminal Fluid Liquid | + immediate | + immediate | + immediate | Not tested |
| 1:10 Seminal Fluid Liquid | + 0:03 | + 0:05 | + 0:02 | Not tested |
| 1:100 Seminal Fluid Liquid | + 0:15 | + 0:10 | + 0:08* | Not tested |
| 1:1,000 Seminal Fluid Liquid | + 1:30 | + 0:58 | +2:23* | Not tested |
| 1:10,000 Seminal Fluid Liquid | - | - | -* | Not tested |
| Negative Control | - | - | -* | Not tested |
| Buffer Only (No AP Added) | Not tested | Not tested | Remained clear | Not tested |

* At approximately 0:30, a yellow color change was observed

Table 5

Acid Phosphatase, Test Card, Microscopy, and Amylase Results for Mock Case Samples

| Sample | RSID™ Card 01051151 Buffer 080811UB | | | | ABAcard® Lot 23200921 | | | | Seratec® Lot F11261 Buffer 11263B | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | AP (on Extract) | Card Result | Sperm Observed | Amylase Results | AP (on Extract) | Card Result | Sperm Observed | Amylase Results | AP (on Extract) | Card Result | Sperm Observed | Amylase Results |
| PCI 24 hr (Semen) | - | + 4:30 | Yes | Low | + 1:00 | - | Yes | Low | - | - | Yes | Low |
| PCI 24 hr (Seminal Fluid) | - | - | Not tested | Low | - | - | Not tested | Low | - | - | Not tested | Low |
| PCI 24 hr, Menstrual Day 1 (Semen) | - | - | Yes | Low | - | - | Yes | Low | - | - | No | Low |
| 1:100 Semen & Saliva | + 1:13 | +4:48 | Yes | Elevated | - | + 0:35 | Yes | Elevated | - | + 0:38 | Yes | Elevated |

Table 6

PBS and Water Extraction Diluted in Supplied Buffers

| Sample | RSID™ Card 01051151 Buffer 080811UB | | ABAcard® Lot 23200921 | | Seratec® Lot F11261 Buffer 11263B | |
|---|---|---|---|---|---|---|
| | PBS | Water | PBS | Water | PBS | Water |
| 1:10 PCI 1 hr (Semen) | + 0:53 | + 0:59 | + 0:25 | + 0:36 | + 0:22 | + 0:25 |
| 1:10 Vaginal (Semen-Free) | - | - | - | - | - | - |
| Negative Control | + 2:00 | + 3:19* | - | - | - | - |

* 2 white bands appeared at both the C and T areas. Test failed.

Table 7

*Amylase Radial Diffusion Test: Controls*

| Well | Description | Clearing (mm) |
|---|---|---|
| 1 | 1:10 α-amylase (ABAcard®) | 26 |
| 2 | 1:100 α-amylase (ABAcard®) | 22 |
| 3 | ABAcard® extraction buffer | 0 |
| 4 | RSID™ Universal buffer | 0 |
| 5 | 1:100 α-amylase (RSID™) | 22 |
| 6 | 1:10 α-amylase (RSID™) | 26 |
| 7 | 1:10 α-amylase (Seratec®) | 26 |
| 8 | 1:100 α-amylase (Seratec®) | 22 |
| 9 | Seratec extraction buffer | 0 |

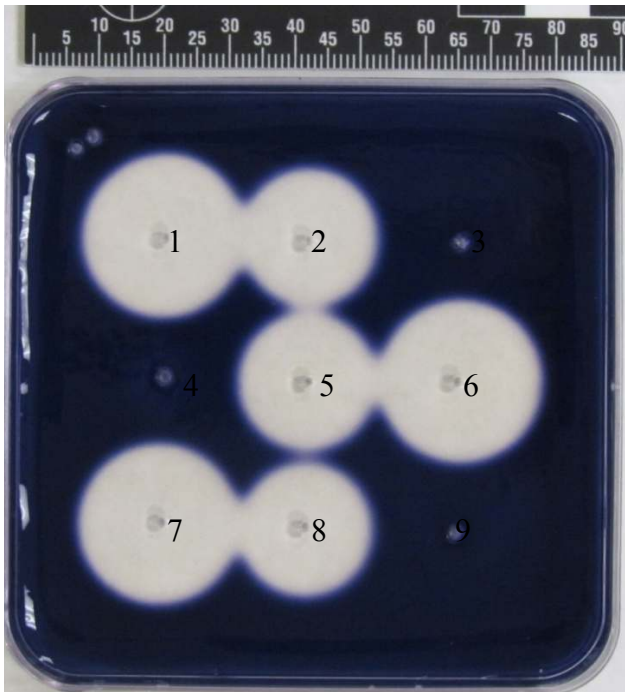


*Figure 1***.** Amylase Radial Diffusion Test: Controls

Table 8

*Amylase Radial Diffusion Test: ABAcard®*

| Well | Description | Clearing (mm) |
|---|---|---|
| 1 | 1:10 α-amylase (ABAcard®) | 26 |
| 2 | 1:100 α-amylase (ABAcard®) | 22 |
| 3 | 1:500 α-amylase (ABAcard®) | 19 |
| 4 | ABAcard® extraction buffer | 0 |
| 5 | Neat α-amylase | 29.5 |
| 6 | Post-Coital Vag (24 hr) w/ Semen | 12 |
| 7 | Semen / Saliva | 24.5 |
| 8 | Post-Coital Vag (24 hr / Menstrual) w/ Semen | 11 |
| 9 | Post-Coital Vag (24 hr) w/ Seminal Fluid | 8 |

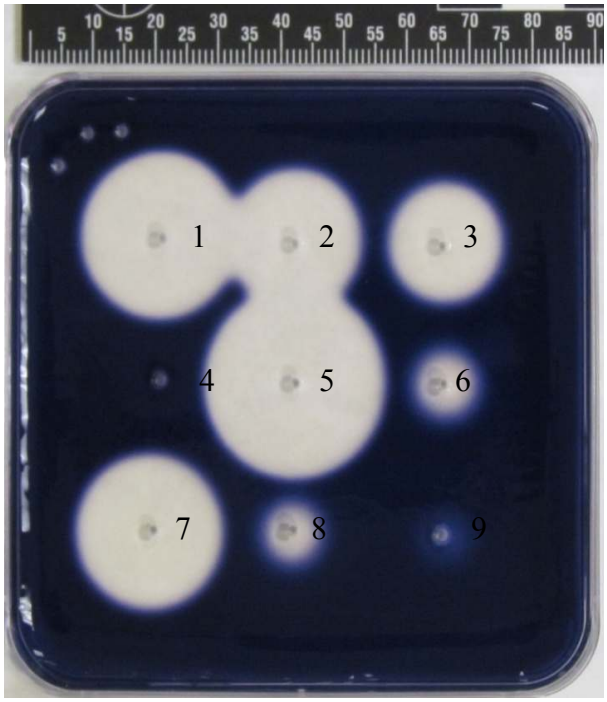


*Figure 2.* Amylase Radial Diffusion Test: ABAcard®

Table 9

*Amylase Radial Diffusion Test: RSID™*

| Well | Description | Clearing (mm) |
|---|---|---|
| 1 | 1:10 α-amylase (RSID™) | 27 |
| 2 | 1:100 α-amylase (RSID™) | 23 |
| 3 | 1:500 α-amylase (RSID™) | 20.5 |
| 4 | RSID™ Universal buffer | 0 |
| 5 | Neat α-amylase | 30.5 |
| 6 | Post-Coital Vag (24 hr) w/ Semen | 13 |
| 7 | Semen / Saliva | 23 |
| 8 | Post-Coital Vag (24 hr / Menstrual) w/ Semen | 12 |
| 9 | Post-Coital Vag (24 hr) w/ Seminal Fluid | 10 |

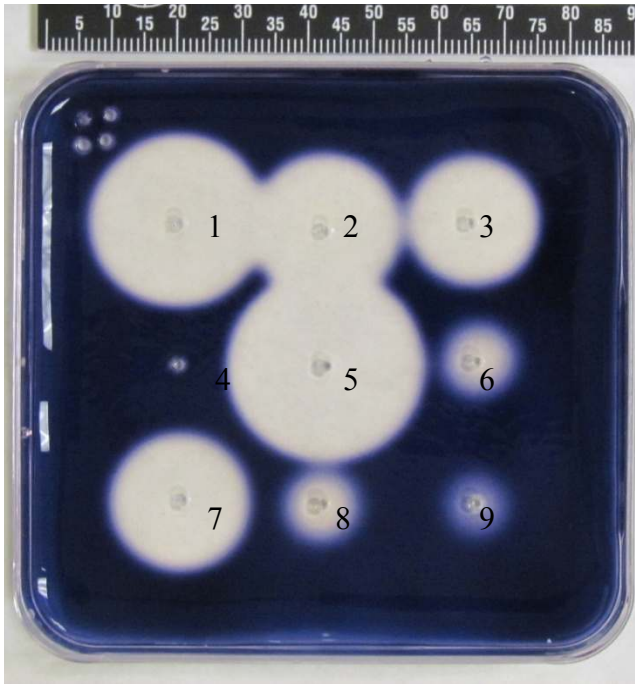


*Figure 3*. Amylase Radial Diffusion Test: RSID™

Table 10

*Amylase Radial Diffusion Test: Seratec®*

| Well | Description | Clearing (mm) |
|---|---|---|
| 1 | 1:10 α-amylase (Seratec) | 27 |
| 2 | 1:100 α-amylase (Seratec) | 23 |
| 3 | 1:500 α-amylase (Seratec) | 18.5 |
| 4 | Seratec extraction buffer | 0 |
| 5 | Neat α-amylase | 30 |
| 6 | Post-Coital Vag (24 hr) w/ Semen | 12 |
| 7 | Semen / Saliva | 23 |
| 8 | Post-Coital Vag (24 hr / Menstrual) w/ Semen | 11 |
| 9 | Post-Coital Vag (24 hr) w/ Seminal Fluid | 9 |

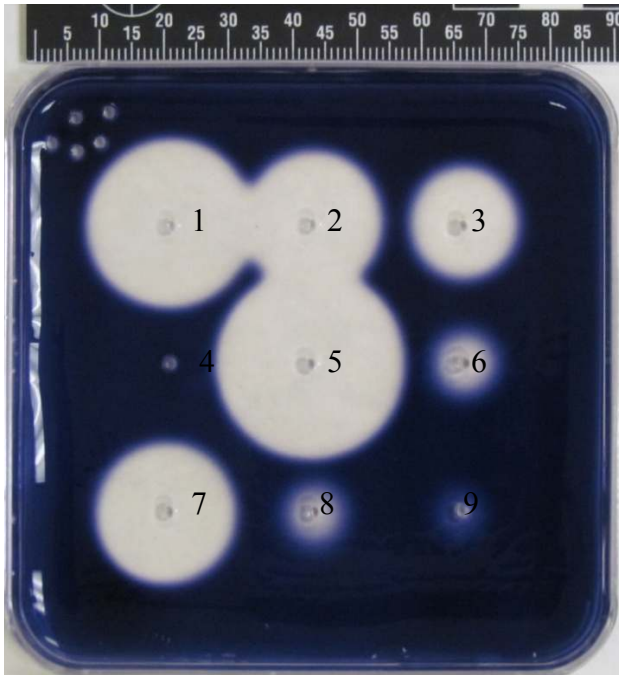


*Figure 4*. Amylase Radial Diffusion Test: Seratec®

Table 11

*pH Levels of Provided Extraction Buffers, PBS, and Water*

| Extractant | PH |
|---|---|
| PBS | 7.40 |
| Ultrapure Autoclaved Water | Not tested |
| RSID™ Universal Buffer | 9.05 |
| ABAcard® Extraction Buffer | 7.50 |
| Seratec® Extraction Buffer | 8.3 |

# CHAPTER 5

## Discussion

The ability to detect seminal fluid is vital in forensic cases involving sexual assaults. Some detection methods for seminal fluid rely on the presence of the prostate specific antigen (PSA) or the acid phosphatase enzyme. Studies have shown that both are not specific for seminal fluid and can be found in other body fluids, including vaginal secretions. The Rapid Stain Identification Test for Semen (RSID™ – Semen) targets semenogelin, a protein said to be specific to seminal fluid. The purpose of this study was to evaluate the use of the RSID™ – Semen Test for the identification of seminal fluid in comparison to ABAcard® p30 and Seratec® PSA. The study also compared the extraction buffers provided by each of the manufacturers.

### *Test Card Sensitivities*

RSID™ – Semen had the ability to detect both normal and vasectomized ejaculates. All three cards had the same sensitivities for liquid semen dilutions. Seratec® and ABAcard® outperformed RSID™ – Semen on liquid seminal fluid dilutions. RSID™ outperformed Seratec® and ABAcard® on dried semen and seminal fluid stains and Seratec® outperformed ABAcard®. The performance of RSID™ on dried stains is particularly important because the majority of forensic samples are received in dried form.

### *Substrate Reactivity*

Two common substrates encountered in forensic samples, cotton cloth and polyester swabs, did not react with any of the test cards. Cotton cloth is commonly

encountered with underwear, t-shirts, and other clothing submissions. Polyester swabs, as well as cotton swabs, may be submitted in sexual assault examination kits.

*Specificity & Reactivity with Other Body Fluids*

All three cards reacted with male urine, which was expected since both urine and seminal fluid exit the body through the same tract. Both ABAcard® and Seratec® alert the user that male urine will react with the cards. ABAcard® and Seratec® did not react with any other body fluids tested. RSID™, on the other hand, reacted with semen-free vaginal fluid and female urine samples. Many sexual assault examinations in the laboratory involve the analysis of swabs from the vaginal area, female external genital area, and female underwear. All of these items can be expected to contain vaginal fluid and female urine, meaning that these samples could potentially react with the RSID™ cards to produce false positive results. This could result in false reports of the detection of seminal fluid.

All test cards gave positive results with semen mixed with non-semen samples that previously tested negative. The cards were able to detect semen in mixtures and, therefore, non-semen samples did not interfere with semen.

*Acid Phosphatase Testing*

Initially, acid phosphatase testing was conducted on liquid semen and seminal fluid samples diluted in each of the three provided buffers. There was no interference with the RSID™ Universal Buffer or the ABAcard® extraction buffer. The sensitivity of samples diluted with the Seratec® extract buffer was 10-fold lower. The yellow color change of the buffer with the acid phosphatase reagent after 30 seconds may have been a contributing factor. The purple color change observed with 1:1,000 dilutions containing

the RSID™ Universal Buffer and the ABAcard® extraction buffer was light. The bright yellow color change of the Seratec® buffer with the acid phosphatase reagent may have masked any purple color change for the 1:1,000 dilution.

*Post-Coital & Semen/Saliva Samples*

Of the three cards, RSID™ was the only test that gave positive results with a vaginal sample taken 24 hours post-coital. The vaginal sample was taken from a subject with a non-vasectomized partner, and sperm cells were identified in the sample. However, the RSID™ card was found to react with semen-free vaginal fluid and female urine samples. Therefore, it is unknown whether the positive result obtained from the 24 hour post-coital vaginal sample was from semenogelin or some other cross-reacting substance. A vaginal sample with a non-vasectomized partner containing menstrual blood did not show positive results with any of the test cards. Additionally, a vaginal sample with a vasectomized partner did not show positive results with any of the test cards.

The semen and saliva mixture samples gave positive results with the three test cards.

For the acid phosphatase test, the vaginal sample with a semen donor extracted in ABAcard® buffer and the semen/saliva mixture extracted in RSID™ Universal Buffer gave positive results.

The reason for the numerous negative results with the acid phosphatase test on the post-coital samples is unknown. The composition and the pH of each of the buffers may have an effect on acid phosphatase activity when samples are extracted for a period of time. Additionally, the test was performed on an extract of the swab, and the extraction

process may have further diluted the acid phosphatase. Samples may not have been evenly distributed on the swabs, especially on post-coital samples where fluids are not evenly distributed in the vaginal cavity. Furthermore, at this post-coital interval, the amount of semen present in the vaginal sample will be significantly less. The reduction of semen in these samples through vaginal drainage and absorption by the body may have also contributed to the negative results.

*Amylase Testing*

There were no significant differences observed with clearing sizes of samples extracted in each buffer with the Amylase Radial Diffusion Test. Each of the buffers did not interfere with this test and forensic samples extracted in these buffers would be suitable for amylase testing.

*Staining for Microscopic Analysis*

No changes in color or form were observed under bright field or phase contrast conditions with for samples extracted with RSID™ Universal Buffer and the ABAcard® extraction buffer. Therefore, samples extracted in these buffers would be suitable for forensic testing. It is unknown if the typical staining colors can be achieved when extracting with the Seratec® buffer. It is hypothesized that changing the staining times may result in achieving the expected colors.

*Concordance with Hypotheses*

RSID™ – Semen was able to detect vasectomized and non-vasectomized ejaculates as predicted. RSID™ – Semen was expected to be more sensitive than the ABAcard® and Seratec® tests. RSID™ – Semen was less sensitive for liquid seminal fluid samples and more sensitive for semen and seminal fluid stains. The same

sensitivities were observed for liquid semen samples. RSID™ – Semen was predicted to be more specific, however, the test reacted with semen-free vaginal and female urine samples. As expected, RSID™ – Semen did not react with polyester or cotton substrates. RSID™ – Semen was also able to detect semen in mixtures with other body fluids as predicted.

No predictions were made for the detection of post-coital vaginal samples, the use of samples extracted in the RSID™ Universal Buffer for additional testing, and the use of other extractants with RSID™ – Semen. RSID™ – Semen was able to detect post-coital vaginal samples. The RSID™ Universal Buffer had no effect on additional tests (acid phosphatase, amylase, and staining for a microscopic examination). Finally, other extractants (autoclaved ultrapure water and PBS) were found to be unsuitable for use with RSID™ – Semen.

*Benefits and Limitations of RSID™ – Semen*

Based on this study, semen and seminal fluid stains extracted in the RSID™ Universal Buffer and used in conjunction with RSID™ – Semen had better sensitivities than ABAcard® p30 and Seratec® PSA. This is important from a forensic standpoint since neat semen or seminal fluid samples are less commonly encountered in casework. Samples typically come in the dry state and when semen or seminal fluid samples are present, they are typically mixed with vaginal fluid or saliva, which dilutes the sample. Furthermore, samples are often collected from victims several hours to days after the sexual assault occurred. The activity of targeted proteins in seminal fluid decrease and are eventually lost from the vaginal cavity due to drainage and other factors. Because of the low level of seminal fluid, the sensitivity of RSID™ – Semen is advantageous for

forensic investigations. RSID™ – Semen was superior over the other two cards for the post-coital vaginal sample from the subject with a non-vasectomized partner.

Conversely, RSID™ – Semen is not recommended for samples that contain vaginal fluid or female urine. The cause of the false positive reactions of these two fluids with RSID™ – Semen is unknown. Semenogelin is said to be specific to semen, therefore, RSID™ – Semen should not react with anything but semen or seminal fluid. It should be noted that RSID™ – Semen recommends further dilution of samples than was actually performed in this study.

A disadvantage of RSID™ – Semen is that a false positive result with PBS and a failed test with ultrapure autoclaved water were observed. This means that samples cannot be extracted and/or diluted solely with these extractants. However, in this study, samples extracted in PBS and water then diluted 1:10 with the Universal Buffer gave the expected results. Since the pH of RSID™ Universal Buffer was much higher than the other provided buffers and PBS the card appears to operate over a range of alkaline pH.

*Conclusions and Recommendations*

Based on the findings of the present study, it is not recommended to use RSID™ – Semen as the sole means of forensic testing for seminal fluid due to the false positive results encountered with male urine, female urine, and vaginal fluid. If forensic laboratories desire, RSID™ – Semen may be used in conjunction with acid phosphatase testing, the ABAcard® p30 test, and/or the Seratec® PSA test. However, this can add significantly to the cost of biology screening as the retail value per test card are as follows: $7.96 (RSID™ – Semen), $4.28 (ABAcard® p30), and $3.00 (Seratec® PSA).

*Future Research*

Further studies should be conducted to determine whether semenogelin is truly specific to seminal fluid. If this is the case, the antibodies used in the RSID™ – Semen cards should be studied to determine the cause of the false positive results.

Since each manufacturer supplies a buffer for use on their specific card, the ability to extract a sample with a single buffer for use on several tests should be studied by laboratories if they plan to use more than one card. The amount of sample consumed in biology screening tests should be minimal. If multiple buffers are required for extraction, this could lessen the amount of sample remaining for DNA analysis. Although in this study, the initial results from samples extracted in PBS or water then diluted 1:10 in the appropriate buffer showed no interference, additional testing should be conducted with other dilutions and other buffers. Lastly, the staining times and/or concentrations of stains for samples extracted with the Seratec® buffer should be studied further to determine if correct colors may be achieved.

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# APPENDIX A

## Reagent Preparation

### *Alpha-Naphthyl Acid Phosphate*

*Citric Acid Buffer*

1. Dissolve and mix 18.9 g citric acid (anhydrous) in 900 mL ultrapure autoclaved water.
2. Adjust pH to 4.9 with NaOH.
3. Adjust the volume to 1 L with ultrapure autoclaved water.

*Working Solution Preparation*

1. Mix 50 mL of citric acid buffer, 50 mg of α-naphthyl phosphate, and 8 mg Fast Garnet GBC salt (GBC).

*Amylase Radial Diffusion*

*Iodine Solution Preparation*

1. Dissolve 1.65 g of KI with 30 mL of ultrapure autoclaved water.
2. Add 1.50 g of $I_2$.
3. Stir for approximately 5 min.
4. Filter and store in the refrigerator.
5. Make a 1:10 dilution to stain a plate.

*Gel Preparation*

1. Dissolve one amylase buffer package (SERI #B116) and 500 mL of ultrapure autoclaved water.
2. Mix 20 mL of prepared buffer and 0.2 g of SERI EA agarose in a flask.
3. Cover and heat to dissolve the agarose.
4. Add purified water, as needed, to maintain the original volume.
5. Pour into a petri dish and allow to cool.

*Nuclear Fast Red Stain*

1. Dissolve and mix 5 g aluminum sulfate and 100 mL of hot autoclaved ultrapure water.
2. Add 0.1 g of nuclear fast red.
3. Cool and filter.

*Picroindigo Carmine Stain*

1. Add 4 g of picric acid in 300 mL of autoclaved ultrapure water.
2. Add 1 g of indigo carmine.
3. Filter.

*Phosphate Buffer Saline*

1. Dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of $Na_2HPO_4$, and 0.24 g of $KH_2PO_4$ in approximately 800 mL of autoclaved ultrapure water.
2. Adjust the pH to 7.4 with concentrated HCl.
3. Autoclave and adjust the volume back to 1000 mL with sterile DI $H_2O$.

## APPENDIX B

Protocols

*Acid Phosphatase Test*

*Protocol*

1. Test the reagents against a semen standard and reagent blank to demonstrate that the reagents are working properly.
2. For an extracted sample, add 5 μL of extract to a piece of filter paper.
3. Add 1 drop of the α-naphthyl acid phosphate working solution.
4. Monitor and record any color changes for up to 5 minutes.

*Interpretation of Results*

Positive: Purple color change was observed within 5 minutes. A presumptive test for acid phosphatase was positive.

Negative. No color change was observed within 5 minutes. No acid phosphatase was detected.

Inconclusive. A color change could not be distinguished due to substrate interference.

*Amylase Radial Diffusion Test*

*Protocol*

1. Punch wells in gel approximately 1.5 cm apart and remove agarose plugs with a vacuum.
2. Fill the wells with 5 μL of sample.
3. Place the gel in a moisture chamber, then into a 37$^{o}$C oven.
4. Incubate for 16 to 24 hours.
5. Flood the gel with 1:10 diluted iodine.
6. Record the ring diameters of each sample and control.

*Interpretation of Results*

Amylase is present in higher levels in saliva, but may be present at lower levels in other body fluids. This test is not conclusive for the presence of saliva.

*Seratec® PSA Semiquant*

*Manufacturer's Protocol*

1. Extract semen stains or swabs in 250 µL of buffer for 2 hours using a shaker.
2. Centrifuge for 1 min at 13,000 g.
3. Add 200 µL (5 drops using provided dropper) of the supernatant to the sample well on the test strip.
4. If the supernatant is too viscous because of a high PSA concentration, it should be diluted.
5. Read the result after 10 minutes at room temperature.
6. To estimate the amount of PSA by comparison with the internal standard, readings should be kept strictly to 10 minutes.

*Notes*

Seminal fluid should be diluted at least 1:500 prior to use because of its extremely high PSA content. For the dilution we strongly recommend to use the provided buffer solution or alternatively a 1 M TRIS solution with a neutral pH of 8.2.

Particle of tissue do not interfere with the test result.

Allow samples to warm up to room temperature before starting the test.

A pH-value below 2 of the specimen can cause false positive or invalid results.

*Interpretation of Results*

*Test result Line (T): reflects PSA concentration of the sample, visible in PSA-positive samples only*

*Internal Standard: color intensity correlates with a concentration of approximately 4 ng/mL PSA*

*Control Line (C): control for possible procedural errors and for the integrity of test components*

Negative result (no PSA in the probe or PSA concentration below detection limit). No test result line (T). Appearance of internal standard line and control line (C) confirm validity of the test. In this case the sample most likely does not contain seminal fluid. Make sure that the dilution of the probe leads to a PSA concentration within the detection range. PSA concentrations that are too low (e.g. due to insufficient extraction) or that are too high (e.g. due to insufficient dilution; 500 µg/mL result in a high dose hook effect) interfere with the formation of the test result.

Positive result (PSA detectable). Test result line (T), internal standard line, and control line (C) appear. In this case it is very likely that the sample contains seminal fluid. If there is the risk of mixing up PSA containing body fluids and seminal fluid you might try to get a more accurate result by testing higher dilutions.

Invalid result. Internal standard line and/or control line (C) are not detectable. The test is invalid and the assay should be repeated with a new test cassette. If the sample contains high amounts of PSA it is possible that the color intensity the control line is only weak.

*ABAcard® p30 Test*

*Manufacturer's Protocol*

1. Frozen specimens/swabs/stains must be thawed completely and brought to room temperature.
2. Extraction of specimens from swab or stain may be performed in 750 µL of Extraction Buffer for 2 hours at 2-8 $^{o}$C. This procedure recovers approximately 99% of the extractable p30 on the swabs.
3. Centrifuge the sample for 3 minutes after the extraction. Remove 300 µL of supernatant for testing purposes. This aliquot may be stored at 2-8 $^{o}$C if not used immediately. Immediately before use, with ABAcard® p30 test, the sample should be brought back to room temperature. Remaining sample may be used for further DNA analysis without affecting the DNA yield.
4. Allow the sample to warm to room temperature if it has been refrigerated.
5. Remove the device and the dropper from the sealed pouch.
6. Add 200 µL (8 drops with the dropper) of sample to the sample well 'S' of the test device.
7. Read result at 10 minutes. Positive results can be seen as early as 1 minute depending on the p30 concentration. For negative results, one must wait the full 10 minutes.

*Interpretation of Results*

Positive. If there are two pink lines, one each in the test area 'T' and in the control area 'C', the test is positive and indicates that the p30 level is at or above 4 ng/mL.

Negative. If there is only one pink line (in the control area 'C'), the test result is Negative. This may indicate that (a) No p30 is present above 4 ng/mL or (b) Presence of "High Dose Hook Effect". If suspected, the sample may be retested using a 10 to 10,000 fold dilution.

Invalid. If there is no pink line visible in the control area 'C', the test is inconclusive. Repeat the test and reexamine the test procedure carefully.

*Limitations*

Positive results may be obtained with male urine, which has reported a p30 mean value of 260 ng/mL. Seminal vesicle specific antigen should not be present when tested with urine. Use of another appropriate test is recommended when male urine is in question.

Appropriate specimen should be used since p30 is detectable in the vaginal tract only up to a maximum of 2 days.

*Rapid Stain Identification of Human Semen*

*Extraction Protocol*

Forensic samples obtained on cotton swabs should be extracted in 200-300 µL of RSID™ – Universal Buffer for 1-2 hours. If a portion of the swab is used, sufficient RSID™ – Universal Buffer should be added to easily cover the sample.

Stains on fabric or paper should be sampled by taking a punch or cutting (~20 $mm^2$) of the item. The punch or cutting should be extracted in 100 µL of RSID™ – Universal Buffer for 1-2 hours.

A general guideline of a maximum of 10% of extract, up to a maximum of 20 µL should be run.

The remainder of the extract can be processed for STR analysis. The buffer provided is STR free and contains a DNA stabilizer. The provided buffers do not interfere with extraction or amplification.

*Test Procedure*

Note: Assays should be performed at room temperature. It is recommended that a positive and negative control be included with every assay.

1. Remove cassette from the foil pouch and discard the silica desiccant.
2. Combine extract aliquot (max of 20 µL) with RSID™ – Universal Buffer to bring test sample to a total volume of 100 µL.
3. Add sample in RSID™ – Universal Buffer to sample window. Start timing at the point the sample is added to the sample window.
4. Due to the High Dose Hook Effect, samples giving a weak positive or negative result should be diluted 1:20 and retested.

5. At 10 minutes, score and record results.

6. Users may add 100 µL from the extraction to the cassette of RSID™ – Semen. This will have little to no effect on the sensitivity or specificity of the test; however, any problems encountered by the high dose hook effect or using a concentrated sample (e.g., altered pH) may be avoided if the extraction is diluted in RSID™ – Universal Buffer.

*Interpretation of Results*

RSID™ – Semen should be evaluated exactly 10 minutes after the addition of sample.

No human semen detected. A visible red line at the Control (C) position only indicated a negative result.

Human semen detected. Visible red lines at both the Control (C) and Test (T) positions indicate a positive result.

Test failure, no conclusion possible. A visible red line at the Test (T) position only indicates a failed test.