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Forensic semen identification in semen-saliva mixtures

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Thesis

FORENSIC SEMEN IDENTIFICATION IN SEMEN-SALIVA MIXTURES

by

COLE REAGAN GIZELBACH

B.S., University of Georgia, 2020

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FORENSIC SEMEN IDENTIFICATION IN SEMEN-SALIVA MIXTURES

COLE REAGAN GIZELBACH

ABSTRACT

Sexual assault evidence composes a large portion of the evidence analyzed by forensic serologists. Key to the processing of sexual assault evidence is the screening of the evidence items for the presence of semen. Due to the intimate nature of a sexual assault, it is very possible that semen is mixed with other body fluids when it is deposited on an item of evidence. One of the body fluids that semen can easily come into contact with during a sexual assault is saliva. Saliva functions as the first step in the human body's digestive system. Due to the digestive system's purpose of breaking down nutrients, it stands to reason that saliva could play a role in breaking down seminal components. To detect semen, specific components of semen are tested for in forensic laboratories. These components are often acid phosphatase, prostate specific antigen, semenogelin, and spermatozoa.

This experiment combined semen from one donor with saliva of seven other donors in a two part survey. In part one, semen was mixed with the saliva of Donors A, B, C, and D at three different ratios of 1:1, 1:2, and 1:10 semen-saliva. Twenty-microliter stains were pipetted onto one inch by one inch squares on twelve cotton swatches to test for acid phosphatase, prostate specific antigen, semenogelin, and spermatozoa. One set of six swatches was allowed to dry and the other set was kept damp. The stains were tested at six timepoints: day zero, day one, week one, week two, week three, and week four. Part two involved incubating the semen with saliva from Donors X, Y, and Z at body temperature

for up to twenty-four hours. The same three ratios used in part one were repeated with the saliva from Donors X, Y, and Z in part two. A twenty microliter stain was pipetted onto a cotton swatch at each of the five timepoints from the start of the incubation period: zero minutes, one hour, five hours, eight hours, and twenty-four hours. Each stain was tested for acid phosphatase, prostate specific antigen, and semenogelin.

The results of part one showed that semen samples that are mixed with saliva but allowed to dry are effectively unaffected by the presence of saliva. On the dry swatches, the stains tested positive for every component of semen at every timepoint for every donor except for Donor D's 1:10 semen-saliva mixture stain, which tested negative for spermatozoa at week 1, but positive for spermatozoa in the subsequent timepoints. The results of the damp swatches suggests that damp environmental factors can prevent the detection of seminal components. By week two, the detection of spermatozoa had completely dropped out in the mixture stains and in the neat semen control stains. Detection of prostate specific antigen ceased in the control by week 3 and had also stopped in all 1:1 semen-saliva mixture stains by week 4. The detection of prostate specific antigen had stopped at week 3 for all donors in the 1:10 semen-saliva mixture stains. Semenogelin was still detectable in the control sample for the duration of the experiment, and it was detected for all donors at week 4 in the 1:1 and 1:2 semen-saliva mixture stains. Detection of semenogelin ceased in Donors B and D in the 1:10 semen-saliva mixture stains by week two.

The results of part two suggested that the detection of acid phosphatase could be affected when semen and saliva have been incubating together at body temperature. Acid

phosphatase was detected at all the timepoints in the neat semen control, but after eight hours, it was no longer detectable in all the mixture ratios of Donors X and Z. Acid phosphatase was no longer detectable in the 1:1 semen-saliva mixture stain of donor Y at eight hours and in the 1:2 semen-saliva mixture stain of Donor Y at twenty-four hours. Acid phosphatase did remain detectable in the 1:10 semen-saliva mixture stain of Donor Y through the twenty-four hour experimental period. Prostate specific antigen and semenogelin remained detectable in all the donors at all three ratios for the duration of the experiment.

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LIST OF ABBREVIATIONS

ALS	Alternate light source
AMY1	Salivary amylase
AP	Acid Phosphatase
Arg	Arginine
DNA	Deoxyribonucleic acid
His	Histidine
hK5	Kallikrein five
H&E	Hematoxylin and eosin
neg	Negative
NISVS	National intimate partner and sexual violence survey
pos	Positive
PSA	Prostate specific antigen
RSID™ Semen	Rapid Stain Identification of Human Semen
rpm	Revolutions per minute
UV	Ultraviolet

1. INTRODUCTION

1.1 Sexual Assault in the United States

Sexual violence is an ongoing issue in the United States. Data collected from the Centers for Disease Control and Prevention for the National Intimate Partner and Sexual Violence Survey (NISVS) in 2015 reports that over 43% of women experienced violent sexual contact in their lives (1). Furthermore, the survey shows that around 13% of women experienced a completed rape within their lifetimes (1). The numbers are lower for men, but still significant. Approximately 25% of men reported experiencing sexual violence (1). NISVS defines rape as "any completed or attempted unwanted vaginal, oral, or anal penetration through the use of physical force or threats to physically harm and includes times when the victim was drunk, high, drugged, or passed out and unable to consent (1)." At the time of writing, the latest statistics available on rape from the Federal Bureau of Investigation's Uniform Crime Reporting Program show that in the year 2018, approximately 139,380 rapes were reported to law enforcement (2). Trends in crime data show that from 2016 to 2018 there has been an increase in reported rapes by approximately 23% (2).

Due to the nature of crimes such as rape, there is often forensically relevant evidence left behind in the form of body fluids. When a victim reports a rape and has a sexual assault kit collected, specially trained nurses or other medical personnel will collect and preserve the biological evidence prior to submitting it to a crime lab for analysis. A study by the United States Department of Justice reported that 34% of sexual assaults by a stranger were not reported to police (3). The reasons for this included the assault being

viewed as a personal matter and fear of reprisal (3). With this in mind and with sexual crimes being so intimate, it is reasonable to infer that victims sometimes delay reporting. The length of the period of delay could lead to an inability to detect forensically relevant evidence. Willot and Allard report that spermatozoa can be detected orally up to six hours after intercourse with buccal swabs (4). Additionally, this study showed that spermatozoa were detected on the lips for up to nine hours after intercourse (4). A more recent study by Casey et al. demonstrated that spermatozoa could be detected orally up to twenty-seven hours after intercourse (5). Both studies highlight the importance of early collection in sexual assault cases for the forensic analysis to be most accurate.

Beyond spermatozoa, the body fluids that are often associated with a sexual crime are semen and saliva. Both fluids contain proteins for which the evidence is screened. Failure to identify these biological markers during serological analysis could result in the stoppage of further analysis of the evidence. Given the hostile environment of the human oral cavity, it is to be expected that evidence deposited in this location could be damaged over time. The oral environment will be further explored in a later section of this document.

1.1.1 Composition of Semen

Human seminal fluid consists of both cellular and protein components. In a single ejaculation event, the average amount of semen is approximately 3.5 milliliters (6). The components of this ejaculate include sperm cells which can number up to 150 million sperm per milliliter of semen or as low as 50 million sperm per milliliter of semen in a healthy male (6, 7).

The prostatic fluid in the ejaculate also contains semenogelin and prostate specific antigen. Semenogelin accounts for approximately twenty to forty percent of seminal plasma with a concentration of approximately 10 to 20 milligrams per milliliter (8). This concentration makes semenogelin the most prevalent protein in semen. Semenogelin's main function is the coagulation of semen and it likely plays a role in reducing the mobility of sperm cells (8).

Prostate specific antigen (PSA) is present in semen with concentrations varying from 0.2 milligrams per milliliter to 5.5. milligrams per milliliter. Being a proteolytic enzyme of the kallikrein family, PSA's main function is to break down semenogelin and return semen to a liquified state (6, 9 – 13).

Acid phosphatase (AP) is another protein present in human semen. Specifically, AP is an enzyme that catalyzes the reaction of cleaving ester-linked phosphates. AP is a very stable enzyme that can retain its primary function for long periods of time under proper storage conditions. Dried AP can remain active for years, and wet AP stored at temperatures of thirty-seven degrees Celsius can remain active for months (6). The concentration of AP in human semen varies, but it is often between 0.3 to 1.0 milligrams per milliliter of semen (6, 14, 15).

1.1.2 Forensic Identification of Semen

Human semen is identified forensically through the detection of spermatozoa, seminal AP, PSA, and semenogelin. Testing for semen often begins with a visual scan of the evidence item. Semen stains manifest as white to off-white stains with a stiff feeling

(6). These characteristics are shared with stains made by other body fluids such as saliva. If a stain is not observed with the naked eye, an alternate light source (ALS) can be used to capitalize on the fluorescence of semen stains (6). Fluorescence can be observed using either ultraviolet (UV) light or blue visible light with a wavelength of about 450 nanometers (6, 18). UV light allows the analyst to avoid the need to use a barrier filter to see the fluorescing stain, but prolonged exposure to UV radiation is known to damage deoxyribonucleic acid (DNA). DNA analysis is often the next step in analyzing evidence after serological testing, therefore using a blue light ALS in conjunction with an orange barrier filter will still exploit the fluorescent nature of semen without damaging the DNA (6, 18).

Once a stain has been located, a presumptive test for AP is conducted. Presumptive testing looks for components that are highly indicative of, but not exclusively belonging to semen. This test capitalizes on the enzyme's ability to cleave ester-linked phosphates (19). When AP is exposed to α-naphthyldiazonuim chloride, the freed naphthol binds with the diazonium compound, and a colored product is formed (19). AP is also naturally present in other body fluids to include vaginal fluid and saliva (6, 16, 17). Because AP is found in these other body fluids, testing for AP does not confirm the presence of semen. A color change that occurs within several minutes indicates semen may be present, however additional testing must be performed to confirm its presence (6, 19 – 21).

Once semen has been presumptively identified via AP testing, the presence of semen can be confirmed with microscopy. This involves eluting spermatozoa from a cutting of the evidence and staining the cells on a microscope slide. There are many

methods of staining, but two of the more common methods are the Christmas Tree method and the hematoxylin and eosin (H&E) method. Both of these methods make the spermatozoa more visible and allow for the comparison of spermatozoa to other cells and debris captured on the slide (6, 22). This is accomplished by the use of different colored stains that stain the nuclear material of cells one color and other cellular material a different color. For example, in the Christmas Tree method sperm heads and epithelial nuclei appear red with green tails and cytoplasm. In the H&E method, sperm heads and nuclei appear purple while the tails and cytoplasm appear pink [Figure 1].

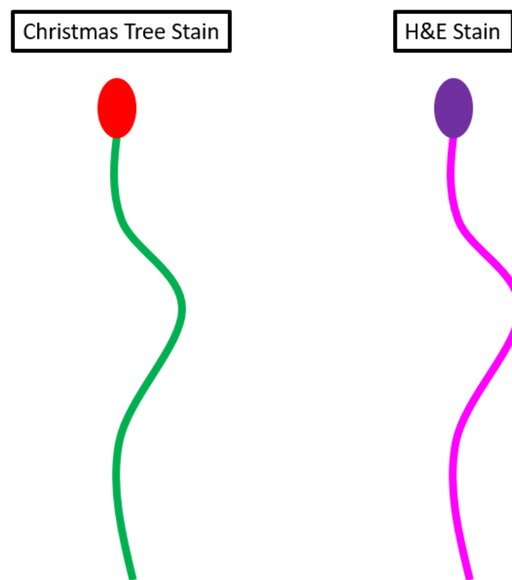

Figure 1 Stained spermatozoa. Illustration showing the appearance of spermatozoa after being stained with Christmas tree stain and H&E stain.

Occasionally an item of evidence will test positive for AP, but no spermatozoa will be observed during microscopic examination. This can occur when the semen being analyzed is from an azoospermic man. Azoospermia can occur because of illness, drugs, nutrient deficiencies, trauma, genetics, or medical procedures (6, 7, 23). Depending on the cause of the azoospermia, the condition may be temporary or permanent. When

azoospermic semen is deposited on an item of evidence or collected in a sexual assault evidence collection kit, the presence of semen can only be presumptively identified using PSA and semenogelin as markers. This is accomplished using lateral flow immunochromatography.

Immunochromatography uses antibodies to identify proteins commonly found in body fluids. Modern immunochromatographic assays employ both monoclonal and polyclonal antibodies (6). The monoclonal antibodies are mobile and will bind to the protein of interest. They also are linked to dye molecules that become visible as the antibody-protein complex accumulates after binding in the test region to immobilized polyclonal antibodies specific to the protein (6). Also present in the assay is a section of immobilized polyclonal antibodies that bind to the mobile antibodies present. The colored line that appears due to the accumulation of antibodies binding at this site functions as a control to ensure the analyst the assay is functioning properly. A successful positive result will have two colored lines (test and control), and a successful negative result will have one colored line in the control area (6). While only microscopy is confirmatory for the presence of semen, the identification of PSA and semenogelin using immunochromatography is highly indicative of the presence of semen, especially following a positive AP result (6). Serology protocols sometimes only require immunochromatographic assays after microscopy has failed to show the presence of spermatozoa [Figure 2].

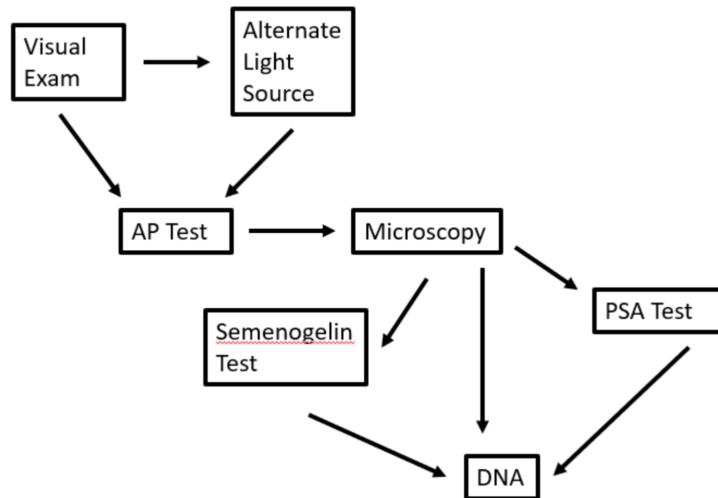

Figure 2 Semen analysis flowchart. Showcases the typical process of analyzing evidence for semen.

1.1.3 Composition of Saliva

Saliva is a fluid excreted in the mouth by the parotid, submaxillary, and sublingual glands. Composed of almost ninety-nine percent water, saliva contains many electrolytes and enzymes (24). The most notable of these enzymes is amylase. Salivary amylase (AMY1) functions to break down starches into monomer carbohydrates (6). Less notable from a forensics perspective but still important salivary proteins are lysozyme, immunoglobulin A, peroxidase, and kallikreins (24). Lysozyme and immunoglobulin A have antimicrobial functions with gram-positive bacteria being more susceptible to lysozyme than gram-negative bacteria (24). Peroxidase is secreted by the salivary glands at a concentration of approximately thirty-two to five hundred sixty-six units per milliliter (25). It catalyzes a reaction that breaks down hydrogen peroxide (26). Hydrogen peroxide is excreted by common oral bacteria (26). Salivary peroxidase when in the presence of hydrogen peroxide and thiocyanate ions will catalyze the reaction that converts the two

precursors into water and hypothiocyanite ions (26). Kallikreins are excreted by multiple human tissues such as the salivary glands and the pancreas, and they have been identified in other body fluids such as sweat and urine (27, 28). Kallikreins are serine proteases that aid in processing precursor proteins into their tertiary forms (29). Saliva is also known to contain AP (30). Salivary AP functions just like the AP found in semen. It catalyzes the reaction of cleaving ester-linked phosphates (30). Because of this, salivary AP is indistinguishable from seminal AP when a colorimetric test is used.

Although saliva has an antimicrobial role, human saliva contains a diverse environment of bacterial flora. Healthy human mouths have been found to have bacteria from six different phyla: *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, and the TM7 phyla. The bacterial flora differs according to location in the oral cavity. Based on this information, it is reasonable to assume that the bacteria found in a saliva sample will be dependent on the areas in the oral cavity with which the saliva came into contact (31). Further, bacteria are known to produce proteolytic enzymes that can be detrimental to biological materials (32).

1.1.4 Potential Effects of Saliva on Sexual Assault Evidence

Due to the proteolytic nature of saliva, it is reasonable to assume that exposing semen to saliva could have a damaging effect on the ability to detect semen. Many of the previously mentioned methods for the detection of semen rely on intact and functional proteins. AP is an enzyme whose detection relies on its Histidine (His) 12 residue and its Arginine (Arg) 11 residue being active (33). The detection of AP is especially important

because it is often the first step that determines if more definitive analyses will be conducted on an evidence item. PSA and semenogelin detection rely on the antibodies being able to bind to the proteins in specific ways. Kallikrein five (hK5) has been shown to be capable of degrading both semenogelin I and II (34). While hK5 is primarily excreted in the prostate, human salivary kallikrein was isolated in 1972 (35). Being an enzyme of the same family as hK5, human salivary kallikrein could possibly exhibit similar degrading functions on the semenogelin proteins. If these important structures are damaged it is entirely possible that these proteins, though present, would not be detected using the aforementioned screening techniques.

Bacteria are capable of altering their environments to make them more suitable for themselves. For example, oral streptococcus bacteria are capable of producing a basic metabolite that in turn raises the pH of the environment. The species does this to protect itself from acidic environments (36). Streptococcus is a member of the *Firmicutes* phyla which is known to exist in the oral cavities of healthy individuals (31). The AP enzyme's active site requires acidic conditions to function (33). In a basic environment, the His12 and Arg11 residues are not able to properly function and catalyze reactions. Some oral bacteria are also capable of making an environment more acidic (37). Chromatographic immunoassays require certain pH ranges to properly function. For example SERATEC® PSA is designed to work within a pH range of five to ten (38). While the range is broad and the system uses a buffer solution to help ensure this pH is achieved, it is still theoretically possible for the pH conditions to be altered to the point that these immunoassays are no longer reliable.

With all of these factors to consider, the purpose of this study is to assess whether or not saliva has an effect on the forensic detection of semen evidence and if so, to what extent. Saliva is often present in sexual assaults when fellatio occurs or when it is used as a lubricant. Because of this, it is important to be aware of how the presence of saliva may affect sexual assault evidence. It is hypothesized that saliva will reduce the detectability of semen due to the enzymes and bacteria present in it.

2. MATERIALS AND METHODS

2.1 Part One: Semen Identification in Stored Semen-Saliva Mixture Stains

2.1.1 Sample Preparation

Purchased semen (LEE Biosolutions, Maryland Heights, MO) was combined with saliva donated by four anonymous volunteers in accordance with a protocol approved by the Boston University Institutional Review Board. These donors were designated as Donor A, Donor B, Donor C, and Donor D. Each donor's saliva was combined with semen at three different ratios. These ratios were 1:1, 1:2, and 1:10 semen to saliva. Two sets of six square cotton swatches (Texwipe, Kernersville, NC) were divided into sixteen one inch by one inch squares. Mixtures were then spotted onto twelve cotton swatches by pipetting 20 microliters into the corresponding labeled squares. Neat semen from the same source and neat saliva from Donor B was also pipetted into squares. These stains along with empty squares labeled "blank" acted as the controls. One set of the six cotton swatches had stains that were allowed to dry and stored in paper manila envelopes (Quality Park, Maple Grove, MN). These swatches were referred to as the dry condition swatches. The other set of six cotton swatches had stains that were not allowed to dry and was stored in plastic zip-top bags (Ziploc®, Racine, WI). These swatches were misted with water after sampling at each time point. This mist replaced lost moisture due to evaporation while outside of the zip-top bags. These swatches were referred to as the damp condition swatches.

2.2 Part One: Semen Identification in Stored Semen-Saliva Mixture Stains

Component Identification

One square from each donor was tested for AP, semenogelin, PSA, and spermatozoa [Figure 4]. The presence of AP was tested using the AP Spot Test (Serological Research Institute, Richmond, CA). The presence of semenogelin was tested using Rapid Stain Identification of Human Semen (RSID™ Semen) (Independent Forensics, Lombard IL). The presence of PSA was tested using SERATEC® PSA Semiquant (SERATEC® Göttingen, Germany). Lastly, spermatozoa was microscopically identified using hematoxylin (Fisher Scientific, Pittsburgh, PA) and eosin (Acros Organics, Geel, Antwerp, Belgium) staining. Each of these tests were completed using a five millimeter by five millimeter cutting of the stain. Each test was completed at six timepoints: day 0 (October 22, 2021), day 1 (October 23, 2021), week 1 (October 29, 2021), week 2 (November 6, 2021), week 3 (November 12, 2021), and week 4 (November 19, 2021) [Figure 3].

Donor A 1:1 Day 0	Donor A 1:1 Day 1	Donor A 1:1 Week 1	Donor A 1:1 Week 2
Donor A 1:1 Week 3	Donor A 1:1 Week 4	Donor B 1:1 Day 0	Donor B 1:1 Day 1
Donor B 1:1 Week 1	Donor B 1:1 Week 2	Donor B 1:1 Week 3	Donor B 1:1 Week 4
Donor C 1:1 Day 0	Donor C 1:1 Day 1	Donor C 1:1 Week 1	Donor C 1:1 Week 2

Donor C 1:1 Week 3	Donor C 1:1 Week 4	Donor D 1:1 Day 0	Donor D 1:1 Day 1
Donor D 1:1 Week 1	Donor D 1:1 Week 2	Donor D 1:1 Week 3	Donor D 1:1 Week 4
Neat Semen Day 0	Neat Semen Day 1	Neat Semen Week 1	Neat Semen Week 2
Neat Semen Week 3	Neat Semen Week 4		

Figure 3 swatches one and two. Diagram of the first and second swatches in a set. Demonstrates the distribution of experimental and control stains.

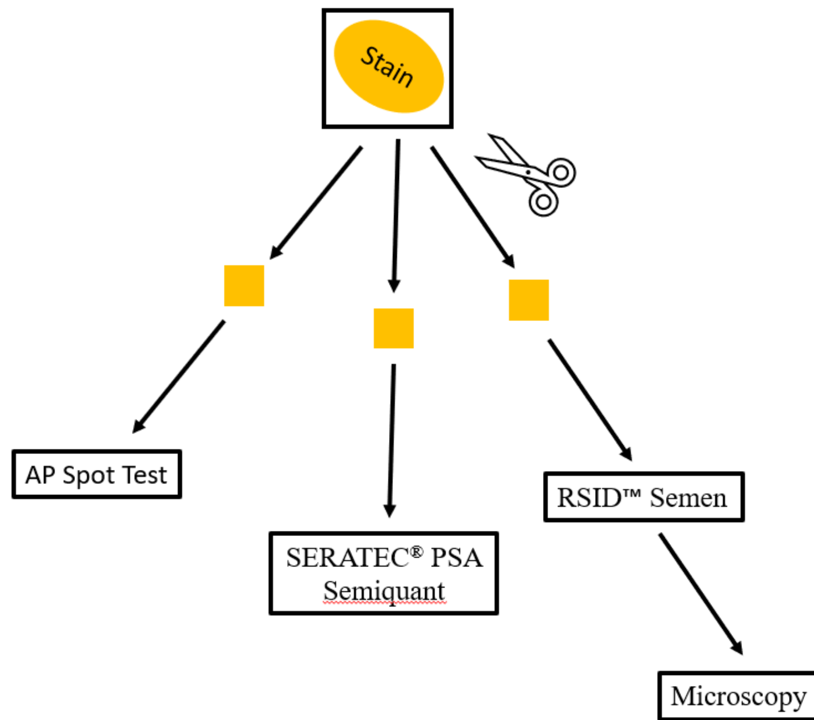

Figure 4 Part one experimental design. Diagram of the part one experimental design. Demonstrates the process each stain went through.

2.2.1 Acid Phosphatase Identification

First, 0.13 grams of AP Spot Test reagent was dissolved in five milliliters of water. Once total dissolution was achieved, one drop was applied to a five millimeter by five millimeter cutting of a prepared stain. A timer was started, and the reaction was allowed to occur for up to five minutes. A positive result was recorded if a purple color appeared within the five minute time frame. The time until the purple color was visible was also recorded. If the cutting did not exhibit a color change after five minutes, a negative result was recorded.

2.2.2 Part One: Semen Identification in Stored Semen-Saliva Mixture Stains Semenogelin Identification

A five millimeter by five millimeter cutting of a stain was placed in a Costar® two milliliter microcentrifuge tube (Corning® Inc., Corning, NY) with three hundred microliters of RSID™ Universal Buffer (Independent Forensics). The tube was placed on a shaker for ten minutes to extract. After the extraction period, one hundred microliters of the extract were added to the sample well of the RSID™ Semen immunoassay card. After ten minutes, the results were recorded. A positive result was noted when a line appeared at both the control line [Figure 5] and the test line. A negative result was recorded when a line appeared only at the control line on the card.

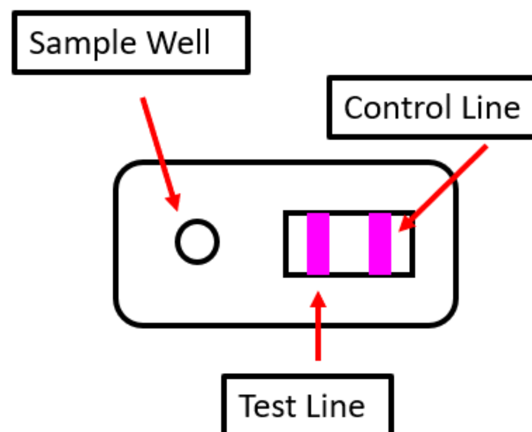

Figure 5 Immunoassay card. Diagram of a typical Immunoassay card. The layout of this figure is very similar to that of an RSID™ Semen Card

2.2.3 Part One: Semen Identification in Stored Semen-Saliva Mixture Stains PSA Identification

A five millimeter by five millimeter cutting of a stain was placed in a Costar® two milliliter microcentrifuge tube with five hundred microliters of SERATEC® PSA Semiquant extraction buffer and placed on a shaker for ten minutes. Using the provided transfer pipette, five to six drops were added to the sample well of the SERATEC® PSA Semiquant cassette. After ten minutes, the results were recorded. A positive result was noted when a line appeared at the control line, the internal standard line, and the test line [Figure 6]. A negative result was noted when a line appeared only at the control line and the internal standard line.

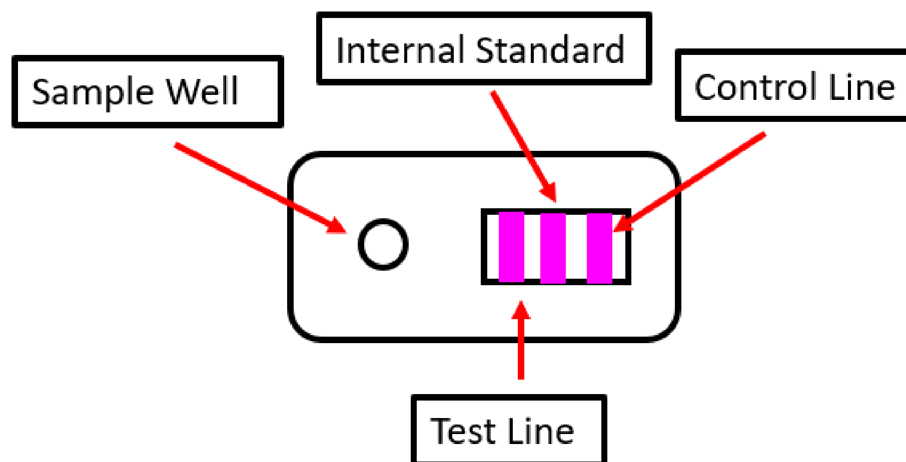

Figure 6 SERATEC® PSA immunoassay card. Illustration of a SERATEC® PSA Semiquant cassette showcasing the internal standard line in addition to the control line and test line.

2.2.4 Part One: Semen Identification in Stored Semen-Saliva Mixture Stains Microscopic Evaluation

Following the removal of one hundred microliters of extract described in section 2.2.2, the tube containing the stain and remaining buffer was returned to the shaker for the remainder of the hour. Throughout the hour, the tube was vortexed approximately every twenty minutes. After the hour was complete, using a clean pair of forceps, the cutting was placed in a Costar® spin basket (Corning® Inc.). The spin basket was reseated in the microcentrifuge tube, and the tube was centrifuged at approximately 14000 revolutions per minute (rpm) for two minutes. The supernatant was discarded. The pellet at the bottom of the tube was resuspended with a micropipette tip, and 3 microliters of the extract was placed on a labeled glass microscope slide. The extract was heat fixed using a hot plate being sure to remove the slide as soon as the extract was dry. Staining the slide was performed with hematoxylin and eosin. First, hematoxylin was added to cover the entire sample. It was left on the slide for three minutes. Afterwards, a gentle stream of methanol (Fisher Chemical™, Pittsburgh, PA) was used to rinse the slide of hematoxylin. Once the slide was dry, eosin was added and remained on the slide for two minutes. Methanol was used to rinse the slide of eosin. Once dry, a single drop of Cytoseal™ 60 mounting media (Thermo Shandon Limited, Cheshire, UK) was added to a glass cover slip (Epredia, Kalamazoo, MI). The cover slip was placed over the sample and allowed to dry. The slide was observed under 400X magnification and any observations were recorded, which included if sperm was present, if the sperm cells appeared damaged or different from the sperm cells in the control, and if possible bacterial or fungal cells were present.

2.3 Part Two: Semen Component Identification in Incubated Semen-Saliva Mixtures

Sample Preparation

Neat semen was mixed with neat saliva from three additional donors (Donor X, Donor Y, and Donor Z) at three different ratios: one to one, one to two, and one to ten semen to saliva. The mixtures along with a neat semen sample which served as the control were incubated at thirty-seven degrees Celsius for twenty-four hours. At the timepoints of zero minutes, one hour, five hours, eight hours, and twenty-four hours, twenty microliters of each mixture were spotted onto a cotton swatch and allowed to dry. The resulting stains were tested for AP, semenogelin, and PSA using the procedures previously described.

3. RESULTS

3.1 Part One: Seminal Protein Identification in Stored Dry Semen-Saliva Mixture Stains

3.1.1 Acid Phosphatase Detection

Table 1. Detection of acid phosphatase in dried control samples. Control samples consisted of neat semen, neat saliva, and blank cotton swatch cuttings. Blue boxes demonstrate a positive (pos) result. Red boxes demonstrate a negative (neg) result.

Dry AP Control						
Day	Oct 22nd	Oct 23rd	Oct 29th	Nov 6th	Nov 12th	Nov 19th
Semen	Pos	Pos	Pos	Pos	Pos	Pos
Saliva	Neg	Neg	Pos	Neg	Pos	Pos
Blank	Neg	Neg	Neg	Neg	Neg	Neg

Table 2. Detection of acid phosphatase in dried 1:1, 1:2, and 1:10 semen-saliva mixture stains. Samples tested on October 22nd were tested immediately upon drying. The results of all three semen-saliva ratios were the same.

Dry 1:1, 1:2, and 1:10 AP						
Day	Oct 22nd	Oct 23rd	Oct 29th	Nov 6th	Nov 12th	Nov 19th
Donor A	Pos	Pos	Pos	Pos	Pos	Pos
Donor B	Pos	Pos	Pos	Pos	Pos	Pos
Donor C	Pos	Pos	Pos	Pos	Pos	Pos
Donor D	Pos	Pos	Pos	Pos	Pos	Pos

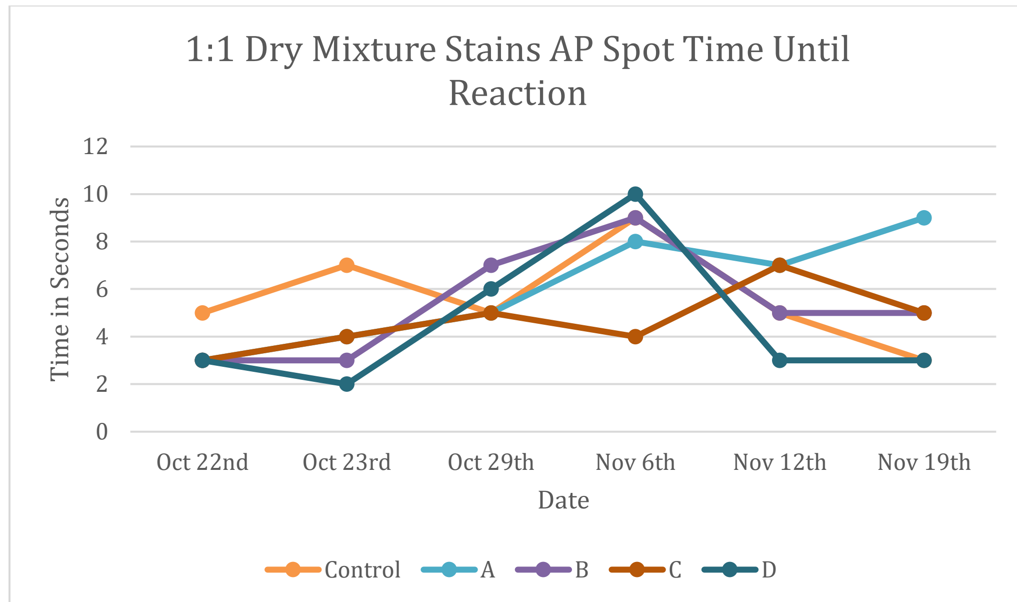

Figure 7 1:1 dry AP reaction time. Time in seconds until a positive reaction for Donors A through D and the control at each timepoint in the dry 1:1 mixture stains.

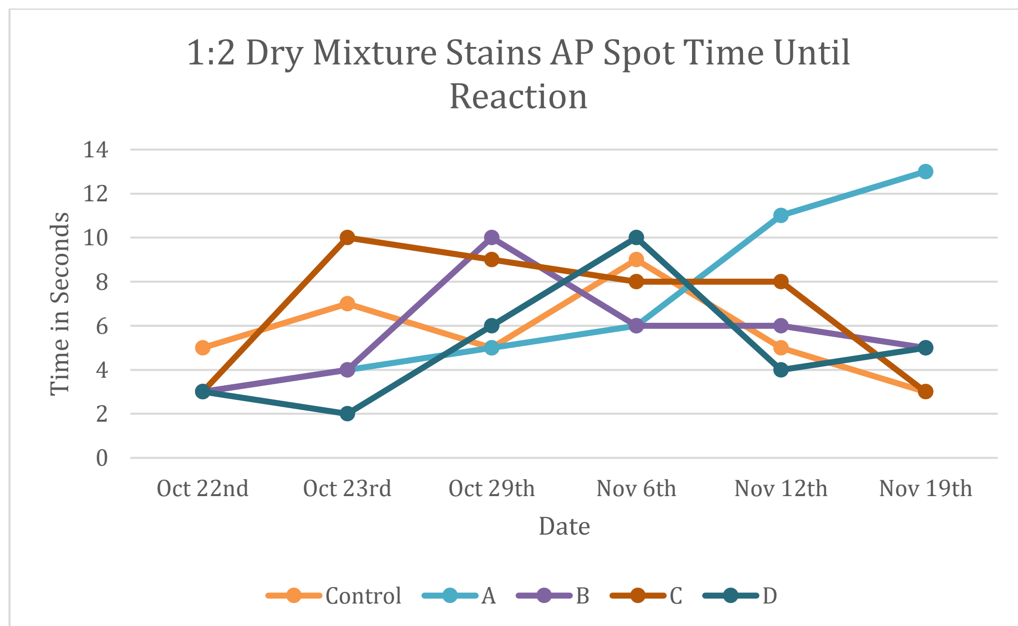

Figure 8 1:2 dry AP reaction time. Time in seconds until a positive reaction for Donors A through D and the control at each timepoint in the dry 1:2 mixture stains.

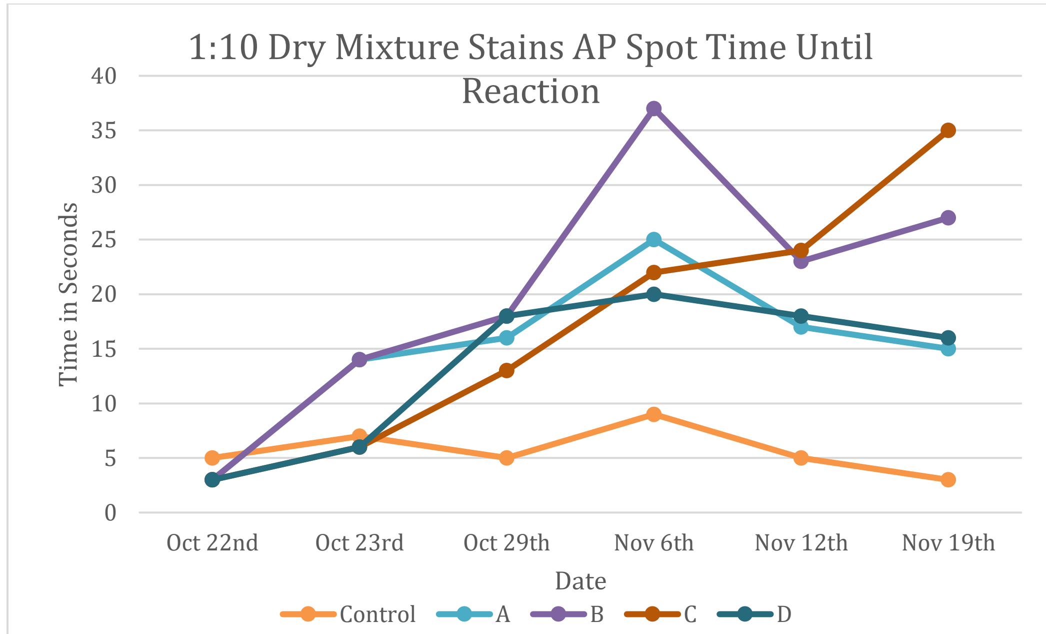

Figure 9 1:10 dry AP reaction time. Time in seconds until a positive reaction for Donors A through D and the control at each timepoint in the dry 1:10 mixture stains.

3.1.2 Prostate Specific Antigen Detection

Table 3. Detection of prostate specific antigen in dry control samples. Control samples include neat semen, neat saliva, and blank cotton swatch

Dry PSA Control						
Day	Oct 22nd	Oct 23rd	Oct 29th	Nov 6th	Nov 12th	Nov 19th
Semen	Pos	Pos	Pos	Pos	Pos	Pos
Saliva	Neg	Neg	Neg	Neg	Neg	Neg
Blank	Neg	Neg	Neg	Neg	Neg	Neg

Table 4. Detection of PSA in dry 1:1 semen-saliva mixture stains. The results of all three semen-saliva ratios were the same.

Dry 1:1, 1:2, and 1:10 PSA						
Day	Oct 22nd	Oct 23rd	Oct 29th	Nov 6th	Nov 12th	Nov 19th
Donor A	Pos	Pos	Pos	Pos	Pos	Pos
Donor B	Pos	Pos	Pos	Pos	Pos	Pos
Donor C	Pos	Pos	Pos	Pos	Pos	Pos
Donor D	Pos	Pos	Pos	Pos	Pos	Pos

3.1.3 Semenogelin Detection

Table 5. Detection of semenogelin in dry control samples. Control samples include neat semen, neat saliva, and blank cotton swatch.

Dry Semenogelin Control						
Day	Oct 22nd	Oct 23rd	Oct 29th	Nov 6th	Nov 12th	Nov 19th
Semen	Pos	Pos	Pos	Pos	Pos	Pos
Saliva	Neg	Neg	Neg	Neg	Neg	Neg
Blank	Neg	Neg	Neg	Neg	Neg	Neg

Table 6. Detection of semenogelin in dry 1:1 semen-saliva mixtures. The results of all three semen-saliva ratios were the same.

Dry 1:1, 1:2, and 1:10 Semenogelin						
Day	Oct 22nd	Oct 23rd	Oct 29th	Nov 6th	Nov 12th	Nov 19th
Donor A	Pos	Pos	Pos	Pos	Pos	Pos
Donor B	Pos	Pos	Pos	Pos	Pos	Pos
Donor C	Pos	Pos	Pos	Pos	Pos	Pos
Donor D	Pos	Pos	Pos	Pos	Pos	Pos

3.2 Part One: Seminal Protein Identification in Stored Damp Semen-Saliva Mixture Stains

3.2.1 Acid Phosphatase Detection

Table 7. Detection of acid phosphatase in damp control samples. Control samples include neat semen, neat saliva, and blank cotton swatch.

Damp AP Control						
Day	Oct 22nd	Oct 23rd	Oct 29th	Nov 6th	Nov 12th	Nov 19th
Semen	Pos	Pos	Pos	Pos	Pos	Neg
Saliva	Pos	Pos	Neg	Pos	Pos	Pos
Blank	Neg	Neg	Neg	Neg	Neg	Neg

Table 8. Detection of acid phosphatase in damp 1:1 semen-saliva mixture stains.

Damp 1:1 AP						
Day	Oct 22nd	Oct 23rd	Oct 29th	Nov 6th	Nov 12th	Nov 19th
Donor A	Pos	Pos	Pos	Pos	Pos	Neg
Donor B	Pos	Pos	Pos	Pos	Pos	Pos
Donor C	Pos	Pos	Pos	Pos	Neg	Pos
Donor D	Pos	Pos	Pos	Pos	Pos	Pos

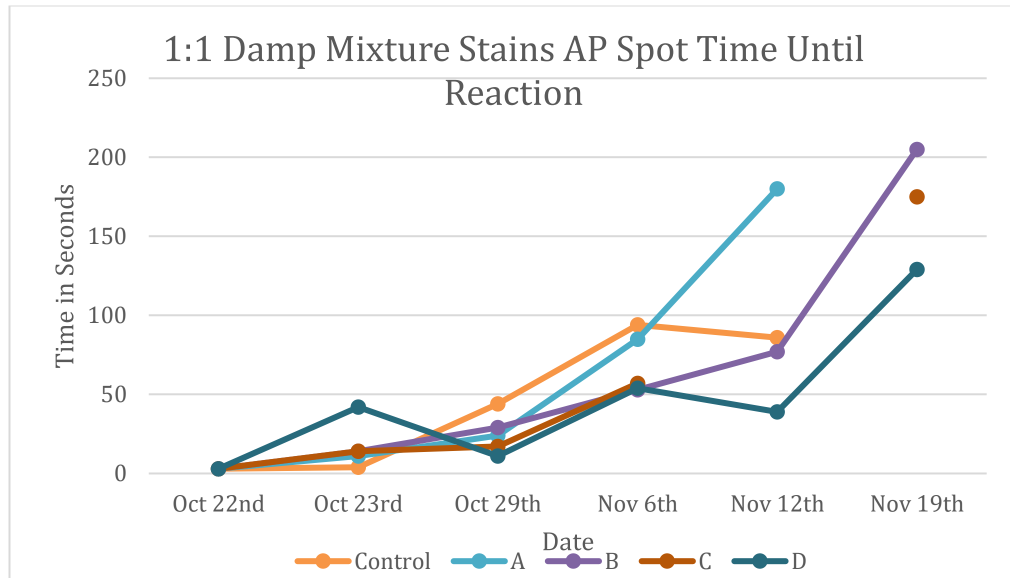

Figure 10 1:1 damp AP reaction time. Time in seconds until a positive reaction for Donors A through D and the control at each timepoint in the damp 1:1 mixture stains. Donor A and the semen control (no saliva) tested negative on Nov 19th and Donor C tested negative on Nov 12th.

Table 9. Detection of acid phosphatase in damp 1:2 semen-saliva mixture stains.

Damp 1:2 AP						
Day	Oct 22nd	Oct 23rd	Oct 29th	Nov 6th	Nov 12th	Nov 19th
Donor A	Pos	Pos	Pos	Pos	Pos	Pos
Donor B	Pos	Pos	Pos	Pos	Pos	Pos
Donor C	Pos	Pos	Pos	Pos	Neg	Pos
Donor D	Pos	Pos	Pos	Pos	Pos	Pos

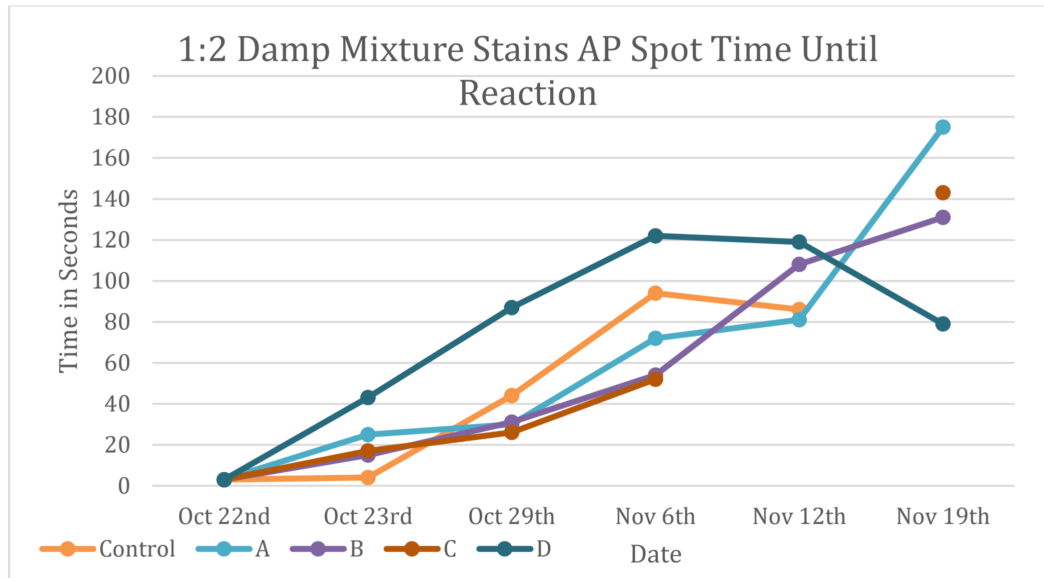

Figure 11 1:2 damp AP reaction time. Time in seconds until a positive reaction for Donors A through D and the control at each timepoint in the damp 1:2 mixture stains. Donor C tested negative on Nov 12th and the semen control (no saliva) tested negative on Nov 19th.

Table 10. Detection of acid phosphatase in damp 1:10 semen-saliva mixture stains.

Damp 1:10 AP						
Day	Oct 22nd	Oct 23rd	Oct 29th	Nov 6th	Nov 12th	Nov 19th
Donor A	Pos	Pos	Pos	Pos	Neg	Pos
Donor B	Pos	Pos	Pos	Pos	Pos	Pos
Donor C	Pos	Pos	Pos	Pos	Pos	Neg
Donor D	Pos	Pos	Pos	Pos	Pos	Pos

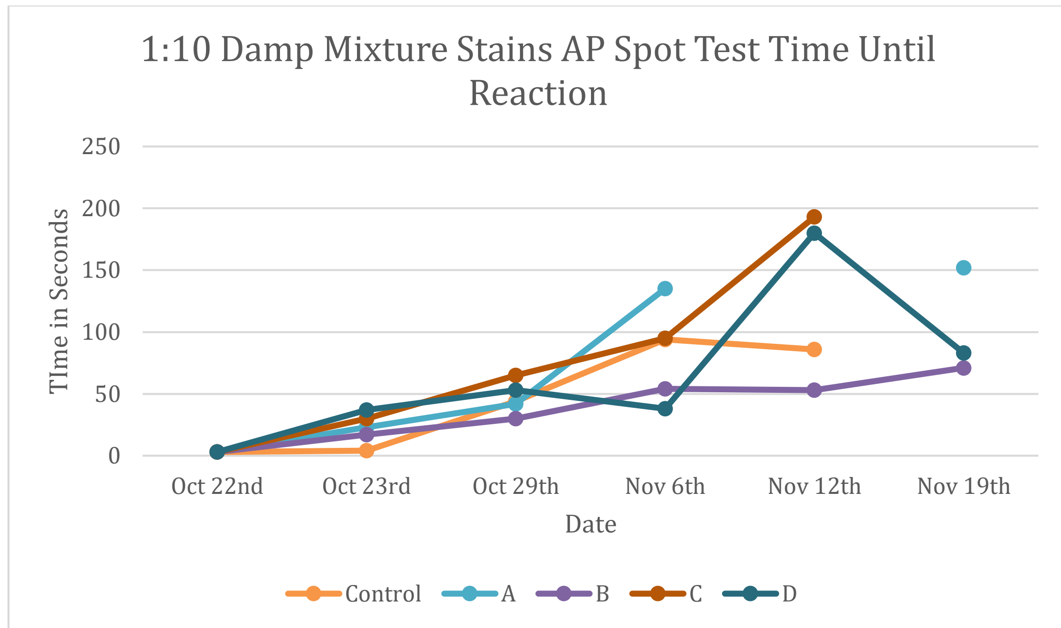

Figure 12 1:10 damp AP reaction time. Time in seconds until a positive reaction for Donors A through D and the control at each timepoint in the damp 1:10 mixture stains. Donor A tested negative on Nov 12th and Donor C and the semen control (no saliva) tested negative on Nov 19th.

3.2.2 Prostate Specific Antigen Detection

Table 11. Detection of prostate specific antigen in damp control samples. Control samples include neat semen, neat saliva, and blank cotton swatch.

Damp PSA Control						
Day	Oct 22nd	Oct 23rd	Oct 29th	Nov 6th	Nov 12th	Nov 19th
Semen	Pos	Pos	Pos	Pos	Neg	Neg
Saliva	Pos	Neg	Neg	Neg	Neg	Neg
Blank	Neg	Neg	Neg	Neg	Neg	Neg

Table 12. Detection of PSA in damp 1:1 semen-saliva mixture stains.

Damp 1:1 PSA						
Day	Oct 22nd	Oct 23rd	Oct 29th	Nov 6th	Nov 12th	Nov 19th
Donor A	Pos	Pos	Pos	Pos	Neg	Neg
Donor B	Pos	Pos	Pos	Pos	Pos	Neg
Donor C	Pos	Pos	Pos	Pos	Neg	Neg
Donor D	Pos	Pos	Pos	Pos	Pos	Neg

Table 13. Detection of PSA in damp 1:2 semen-saliva mixture stains.

Damp 1:2 PSA						
Day	Oct 22nd	Oct 23rd	Oct 29th	Nov 6th	Nov 12th	Nov 19th
Donor A	Pos	Pos	Pos	Pos	Pos	Pos
Donor B	Pos	Pos	Pos	Pos	Pos	Pos
Donor C	Pos	Pos	Pos	Pos	Pos	Pos
Donor D	Pos	Pos	Pos	Neg	Neg	Pos

Table 14. Detection of PSA in damp 1:10 semen-saliva mixture stains.

Damp 1:10 PSA						
Day	Oct 22nd	Oct 23rd	Oct 29th	Nov 6th	Nov 12th	Nov 19th
Donor A	Pos	Pos	Pos	Neg	Neg	Neg
Donor B	Pos	Pos	Pos	Pos	Neg	Neg
Donor C	Pos	Pos	Pos	Pos	Neg	Neg
Donor D	Pos	Pos	Pos	Pos	Neg	Neg

3.2.3 Detection of Semenogelin

Table 15. Detection of semenogelin in damp control samples. Samples include neat semen, neat saliva, and blank cotton swatch.

Damp Semenogelin Control						
Day	Oct 22nd	Oct 23rd	Oct 29th	Nov 6th	Nov 12th	Nov 19th
Semen	Pos	Pos	Pos	Pos	Pos	Pos
Saliva	Neg	Neg	Neg	Neg	Neg	Neg
Blank	Neg	Neg	Neg	Neg	Neg	Neg

Table 16. Detection of semenogelin in damp 1:1 semen-saliva mixture stains.

Damp 1:1 Semenogelin						
Day	Oct 22nd	Oct 23rd	Oct 29th	Nov 6th	Nov 12th	Nov 19th
Donor A	Pos	Pos	Pos	Pos	Pos	Pos
Donor B	Pos	Pos	Pos	Pos	Pos	Pos
Donor C	Pos	Pos	Pos	Pos	Pos	Pos
Donor D	Pos	Pos	Pos	Neg	Pos	Pos

Table 17. Detection of semenogelin in damp 1:2 semen-saliva mixture stains.

Damp 1:2 Semenogelin						
Day	Oct 22nd	Oct 23rd	Oct 29th	Nov 6th	Nov 12th	Nov 19th
Donor A	Pos	Pos	Pos	Pos	Pos	Pos
Donor B	Pos	Pos	Pos	Pos	Neg	Pos
Donor C	Pos	Pos	Pos	Pos	Pos	Pos
Donor D	Pos	Pos	Pos	Pos	Pos	Pos

Table 18. Detection of semenogelin in damp 1:10 semen-saliva mixture stains.

Damp 1:10 Semenogelin						
Day	Oct 22nd	Oct 23rd	Oct 29th	Nov 6th	Nov 12th	Nov 19th
Donor A	Pos	Pos	Pos	Neg	Pos	Pos
Donor B	Pos	Pos	Pos	Neg	Neg	Neg
Donor C	Pos	Pos	Pos	Pos	Pos	Pos
Donor D	Pos	Pos	Pos	Neg	Neg	Neg

3.3 Part One: Semen Identification Via Microscopy With Semen-Saliva Mixture Stains

3.3.1 Control Stains

Table 19. Spermatozoa identification in neat semen controls. Blue colored cells with "Pos" written inside indicate stains in which spermatozoa were seen microscopically. Red colored cells with "Neg" written inside indicate stains in which spermatozoa were not seen microscopically.

Microscopy Controls						
Day	Oct 22nd	Oct 23rd	Oct 29th	Nov 6th	Nov 12th	Nov 19th
Dry	Pos	Pos	Pos	Pos	Pos	Pos
Damp	Pos	Pos	Pos	Neg	Neg	Neg

3.3.2 Dry Semen-Saliva Mixture Stains

Table 20. Spermatozoa identification in dry 1:1 semen-saliva mixture stains.

Dry 1:1 Sperm						
Day	Oct 22nd	Oct 23rd	Oct 29th	Nov 6th	Nov 12th	Nov 19th
Donor A	Pos	Pos	Pos	Pos	Pos	Pos
Donor B	Pos	Pos	Pos	Pos	Pos	Pos
Donor C	Pos	Pos	Pos	Pos	Pos	Pos
Donor D	Pos	Pos	Pos	Pos	Pos	Pos

Table 21. Spermatozoa identification in dry 1:2 semen-saliva mixture stains.

Dry 1:2 Sperm						
Day	Oct 22nd	Oct 23rd	Oct 29th	Nov 6th	Nov 12th	Nov 19th
Donor A	Pos	Pos	Pos	Pos	Pos	Pos
Donor B	Pos	Pos	Pos	Pos	Pos	Pos
Donor C	Pos	Pos	Pos	Pos	Pos	Pos
Donor D	Pos	Pos	Pos	Pos	Pos	Pos

Table 22. Spermatozoa identification in dry 1:10 semen-saliva mixture stains.

Dry 1:10 Sperm						
Day	Oct 22nd	Oct 23rd	Oct 29th	Nov 6th	Nov 12th	Nov 19th
Donor A	Pos	Pos	Pos	Pos	Pos	Pos
Donor B	Pos	Pos	Pos	Pos	Pos	Pos
Donor C	Pos	Pos	Pos	Pos	Pos	Pos
Donor D	Pos	Pos	Neg	Pos	Pos	Pos

3.3.3 Damp Semen-Saliva Mixture Stains

Table 23. Spermatozoa identification in damp 1:1 semen-saliva mixture stains.

Damp 1:1 Sperm						
Day	Oct 22nd	Oct 23rd	Oct 29th	Nov 6th	Nov 12th	Nov 19th
Donor A	Pos	Pos	Pos	Neg	Neg	Neg
Donor B	Pos	Pos	Pos	Neg	Neg	Neg
Donor C	Pos	Pos	Pos	Neg	Neg	Neg
Donor D	Pos	Pos	Neg	Neg	Neg	Neg

Table 24. Spermatozoa identification in damp 1:2 semen-saliva mixture stains.

Damp 1:2 Sperm						
Day	Oct 22nd	Oct 23rd	Oct 29th	Nov 6th	Nov 12th	Nov 19th
Donor A	Pos	Pos	Pos	Neg	Neg	Neg
Donor B	Pos	Pos	Neg	Neg	Neg	Neg
Donor C	Pos	Pos	Neg	Neg	Neg	Neg
Donor D	Pos	Pos	Neg	Neg	Neg	Neg

Table 25. Spermatozoa identification in damp 1:10 semen-saliva mixture stains.

Damp 1:10 Sperm						
Day	Oct 22nd	Oct 23rd	Oct 29th	Nov 6th	Nov 12th	Nov 19th
Donor A	Pos	Pos	Pos	Neg	Neg	Neg
Donor B	Pos	Pos	Pos	Neg	Neg	Neg
Donor C	Pos	Pos	Neg	Neg	Neg	Neg
Donor D	Pos	Pos	Pos	Neg	Neg	Neg

3.4 Part Two: Semen Component Identification in Incubated Semen-Saliva Mixtures

Table 26. Incubated neat semen control. Blue cells indicate the component was detected. Red cells indicated the component was not detected.

Neat Semen			
Time	AP	PSA	Semenogelin
0 minutes	Pos	Pos	Pos
1 hour	Pos	Pos	Pos
5 hours	Pos	Pos	Pos
8 hours	Pos	Pos	Pos
24 hours	Pos	Pos	Pos

Table 27. Semen component identification from incubated donor X mixtures.

Donor X 1:1			
Time	AP	PSA	Semenogelin
0 minutes	Pos	Pos	Pos
1 hour	Pos	Pos	Pos
5 hours	Pos	Pos	Pos
8 hours	Neg	Pos	Pos
24 hours	Neg	Pos	Pos
Donor X 1:2			
Time	AP	PSA	Semenogelin
0 minutes	Pos	Pos	Pos
1 hour	Pos	Pos	Pos
5 hours	Pos	Pos	Pos
8 hours	Neg	Pos	Pos
24 hours	Neg	Pos	Pos
Donor X 1:10			
Time	AP	PSA	Semenogelin
0 minutes	Pos	Pos	Pos
1 hour	Pos	Pos	Pos
5 hours	Pos	Pos	Pos
8 hours	Neg	Pos	Pos
24 hours	Neg	Pos	Pos

Table 28. Semen component identification from incubated donor Y mixtures.

Donor Y 1:1			
Time	AP	PSA	Semenogelin
0 minutes	Pos	Pos	Pos
1 hour	Pos	Pos	Pos
5 hours	Pos	Pos	Pos
8 hours	Neg	Pos	Pos
24 hours	Neg	Pos	Pos
Donor Y 1:2			
Time	AP	PSA	Semenogelin
0 minutes	Pos	Pos	Pos
1 hour	Pos	Pos	Pos
5 hours	Pos	Pos	Pos
8 hours	Pos	Pos	Pos
24 hours	Neg	Pos	Pos
Donor Y 1:10			
Time	AP	PSA	Semenogelin
0 minutes	Pos	Pos	Pos
1 hour	Pos	Pos	Pos
5 hours	Pos	Pos	Pos
8 hours	Pos	Pos	Pos
24 hours	Pos	Pos	Pos

Table 29. Semen component identification from incubated donor Z mixtures.

Donor Z 1:1			
Time	AP	PSA	Semenogelin
0 minutes	Pos	Pos	Pos
1 hour	Pos	Pos	Pos
5 hours	Pos	Pos	Pos
8 hours	Neg	Pos	Pos
24 hours	Neg	Pos	Pos
Donor Z 1:2			
Time	AP	PSA	Semenogelin
0 minutes	Pos	Pos	Pos
1 hour	Pos	Pos	Pos
5 hours	Pos	Pos	Pos
8 hours	Neg	Pos	Pos
24 hours	Neg	Pos	Pos
Donor Z 1:10			
Time	AP	PSA	Semenogelin
0 minutes	Pos	Pos	Pos
1 hour	Pos	Pos	Pos
5 hours	Pos	Pos	Pos
8 hours	Neg	Pos	Pos
24 hours	Neg	Pos	Pos

4. DISCUSSION

4.1 Part One: Seminal Protein Identification in Stored Dry Semen-Saliva Mixture Stains

4.1.1 Acid Phosphatase Detection

The AP Spot Test yielded positive results throughout the duration of the experiment in the neat semen control and in all of the semen-saliva mixture samples (Tables 1 and 2). The saliva control yielded a positive result in three of the tests, occurring on week one, week three, and week four (Table 1). Saliva has previously been shown to contain low but detectable levels of AP (30). The positive detection of AP in the saliva control is due in part to this excreting of AP in saliva in the human mouth (30). It could also be the result of AP excretion by common oral bacteria such as *Streptococcus* (39). With this knowledge in mind, it is possible for saliva to produce a color reaction when exposed to the AP Spot Test.

The time elapsed until a positive AP reaction occurs is a direct reflection of the concentration of AP in the stain (21). Studies have shown that by allowing more time for the reaction to occur, a positive result becomes more likely in instances of low AP concentration (20, 21). In addition to extended periods of time indicating a lower concentration, weaker color reactions also indicate that a stain has a lower concentration of AP. One study showed that the colored product in an AP test becomes progressively

lighter as semen stains become more dilute, culminating in a negative AP test lacking a color change once the semen had been diluted to one in five hundred twelve (39).

The maximum time allowed for a positive result for the current study was five minutes, therefore it is understandable that positive AP results would occur for the dilutions tested because even stains with lower concentrations of AP had time to produce a visible color reaction. The results for the mixed stains suggest AP is easily detected in dried mixture stains even when semen is the minor component. The time required for a positive reaction to occur for the 1:1 and 1:2 mixture stains followed a very similar pattern to the neat semen control [Figures 7 and 8]. The 1:10 mixture stains had consistently longer positive reaction times [Figure 9]. This is likely due to the increased dilution level of the semen and thus the increased dilution level of the AP in the stains. These results suggest that the enzymatic activity of saliva likely does not have a significant impact on the detection of AP, however the presence of saliva will dilute the sample causing increased reaction times. These results are concordant with other published works that show diluted semen will require longer reaction times depending the level of dilution (20, 21).

4.1.2 Prostate Specific Antigen Detection

It was hypothesized that the proteases in the saliva would damage the PSA protein, possibly rendering it undetectable by the immunoassay cards used. Other ways the protein could potentially go unnoticed would be by salivary bacteria damaging the protein in some way, or salivary bacteria altering the pH enough so that the immunoassay cards no longer functioned properly, however these specific factors were not evaluated. Throughout the

course of the four week experiment, PSA was detected in every test in the neat semen control and the mixture stains at all six timepoints (Tables 3 and 4). As with the AP tests, the results suggest that saliva does not have a negative effect on the ability to detect PSA in semen when the stains are dry. This is reassuring news for forensic laboratories because semen stains are most often stored dried at room temperature in paper envelopes and may be mixed with other body fluids including saliva (41).

4.1.3 Semenogelin Detection

It was also thought that the proteases of saliva might damage semenogelin in such a way that it would no longer be detectable via immunochromatography. Given that kallikrein enzymes are present in saliva, and it is known that some kallikreins can cleave both semenogelin I and II, there is evidence to suggest that exposing semen to saliva would result in the breakdown of semenogelin (34). However, contrary to the hypothesis, the results showed that semenogelin was detected by the immunoassay cards in every mixed stain and at every timepoint (Table 6). The results of this experiment show that in dried stains containing mixtures of semen and saliva, semenogelin can still be detected via conventional means. This was true even when the volume of semen in the stain represented only one tenth of the semen-saliva mixture. The use of multiple donors provided a range of oral environments with potentially different characteristics, however, none of the saliva samples seemed to have an effect on the immunoassay's ability to function. It is also possible that the semenogelin was cleaved by the kallikrein in the saliva, but the binding

site of the antigen remained intact. Based on the results of this experiment, the exposure of semen to saliva does not impair the ability to detect semenogelin.

4.2 Part One: Seminal Protein Identification in Stored Damp Semen-Saliva Mixture Stains

4.2.1 Acid Phosphatase Detection

There was a noticeable increase in the time it took for a positive reaction to occur when comparing the damp samples to the dry samples. This longer time period was consistent between the neat semen control and the mixture stains [Figures 10-12], thus this increase in time is most likely due to the damp conditions and is not caused by the saliva itself. The negative results seen in the neat semen control (Table 7), in donors A and C in the 1:1 semen-saliva mixture stains (Table 8), donor C in the 1:2 semen-saliva mixture stains (Table 9), and in donors A and C in the 1:10 semen-saliva mixture stains were also most likely the result of the damp conditions. The overall results of this experiment suggest that exposing semen to saliva in a damp environment can result in longer periods of time for a positive AP result to occur, but this slowed reaction is ultimately caused by the damp environment and not a factor of being exposed to saliva. Water is known to facilitate the unfolding of proteins (42). The damp environment will expose the AP to water and cause it to break down. The damp environment and the addition of saliva both contribute to the dilution of semen in the stain. Although AP is present in saliva from both human and bacterial sources, it is not as concentrated as the AP in semen (30, 39). Studies previously mentioned have shown that reliability of colorimetric AP tests is directly correlated to the

concentration of AP in the stain (40, 20, 21). The environment in which the damp stains were stored likely resulted in the stains becoming further diluted. This led to the increase in reaction time and to the negative results in stains from Donors A and C.

4.2.2 Prostate Specific Antigen Detection

Detection of PSA changed from being positive under dry conditions to being negative under damp conditions. In the neat semen controls (Table 11) the stains stopped testing positive after the fourth timepoint. After three weeks of being stored in the damp condition, PSA was no longer detected in neat semen samples. In the mixture samples, there was a negative result as early as the second week in the 1:2 semen-saliva mixtures for Donor D (Table 13). This also occurred with the 1:10 semen-saliva mixture stains for Donor A (Table 14). Positive detection of PSA completely dropped out in the 1:1 semen-saliva mixtures by the fourth week (Table 12) and positive detection of PSA completely stopped in the 1:10 semen-saliva mixtures by the third week. Because the results of the mixture stains closely resembles the results of the control stains, it is likely that the drop out of PSA detection occurred because of the damp environment. Had the introduction of saliva to the semen been the cause of the loss of PSA detection, the control sample consisting of semen dampened with water would have likely tested positive for PSA through the fourth week. The results of this experiment show that even in damp conditions, the enzymatic activity of saliva does not appear to have a negative effect on the forensic detection of semen in PSA, however the addition of saliva could dilute the sample to a non-detectable level. Being unable to detect PSA in a damp sample is likely because the sample

was stored in moist conditions for an extended period of time. The results also show that it is still possible to detect PSA in some semen stains after four weeks of damp conditions because all four of the 1:2 semen-saliva mixture stains tested positive for PSA after four weeks (Table 13).

4.2.3 Semenogelin Detection

Semenogelin was consistently detected in the neat semen control (Table 15), however it was not detected in all of the damp mixed stains. Seven of the nine negative results were from the 1:10 mixture stains, primarily from Donors B and D, however, there was at least one positive test for semenogelin at each time point in the 1:10 mixture stains (Table 18). This suggests that semenogelin can be detected in some semen stains at least four weeks after deposition and when stored in damp conditions. The negative results in the 1:1, 1:2, and 1:10 mixture stains and the results from the damp PSA experiment suggests that the negative results that do occur could be a result of the combination of storage conditions and semen being mixed with saliva (Tables 16-18). It is possible that kallikrein and other proteases in saliva were breaking down the semenogelin. The higher levels of kallikrein proteins in the 1:10 mixture stains would support the occurrence of negative results more consistently at the two week time point and beyond in the 1:10 mixture stains (Table 18). Salivary kallikrein levels have been shown to vary in individuals with levels being generally higher in females than in males (41). Levels also increase as people age (43). This variation could explain why certain donors had a higher frequency of negative results than others. Overall the results of this experiment suggest that

semenogelin can be reliably identified for up to one week when mixed with saliva in damp conditions, and some samples can be positive up to at least four weeks in those same conditions.

4.3 Part One: Semen Identification Via Microscopy With Semen-Saliva Mixture Stains

4.3.1 Controls

The dry neat semen control stains yielded a positive identification of spermatozoa at all six timepoints (Table 19). The damp neat semen controls were negative for spermatozoa after one week. Heavy bacterial or fungal growth could be seen on these slides, but no spermatozoa were visible (Table 19). This suggests that the damp condition was responsible for the lack of visible spermatozoa on the microscope slides. The spermatozoa that were visible under both conditions throughout the experiment maintained the same appearance. Neither the damp nor dry conditions appeared to have an effect on the morphology of the spermatozoa.

4.3.2 Dry Semen-Saliva Mixtures

Spermatozoa were positively identified throughout the experiment from all donors and at all timepoints except one (Tables 20-22). Only in Donor D's 1:10 semen-saliva mixture stain at one week was there a failure to identify spermatozoa. This is most likely the result of the semen being diluted or pipetting discrepancies and not a reflection of the semen's exposure to saliva. The spermatozoa observed in the experimental mixture stains

matched in physical appearance to the spermatozoa seen in the dry control stains. This suggests that the saliva did not alter the morphology of the sperm cells. Based on these results, it can be assumed that spermatozoa can confidently be identified via microscopy when a semen stain has been mixed with saliva.

4.3.3 Damp Semen-Saliva Mixtures

The results of the mixture stains stored under damp conditions reflected the results of the neat semen control stored in damp conditions. In the 1:1 semen-saliva mixtures stains, Donor D began exhibiting negative results for spermatozoa at one week, followed by the remainder of the donors at two weeks (Table 23). The 1:2 semen-saliva mixture stains began testing negative for spermatozoa at one week (Table 24). Only Donor A tested positive at one week, but was negative at all subsequent timepoints (Table 24). All donors in the 1:10 semen-saliva mixture stains began testing negative for spermatozoa at two weeks (Table 25), while Donor C of the 1:10 stains began testing negative at one week (Table 25). At two weeks the slides showed large amounts of bacterial or fungal growth. The spermatozoa that were seen in the early timepoints of the experiment were visually similar to the spermatozoa from the damp and dry controls, again suggesting that the exposure to saliva did not have an effect on the morphology of the sperm cells. The results of this experiment imply that spermatozoa can be detected with microscopy after being

mixed with saliva in damp conditions. However, if the stain remains damp for an extended period of time exceeding one week, the likelihood of detecting spermatozoa decreases.

4.4 Part Two: Semen Component Identification in Incubated Semen-Saliva Mixtures

To further explore whether saliva has a damaging effect on the ability to detect the components of semen, an experiment was designed to simulate a mixture of semen and saliva being kept at body temperature or 37 °C. Liquid semen and saliva were combined at various ratios and incubated at 37 °C for up to 24 hours prior to testing.

4.4.1 Acid Phosphatase Detection

AP detection was constant over five timepoints in the neat semen control demonstrating that semen incubated at body temperature for twenty-four hours will test positive for AP (Table 26). When testing for AP in the Donor X and Donor Z mixed stains, AP was no longer detected after eight hours of incubation with saliva. This remained true for all three mixture ratios (Table 27), suggesting that some component of saliva was responsible for inhibiting the detection of AP using the AP Spot Test. In the Donor Y stains, AP was detected in all samples except at eight and twenty-four hours in the 1:1 semen-saliva mixture and twenty-four hours in the 1:2 semen-saliva mixture. Remarkably, AP was detected at every timepoint in the 1:10 semen-saliva mixture stains (Table 28). Collectively, these results indicate that saliva, when incubated with semen at body temperature, could negatively affect a forensic analyst's ability to detect AP. Given that AP is often the first seminal component for which a sexual assault swab or stain is tested,

there are significant consequences to a false negative. A negative presumptive test for AP can result in stopping subsequent analysis for semen.

4.4.2 Prostate Specific Antigen Detection

The neat semen control tested positive for PSA at all five timepoints (Table 26). Donors X, Y and Z also tested positive for PSA at all five timepoints in all three semen-saliva ratios (Tables 27-29). These results suggest that exposing semen to saliva in a liquid-liquid mixture for a twenty-four hour period does not affect the ability to detect PSA using immunochromatography.

4.4.3 Semenogelin Detection

Detection of semenogelin mirrored the results for PSA. The neat semen control sample tested positive at all six timepoints as did all of the semen stains containing saliva from Donors X, Y, and Z (Tables 26-29). These results were surprising because it was hypothesized that semenogelin would be broken down by the kallikrein proteins in saliva, especially in the 1:10 semen-saliva mixture stains. A previous study showed that hK5 is capable of breaking down semenogelin when the two proteins are incubated together at body temperature (34). The results suggest semenogelin detection can be relied upon by forensic analysts even when semen and saliva are mixed together and incubated for up to a twenty-four hour period. The outcome of this experiment might be because the kallikrein enzymes in saliva do not have the same effect as hK5 in the prostate, or that the concentration of kallikrein in saliva is too low to degrade the semenogelin enough so that

the immunoassay cannot detect it. Salivary kallikrein and hK5 have slightly different molecular weights; this could mean the two proteins interact with semenogelin differently (44).The continued detection of semenogelin could also relate back to the fact that older individuals around the age of sixty five have higher levels of kallikrein in their saliva than younger individuals around the age of forty four and younger (43). Perhaps if saliva from older individuals was mixed with semen, the semenogelin would be more degraded.

5. CONCLUSIONS

The overall conclusion that can be drawn from this study is that saliva may have some effect on the ability to detect the components of semen, but this effect is limited and may be primarily due to dilution of seminal components. Semen in semen-saliva mixed stains that have been quickly deposited onto a surface is easily detectable using methods that test for AP, spermatozoa, PSA and semenogelin. This is especially true when is the stain is allowed to thoroughly dry as most of the hinderances in detecting seminal components on fabric occurred in the stains stored in damp conditions. It appears that storing a semen-saliva mixture stain in damp conditions for an extended period of time does result in the protein components becoming no longer detectable.

The detection of PSA and semenogelin went unhindered by incubating the two body fluids together in a liquid state for twenty-four hours, however, AP was undetectable after eight hours in most samples (Tables 27-29). This suggests that saliva may be capable of breaking down the proteins in semen while in the body. Perhaps future steps could involve repeating part two of the experiment over a longer period of time to see if the extended exposure would lead to the inability to detect PSA and semenogelin via immunoassay. Overall, the current methods for detecting semen in forensic evidence are very reliable. However, it may be of use to forgo the preliminary test for AP in an evidence item such as a swab that was collected from an individual's mouth or pool of spittle. Moving directly to microscopy or immunochromatography may more reliable in those specific circumstances.

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