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# Migration patterns of seminal fluid components and spermatozoa in semen stains exposed to water and blood

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BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

**MIGRATION PATTERNS OF SEMINAL FLUID COMPONENTS AND  
SPERMATOOZOA IN SEMEN STAINS EXPOSED TO WATER AND BLOOD**

by

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B.S., Marshall University, 2014

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**LYNDSEY TAYLOR BROWN**

**ABSTRACT**

Typically, semen testing involves presumptive and confirmatory tests to determine the region in which a semen stain has been deposited prior to initiating DNA analysis. However, previous research showed that the soluble components of seminal fluid, but not spermatozoa, migrated from their original location on cotton cloth upon exposure to porcine decomposition fluids and rainfall/dew<sup>6</sup>. This indicates that preliminary testing and detection techniques may result in areas being sampled that will not yield a successful DNA profile. The present study assesses how various amounts of water or blood affect migration patterns of seminal fluid components using traditional serological screening methods as well as DNA analysis. The effects of exposing a semen stain to water over the course of several days are also investigated. The final component of the study evaluates whether the presence of acid phosphatase (AP) Spot reagent had any detrimental effects on subsequent antigen P30 (P30) testing, Kernechtrot Picroindigocarmine (KPIC) sperm staining or DNA analysis.

Neat semen was deposited onto swatches from cotton sheets and allowed to dry before being sprayed with 2 mL, 5 mL, or 10 mL of water or blood. The swatches were allowed to dry while lying flat, at 45°, or at 90°. Three of the swatches were sprayed directly with AP Spot reagent to determine any potential interference with subsequent

P30 and DNA testing. After the water or blood was dry, the swatches were viewed with an alternate light source (ALS) at 450 nm using orange barrier filter goggles. Three-millimeter fabric punches were collected from each swatch in at least thirteen locations (one from the center of the stain and four at 1 cm, 4 cm, and 7 cm from the perimeter of the stain in multiple directions), and were extracted for two hours prior to testing for the presence of P30. Additional fabric punches were collected from each P30 positive location to be used for DNA analysis.

AP testing showed positive results beyond the original semen stain with an average distance of 1-3 cm from the perimeter of the original region of deposition (ORD) for all swatches except those moistened with blood. AP mapping was performed on the swatches moistened with blood and negative results were obtained. Positive P30 results were obtained for all swatches with an average distance of 1-3 cm from the ORD. The angle at which the swatch was positioned influenced the direction(s) that the soluble components migrated; however the amount of water (or blood) the swatch was exposed to had a much greater effect on the distance of migration.

Microscopic examination of slides made from the extracts of each fabric punch revealed minimal spermatozoa migration for all swatches; the majority of the samples outside of the ORD showed no spermatozoa, although a few showed a single sperm cell. These findings demonstrate that the soluble components of semen stains that often aid in detection migrated when exposed to moisture, while sperm cells containing genetic material largely remained in their original location. The DNA analysis results confirmed the lack of spermatozoa migration. Full DNA profiles were obtained from within the

ORD of the flat and 90° swatches. The samples from outside of the ORD produced either partial profiles (maximum dropout rate of 97%) or no profile. If case circumstances suggest that evidence has been exposed to water, multiple regions should be tested in order to maximize the possibility of identifying semen and obtaining a DNA profile.

AP Spot reagent was not found to have detrimental effects on P30 testing, sperm staining or DNA analysis. Therefore, direct application of AP Spot reagent could be used for larger pieces of evidence where the location of a stain is unknown. This would eliminate the careful documentation needed for chemical mapping and the reliance on the transfer of acid phosphatase from one substrate to another.

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## LIST OF ABBREVIATIONS

μL	Microliters
°C	Degrees Celsius
Ab	Antibody
Ab*	Dye-labeled antibody
Ag	Antigen
ALS	Alternate Light Source
AP	Acid Phosphatase
cm	Centimeters
DI	Deionized
e.g.	<i>Exempli Gratia</i> , (for example)
g	Grams
i.e.	<i>Id est</i> (that is)
KPIC	Kernechtrot Picroindigocarmin
mL	Milliliters
mm	Millimeters
ng	Nanograms
nm	Nanometers
ORD	Original Region of Deposition
P30	Prostate Specific Antigen
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism

RFU	Relative Fluorescence Units
r.p.m.	Revolutions Per Minute
STR	Short Tandem Repeat
TE	Tris-EDTA buffer
VNTR	Variable Number of Tandem Repeats

## **1. INTRODUCTION**

The methods for processing sexual assault evidence are well documented in forensic literature<sup>1-4</sup>. However, the results of these serological tests can be affected by the environmental conditions to which the evidence was exposed prior to collection and testing. A significant portion of the literature regarding evidence exposed to adverse environmental conditions (*e.g.* exposure to sunlight) or environmental contamination (*e.g.* exposure to soil) focuses on how the resulting DNA profiles are affected<sup>5</sup>, not the serological screening tests. Furthermore, there are few, if any, authors who address semen evidence exposed to water in manners other than machine-washing (*e.g.* environmental moisture such as rainfall, snow or dew).

The effects of water or blood on dried semen stains can be important to the processing of sexual assault evidence. Previous research found that upon exposure to porcine decomposition fluids and environmental moisture (*i.e.* rainfall/dew), the soluble components of seminal fluid migrated from their original location on cotton cloth but the spermatozoa did not<sup>6</sup>. This can impact the interpretation of serological testing results and may lead to sampling an area that is unlikely to yield a DNA profile.

### **1.1 Effects of Water on Semen Stains**

In forensic casework, evidence is not always recovered or received in a pristine manner. In the case of a sexual assault, items of clothing or bedding may have been machine-washed either in an attempt to destroy evidence or because the victim did not report the incident immediately. There are several studies regarding the effects of

machine-washing on the detection of semen and DNA recovery. Kafarowski, Lyon and Sloan found that after machine-washing a pair of semen-stained underwear, acid phosphatase (AP) activity was no longer detectable but a significant number of spermatozoa were retained on the fabric<sup>7</sup>. In a similar study conducted by Crowe, Moss and Elliot, semen stains were deposited on twelve cotton T-shirts before being machine-washed under various conditions<sup>8</sup>. After washing, AP was only detectable on one shirt (cold wash with no detergent); however, sufficient spermatozoa were present on all of the shirts to give a DNA profile<sup>8</sup>. Another study conducted by Brayley-Morris *et al.* focused on the recovery of DNA from several items of clothing machine-washed under various conditions 8 months after semen deposition<sup>9</sup>. Each item generated full DNA profiles, even after some had been subjected to washing multiple times<sup>9</sup>.

In addition to machine-washing, evidence could also come into contact with water via submersion. In a study by Joshi, Subhedar and Saraf, pieces of semen-stained cotton cloth submerged for various amounts of time in Petri dishes filled with water were tested for the presence of AP and spermatozoa<sup>10</sup>. After 144 hours (6 days) of submersion, AP activity was still detectable within 2-3 minutes and sperm heads were still identifiable<sup>10</sup>.

Another study of interest was conducted by Akkaya *et al.* in which they examined the effects of different types of water on liquid semen<sup>11</sup>. Liquid semen was placed into flasks containing sea water, river water, tap water, or distilled (DI) water from which samples were taken daily and the number of sperm were counted<sup>11</sup>. The results showed that sperm were detectable for long periods from each type of water (1128 hours for tap water, 1008 hours for DI water, 792 hours for river water and 888 hours for sea water)

but that the sea and river water samples showed a faster decrease in sperm concentration<sup>11</sup>. This can be of importance if sexual assault evidence is found in or nearby a body of water because sperm may still be present on the item in spite of a lengthy immersion.

Based on these studies, it is apparent that evidence thought to contain semen should not be dismissed without testing because of exposure to water. However, there are no studies, to the author's knowledge, that examine migration patterns of the soluble components of seminal fluid and spermatozoa upon exposure to moisture.

## **1.2 Semen Screening**

Semen is made up of two main components: the cellular fraction (*i.e.* spermatozoa and epithelial cells) and the seminal plasma (*i.e.* the fluid made up of secretions from several glands)<sup>12</sup>. Semen is typically about 90% seminal plasma and 10% cellular components<sup>12</sup>, and both of these components are used for forensic semen testing. The seminal plasma contains AP, antigen P30 (P30) as well as other enzymes and proteins. The cellular fraction is of critical importance in forensic cases because the spermatozoa can be used to obtain a DNA profile.

Semen screening can require up to four steps, beginning with stain identification. Locating semen stains is critical to the examination of sexual assault evidence. If the stain is not readily visible to the naked eye, an alternate light source (ALS) may be utilized. There are numerous ALS devices described in the literature, however each one utilizes semen's fluorescence excitation spectrum first described by Stoilovic<sup>13</sup>. This spectrum

extends from 300 nm to 495 nm, while the emission spectrum ranges from 400 nm to 700 nm<sup>13</sup>. The ideal excitation wavelength for semen has been reported to be 450 nm when used with an orange barrier filter<sup>14</sup>. However, since other body fluids as well as detergents, lotions, and other non-biological substances can fluoresce, ALS examination is considered a presumptive screening tool<sup>14</sup>.

After locating the prospective semen stain, presumptive testing is performed by detecting seminal AP, an enzyme that catalyzes the hydrolysis of organic phosphates and was formerly used as a marker for prostate cancer<sup>15,16</sup>. A common method for detecting AP is the AP Spot test, based on the Brentamine Fast Blue-B reaction. In the presence of AP and  $\alpha$ -naphthol phosphate, a free naphthol will be formed which will couple with a diazonium salt (in this case Fast Blue B) to produce a violet-colored product<sup>17-20</sup>. There is the possibility of a false positive result from other body fluids, vaginal secretions, and certain plant materials, therefore subsequent testing is necessary to confirm the presence of semen<sup>21</sup>.

The best confirmation of semen is the microscopic identification of spermatozoa because these cells are unique to semen. Common practice is to place a small amount of a suspected semen extract onto a microscope slide and stain the sample with Kernechtrot Picroindigocarmine (KPIC) stain. This staining procedure utilizes two dyes: nuclear fast red (stains sperm heads and other nuclei red and the acrosomal caps appear white or pale pink) and picroindigocarmine (stains the sperm tails and epithelial cell cytoplasm green)<sup>3</sup>. The presence of semen is confirmed if spermatozoa are visualized because these cells are not found in any other body fluid. However, in a vasectomized or otherwise

azoospermic male, this test will be ineffective and another confirmatory test will be necessary to indicate the presence of semen.

If no spermatozoa are detected, a lateral flow immunoassay card test may be conducted to detect the presence of P30, a serine protease involved in liquefying seminal fluid<sup>22</sup>. This protein is found in semen in high concentrations (200,000-5,500,000 ng/mL of semen), even in vasectomized or azoospermic males<sup>23</sup>. Human P30 antigen (Ag) binds to a dye-labeled mobile monoclonal anti-P30 antibody (Ab\*) in the sample well of the card. The Ab\*-Ag complex migrates to the test region where it binds to an immobilized anti-P30 antibody (Ab), forming an Ab\*-Ag-Ab sandwich. This results in the formation of a pink band in the test region. If no P30 is present, this band will not form. Excess unbound Ab\* migrates to the control region and binds to the anti-P30 immunoglobulin present, forming a pink line, and indicates the test is working properly. These immunoassay cards are faster and simpler than methods used previously to detect P30<sup>22,24</sup>.

### **1.3 DNA Analysis**

Individualizing biological evidence from crime scenes can be a critical component of an investigation. Determining if the sample came from the victim, perpetrator, or someone else can not only help corroborate or refute stories regarding what took place but can also be an important part of reconstructing the crime scene and determining the sequence of events. When DNA analysis was first introduced to forensic science, sample individualization was accomplished using variable number of tandem repeats (VNTRs)

and restriction fragment length polymorphisms (RFLPs)<sup>4</sup>. However, this technique required a large amount of intact, high-molecular weight DNA and did not work well with the degraded or contaminated samples often encountered in forensic casework<sup>4,5</sup>.

Today, short tandem repeat (STR) techniques are used to individualize samples. Because these are much shorter repeat units than VNTRs and the kits require much less DNA than the RFLP technique, STRs are better suited for forensic casework<sup>4</sup>. STR loci are amplified using multiplex polymerase chain reaction (PCR) kits, which utilize a fluorescent detection system<sup>4</sup>. The PCR products are separated using capillary electrophoresis and all amplification products labeled with a fluorescent dye are detected<sup>4</sup>. This fluorescent detection system allows for the simultaneous analysis of 16 or more loci, giving the resulting DNA profile a high power of discrimination<sup>4</sup>.

Although more resistant to degradation than previous DNA analysis methods, STRs can still be damaged by environmental factors including UV light, extreme temperatures, and soil<sup>25</sup>. Evidence may be exposed to all of these factors as well as moisture that can cause dilution of the sample. The extent of this damage on the resulting STR profile can be moderate (some allelic dropout) to severe (no profile generated). Other factors, such as the presence of heme, can have an inhibitory effect on sample processing, particularly during amplification<sup>26,27</sup>.

#### **1.4 Study Objectives**

The first goal of this study was to determine how exposure to water or blood affects migration of seminal components and thus, downstream serological testing and

DNA analysis methods. The second goal was to examine the effect of the presence of AP Spot reagent, via direct AP mapping, on subsequent semen testing methods and DNA analysis.

## **2. MATERIALS AND METHODS**

### **2.1 Sample Preparation**

One set of twin size, pale blue-green 100% cotton Cynthia Rowley (Cynthia Rowley, New York, NY) sheets was purchased and pre-washed using a 30 minute “quick wash” cold setting (Whirlpool® front-loading washing machine) and approximately 2 fluid ounces of Gain® Original HE detergent (Procter & Gamble, Cincinnati, OH). Eighteen swatches measuring approximately 10 inches by 10 inches were cut from the sheets and an “x” was placed directly in the middle of each swatch using a pencil, designating where the semen sample would be placed.

Approximately 7 mL of human semen was obtained from BioreclamationIVT (Westbury, NY) and aliquoted into thirteen microcentrifuge tubes for long-term storage. Thirty microliters of semen was dispensed onto each swatch by placing the pipette tip directly on the “x” in the middle of the swatch. Once the semen stain had spread to its maximum diameter, it was outlined with pencil to mark the original region of deposition (ORD). The stains were allowed to dry before the swatches were packaged in separate cardboard boxes and stored at room temperature for the remainder of the experiment.

#### **2.1.1 Samples Exposed to Various Amounts of Water at Different Angles**

Nine swatches were created in the manner described above. Two days later, each swatch was sprayed with one of three amounts of water while positioned at one of three angles. Six of the swatches were placed flat on the bench top and three of the swatches were positioned at 90°. This was accomplished by clipping the swatch to a clipboard that

was attached to a ring stand (the center of the ORD was approximately 9 inches from the bench top). The swatches were then sprayed with 2, 5, or 10 mL of DI water using a spray bottle positioned approximately 18 inches from the swatch. Each spray emitted approximately 1 mL of water. For the swatches positioned at 90°, the neck of the spray bottle was clamped to a second ring stand so that the nozzle was approximately 11 inches from the bench top. For the swatches that were laid flat on the bench top, the spray bottle was held horizontally between two ring stands (one holding the bottom of the bottle and one holding the top of the bottle) and positioned over the swatches. After three of the flat swatches were sprayed with the appropriate amount of water, they were immediately placed on a clipboard positioned at 45° to dry. This resulted in one swatch at each angle and each amount of water. The swatches were allowed to dry completely while positioned at the appropriate angle before being repackaged into their cardboard boxes. The swatches were named based on their angle and amount of water (e.g. 90A represents the swatch positioned at 90° sprayed with 2 mL of water).

#### 2.1.2 AP Spot Pre-treatment

Three additional swatches were prepared in the same manner described previously. Each was sprayed with one of the volumes of water (2, 5, or 10 mL) while flat on the bench top. After drying, the swatches were sprayed with AP Spot reagent prior to testing for P30 and conducting a microscopic examination for sperm. The AP Spot solution was prepared by adding 0.39 g of AP Spot reagent (SERI, Richmond, CA) to 15 mL of DI water and swirling the solution until the reagent was dissolved. Each swatch

was sprayed 10 times with the solution: 2 in the center, 4 around the ORD in a circular fashion and 4 in the same circular fashion farther out. Photographs were taken 90 seconds and 5 minutes after application of the reagent, using a ruler to determine the extent of AP migration outside of the ORD. A positive result was indicated by a violet color change and a negative result was indicated by no color change within the 5-minute observation period. The AP Spot reagent was allowed to dry before the swatches were repackaged in their cardboard boxes. The swatches were named based on the fact that they were pre-treated with AP Spot and the amount of water they were sprayed with (e.g. AP-A represents the AP Spot pre-treated swatch sprayed with 2 mL of water).

### 2.1.3 Swatch Moistened over Multiple Days

To test the effects of applying water over several days, one swatch was prepared in the same manner described previously. After the semen stain dried and was outlined, the swatch was positioned at 90° in the same manner as the previous 90° samples. The swatch was sprayed with approximately 2.5 mL of water every day for 4 days, creating a total of approximately 10 mL of water. The swatch was allowed to dry each day before being repackaged in its cardboard box. Because samples were taken from this swatch on day 2 (after applying 5 mL of water) and day 4 (after applying 10 mL of water), this generated two sample sets designated as multi-spray (MS) 5 and MS 10.

#### 2.1.4 Swatches Contaminated with Blood

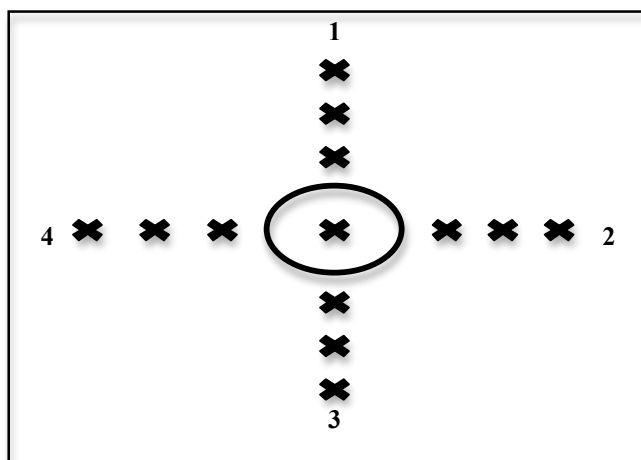
To test the effects of wetting the semen stains with blood instead of water, three additional swatches were prepared in the manner described above. Two days after the semen deposition, the swatches were placed flat on the bench top and 2, 5, or 10 mL of whole bovine blood with K<sup>2</sup>-EDTA was poured over each swatch using a graduated cylinder. The swatches were allowed to dry before being repackaged in their cardboard boxes. These swatches were named based on the amount of blood they were exposed to (e.g. blood A represents the swatch that had 2 mL of blood poured on it).

## 2.2 Semen Testing

Each swatch was visually inspected using a CrimeLite® (Foster and Freeman, Evesham, Worcestershire, UK) ALS at 430-470 nm with orange filter goggles prior to any testing. Photographs were taken using an orange camera filter and a ruler to determine the extent of fluorescence migration outside of the ORD. For the swatch sprayed over multiple days, this was performed on all four days of water exposure.

Three-millimeter Harris punches (Ted Pella Inc., Redding, CA) were initially taken from each swatch in thirteen locations: one from the center of the ORD and then at 1, 4, and 7 cm from the perimeter of the ORD in four directions (Figure 1). Between each sample collection, the Harris punch was cleaned with a 70% isopropanol wipe (Fisher, Pittsburgh, PA) and a “blank” was punched from a piece of filter paper. The cutting mat was also cleaned between swatches with a 70% isopropanol solution and a clean piece of butcher paper was placed between the swatch and the cutting mat. For the swatch sprayed

over multiple days, punches were taken on day 2 (after approximately 5 mL of water) and day 4 (after approximately 10 mL of water).



**Figure 1. Schematic of samples taken from each swatch.** The circle represents the perimeter of the ORD for the semen stain while each “x” represents a 3 mm Harris punch collected from the center of the ORD and from 1, 4, and 7 cm from the perimeter of the stain. The numbers designate each direction (1 represents punches above the stain, 2 represents punches to the right of the stain, etc.).

Each fabric punch was placed in a labeled 2 mL microcentrifuge tube with 450  $\mu$ L of ABACard® P30 extraction buffer (Abacus Diagnostics, West Hills, CA). The samples were allowed to extract on an orbital shaker at room temperature for two hours, with brief vortexing every 30 minutes. After the extraction period, each tube was vortexed before each fabric punch was removed from the buffer and placed into a Spin-X® insert spin basket (Costar, Corning, NY), which was placed back into the microcentrifuge tube. The tubes were then centrifuged at approximately 13,000 rpm for five minutes. The fabric punch and spin basket were set aside and approximately 400  $\mu$ L of the supernatant was removed without disturbing the pellet of cellular material at the bottom of the tube. This

volume was dispensed into a second labeled microcentrifuge tube to be used for P30 testing.

The supernatant from each fabric punch was tested for the presence of P30 using the ABACard® P30 immunoassay cards (Abacus Diagnostics, West Hills, CA). Two hundred microliters of supernatant from each sample was added to a labeled P30 immunoassay card and allowed to diffuse up the test strip for 10 minutes. A positive result was indicated by the presence of two pink bands and a negative result was indicated by the appearance of a pink band only in the control region. If a sample taken 1 cm from the perimeter of the ORD tested positive for P30 but the 4 cm sample in that same direction tested negative, fabric punches at 2 and 3 cm were collected and tested. Similarly, if a 4 cm sample tested positive and the 7 cm sample in that direction tested negative, punches were taken at 5 and 6 cm. The same extraction and slide preparation procedure was performed on these samples.

Each pellet was resuspended in the remaining 50  $\mu$ L volume by vortexing and mixing with the pipette tip. Three microliters of the resuspended pellet solution was pipetted into designated sample areas on glass microscope slides, and the samples were heat-fixed on a heating block until dry. The slides were then stained using nuclear fast red KPIC stain (SERI, Richmond, CA) by applying 1-2 drops of the stain to the samples and allowing it to absorb for approximately 10 minutes. The stain was then rinsed with a gentle stream of water until the stream ran clear and was allowed to air dry. Only the nuclear fast red stain was utilized in this study since no epithelial cells were expected to be present. When completely dry, one drop of Cytoseal™ (ThermoFisher Scientific,

Waltham, MA) was added to a glass coverslip, which was then placed directly onto the sample area. The prepared slides were examined for the presence of spermatozoa at 400x magnification using a compound microscope. A rating scale was used to assess the number of sperm observed within each sample (Table 1).

**Table 1. Rating scale for spermatozoa.** Guidelines for assessing the number of sperm present on a slide.

<b>Rating</b>	<b>Description</b>
0	No sperm observed on the entire slide
1-10 count	Total number of sperm observed on the entire slide
1+	Few sperm on the entire slide, difficult to locate; greater than 10 total
2+	At least one sperm in most fields
3+	Several sperm in most fields, easy to locate
4+	Many sperm in most fields

Each swatch was sprayed with AP Spot reagent, which was prepared and applied as described previously. Photographs were taken 90 seconds and 5 minutes after applying the reagent. A ruler was included in the photographs to allow the distance of AP migration to be determined.

The above testing procedure (ALS examination, P30 testing, and microscopic examination) was followed for all swatches. The swatches moistened with blood instead of water had AP mapping performed via the transfer method instead of directly spraying the swatch with AP Spot due to the blood interfering with visualizing the color change. Pieces of filter paper were sprayed with water until damp and then pressed against the swatches firmly for 15 seconds. The filter paper was then removed and sprayed with the AP Spot reagent.

## 2.3 DNA Analysis

### 2.3.1 DNA Testing Procedure

New 3 mm fabric punches were taken from the flat and 90° swatches based on the results obtained from presumptive and confirmatory testing. These punches were placed in labeled sterile 0.2 mL PCR tubes and stored at room temperature until extraction. The extraction followed a modified version of the *forensicGEM* Saliva (ZyGEM, Hamilton, New Zealand) extraction protocol which included the use of an additional protease<sup>28,29</sup>. Ten microliters of DI water and 30 µL of protease were added to each PCR tube. The samples were briefly vortexed for 10 seconds before placing the samples in the thermal cycler and incubating at 37°C for 60 minutes, pausing every 10 minutes to briefly vortex the samples. After a 70°C incubation for 10 minutes, the samples were cooled to 25°C before adding five microliters of 10x Buffer BLUE, 4.5 µL of DNA-free water, and 0.5 µL of *forensicGEM* enzyme to each PCR tube for a final volume of 50 µL. The samples were briefly vortexed for 10 seconds and were placed in the thermal cycler to incubate at 75°C for 15 minutes and 95°C for 5 minutes. After removing the samples from the thermal cycler, the samples were centrifuged at approximately 15,000 rpm for 5 minutes. The fabric punch was then moved onto the wall of the tube using the pipette tip and the supernatant was transferred to a clean 0.2 mL PCR tube. The punch was then placed back to the bottom of the tube and pressed with the pipette tip to remove some of the supernatant trapped in the fabric. Samples were then stored at -20°C until quantification.

Quantification was performed using the ABI Quantifiler Duo® kit (Life Technologies, Grand Island, NY) protocol. A master mix was prepared using 10.5 µL

Duo Primer Mix per sample and 12.5  $\mu\text{L}$  Duo Reaction Mix per sample. The master mix was briefly vortexed prior to pipetting 23  $\mu\text{L}$  into each of the 0.2 mL optical tubes, followed by 2  $\mu\text{L}$  of each DNA sample. The tubes were capped, vortexed, and placed into the ABI 7500® Real-Time PCR system (Life Technologies, Grand Island, NY).

Since all of the quantification values were low, only those samples that also gave a strong positive AP Spot result were chosen for amplification. Amplification was performed using the AmpFISTR® Identifiler® Plus PCR Amplification Kit (Life Technologies, Grand Island, NY). Using a target of 1 ng of DNA and the quantification data, the samples were diluted using Tris EDTA (TE) buffer when necessary. For any samples where 1 ng could not be targeted using the maximum volume for amplification, 10  $\mu\text{L}$  of the sample extract was added to the amplification reaction.

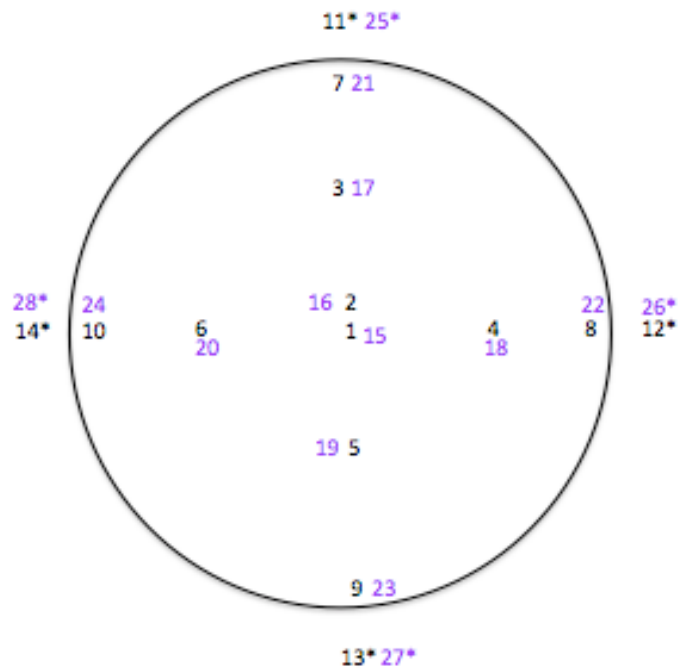
A master mix was prepared by combining 10  $\mu\text{L}$  of Identifiler® Plus Master Mix per sample and 5  $\mu\text{L}$  of the Identifiler® Plus primer set per sample. Fifteen microliters of the master mix was added to each tube in the 0.2 mL PCR strip followed by 10  $\mu\text{L}$  of the DNA sample. If the volume of DNA needed was less than 10  $\mu\text{L}$ , DI water was added to bring the final volume to 10  $\mu\text{L}$ . The final volume of each tube was 25  $\mu\text{L}$ . Positive and negative controls were prepared by adding 10  $\mu\text{L}$  of control DNA to the positive control tube and 10  $\mu\text{L}$  of DI water to the negative control tube. The tubes were capped, vortexed, and placed into the thermal cycler. The following thermal cycler parameters were used: 95°C for 11 minutes, 94°C for 20 seconds and 59°C for 3 minutes (for 28 cycles), and 60°C for 10 minutes followed by a 4°C hold.

Capillary electrophoresis was performed using an ABI Prism 3130 Genetic Analyzer (Life Technologies, Grand Island, NY). A master mix was created by combining 9.5  $\mu$ L of formamide per sample with 0.5  $\mu$ L of LIZ 600 internal size standard per sample. Ten microliters of the master mix was added to each well of the 96-well plate followed by 1  $\mu$ L of either allelic ladder or sample. The plate was sealed with a clean septa, vortexed, and denatured at 95°C for 3 minutes. The plate was then cooled for at least 3 minutes at 4°C. Once placed in the genetic analyzer, the samples were injected for 10 seconds.

Analysis was performed using GeneMapper® ID, v.3.2 (Applied Biosystems, Foster City, CA) using the Identifiler® Plus analysis method with stutter filters, LIZ 600 size standard, and a threshold of 30 relative fluorescence units (RFU) for both the samples and the ladder. Average peak height per locus was calculated for each profile. For heterozygous loci, this was done by averaging the peak heights of both alleles at each locus. For homozygous loci, the peak height was divided by two. Peak height ratios were calculated for each heterozygous locus by dividing the smaller peak height by the larger peak height, producing a value less than or equal to one. Average peak height ratios and average peak heights were then calculated for each profile. Heterozygous loci that showed dropout of one or both alleles and homozygous loci were not used in the PHR calculation. Allelic dropout was designated as a known allele not appearing in a sample that displayed other correct allele calls.

### 2.3.2 Effects of AP Spot Reagent on DNA Analysis

To examine the effects of AP Spot reagent on DNA analysis, a swatch was prepared with 30  $\mu$ L of semen in the same manner described previously. Once the stain was dry and outlined, ten 3 mm fabric punches were taken and placed into labeled sterile 0.2 mL PCR tubes. After these punches were collected, the swatch was sprayed with AP Spot reagent, prepared and applied as described previously. Ten additional fabric punches were then collected adjacent to the first set of punches and placed into labeled sterile 0.2 mL PCR tubes (Figure 2). Between each sample collection, the Harris punch was cleaned with a 70% isopropanol wipe (Fisher, Pittsburgh, PA) and a “blank” was punched from a piece of filter paper. The procedures detailed previously for extraction through capillary electrophoresis and analysis were also followed for these samples.



**Figure 2. Schematic of samples used for testing the effects of AP Spot reagent on DNA analysis.** The circle represents the semen stain and each number represents the location of a 3 mm punch. The purple color indicates which punches were taken after the AP Spot reagent was applied. (This figure is not to scale and is enlarged to show the areas of the stain from which each punch was taken.) \*Samples 11-14 and 25-28 were taken 1 cm out from the perimeter of the semen stain however, they were not carried on to amplification because their quantification values were 0 ng/ $\mu$ L.

### 2.3.3 Swatches Contaminated With Blood

New fabric punches were taken from each of these swatches based on the presumptive and confirmatory results. The extraction and quantification procedures discussed previously were followed with these samples. Because heme is known to be a PCR inhibitor<sup>26,27</sup>, a 1:10 dilution of the extract from the center of the ORD for each swatch was made and quantified. The 1:10 dilution samples were carried on to amplification and capillary electrophoresis, using the previously discussed procedures. For the capillary electrophoresis, injection times of 5 and 10 seconds were used and the 5-second injection time electropherograms were used for further analysis.

### **3. RESULTS**

#### **3.1 Semen Testing Results**

Each sample was subjected to the same testing procedure however, most sample sets showed some differences in the results obtained, likely due to the different variable(s) examined within each sample set.

##### **3.1.1 Samples Exposed to Various Amounts of Water at Different Angles**

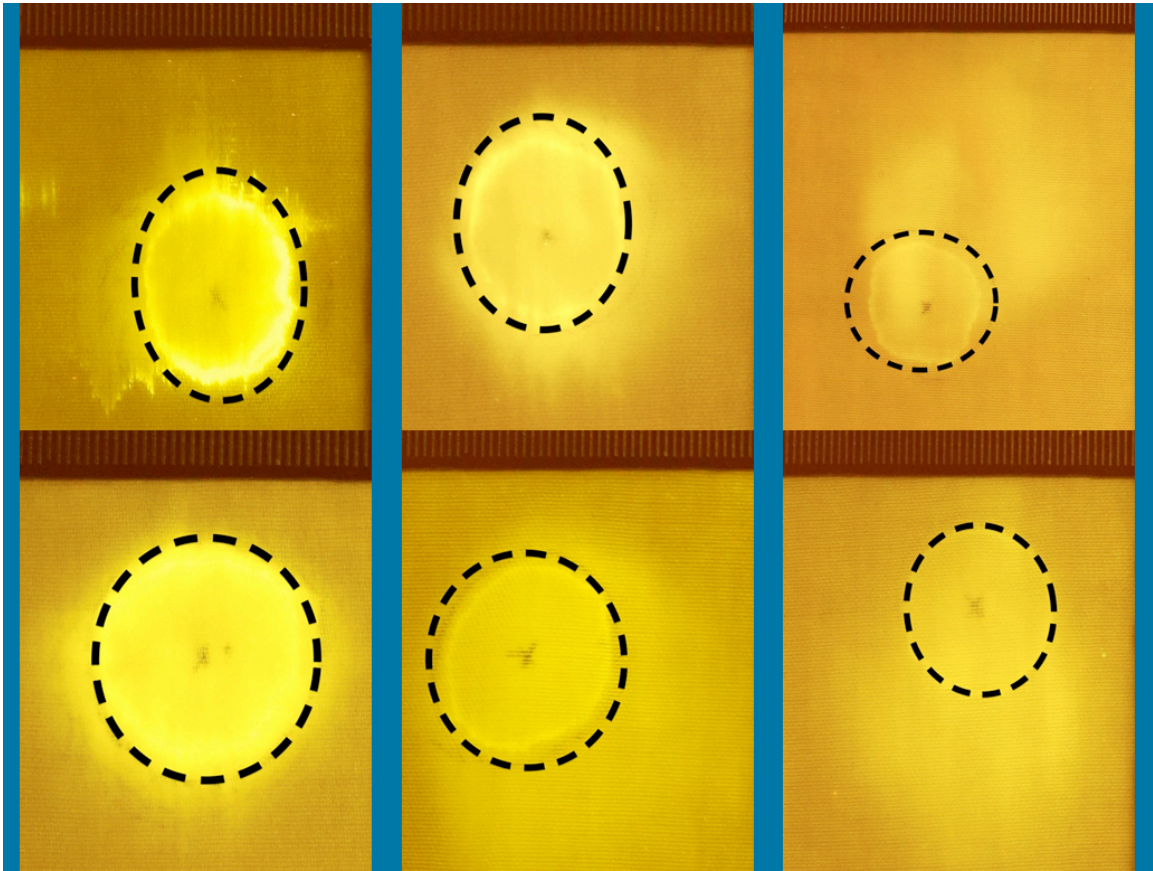
Each of the swatches appeared visually the same before and after the water was applied. However, for the majority of the swatches, the semen stain could only be located using the pencil outline of the ORD.

Upon examination with the ALS, fluorescence was noted both inside and outside of the ORD on all nine swatches from this sample set (Table 2). On all of the swatches sprayed with 2 mL or 5 mL of water, the edge of the ORD displayed brighter fluorescence than the interior of the stain or any fluorescence outside of the ORD. On the swatches sprayed with 10 mL, the edge of the ORD was still brighter than the interior or any fluorescence outside the ORD but it was much fainter than the edges of the ORDs on the 2 and 5 mL swatches (Figure 3).

The farthest fluorescence migration noted was approximately 3 cm from the perimeter of the ORD. This was only observed on the 90° swatch that was exposed to 10 mL of water. The majority of samples did not show any fluorescence beyond 1-2 cm from the perimeter of the ORD. Fluorescence migration was noted in at least 2 directions away from the ORD for each swatch.

**Table 2. ALS results for swatches at various angles and amounts of water.** Results were designated as “+” when fluorescence was observed or “-” when no fluorescence was observed. Each column represents one swatch. Each row represents the distance at which the result occurred: the first number represents the direction of the result (Figure 1) and the second number represents the distance in centimeters from the perimeter of the ORD.

Sample position	Flat 2ml	Flat 5ml	Flat 10ml	45° 2ml	45° 5ml	45° 10ml	90° 2ml	90° 5ml	90° 10ml	Control (no water)
ORD	+	+	+	+	+	+	+	+	+	+
1-1 cm	+	+	+	-	-	-	+	+	+	-
1-2 cm	-	-	+	-	-	-	-	-	-	-
1-3 cm	-	-	-	-	-	-	-	-	-	-
1-4 cm	-	-	-	-	-	-	-	-	-	-
2-1 cm	-	+	+	+	+	+	+	+	-	-
2-2 cm	-	-	+	-	-	+	-	+	-	-
2-3 cm	-	-	-	-	-	-	-	-	-	-
2-4 cm	-	-	-	-	-	-	-	-	-	-
3-1 cm	-	+	-	+	-	+	+	+	+	-
3-2 cm	-	-	-	+	-	+	-	+	+	-
3-3 cm	-	-	-	-	-	-	-	-	+	-
3-4 cm	-	-	-	-	-	-	-	-	-	-
4-1 cm	+	-	-	+	+	-	+	-	-	-
4-2 cm	-	-	-	-	-	-	-	-	-	-
4-3 cm	-	-	-	-	-	-	-	-	-	-
4-4 cm	-	-	-	-	-	-	-	-	-	-



**Figure 3. ALS photographs of flat and 90° swatches sprayed with 2, 5 and 10 mL of water.** The dotted line indicates the perimeter of the ORD. Top row (left to right): 90° swatches sprayed with 2, 5 and 10 mL of water. Bottom row (left to right): flat swatches sprayed with 2, 5 and 10 mL of water.

The center of the ORD as well as some areas outside of the ORD tested positive for P30 for all nine swatches in this sample set (Table 3). On the 2 mL swatches, the majority of the P30 migration extended only 1 cm from the perimeter of the ORD with a maximum of 2 cm. On the 5 mL swatches, the majority of the P30 migration also extended 1 cm from the perimeter of the ORD with a maximum of 3 cm. On the 10 mL swatches, the majority of the P30 migration extended 2-3 cm from the perimeter of the ORD with a maximum of 6 cm. The 7 cm punch tested negative in all directions for all swatches.

**Table 3. P30 results for swatches at various angles and amounts of water.** Results are designated as “+” for a positive result within 10 minutes or “-” for a negative result. “+<sup>†</sup>” signifies a positive result also occurred at 5 and 6 cm in the indicated direction. “n/a” signifies that the indicated sample was not tested.

Sample position	Flat 2ml	Flat 5ml	Flat 10ml	45° 2ml	45° 5ml	45° 10ml	90° 2ml	90° 5ml	90° 10ml	Control (no water)
ORD	+	+	+	+	+	+	+	+	+	+
1-1 cm	+	+	+	-	+	-	-	+	+	-
1-2 cm	+	-	+	n/a	-	n/a	n/a	-	n/a	n/a
1-3 cm	-	n/a	+	n/a	n/a	n/a	n/a	n/a	n/a	n/a
1-4 cm	-	-	-	-	-	-	-	-	+ <sup>†</sup>	-
2-1 cm	-	+	+	-	+	+	+	+	-	-
2-2 cm	n/a	-	-	n/a	-	+	-	+	n/a	n/a
2-3 cm	n/a	n/a	n/a	n/a	n/a	-	n/a	-	n/a	n/a
2-4 cm	-	-	-	-	-	-	-	-	-	-
3-1 cm	-	+	-	+	+	+	+	+	+	-
3-2 cm	n/a	-	n/a	-	-	+	-	+	+	n/a
3-3 cm	n/a	n/a	n/a	n/a	n/a	-	n/a	+	+	n/a
3-4 cm	-	-	-	-	-	-	-	-	-	-
4-1 cm	+	+	-	+	+	+	+	-	+	-
4-2 cm	-	-	n/a	-	-	-	-	n/a	-	n/a
4-3 cm	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
4-4 cm	-	-	-	-	-	-	-	-	-	-

Spermatozoa were noted in the center of the ORD for all nine swatches with an average rating of 2+. The majority of the swatches showed few or no spermatozoa outside of the ORD (Table 4). No sperm were noted outside of the ORD for any of the 2 mL swatches. Sperm were infrequently observed outside of the ORD on the 5 mL swatches positioned at 45° and 90°. Each swatch had two slides on which a single sperm was observed. Minimal sperm migration was also observed on the 10 mL swatches. The flat and 45° swatches each had one slide exhibiting a single sperm and the 90° swatch had two slides exhibiting a single sperm.

**Table 4. Sperm search results for swatches at various angles and amounts of water.** The amount of microscopically observed sperm is rated based on the values from Table 1. “n/a” means that the indicated sample was not tested.

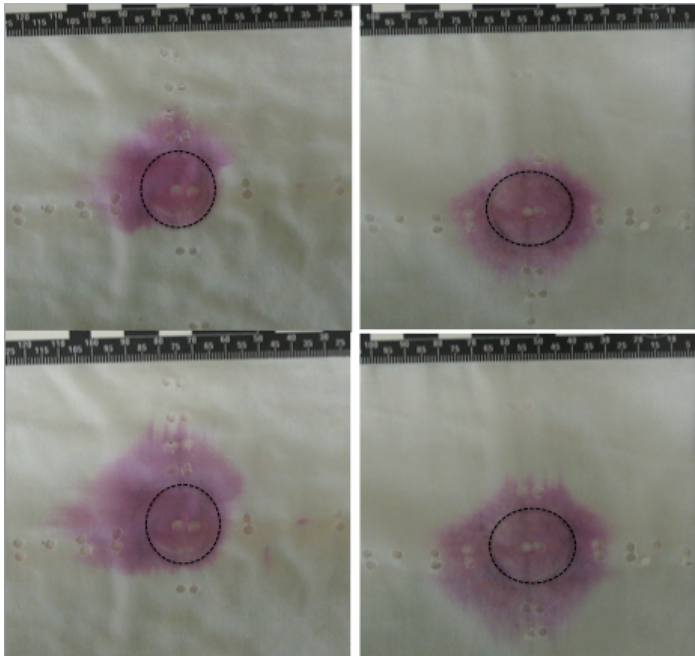
Sample position	Flat 2ml	Flat 5ml	Flat 10ml	45° 2ml	45° 5ml	45° 10ml	90° 2ml	90° 5ml	90° 10ml	Control (no water)
ORD	1+- 2+	1+	1+-2+	3+- 4+	2+-3+	1+-2+	1+- 2+	1+-2+	2+-3+	1+-2+
1-1 cm	0	0	0	0	0	0	0	0	0	0
1-2 cm	0	0	0	n/a	0	n/a	n/a	0	n/a	n/a
1-3 cm	0	n/a	0	n/a	n/a	n/a	n/a	n/a	n/a	n/a
1-4 cm	0	0	0	0	1 sperm	0	0	0	1 sperm	0
1-7 cm	0	0	0	0	0	0	0	0	0	0
2-1 cm	0	0	0	0	0	1 sperm	0	0	0	0
2-2 cm	n/a	0	0	n/a	0	0	0	0	n/a	n/a
2-3 cm	n/a	n/a	n/a	n/a	n/a	0	n/a	0	n/a	n/a
2-4 cm	0	0	0	0	0	0	0	4 sperm	0	0
2-7 cm	0	0	0	0	0	0	0	0	0	0
3-1 cm	0	0	0	0	0	0	0	0	0	0
3-2 cm	n/a	0	n/a	0	0	0	0	1 sperm	1 sperm	n/a
3-3 cm	n/a	n/a	n/a	n/a	n/a	0	n/a	0	0	n/a
3-4 cm	0	0	0	0	0	0	0	0	0	0
3-7 cm	0	0	0	0	0	0	0	0	0	0
4-1 cm	0	0	1 sperm	0	0	0	0	0	0	0
4-2 cm	0	0	n/a	0	1 sperm	0	0	n/a	0	n/a
4-3 cm	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
4-4 cm	0	0	0	0	0	0	0	0	0	0
4-7 cm	0	0	0	0	0	0	0	0	0	0

All nine swatches showed positive AP Spot reactions both inside and outside of the ORD (Table 5). For all of the 2 mL swatches, AP migration extended 1-2 cm beyond the perimeter of the ORD within 90 seconds (Figure 4). For the 5 mL swatches, the majority of the AP migration extended 1 cm beyond the perimeter of the ORD with a

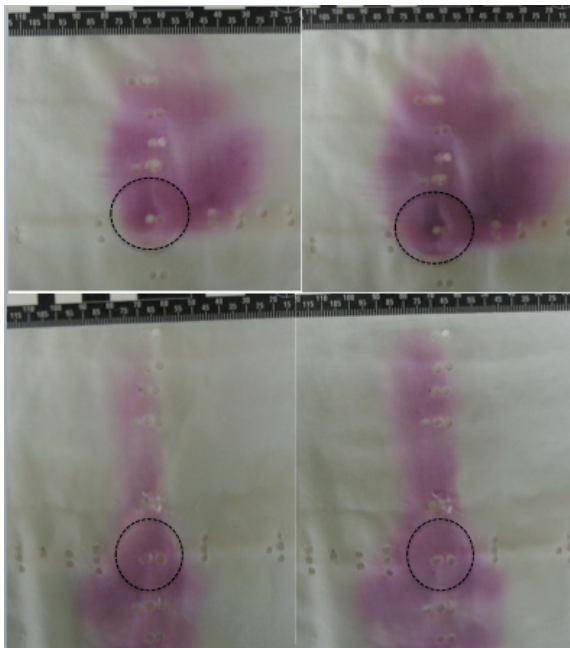
maximum of 3 cm within 90 seconds. The flat and 45° 10 mL swatches showed AP migration primarily to 2 cm from the perimeter of the ORD with a maximum of 4 cm (Figure 5). The 90° 10 mL swatch showed the farthest AP migration with positive AP Spot results observed up to 6 cm above the ORD, 3 cm below the ORD, and 1 cm to the right of the ORD within 90 seconds (Figure 5). The positive AP Spot results extended an additional centimeter for many of the samples after 5 minutes.

**Table 5. AP Spot results for swatches at various angles and amounts of water.** Results are designated as “+” for a violet color change within 90 seconds, “+\*” for a violet color change within 5 minutes, or “-” for no color change within 5 minutes. “+<sup>†</sup>” indicates a positive result also occurred at 1-5 and 1-6 within 90 seconds.

Sample position	Flat 2ml	Flat 5 ml	Flat 10 ml	45° 2ml	45° 5ml	45° 10ml	90° 2ml	90° 5ml	90° 10ml	Control (no water)
ORD	+	+	+	+	+	+	+	+	+	+
1-1 cm	+	+	+	+*	+	+*	+	+	+	+*
1-2 cm	+	-	+	-	+*	-	-	+*	+	-
1-3 cm	-	-	+	-	-	-	-	-	+	-
1-4 cm	-	-	+	-	-	-	-	-	+ <sup>†</sup>	-
1-7 cm	-	-	-	-	-	-	-	-	+*	-
2-1 cm	-	+	+	+	+	+	+	+	-	+*
2-2 cm	-	+*	+	-	+*	+	-	+	-	-
2-3 cm	-	-	+*	-	-	+*	-	-	-	-
2-4 cm	-	-	-	-	-	-	-	-	-	-
2-7 cm	-	-	-	-	-	-	-	-	-	-
3-1 cm	-	+	-	+	+	+	+	+	+	+*
3-2 cm	-	+*	-	+	+*	+	+*	+	+	-
3-3 cm	-	-	-	+*	-	+*	-	+	+	-
3-4 cm	-	-	-	-	-	-	-	+*	+*	-
3-7 cm	-	-	-	-	-	-	-	-	-	-
4-1 cm	+	+	+*	+	+	+	+	+	+	+*
4-2 cm	+*	+*	-	+	+*	+*	+*	-	+*	-
4-3 cm	-	-	-	+*	-	-	-	-	-	-
4-4 cm	-	-	-	-	-	-	-	-	-	-
4-7 cm	-	-	-	-	-	-	-	-	-	-



**Figure 4. AP Spot results for flat and 90° swatches sprayed with 2 mL of water.** Top left: flat swatch at 90 seconds; bottom left: flat swatch at 5 minutes; top right: 90° swatch at 90 seconds; bottom right: 90° swatch at 5 minutes. The black circles indicate the perimeter of the ORD.



**Figure 5. AP Spot results for flat and 90° swatches sprayed with 10 mL of water.** Top left: flat swatch at 90 seconds; top right: flat swatch at 5 minutes; bottom left: 90° swatch at 90 seconds; bottom right: 90° swatch at 5 minutes. The black circles indicate the perimeter of the ORD.

### 3.1.2 AP Spot Pre-treatment

The AP Spot pre-treated swatches were examined with the ALS prior to being sprayed with AP Spot reagent. The results were similar to those obtained for the flat swatches (Table 2).

Spraying swatches with AP Spot reagent prior to P30 testing appeared to cause no detrimental effects (Table 6). The 2 mL swatch demonstrated P30 migration extending 1 cm in all 4 directions. The 5 mL swatch also demonstrated P30 migration extending to 1 cm, with a maximum of 2 cm in at least one direction. The 10 mL swatch demonstrated P30 migration in only two directions away from the ORD, extending 1 cm and 3 cm respectively. The 7 cm samples were negative in all directions for all three swatches.

**Table 6. P30 results for AP Spot pre-treated swatches.** Results are designated as “+” for a positive result or “-” for a negative result. “n/a” means that the indicated distance was not tested.

	AP 2ml	AP 5ml	AP 10ml
ORD	+	+	+
1-1 cm	+	+	-
1-2 cm	-	+	n/a
1-3 cm	n/a	-	n/a
1-4 cm	-	-	-
2-1 cm	+	+	+
2-2 cm	-	-	-
2-3 cm	n/a	n/a	n/a
2-4 cm	-	-	-
3-1 cm	+	-	+
3-2 cm	-	n/a	+
3-3 cm	n/a	n/a	+
3-4 cm	-	-	-
4-1 cm	+	+	-
4-2 cm	-	-	n/a
4-3 cm	n/a	n/a	n/a
4-4 cm	-	-	-

Pre-treatment with AP Spot reagent also had no negative effects on the staining of spermatozoa using nuclear fast red. The three swatches showed an average spermatozoa rating of 2+ in the center of the ORD. Similar to the previously discussed sample set, there was minimal spermatozoa migration (Table 7). The 5 mL swatch did not display any spermatozoa outside of the ORD in the areas tested. The 2 mL and 10 mL swatches each had one slide demonstrating a single sperm outside of the ORD.

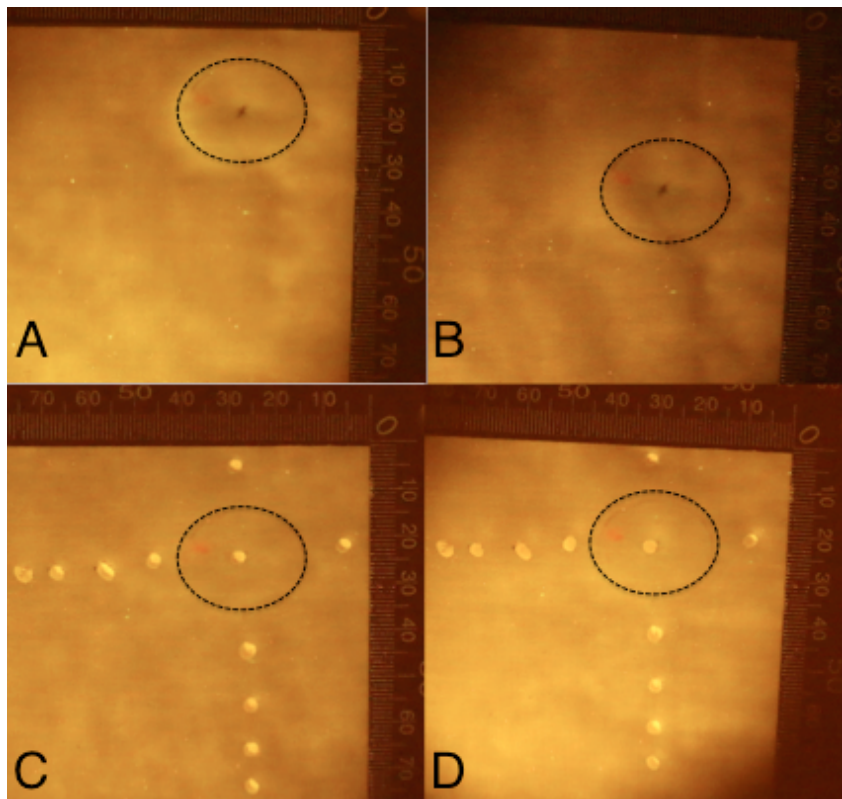
**Table 7. Sperm search results for AP Spot pre-treated swatches.** The amount of sperm present is rated based on the values from Table 1. “n/a” means that the indicated distance was not tested.

	AP 2ml	AP 5ml	AP 10ml
ORD	1+-2+	2+	1+-2+
1-1 cm	0	0	1 sperm
1-2 cm	0	0	n/a
1-3 cm	n/a	0	n/a
1-4 cm	0	0	0
1-7 cm	0	0	0
2-1 cm	0	0	0
2-2 cm	0	0	0
2-3 cm	n/a	n/a	n/a
2-4 cm	0	0	0
2-7 cm	0	0	0
3-1 cm	1 sperm	0	0
3-2 cm	0	0	0
3-3 cm	n/a	n/a	0
3-4 cm	0	0	0
3-7 cm	0	0	0
4-1 cm	0	0	0
4-2 cm	0	0	n/a
4-3 cm	n/a	n/a	n/a
4-4 cm	0	0	0
4-7 cm	0	0	0

### 3.1.3 Swatch Moistened over Multiple Days

The ALS examination of this swatch revealed faint fluorescence on all four days. Fluorescence inside and outside of the ORD became very difficult to see following day 2

(approximately 5 mL of water). Fluorescence extending approximately 1 cm from the perimeter of the ORD was observed on day 2. Any additional migration that may have occurred was too faint to be visualized (Figure 6).



**Figure 6. ALS photographs of the swatch moistened over multiple days.** A: day 1 (approximately 2.5 mL of water); B: day 2 (approximately 5 mL of water); C: day 3 (approximately 7.5 mL of water); D: day 4 (approximately 10 mL of water). The black circles indicate the perimeter of the ORD.

Fabric punches taken on day 2 (after approximately 5 mL of water) and day 4 (after approximately 10 mL of water) were tested for the presence of P30. Both sample sets showed positive P30 results inside and outside of the ORD (Table 8). The 5 mL samples exhibited an average P30 migration of 2 cm from the perimeter of the ORD with a maximum of 3 cm. The 10 mL samples exhibited an average P30 migration of 2-3 cm

from the perimeter of the ORD with a maximum of 4 cm. The 7 cm punches were negative in all directions for both sample sets.

**Table 8. P30 results for the swatch moistened over multiple days.** Samples were tested on day 2 (~ 5 mL of water) and day 4 (~10 mL of water). Results were designated as “+” for a positive result or “-” for a negative result. “n/a” means the sample at the indicated distance was not tested.

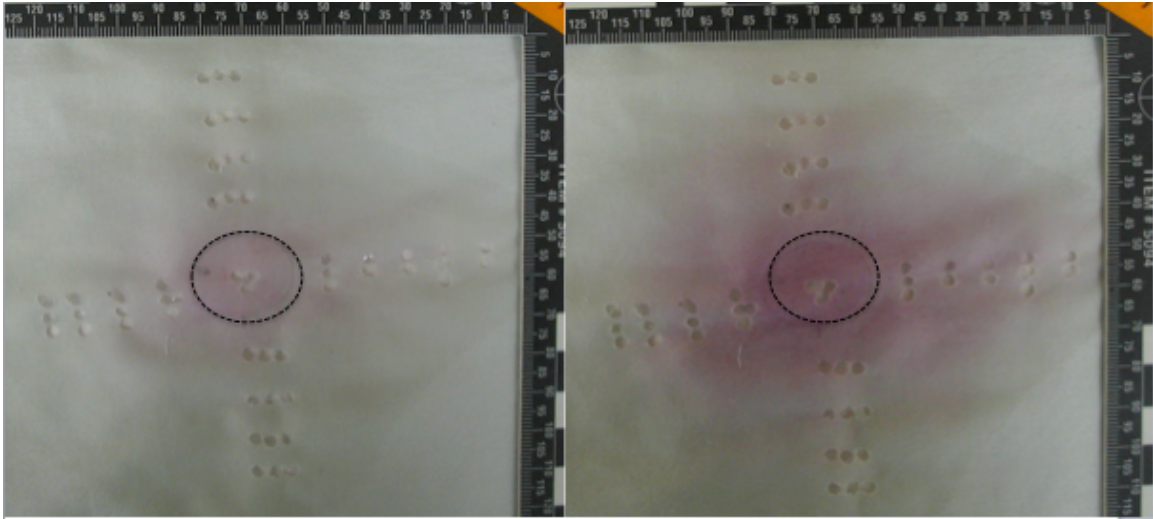
	Multi-Spray 5 mL	Multi-Spray 10 mL
ORD	+	+
1-1 cm	+	+
1-2 cm	+	+
1-3 cm	-	-
1-4 cm	-	-
2-1 cm	+	+
2-2 cm	+	n/a
2-3 cm	+	n/a
2-4 cm	-	+
2-5 cm	n/a	-
3-1 cm	+	+
3-2 cm	+	+
3-3 cm	-	-
3-4 cm	-	-
4-1 cm	+	+
4-2 cm	+	+
4-3 cm	-	+
4-4 cm	-	-

Spermatozoa were observed in the center of the ORD for both day 2 (approximately 5 mL of water applied) and day 4 (approximately 10 mL of water applied), with an average rating of 2+. Both sets of samples showed minimal sperm migration, each having only one slide from outside of the ORD that showed any spermatozoa (Table 9).

**Table 9. Sperm search results for the swatch moistened over multiple days.** The amount of sperm present is rated based on the values from Table 1. “n/a” means that the indicated sample was not tested.

	Multi-Spray 5 mL	Multi-Spray 10 mL
ORD	1+-2+	2+-3+
1-1 cm	2 sperm	0
1-2 cm	0	0
1-3 cm	0	0
1-4 cm	0	0
1-7 cm	0	0
2-1 cm	0	0
2-2 cm	0	n/a
2-3 cm	0	n/a
2-4 cm	0	0
2-5 cm	n/a	0
2-7 cm	0	0
3-1 cm	0	1 sperm
3-2 cm	0	0
3-3 cm	0	0
3-4 cm	0	0
3-7 cm	0	0
4-1 cm	0	0
4-2 cm	0	0
4-3 cm	0	0
4-4 cm	0	0
4-7 cm	0	0

The AP Spot reagent gave positive results only in the center of the ORD and 1 cm to the left and right of the ORD within 90 seconds. However, within 5 minutes, a positive result was obtained in all four directions at the following distances: 2 cm above the ORD, 4 cm to the left of the ORD, 1 cm below the ORD, and 3 cm to the right of the ORD. The appearance of the positive AP Spot results was much fainter than those obtained for the swatches exposed to water on only one occasion (Figure 7).



**Figure 7. AP Spot results for the swatch moistened over multiple days.** Positive results for AP Spot are indicated by a violet color change. Left: AP Spot results after 90 seconds and right: AP Spot results after 5 minutes.

#### 3.1.4 Swatches Contaminated with Blood

The ALS examination of these swatches did not provide much information because the blood obscured much of the fluorescence. Faint fluorescence was visible inside and outside of the ORD of the swatch moistened with 2 mL of blood. The observable fluorescence migration was minimal, extending approximately 0.5 cm from the perimeter of the ORD. No fluorescence was visible on the swatches moistened with 5 mL and 10 mL of blood.

All three swatches showed positive P30 results inside and outside of the ORD (Table 10). The 2 mL swatch demonstrated positive P30 results 1-2 cm from the perimeter of the ORD. The 5 mL swatch demonstrated positive P30 results in two directions, extending 4 cm and 5 cm respectively. The 10 mL swatch demonstrated positive P30 results in three directions, extending 2 cm, 6 cm and 9 cm respectively.

**Table 10. P30 results for swatches contaminated with blood.** Results are designated as “+” for a positive result or “-” for a negative result. “n/a” means the sample at the indicated distance was not tested.

	Blood 2ml	Blood 5ml	Blood 10ml
ORD	+	+	+
1-1 cm	+	-	+
1-2 cm	-	n/a	+
1-3 cm	n/a	n/a	-
1-4 cm	-	-	-
1-7 cm	n/a	n/a	n/a
2-1 cm	+	-	-
2-2 cm	-	n/a	n/a
2-3 cm	n/a	n/a	n/a
2-4 cm	-	-	-
2-7 cm	n/a	n/a	n/a
3-1 cm	+	+	+
3-2 cm	+	n/a	n/a
3-3 cm	-	n/a	n/a
3-4 cm	-	+	+
3-5 cm	n/a	+	+
3-6 cm	n/a	-	+
3-7 cm	n/a	n/a	n/a
4-1 cm	+	+	+
4-2 cm	+	n/a	n/a
4-3 cm	-	n/a	n/a
4-4 cm	-	+	+
4-5 cm	n/a	-	+
4-6 cm	n/a	n/a	+
4-7 cm	n/a	n/a	+
4-8 cm	n/a	n/a	+
4-9 cm	n/a	n/a	+
4-10 cm	n/a	n/a	-

The presence of blood on the fabric punches did not affect sperm staining. The slides made from the center of the ORD showed less sperm than the center slides from the other sample sets, with an average of 1+ and 2+ respectively. The slides made from the other distances still showed minimal sperm migration (Table 11). The 5 mL swatch

did not have any slides from outside of the ORD with sperm present. However, the 2 and 10 mL swatches each had two slides with a single sperm present.

**Table 11. Sperm search results for swatches contaminated with blood.** The amount of sperm present is rated based on the values from Table 1. “n/a” indicates that the sample at the indicated distance was not tested.

	Blood 2ml	Blood 5ml	Blood 10ml
ORD	1+	1+-2+	1+-2+
1-1 cm	1 sperm	0 sperm	0 sperm
1-2 cm	0 sperm	n/a	0 sperm
1-3 cm	n/a	n/a	0 sperm
1-4 cm	0 sperm	0 sperm	0 sperm
1-7 cm	n/a	n/a	n/a
2-1 cm	0 sperm	0 sperm	0 sperm
2-2 cm	0 sperm	n/a	n/a
2-3 cm	n/a	n/a	n/a
2-4 cm	1 sperm	0 sperm	0 sperm
2-7 cm	n/a	n/a	n/a
3-1 cm	0 sperm	0 sperm	1 sperm
3-2 cm	0 sperm	n/a	n/a
3-3 cm	n/a	n/a	n/a
3-4 cm	0 sperm	0 sperm	0 sperm
3-5 cm	n/a	0 sperm	0 sperm
3-6 cm	n/a	0 sperm	
3-7 cm	n/a	n/a	n/a
4-1 cm	0 sperm	0 sperm	0 sperm
4-2 cm	0 sperm	n/a	n/a
4-3 cm	n/a	n/a	n/a
4-4 cm	0 sperm	0 sperm	1 sperm
4-5 cm	n/a	0 sperm	0 sperm
4-6 cm	n/a	n/a	0 sperm
4-7 cm	n/a	n/a	0 sperm
4-8 cm	n/a	n/a	0 sperm
4-9 cm	n/a	n/a	0 sperm
4-10 cm	n/a	n/a	0 sperm

## 3.2 DNA Analysis Results

### 3.2.1 Samples Exposed to Various Amounts of Water at Different Angles

New fabric punches were taken from each of the flat and 90° swatches adjacent to samples that gave a positive P30 result. The results of the quantification step revealed almost no DNA in any of the samples from outside of the ORD (Table 12). The amount of DNA extracted from each sample was calculated utilizing the cycle threshold ( $C_T$ ) value from the HID Real-Time PCR software and the following equation:

$$\text{DNA mass (ng/}\mu\text{L)} = 10^{(\text{CT-y intercept})/\text{slope}}$$

The slope and y-intercept are generated from standard curves. Because the samples from outside of the ORD had low quantification values, only those samples that also displayed a strong positive AP Spot result within 90 seconds were chosen for amplification and capillary electrophoresis.

**Table 12. Amounts of DNA obtained at various distances from the ORD.** New punches from the locations on the flat and 90° swatches that exhibited a positive P30 result were extracted and quantified to determine the amount of DNA present.

Sample Angle	Amount of Water	Distance from ORD	Amount of DNA obtained (ng/ $\mu$ L)
Flat	2 mL	0 cm	0.338
Flat	2 mL	1 cm	0.001
Flat	2 mL	2 cm	0
Flat	2 mL	1 cm	0.002
Flat	5 mL	0 cm	0.945
Flat	5 mL	1 cm	0
Flat	5 mL	1 cm	0
Flat	5 mL	1 cm	0
Flat	5 mL	1 cm	0
Flat	10 mL	0 cm	1.519
Flat	10 mL	1 cm	0.001

<b>Sample Angle</b>	<b>Amount of Water</b>	<b>Distance from ORD</b>	<b>Amount of DNA obtained (ng/<math>\mu</math>L)</b>
Flat	10 mL	2 cm	0
Flat	10 mL	3 cm	0
Flat	10 mL	1 cm	0
90°	2 mL	0 cm	1.735
90°	2 mL	1 cm	0
90°	2 mL	1 cm	0
90°	2 mL	1 cm	0
90°	5 mL	0 cm	1.228
90°	5 mL	1 cm	0
90°	5 mL	1 cm	0
90°	5 mL	2 cm	0
90°	5 mL	1 cm	0.003
90°	5 mL	2 cm	0
90°	5 mL	3 cm	0
90°	10 mL	0 cm	0.444
90°	10 mL	1 cm	0
90°	10 mL	4 cm	0.001
90°	10 mL	5 cm	0
90°	10 mL	6 cm	0
90°	10 mL	1 cm	0.001
90°	10 mL	2 cm	0.002
90°	10 mL	3 cm	0
90°	10 mL	1 cm	0

Samples were designated as producing a full, partial or no profile (Table 13). A full profile means that all of the correct allele calls were made at all 16 of the Identifiler® Plus loci. A partial profile means that at least one correct allele call for at least one locus was present. No profile means that no correct allele calls were present above the 30 RFU analytical threshold.

**Table 13. Type of DNA profile obtained for flat and 90° swatches.** DNA analysis results were classified as “full”- all alleles at all loci are present, “partial”- at least one correct allele for the sample is present, or “none”- no correct allele calls were made for the sample. The number of correct alleles present out of 30 for each partial profile is given.

<b>Sample Angle</b>	<b>Amount of Water</b>	<b>Distance from ORD</b>	<b>Type of Profile</b>
Flat	2 mL	0 cm	Full
Flat	2 mL	1 cm	None
Flat	5 mL	0 cm	Full
Flat	5 mL	1 cm	None
Flat	5 mL	1 cm	None
Flat	10 mL	0 cm	Full
Flat	10 mL	1 cm	Partial (14/30)
Flat	10 mL	2 cm	Partial (8/30)
Flat	10 mL	3 cm	None
Flat	10 mL	1 cm	None
90	2 mL	0 cm	Full
90	2 mL	1 cm	None
90	5 mL	0 cm	Full
90	5 mL	1 cm	None
90	5 mL	1 cm	Partial (9/30)
90	10 mL	0 cm	Full
90	10 mL	1 cm	Partial (7/30)
90	10 mL	2 cm	Partial (1/30)
90	10 mL	3 cm	None

Regardless of angle and amount of water, the center of the ORD produced a full profile for all of the flat and 90° swatches. However, none of the samples from outside of the ORD gave a full profile. From the six swatches used for DNA analysis, there were 13 total samples outside of the ORD that were carried on to amplification. Seven of these samples came from the flat swatches, five of which gave no profile. The other six samples came from the 90° swatches, three of which gave no profile. The majority of the

samples outside of the ORD that gave a partial profile came from the swatches sprayed with 10 mL of water and from a distance of 1-2 cm from the perimeter of the ORD.

The samples taken from the center of the ORD had much higher peak heights than those from outside of the ORD, with averages ranging from approximately 2300-5000 RFU to approximately 30-50 RFU, respectively. The average peak height per locus was calculated for each profile, excluding the loci that exhibited dropout of one or both alleles.

Of the five samples outside of the ORD that gave partial profiles, only three were used to calculate average peak height per profile. One of the remaining two samples only produced one allele above threshold while the other only had one full locus present in the profile.

The amount of water the swatches were sprayed with did not seem to have an effect on the average peak heights of the samples. However, the distance from the ORD had a substantial effect on the average peak height. The samples from the center of the ORD had much higher average peak heights than the samples from outside of the ORD. The average PHR values for the samples from the center of the ORD were slightly higher than from 1 or 2 cm outside of the ORD. PHRs were only calculated for one of the samples outside of the ORD because the other four samples exhibited substantial dropout (Table 14).

**Table 14. DNA results from samples exposed to various amounts of water at different angles.** Average peak heights and peak height ratios were calculated for each full profile. Average peak heights were calculated for all but one partial profile, using any homozygous and complete heterozygous loci present. No average peak height ratio values were calculated for the partial profiles due to the large amount of dropout, indicated by n/a. ‡ indicates that because the quantification value for this sample was 0 ng/μL, the ng amplified was calculated to be 0. \*This sample only contained one allele in the entire profile so no average peak height value was calculated.

<b>Sample Angle</b>	<b>Amount of Water</b>	<b>Distance from ORD</b>	<b>Total DNA mass obtained</b>	<b>ng Amplified</b>	<b>Average profile peak height</b>	<b>Average profile peak height ratio</b>
Flat	2 mL	0 cm	16.9 ng	1	2346	0.931
Flat	5 mL	0 cm	47.3 ng	1	3566	0.926
Flat	10 mL	0 cm	76 ng	1	4729	0.921
Flat	10 mL	1 cm	0.05 ng	0.01	49.3	0.838
Flat	10 mL	2 cm	0 ng	0 <sup>‡</sup>	33.3	n/a
90	2 mL	0 cm	86.75 ng	1	4095	0.931
90	5 mL	0 cm	61.4 ng	1	5004	0.939
90	5 mL	1 cm	0.15 ng	0.03	31.7	n/a
90	10 mL	0 cm	22.2 ng	1	2494	0.914
90	10 mL	1 cm	0.05 ng	0.01	67	n/a
90	10 mL	2 cm	0.025 ng	0.02	n/a*	n/a

### 3.2.2 Effect of AP Spot reagent on DNA Analysis

The profiles from before and after AP Spot application appear similar in profile quality. Every sample from inside the semen stain gave a full profile and the majority of the samples from the edge of the stain gave partial profiles (maximum dropout rate of 13%) both before and after AP Spot treatment (Table 15).

**Table 15. Type of DNA profile obtained from samples with and without AP Spot reagent.** DNA analysis results were classified as “full”- all alleles at all loci are present, or “partial”- at least one correct allele for the sample is present. The number of correct alleles present in each partial profile is given. The sample numbers refer to the schematic in Figure 2, which depicts the location of fabric punches collected from the center of the semen stain, edge of the semen stain and halfway between the center and edge of the semen stain. Samples 1-10 do not have AP Spot reagent present and samples 15-24 have AP Spot reagent present.

<b>Sample Number</b>	<b>Sample Position</b>	<b>Type of Profile</b>
1	Center	Full
2	Center	Full
3	Halfway	Full
4	Halfway	Full
5	Halfway	Full
6	Halfway	Full
7	Edge	Partial (28/30)
8	Edge	Partial (27/30)
9	Edge	Partial (26/30)
10	Edge	Full
15	Center	Full
16	Center	Full
17	Halfway	Full
18	Halfway	Full
19	Halfway	Full
20	Halfway	Full
21	Edge	Partial (27/30)
22	Edge	Partial (26/30)
23	Edge	Partial (29/30)
24	Edge	Full

The average peak height was calculated for each profile. There did not seem to be any effect attributable to the presence of AP Spot reagent; the peak height values ranged from approximately 2000 RFU to approximately 4000 RFU for both the samples with and without AP Spot pre-treatment. The PHR values were consistently above 0.875 for

all samples containing at least 0.25 ng/ $\mu$ L of DNA, regardless of the presence of AP Spot reagent (Table 16).

**Table 16. Comparison of DNA results for samples before and after AP Spot reagent application.** Sample 1 indicates the samples without AP Spot reagent present; Sample 2 indicates the samples with AP Spot reagent present. The average peak height and peak height ratio values were calculated for each profile.

Sample 1 (No AP)	Sample 2 (AP)	ng Amplified Sample 1	ng Amplified Sample 2	Avg. Peak Height Sample 1	Avg. Peak Height Sample 2	Avg. PHR Sample 1	Avg. PHR Sample 2
1 (center)	15 (center)	1	1	3840	4261	0.908	0.905
2 (center)	16 (center)	1	1	3382	3544	0.933	0.917
3 (halfway)	17 (halfway)	1	1	3545	2538	0.938	0.908
4 (halfway)	18 (halfway)	1	1	4045	2265	0.905	0.876
5 (halfway)	19 (halfway)	1	0.82	3098	2024	0.888	0.913
6 (halfway)	20 (halfway)	1	1	2691	2540	0.899	0.926
7 (edge)	21 (edge)	0.07	0.03	94.3	61.2	0.859	0.861
8 (edge)	22 (edge)	0.03	0.02	123	82	0.810	0.772
9 (edge)	23 (edge)	0.11	0.07	127	103	0.856	0.771
10 (edge)	24 (edge)	0.13	0.11	266	168	0.851	0.821

### 3.2.3 Swatches Contaminated with Blood

The large amount of blood present on these samples greatly affected DNA analysis, likely due to the absence of a cleanup step in the extraction protocol. Because inhibition of the quantification process was anticipated, a sample from each center punch was diluted 1:10 and tested. These were the only samples that did not display inhibition and were carried on to amplification and capillary electrophoresis. The DNA from the

blood was not detected by the quantification or amplification kits because it was non-human.

The diluted samples did not show any inhibition when amplified. Each sample produced a full profile with an average peak height ranging from 1445-2211 RFU and an average PHR ranging from 0.88-0.93 (Table 17).

**Table 17. Average peak heights and peak height ratios for samples from blood swatches.** Average peak height ratios were calculated using all heterozygous loci that did not exhibit any allelic dropout.

<b>Sample Name</b>	<b>Average profile peak height (RFU)</b>	<b>Average PHR</b>	<b>Amount of DNA obtained (ng/<math>\mu</math>L)</b>
Blood 2 ORD 1:10	2211	0.88	0.339
Blood 5 ORD 1:10	1381	0.89	0.327
Blood 10 ORD 1:10	1445	0.93	0.363

## **4. DISCUSSION**

### **4.1 Samples Exposed to Various Amounts of Water at Different Angles**

Each swatch in this sample set demonstrated positive presumptive and confirmatory results both inside and outside of the ORD. As predicted, the soluble components of seminal fluid (*i.e.* the fluorescing component(s), AP, and P30) demonstrated migration outside of the ORD up to several centimeters each while the spermatozoa primarily stayed in their original location. Though the angle of the swatch appeared to affect the direction(s) of migration on some samples, the amount of water each swatch was exposed to had a more significant overall effect on the extent of migration that occurred for each soluble component tested. The samples positioned at 45° or 90° mostly showed migration patterns for fluorescence, AP, and P30 that correlated with gravity (*i.e.* the majority of the migration was directed below and to the sides of the ORD). Although an analyst would not necessarily know what position an item of evidence has been in, it was important to evaluate the migration patterns under different conditions. The three angles used in this study were chosen to represent a range of positions that an evidentiary item might be in during contact with water.

The results of the ALS examination revealed fluorescence extending at least one centimeter outside of the ORD on all swatches; however, the fluorescence did extend farther on the swatches exposed to more water. The amount of water also affected the intensity of the fluorescence. The swatches sprayed with more water displayed fainter fluorescence than the swatches sprayed with less water, as expected because these stains became more diluted.

Another observation made during the ALS examination was that the perimeter of the ORD was brighter than any fluorescence inside or outside of the ORD for the 2 and 5 mL swatches. This is not an uncommon finding for diluted semen stains, as this phenomenon was also noted in a study regarding the ALS detection of body fluid stains under various conditions (e.g. serial dilutions and different fabric types)<sup>14</sup>. This observation can be useful when examining evidence that may have been exposed to water as it may help differentiate the original stain (and the location of the spermatozoa) from seminal fluid migration.

Similarly, P30 was detected inside and outside of the ORD for all swatches. Like the results of the ALS examination, farther migration was seen on the samples exposed to more water. The majority of the samples that gave a positive P30 result also gave a positive AP Spot result and exhibited fluorescence. However, these samples also showed minimal or no spermatozoa, and could cause an analyst to erroneously conclude that the semen stain is very dilute, degraded or from an azoospermic male. If case circumstances suggest that sexual assault evidence was exposed to water either through washing, precipitation or submersion, the analyst should exercise caution when interpreting presumptive and confirmatory test results for semen.

The AP Spot results for all of these swatches show a difference in the results from 90 seconds and 5 minutes after reagent application. This can be attributed to a few factors. The edges of the AP migration may be weaker and need more time to react. Many laboratories use a 2 minute or less cutoff for AP testing, however, it has been shown that there is no scientific support for such a short cutoff and that this may be

insufficient for more dilute stains<sup>18,20</sup>. In one study, 57% of samples (ranging from neat semen to a 1:1000 dilution) gave a positive result after 2 minutes<sup>18</sup>. In another study using the transfer method (wetting a piece of filter paper before pressing it against the semen-stained fabric and then testing the paper), dilutions of greater than 1:40 required 5-10 minutes to be detected<sup>20</sup>. If sexual assault evidence is known or suspected to have come into contact with water, it may be necessary to increase the reaction cutoff time to ensure the best chances of detecting diluted semen. Although it is noted that increasing the reaction time may also increase the chance of a false positive result (e.g. from vaginal secretions)<sup>20</sup>, those samples would be identified as such through further testing.

Interestingly, positive P30 and AP results extended 6 cm above the ORD for the 90° swatch sprayed with 10 mL of water. This is the opposite of what one would expect based on the direction of flow due to gravity. There are a few possible explanations for these results. It is possible that although the position of the spray bottle was checked with a level prior to spraying, it became slightly angled upon manipulation while spraying. It is also possible that the holes in the nozzle are not perfectly straight, causing a slightly angled spray. A third possibility stems from the wicking properties of the fabric. Capillary action is a force that pulls the liquid through the fabric structure without any external force<sup>30</sup>. Cotton fibers were previously found to have a high flow rate due to several factors including high porosity, large diameter of the fiber and low surface tension<sup>30</sup>. It has also been shown that water moves quickly along a fabric initially and then slows<sup>31</sup>. Because the wicking properties of each fabric are different, this is a factor

to consider if sexual assault evidence exposed to water is received. Table 18 provides a summary of the serology results for this sample set.

**Table 18. Summary of serology results for samples exposed to various amounts of water at different angles.** The positive results obtained at each listed distance in any direction were combined. Samples from all angles at each amount of water were also combined, producing a maximum total number of samples at each distance of 12. “F” indicates fluorescence, “P” indicates P30, “S” indicates spermatozoa, and “A” indicates acid phosphatase.

<b>Sample position</b>	<b>2 mL Water</b>	<b>5 mL Water</b>	<b>10 mL Water</b>
ORD	F (3/3) P (3/3) S (3/3) A (3/3)	F (3/3) P (3/3) S (3/3) A (3/3)	F (3/3) P (3/3) S (3/3) A (3/3)
1 cm	F (9/12) P (7/12) S (0/12) A (10/12)	F (8/12) P (11/12) S (0/12) A (12/12)	F (6/12) P (9/12) S (2/12) A (10/12)
2 cm	F (1/12) P (1/7) S (0/7) A (6/12)	F (2/12) P (2/11) S (2/11) A (10/12)	F (5/12) P (4/7) S (1/7) A (8/12)
3 cm	F (0/12) P (0/1) S (0/1) A (2/12)	F (0/12) P (1/2) S (0/2) A (3/12)	F (1/12) P (2/4) S (0/4) A (6/12)
4 cm	F (0/12) P (0/12) S (0/12) A (0/12)	F (0/12) P (0/12) S (2/12) A (1/12)	F (0/12) P (1/12) S (1/12) A (3/12)
7 cm	F (0/12) P (0/12) S (0/12) A (0/12)	F (0/12) P (0/12) S (0/12) A (0/12)	F (0/12) P (0/12) S (0/12) A (1/12)

The DNA results confirmed that there was minimal spermatozoa migration. Full profiles were obtained from the center of each ORD, however the samples from outside of the ORD either gave a partial or no profile. The best profile obtained from outside of

the ORD had dropout of 16 of 30 alleles. The high rate of dropout could present an issue with analysis because loci with only one allele present could be mistakenly interpreted as homozygous.

The majority of the samples from outside of the ORD that gave partial profiles were from swatches sprayed with 10 mL of water and were located 1 cm from the perimeter of the ORD, suggesting that being exposed to more water facilitated some sperm migration, but not very much compared to the water soluble components of semen. Although the amount of water, if any, that a piece of evidence is exposed to will likely be unknown, it may be possible to estimate based on case circumstances, and it is suggested that multiple areas are sampled for DNA analysis.

The amount of water sprayed on the swatch did not seem to affect the PHR and peak heights obtained. As expected, the samples from outside of the ORD gave significantly lower peak heights compared to those from the center of the ORD. This information as well as the low quantification values confirms minimal spermatozoa migration.

#### **4.2 AP Spot Pre-treatment**

Pre-treating samples with AP Spot reagent was found not to have any detrimental effects on P30 testing or KPIC sperm staining. The P30 results from the flat swatches exposed to AP Spot reagent were similar to those obtained from the flat swatches without AP Spot reagent. The slide ratings for the center of each ORD for the AP Spot pre-treated swatches were also similar to those from the center slides from the flat swatches.

Perhaps more importantly, AP Spot reagent had no detrimental effects on DNA analysis. A full profile was obtained from every sample inside of the stain, regardless of the presence of AP Spot. The only samples that did not provide a full profile were from the edge of the stain. The PHR and peak height values before and after AP Spot application were very similar. The samples exhibiting lower peak heights and PHRs came from the edge of the stain and had much less DNA present.

These findings are somewhat in contrast with previous research, which found that direct application of AP reagent had a detrimental effect on some semen stains, exhibited by lower peak areas and profile quality<sup>19</sup>. Differences in AP reagent preparation and application and/or DNA extraction and amplification buffers or kits may explain this disparity.

Treating evidence directly with AP Spot reagent is not common practice in order to preserve the integrity of the evidence. However, the current study has shown that the reagent has no detrimental effects on downstream testing methods, suggesting that direct AP reagent application be utilized when semen stains cannot otherwise be readily located. One consideration is the potential of causing AP migration to occur from exposure to the test reagent itself. A study by Davidson and Jalowiecki showed that when fabric was directly sprayed with AP reagent, the AP positive area was slightly extended due to the solubility of acid phosphatase<sup>17</sup>. This phenomenon was observed in the current study when spraying the control swatch (made in the same way as the other swatches but without any water added) with AP Spot reagent; positive results extended approximately a centimeter outside of the ORD. This can also be demonstrated by comparing the area of

the positive AP result after 90 seconds with the positive area after 5 minutes. As shown in Figures 4, 5, and 7, the area yielding a positive AP result extends farther away from the original stain over time. However, as long as the analyst did not exclusively sample from the edge of the AP positive region, downstream testing and obtaining a DNA profile is likely to be successful.

#### **4.3 Swatch Moistened over Multiple Days**

This swatch displayed some differences from the previous sample sets. The ALS examination was much more challenging because even the fluorescence inside the ORD became difficult to see after day 2. This suggests that adding water incrementally has more of a diluting effect on the fluorescence than adding water all at once. Similar observations were reported in the study regarding the effects of porcine decomposition fluid on biological screening methods. Upon exposure to the porcine decomposition fluid and environmental moisture, multiple regions of faint fluorescence were noted outside of the region where the samples were deposited<sup>6</sup>. The fact that there appears to be a difference in fluorescence migration between the swatches moistened once and the swatch moistened several times suggests that evidence exposed to moisture on multiple occasions may be more likely to have no visible fluorescence, even when semen is present.

There were no drastic differences between the P30 and sperm search results for days 2 and 4. However, there were some differences compared to the 5 and 10 mL 90° swatches, suggesting that the exposure to water over several days does have an impact.

The P30 results for days 2 and 4 of the multi-day swatch are slightly farther away from the ORD than those for the 5 and 10 mL 90° swatches (extending an additional centimeter in most cases).

This swatch also exhibited differences in AP Spot results. The appearance of the violet color change was much fainter and had a more speckled appearance. This is unlike any other sample tested, suggesting that being moistened (and dried) over multiple days had a more diluting effect than being moistened just once. There was a marked difference between the results at 90 seconds and 5 minutes, again signifying that the sample was much more dilute and needed a longer reaction time. This reinforces the recommendation that evidence that may have been washed or otherwise dampened should be sampled and tested in multiple areas.

#### **4.4 Swatches Contaminated with Blood**

The swatches moistened with blood posed a challenge during several steps of the analysis. The ALS examination was difficult because the blood obscured much of the fluorescence on all of the swatches. This is not an uncommon observation and concurs with a study that attempted to improve fluorescence detection on casework samples<sup>32</sup>. One potential solution to this issue would be to try different wavelengths within the semen excitation spectrum.

The presence of blood did not have any effect on P30 testing. An interesting observation is that the P30 migration extended further on the swatch moistened with 10 mL of blood compared to the flat swatches moistened with 10 mL of water. This could

possibly be due to the makeup of the blood (*i.e.* the presence of cellular material and different viscosity) compared to water.

The presence of blood also did not affect sperm staining. However, the 3 slides from the center of each ORD had less sperm than the 9 center slides from the other samples. One possible explanation is that exposure to blood caused the sperm to adhere more strongly to the fabric, possibly requiring a different extraction procedure or longer extraction time for these types of samples.

There was also some difficulty with AP Spot testing. Due to the dark color of the blood, these swatches could not be sprayed directly with the reagent like the other sample sets. AP mapping via the transfer method was performed for these swatches and negative results were obtained. This could possibly be due to the blood layered on top of the semen, preventing the AP from transferring to the filter paper. This could potentially be remedied by moistening the fabric directly with water or pressing the filter paper on the fabric for a longer period of time. Another potential solution could be turning the fabric over and testing the back side either directly or by mapping. It is possible that the semen stain absorbed completely through the fabric and could be detectable on the other side.

The DNA testing of the semen stains contaminated with blood also presented some difficulties. Because heme is a PCR inhibitor<sup>26,27</sup>, the majority of the samples did not amplify in the quantification step. The only samples not exhibiting inhibition were 1:10 dilutions of the extracts from the center of each ORD. These diluted samples all had high average peak heights and PHR values, indicating that diluting the samples allowed for successful amplification despite the presence of heme. Because only the extracts from

the center of each ORD were diluted and successfully processed, no conclusions can be definitively made regarding the potential to obtain DNA from outside of the ORD on the samples contaminated with blood. However, due to the lack of sperm migration with these samples, it is likely that results similar to those outside of the ORD on the other swatches would be obtained. Casework samples that contain concentrated bloodstains should be diluted or a more suitable extraction method utilized.

## **5. CONCLUSIONS**

Sexual assault evidence that has been exposed to water or blood flow should be analyzed with caution. When dried semen stains are moistened with water or blood, the soluble components can migrate up to several centimeters from where the stain is deposited, causing fluorescence as well as AP and P30 activity in locations containing very few or no spermatozoa. This could result in the stain being misinterpreted as originating from an azoospermic male, being too dilute or degraded for successful DNA analysis or a false positive result for semen. If case circumstances suggest that evidence has been exposed to water, analysts should not limit testing to the edge of a fluorescing or AP positive region. Multiple regions in the area of the suspected semen stain should be tested to ensure the chances of finding spermatozoa and obtaining a DNA profile are maximized.

The presence of AP Spot reagent on an item of evidence was found to have no negative effects on downstream testing for P30, microscopic sperm staining or conducting DNA analysis. Direct application of AP reagent could be useful when testing a large item where the location of the stain is unknown because the careful documentation needed for mapping will be eliminated and the transfer of acid phosphatase from one substrate to another would not be required.

## **6. Future Directions**

A limitation of this study was that only one swatch was created for each condition. A future study should replicate these parameters in a larger sample set enabling more definitive conclusions to be drawn, particularly for stains wetted over multiple days. It would be of interest to observe the effects of different amounts of water over different periods of time (e.g. 10 mL over 10 days instead of 4). Additionally, vaginal secretions should be mixed with the semen to simulate sexual assault samples.

It would also be important to investigate the effects of semen moistening a dried semen stain to simulate multiple sexual assaults. Similarly, it would be of value to assess whether there were any differences between donors or the effects of moistening a dried semen stain with semen from another donor (e.g. to simulate a sexual assault by multiple assailants).

Finally, observing how wet semen stains behave under these conditions would be helpful in evaluating optimal semen screening and testing methods for casework. Swatches with wet semen stains could be subjected to the same conditions tested in this study to identify any trends or differences in migration patterns that may arise.

## LIST OF JOURNAL ABBREVIATIONS

Can Soc Forensic Sci J	Canadian Society of Forensic Science Journal
Forensic Sci Int: Genet	Forensic Science International: Genetics
Forensic Sci Int	Forensic Science International
Forensic Sci Med Pathol	Forensic Science, Medicine and Pathology
J Clin Pathol: Mol Pathol	Journal of Clinical Pathology: Molecular Pathology
J Forensic Sci	Journal of Forensic Sciences
J Legal Med	Journal of Legal Medicine
Proteomics Clin Appl	Proteomics- Clinical Applications
Sci Justice	Science and Justice
Text Res J	Textile Research Journal
Z Rechtsmed	Zeitschrift fur Rechtsmedizin (Journal of Legal Medicine)

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## CURRICULUM VITAE

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### Education

- **Boston University School of Medicine, Boston MA**
  - Master of Science in Biomedical Forensic Sciences Expected May 2016
  - GPA: 3.84
- **Marshall University, Huntington WV**
  - Bachelor of Science in Forensic Chemistry, Magna Cum Laude May 2014
    - Minors in Biology, Psychology, and Integrated Science & Technology
  - GPA: 3.69

### Technical Skills

- General: pipetting, microscopy
- Serology: alternate light source, blood screening (Kastle-Meyer, tetramethylbenzidine, leucomalachite green, ortho-tolidine, ABACard Hematrace, Ouchterlony gel), semen screening (acid phosphatase (AP Spot), ABACard P30, KPIC staining/sperm searching), saliva screening (starch-iodine gel, RSID-Saliva, Saligae, Phadebas paper)
- DNA analysis: gel electrophoresis, Sanger sequencing, extraction, quantification (Quantifiler Duo kit and Applied Biosystems 7500 RealTime PCR system), amplification (Identifiler Plus kit and Applied Biosystems PCR System 9700 thermal cycler), capillary electrophoresis (Applied Biosystems 3130 Genetic Analyzer), profile analysis (CodonCode Aligner and GeneMapper ID)

### Relevant Coursework

- Marshall University: statistics, genetics, biochemistry, cellular biology/lab, forensic DNA analysis/lab, organic chemistry/lab, analytical chemistry/lab, modern instrument methods/lab, physical chemistry/lab, physics/lab, criminal procedure, crime scene investigation
- Boston University: forensic biology/lab, forensic DNA analysis/lab, advanced DNA, crime scene investigation and mock court

### Relevant Experience

- Intern, Boston Police Department Crime Laboratory February 2016-Present
  - Review sexual assault case files and compile data
  - Determine trends for 2015 sexual assault cases
- Forensic Biology Laboratory/Teaching Assistant, Boston University July 2015-Present
  - In charge of maintaining cleanliness and inventory of the forensic biology laboratory as well as preparing necessary reagents/chemicals

- Responsible for preparing the laboratory exercises and mock evidence items for the class
- Assist in grading/reading students' laboratory and case reports
- Tutor, Marshall University Buck Harless Student Athlete Program February 2012-May 2013
  - Chemistry, biology, psychology, and Spanish

#### **Research Experience**

- Thesis research - Boston University School of Medicine June 2015-Present
  - Evaluation of the migration patterns of acid phosphatase, P30 and spermatozoa when semen stains are exposed to moisture
  - Utilized various forensic serology and DNA testing methods
  - Poster presented at 68<sup>th</sup> Annual AAFS meeting, Las Vegas NV, February 2016.
- 2-year summer fellowship - Masonic Medical Research Laboratory, Utica NY 2012-2013
  - Responsible for Sanger sequencing of Brugada Syndrome patient DNA samples to determine if a mutation or polymorphism was present in specific genes
  - The goal of this research was to possibly identify new genes of interest for Brugada Syndrome

#### **Affiliations/Memberships**

- Delta Delta Epsilon Forensic Science Honor Society August 2015-Present
  - Nu Chapter
- Boston University Forensic Science Society September 2014-Present
  - Elected Event and Outreach Coordinator for 2015
- Marshall University Honors College June 2011-May 2014
- National Society of Collegiate Scholars January 2011-Present
  - Elected President of the Marshall University chapter for 2013-2014
- Golden Key International Honors Society November 2011-Present
  - Elected Social Director of the Marshall University chapter for 2013-2014
- Gamma Beta Phi Honors Society October 2011-Present
- Alpha Chi Sigma Chemistry Fraternity April 2012-Present
  - Gamma Eta chapter
  - Elected Master of Ceremonies- Spring 2013
  - Elected Vice Master Alchemist (vice president)- Fall 2013
  - Elected Master Alchemist (president)- Spring 2014