

2022

Efforts toward optimization of sexual assault swab processing

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Thesis

**EFFORTS TOWARD OPTIMIZATION OF
SEXUAL ASSAULT SWAB PROCESSING**

by

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B.A., Rutgers University, 2020

Submitted in partial fulfillment of the
requirements for the degree of
Master of Science

2022

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ACKNOWLEDGMENTS

I am very grateful to Professor Brodeur and Dr. Cotton for their guidance during the research process. Thank you to my family, friends, and classmates for your constant encouragement and support. A special acknowledgment goes to the Master of Science in Statistical Practice (MSSP) consulting group for their thorough statistical analysis.

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ABSTRACT

One way to address the sexual assault kit (SAK) backlog in the United States is by improving the efficiency of SAK swab processing. Three areas which could benefit from optimization were addressed in this study. Swab elution time and number of centrifugations varies among forensic labs, therefore, precision in this step would reduce time spent performing these steps for longer than is necessary. If successful, staining spermatozoa pellets while in the tube, rather than on the microscope slide, would speed up the slide preparation process. Finally, using the same swab cutting tested for acid phosphatase (AP) during microscope slide preparation, immunoassay card testing, and deoxyribonucleic acid (DNA) analysis, would reduce the amount of consumed evidence and simplify the process.

Determination of ideal elution time and number of centrifugations was done by calculating the relative percentage of eluted spermatozoa after a first and second elution and comparing these values between four elution time categories. A procedure for in-tube spermatozoa staining was experimentally developed and evaluated by examination of microscope slides. Sets of experimental (exposed to AP Spot reagent) semen-only and semen-saliva swabs of various dilutions were compared to sets of control swabs by means of their microscope slides, immunoassay card test results and DNA profiles. Additionally,

the quantities of DNA extracted from post-eluted swabs were compared to those from spermatozoa pellets.

An elution time of 30 minutes was found to be just as adequate as longer elution times, and a single centrifugation following elution was determined to be sufficient for spermatozoa detection. The in-tube spermatozoa staining method had a negative effect on the number of spermatozoa present on the microscope slides, therefore it was not incorporated into subsequent procedures. AP Spot reagent seemed to have no effect on swab processing and downstream testing, and the amount of DNA remaining on post-eluted swabs is significant; thus, swabs should be recombined with their cellular pellets prior to DNA analysis.

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LIST OF ABBREVIATIONS

μL	Microliters
°C	Degrees Celsius
AP	Acid Phosphatase
ANOVA	Analysis of Variance
BPD	Boston Police Department
DNA	Deoxyribonucleic Acid
g	Grams
H&E	Hematoxylin and Eosin
i.e.	<i>Id est</i> (that is)
KPIC	Kernechtrot Picroindigocarmin
mL	Milliliters
ng	Nanograms
PCR	Polymerase Chain Reaction
PHR	Peak Height Ratio
PSA/p30	Prostate Specific Antigen/Protein 30
qPCR	Quantitative/Real Time Polymerase Chain Reaction
RFU	Relative Fluorescent Units
RPM	Revolutions Per Minute
RSID	Rapid Stain Identification Series
SAK	Sexual Assault Kit
SOP	Standard Operating Procedure

1. INTRODUCTION

1.1 Sexual Assault Kits

1.1.1 Backlog of Sexual Assault Kits

The backlog of sexual assault kits (SAKs) across the United States is a problem that is well known to the forensic community as well as to the public. This issue has been addressed on several levels; from the perspective of laboratory efficiency, the National Institute of Justice has recommended that laboratories change from the traditional SAK processing workflow to a Direct-to-DNA analysis process (1). However, laboratories may prefer a simpler way to increase efficiency in SAK processing by modifying their current Standard Operating Procedures (SOPs) rather than by replacing them entirely.

1.1.2 Traditional Processing of Sexual Assault Kit Swabs

The traditional processing of sexual assault swabs begins with an acid phosphatase (AP) presumptive test for semen (2,3). Present at a high concentration in semen, the enzyme AP hydrolyzes the test reagent alpha-naphthyl phosphate to alpha-naphthol, which combines with the test's diazonium salt to produce a colored dye (4). The AP Spot Test contains these test reagents (5). If the swab tests positive for AP, the analyst attempts to elute spermatozoa from the swab and prepares a microscope slide from the eluate.

To aid in the visualization of spermatozoa, staining reagents can be applied to the slide, the most commonly used being the Kernechtrot picroindigocarmine (KPIC) stain, also known as the Christmas Tree Stain, which was developed specifically for the purpose of staining spermatozoa in 1969 (6,7). Another staining technique is hematoxylin and eosin

(H&E), which was first described for forensic use in 1956 (7), and is extensively used by histologists to stain other types of cells (8). A study by Allery et al. compared the staining ability of KPIC, H&E, and a third stain called alkaline fuchsin. They found that KPIC and H&E both effectively stained spermatozoa and concluded that KPIC made the spermatozoa more discernable than H&E (9). The presence of spermatozoa is the only confirmatory test for semen (4,9).

Further testing for proteins that indicate the presence of semen may be performed using the supernatant produced by the swab elution. This may be done when the initial AP screening test is positive but no spermatozoa are visualized because it is possible that the semen originated from an azoospermic or vasectomized male, therefore lacking spermatozoa (4,10). Prostate specific antigen (PSA or p30) is one of the main proteins found in semen and can be detected using a lateral flow immunochromatographic assay such as the ABACard® p30 test (4,6). If it is suspected that a swab contains saliva as well, the supernatant can also be tested for amylase; the RSID™-Saliva immunochromatographic assay that detects human salivary alpha amylase is a testing option (4).

1.2 Procedural Targets for Optimization

1.2.1 Elution Time and Number of Centrifugations

In this study, three areas in sexual assault swab processing that could benefit from optimization were addressed, the first being elution time and centrifugation of swabs that presumptively test positive for semen. The length of time prescribed for spermatozoa

elution varies between forensic laboratories; among protocols from a sample of four laboratories, the time values include 2 hours to overnight, 1 hour, 30 minutes, and 10 minutes (11–14). Furthermore, the language used in these SOPs tends to be vague, modifying the recommended duration with a phrase such as, “at least,” or, “a minimum of,” seemingly indicating an inexact knowledge of the length of time that is sufficient. Therefore, if it were known with precision how much time is necessary for spermatozoa elution, efficiency could be improved. Furthermore, the Boston Police Department (BPD) SOP instructs analysts to perform an additional wash-and-centrifugation step to aid in the removal of spermatozoa from the swab (15), but the necessity of this step is unknown.

1.2.2 Spermatozoa Staining

This study’s second area of focus was an attempt to simplify spermatozoa staining by applying stain reagents to the spermatozoa eluate while in the tube, rather than to the microscope slide. A recently published study discovered that it is possible to stain spermatozoa with hematoxylin while in a microcentrifuge tube (16). If this concept could be successfully applied to slide preparation of spermatozoa eluate, time would likely be reduced and efficiency improved. It is unknown whether staining spermatozoa eluate in this manner would hinder downstream deoxyribonucleic acid (DNA) analysis. It has been found that H&E staining of vaginal and post-coital smear slides did not affect DNA analysis done on cells of the fixed slides (17). When DNA testing was done on archived slide-mounted epithelial cells stained with either KPIC or H&E, it was found that polymerase chain reaction (PCR) was not inhibited using either staining type, but while

full DNA profiles were generated from the H&E-stained cells, sometimes allele dropout occurred in the profiles of the KPIC-stained cells (18). Therefore, it would need to be determined if KPIC-stained spermatozoa eluates in particular have incomplete DNA profiles.

1.2.3 AP Spot Test and Post-Elution DNA on Swabs

The third opportunity for optimization addressed by this study concerned the typical practice of labs to perform the AP Spot test using an initial cutting of the evidence swab and, following a positive result, to take another cutting for elution of spermatozoa and further downstream testing (19). It is a principle of forensic science standards that testing of evidence should be minimized and chosen carefully in order to maintain sample integrity as much as possible and to save part of it for potential future testing (20–22). Less evidence would be consumed and efficiency improved if the same cutting used for the AP Spot test could be used for spermatozoa elution, immunoassay card testing and DNA analysis. In this laboratory, it has previously been found that when swatches of neat semen sprayed with AP Spot Test reagent were processed, staining of spermatozoa with KPIC, p30 testing, and DNA analysis were not affected (23). However, another study found that the electropherogram peaks of DNA profiles generated from swatches of various semen dilutions sprayed or aerosolized with AP reagent were lower than peaks from the samples to which AP reagent was not applied (3). Therefore, clarification was necessary to determine if AP Spot Test reagent has an effect on the processing of diluted semen stains.

Another point of interest investigated at the same time as determining the effect of AP Spot Test reagent was the amount of DNA remaining on swabs after elution compared to that contained in the eluted pellet. It has already been established that cotton swabs retain cellular material and DNA after extraction. Swabs of saliva have been found to retain 50%, and swabs of blood 80%, of DNA after extraction (24). A previous study in this lab compared the amount of DNA recovered from semen swabs after trypsin-ZyGEM extraction and a subsequent direct lysis. It was found that only about 10% of the DNA recovered after both extractions was recovered after the initial extraction (25). Also, a recent study found that the Qiagen EZ1® Advanced extraction method used to extract eluted swabs leaves significant amounts of DNA on the swabs (26).

1.3 Study Objectives

The first part of this study aimed to discover an effective length of time for spermatozoa elution and whether a second wash-and-centrifugation step is necessary. In the second part of this study, the goal was to determine if time can be saved by adding spermatozoa staining reagents directly to the tube containing the spermatozoa eluate without having a negative effect. The objective of the third component of this study was to discover if AP Spot Test reagent affects downstream testing, and therefore, if all serological and DNA testing can be conducted on a single cutting from a sexual assault swab. Furthermore, the third part of this study intended to discover if a significant quantity of DNA remains on an eluted swab.

2. MATERIALS AND METHODS

2.1 Elution Time and Number of Centrifugations

2.1.1 Preparation of Replicates

For each of the four elution time categories (1 minute initial elution, 30 minute initial elution, 1 hour initial elution, and 2 hours elution), ten replicate samples were prepared from two and a half swabs, for a total of forty replicates from a single semen donor. Each swab was prepared by pipetting 50 μ L of neat semen onto the tip of the sterile swab and allowing the swab to air dry for a minimum of 1 hour. The dried swab was packaged and remained at room temperature for approximately 24-72 hours before being processed. Each swab was cut in half with a scalpel and then into quarters with a pair of scissors in order to create four replicate samples of approximately the same size.

2.1.2 Processing of Replicates

Each quarter swab was placed into a microcentrifuge tube and 250 μ L of Rapid Stain Identification Series (RSID) Universal Buffer (Independent Forensics, Lombard, Illinois) was added, followed by elution on an orbital shaker for the specified length of time. After elution, the quarter swab was placed into a Costar® Spin-X® centrifuge tube filter (Corning®, Corning, New York) and the microcentrifuge tube was centrifuged for 5 minutes at 13,200 revolutions per minute (rpm). Following centrifugation, the quarter swab was transferred to a new microcentrifuge tube with new buffer for a second elution of 2 hours minus the time of the first elution. In the case of the 2 hours elution time category samples, the quarter swab was moved to a new microcentrifuge tube with new buffer and

processing continued immediately without the tube being placed on the orbital shaker (Figure 1). Next, 225 μL of supernatant was removed from each eluate and 3 μL of the remaining pellet was pipetted onto a 12-well 6 mm microscope slide. Each slide was heat fixed using a hot plate and stained using hematoxylin (Thermo Fisher Scientific, Waltham, Massachusetts) and eosin Y (Acros Organics (Thermo Fisher Scientific), Waltham, Massachusetts). Slide covers were affixed using Cytoseal-60 (Thermo Scientific, Swedesboro, New Jersey).

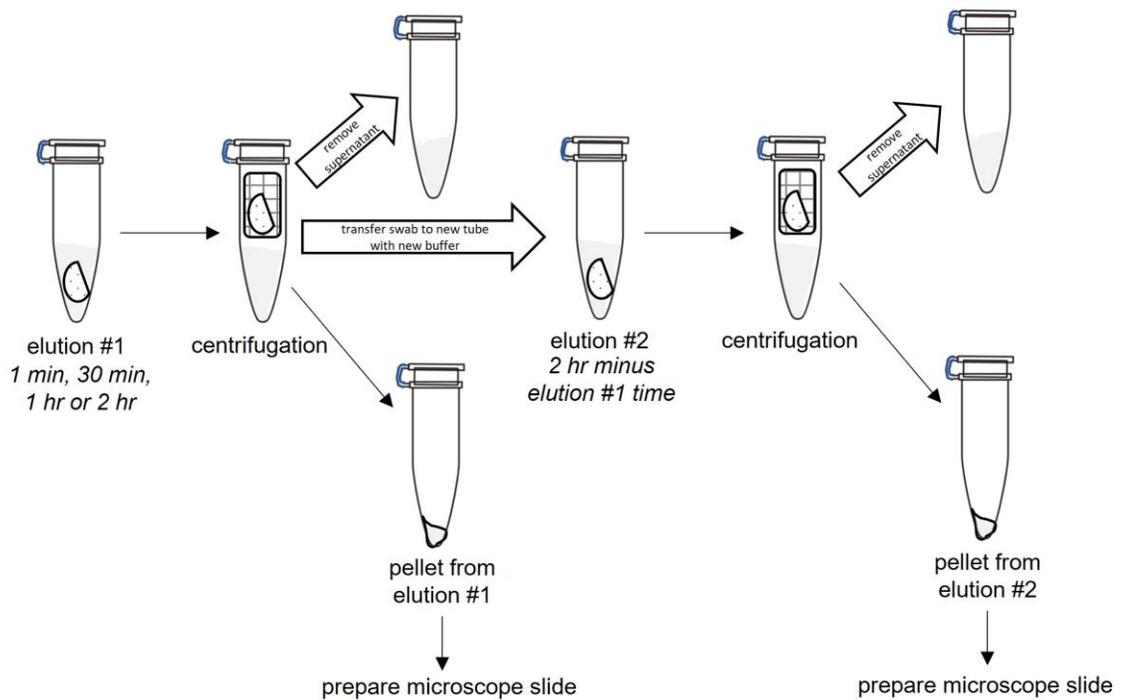


Figure 1. Elution time experiment processing of replicates. In the processing of the 2 hour elution samples, the quarter swabs were transferred to new tubes with new buffer and immediately centrifuged (elution #2 was skipped).

The microscope slides were examined at 400X magnification. Spermatozoa were counted with the assistance of a manual cell counter (Thermo Fisher Scientific, Waltham, Massachusetts) and the numbers of spermatozoa in clumps were estimated.

2.1.3 Calculation of Relative Amount of Eluted Spermatozoa

The relative percentages of spermatozoa observed after the first elution for each replicate were calculated (Figure 2), and relative percentages of spermatozoa observed after the second elutions were similarly calculated.

$$\text{Relative Percentage of Spermatozoa After First Elution} = \frac{\text{Number of Spermatozoa Observed After First Elution}}{\text{Sum of Spermatozoa Observed After Both Elutions}}$$

Equation 1. Calculation of relative percentage of eluted spermatozoa.

2.2 Spermatozoa Staining In-Tube

2.2.1 Preliminary Work

The final procedure was determined through experimentation with the following variables: volume of stain, use of one or two stains, use of H&E or KPIC, length of time for staining, when to remove supernatant, and use of a wash.

2.2.2 Final Procedure

Fifty microliters of diluted liquid semen (1:5, 1:10, 1:50) from the same donor whose semen was used in the Elution Time experiment was pipetted into a microcentrifuge

tube and one drop of the first staining reagent was added. Staining was allowed to occur for a prescribed length of time: 1 minute for hematoxylin, when staining with H&E, and 5 minutes for Xmas Tree Stain A (Serological Research Institute, Richmond, California), when staining with KPIC. Two hundred microliters of water were added, and the tube was centrifuged for 2 minutes. The supernatant was removed and 1 drop of the second staining reagent was added and allowed to stain for a certain length of time: 1 minute for either eosin Y or Xmas Tree Stain B (Serological Research Institute, Richmond, California). Two hundred microliters of water were added, the tube was centrifuged for 2 minutes, and the supernatant was removed. Three microliters of the pellet were pipetted onto a slide and heat fixed. The coverslip was attached using Cytoseal-60. The microscope slides were examined at 400X magnification.

2.3 AP Spot Test and Post-Elution DNA on Swabs

2.3.1 Semen-Only Swabs

For each of the four sample type categories (neat, 1:25, 1:100, and 1:250), two swabs were prepared using 50 μ L of semen or semen dilution from the same semen donor as in the Elution Time experiment. Similar to the Elution Time experiment, each swab was divided into four replicates and packaged for 24-72 hours before processing. One set of replicates was tested using the experimental method (tested with AP Spot Test) and one set of replicates was tested using the control method (not tested with AP Spot Test). AP Spot Test working solution was prepared daily from 0.13 g AP Spot Test (Serological Research Institute, Richmond, CA) and 5 mL Millipore water.

The samples were processed similarly to the samples in the Elution Time experiment, with the following exceptions: elution time was always 30 minutes, one centrifugation was performed for 2 minutes, and the slides were stained using KPIC. The microscope slides were examined at 400X magnification and spermatozoa scores were assigned (Table 1). The supernatants were frozen to be tested later with ABACard® p30 cards (Abacus Diagnostics, Inc., West Hills, CA). ABACard® p30 testing was performed by pipetting 200 µL of supernatant into the sample well and waiting 10 minutes to read the results (27). Before testing the experimental and control replicates for p30, a set of four swabs (neat, 1:25, 1:100, and 1:250) was tested for p30 to confirm that p30 could be detected at all of the dilution levels.

Table 1. Spermatozoa score values.

0	no spermatozoa observed on slide
<1+	fewer than 10 spermatozoa observed on slide
1+	a few spermatozoa in some fields; difficult to locate
2+	at least one spermatozoon in most fields
3+	several spermatozoa in most fields; easy to locate
4+	many spermatozoa in most fields

2.3.2 Semen-Saliva Swabs

For each of the four sample type categories (neat, 1:25, 1:100, and 1:250), two swabs were prepared using 25 μL of semen or semen dilution from the same semen donor as in the Elution Time experiment and 25 μL of saliva or saliva dilution from a single female donor. Two quarter-swab replicates were used from each swab; one set of replicates was tested according to the experimental method and one set according to the control method. Swab preparation and processing was similar to that for the semen-only swabs. In addition to ABACard® p30 testing, RSID™-Saliva card testing was carried out by adding 80 μL of RSID Universal Buffer to 20 μL of sample extract, pipetting the resulting volume of 100 μL into the sample well and waiting 10 minutes to read the results (28).

2.3.3 DNA Analysis

DNA was extracted from the frozen pellets (the same used to prepare the microscope slides) and processed quarter swabs using the Qiagen QIAamp DNA Investigator Kit (Qiagen, Hilden, Germany). The semen-saliva swabs required an extra series of steps to first separate the epithelial cells from the spermatozoa. Five hundred microliters of 56 °C ATL buffer and 20 μL proteinase K were added to the samples, which were then incubated at 37 °C for 2 hours with vortexing at 15-minute intervals. The swab samples were placed in Spin-X baskets and then all the samples were centrifuged at 14,000 rpm for 1 minute. Another centrifugation was done at 15,000 rpm for 5 minutes, after which the supernatants, now designated as the epithelial cell extracts, were removed from the spermatozoa pellet. The epithelial cell extracts were then vortexed in 300 μL of 56 °C

AL buffer, and their processing was continued with the 70°C incubation below. The spermatozoa pellets were twice re-suspended in 700 µL of 56 °C ATL buffer, centrifuged at 14,000 rpm for 5 minutes, and had the supernatants removed.

The following steps were carried out for both the semen-only and semen-saliva swab samples. Each sample was vortexed in 280 µL of 56 °C ATL buffer and then mixed with 10 µL proteinase K and 10 µL 1M DTT (Sigma-Aldrich Darmstadt, Germany). The samples were then incubated at 56 °C for 2 hours with vortexing at 15-minute intervals. The samples were vortexed in 300 µL of 56 °C AL buffer and incubated at 70 °C for 10 minutes with vortexing at 3-minute intervals. The samples were centrifuged at 14,000 rpm for 1 minute, and in the case of the swabs (of the semen-only samples), the swabs were first placed in Spin-X baskets. The supernatants were vortexed in 150 µL of 200 proof ethanol (Sigma-Aldrich, Darmstadt, Germany) and transferred to QIAamp MinElute columns in collection tubes. The samples were centrifuged at 8,000 rpm for 1 minute and the columns were transferred to new collection tubes. Five hundred microliters of AW1 buffer were added to each column, followed by centrifugation at 8,000 rpm for 1 minute and transfer of the columns to new collection tubes. Similar steps were carried out using 700 µL AW2 buffer and 700 µL 200 proof ethanol. The samples were centrifuged at 14,000 rpm for 3 minutes and the columns were placed in new tubes. Following a room temperature incubation of 10 minutes, 50 µL ATE buffer were added to the columns. After a room temperature incubation of 5 minutes, the samples were centrifuged at 14,000 rpm for 1 minute.

DNA quantitation was performed using the Applied Biosystems™ Quantifiler™ Trio DNA Quantification Kit (Thermo Fisher Scientific, Waltham, Massachusetts). Five standards were prepared via a serial dilution, or a virtual standard curve was used. Master mix was prepared using 8 μL Quantifiler Trio Primer Mix and 10 μL Quantifiler Trio PCR Reaction Mix per sample. To each reaction well, 18 μL of master mix and 2 μL of sample or standard (or master mix, in the case of the No Template Control well) were added. When a virtual standard curve was used, a positive control was prepared from 95 μL of DNA dilution buffer and 5 μL of DNA standard, and 2 μL were added to a reaction well. Quantitative PCR (qPCR) was performed using the ABI PRISM® 7500 Real-Time PCR System (Thermo Fisher Scientific, Waltham, Massachusetts) in conjunction with the HID Real Time PCR Analysis Software v.1.3.

The replicates which were extracted from the swabs (and not those extracted from the pellets) were amplified using the GlobalFiler™ PCR Amplification Kit (Thermo Fisher Scientific, Waltham, Massachusetts). Master mix was prepared using 7.5 μL GlobalFiler™ Master Mix and 2.5 μL GlobalFiler™ Primer Set per sample. The volumes of DNA to be amplified were calculated based on a maximum volume of 15 μL and a target mass of 0.75 ng (although for samples with smaller quantities, this target mass was not reached). Samples were diluted with TE buffer if necessary. To each reaction well, 10 μL of master mix and the appropriate volumes of DNA sample and TE buffer were added (for a total volume of 25 μL per well). The negative control contained 15 μL of TE buffer, and the positive control contained 10 μL of TE buffer and 5 μL of DNA Control 007. Amplification was performed using the Applied Biosystems Veriti 96 well thermal cycler

(Thermo Fisher Scientific, Waltham, Massachusetts) according to the following cycling parameters: 95 °C for 1 minute, 94 °C for 10 seconds and 59 °C for 90 seconds (for 29 cycles), 60 °C for 10 minutes, and a hold at 4 °C.

Capillary electrophoresis was performed using the Applied Biosystems SeqStudio™ genetic analyzer (Thermo Fisher Scientific, Waltham, Massachusetts). Master mix was prepared using 9.4 µL of Hi-Di formamide and 0.6 µL of GeneScan™ 600 LIZ™ size standard (Thermo Fisher Scientific, Waltham, Massachusetts) per sample. To each well of the 96-well MicroAmp reaction plate, 10 µL of master mix and 1 µL of amplicon or GlobalFiler™ allelic ladder were added. A septum was placed on top of the plate, which was then vortexed, centrifuged, heated to 95 °C for 3 minutes and chilled at -20 °C for at least 3 minutes. The plate setup was done using the Applied Biosystems Plate Manager software according to the following parameters: HID application type, GS600-Liz (60-460) size standard, J6 (DS-36) dye set, and HIDAnalysis with a 5 second injection time as the run module.

Analysis was performed using GeneMapper™ ID-X v.1.6 (Thermo Fisher Scientific, Waltham, Massachusetts) with the GlobalFiler™ SeqStudio™ analysis method, which included an analytical threshold of 100 relative fluorescence units (RFU), and a stutter filter.

3. RESULTS

3.1 Elution Time and Number of Centrifugations Results

3.1.1 Spermatozoa Counts and Relative Percentages

Spermatozoa were detected in all replicates at all elution times, including at the 1-minute mark. The percentage of spermatozoa collected from the first elution was greater than the second elution at the 2-hour mark for all but two samples. This was particularly true for the 30 minute, 1 hour and 2 hour elution times. Spermatozoa counts were used to calculate relative percentages of spermatozoa released after the first and second elutions (Tables 2-5). The relative percentages of spermatozoa released during the initial elutions seemed to increase between the 1 minute and 30 minute elution times, but not when eluting for longer than 30 minutes.

Table 2. One-minute initial elution spermatozoa counts and relative percentages. Replicate #4 was the only sample that had a larger relative percentage of spermatozoa observed after the second elution than after the first elution. R=replicate E= elution

Sample	Spermatozoa Count	Total Spermatozoa of Replicate	Relative Percentage
R#1E#1	98	113	86.7%
R#1E#2	15		13.3%
R#2E#1	37	56	66.1%
R#2E#2	19		33.9%
R#3E#1	34	46	73.9%
R#3E#2	12		26.1%
R#4E#1	28	61	45.9%
R#4E#2	33		54.1%
R#5E#1	55	90	61.1%
R#5E#2	35		38.9%
R#6E#1	31	50	62.0%
R#6E#2	19		38.0%
R#7E#1	64	77	83.1%
R#7E#2	13		16.9%
R#8E#1	59	63	93.7%
R#8E#2	4		6.3%
R#9E#1	281	299	94.0%
R#9E#2	18		6.0%
R#10E#1	264	280	94.3%
R#10E#2	16		5.7%

**Table 3. Thirty-minute initial elution spermatozoa counts and relative percentages. R=replicate
E=elution**

Sample	Spermatozoa Count	Total Spermatozoa of Replicate	Relative Percentage
R#1E#1	484	493	98.2%
R#1E#2	9		1.8%
R#2E#1	286	323	88.5%
R#2E#2	37		11.5%
R#3E#1	447	489	91.4%
R#3E#2	42		8.6%
R#4E#1	165	170	97.1%
R#4E#2	5		2.9%
R#5E#1	778	784	99.2%
R#5E#2	6		0.8%
R#6E#1	571	598	95.5%
R#6E#2	27		4.5%
R#7E#1	483	514	94.0%
R#7E#2	31		6.0%
R#8E#1	466	479	97.3%
R#8E#2	13		2.7%
R#9E#1	364	385	94.5%
R#9E#2	21		5.5%
R#10E#1	270	271	99.6%
R#10E#2	1		0.4%

Table 4. One-hour initial elution spermatozoa counts and relative percentages. R=replicate E=elution

Sample	Spermatozoa Count	Total Spermatozoa of Replicate	Relative Percentage
R#1E#1	121	130	93.1%
R#1E#2	9		6.9%
R#2E#1	82	93	88.2%
R#2E#2	11		11.8%
R#3E#1	152	160	95.0%
R#3E#2	8		5.0%
R#4E#1	121	126	96.0%
R#4E#2	5		4.0%
R#5E#1	9	10	90.0%
R#5E#2	1		10.0%
R#6E#1	240	243	98.8%
R#6E#2	3		1.2%
R#7E#1	206	219	94.1%
R#7E#2	13		5.9%
R#8E#1	156	166	94.0%
R#8E#2	10		6.0%
R#9E#1	66	73	90.4%
R#9E#2	7		9.6%
R#10E#1	143	177	80.8%
R#10E#2	34		19.2%

Table 5. Two-hour elution spermatozoa counts and relative percentages. Replicate #8 was the only sample that had a larger relative percentage of spermatozoa observed after the second elution than after the first elution. R=replicate E=elution

Sample	Spermatozoa Count	Total Spermatozoa of Replicate	Relative Percentage
R#1E#1	35	39	89.7%
R#1E#2	4		10.3%
R#2E#1	818	827	98.9%
R#2E#2	9		1.1%
R#3E#1	75	89	84.3%
R#3E#2	14		15.7%
R#4E#1	556	574	96.9%
R#4E#2	18		3.1%
R#5E#1	236	239	98.7%
R#5E#2	3		1.3%
R#6E#1	88	89	84.3%
R#6E#2	1		15.7%
R#7E#1	300	314	95.5%
R#7E#2	14		4.5%
R#8E#1	12	26	46.2%
R#8E#2	14		53.8%
R#9E#1	612	658	93.0%
R#9E#2	46		7.0%
R#10E#1	145	147	98.6%
R#10E#2	2		1.4%

3.1.2 Test for Statistical Significance Between Elution Time Categories

A one-way analysis of variance (ANOVA) test was conducted to test the null hypothesis that there was no statistically significant difference between the relative percentages of eluted spermatozoa for the four elution time categories. First, the natural

log ratio of each pair of first and second elution spermatozoa count values was calculated. This allowed for visualization of the data in a boxplot (Figure 2) to investigate if it seemed that one or more of the categories had a mean that was different from that of the other categories. It is apparent that the mean log ratio of the 1 minute initial elution group seems to be lower than that of the other groups.

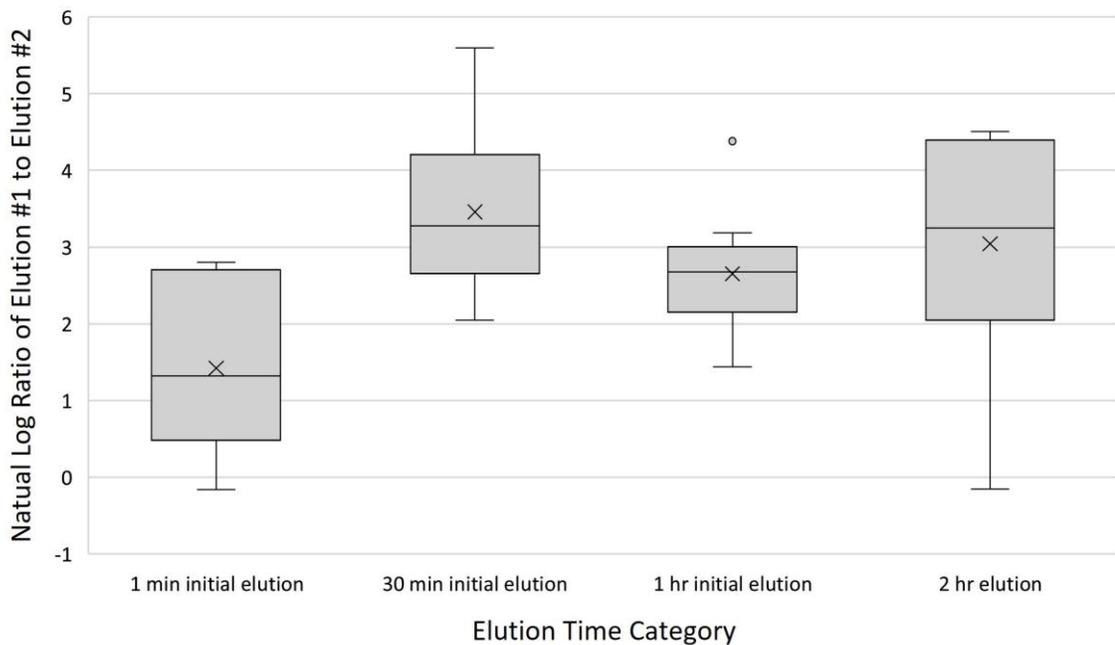


Figure 2. Boxplot of ratios between first and second elutions. The 1 minute initial elution category appears to have a lower mean log ratio than the other three categories.

Prior to performing the ANOVA test, it was determined that the assumptions for a one-way ANOVA test (that the data were normally distributed and the variance across groups was homogenous) were met. The ANOVA test was carried out using R statistical software (Table 6) and was validated using the Shapiro-Wilk normality test of the residuals. The chosen significance value, or alpha value, was 0.05, and if the p-value was larger than the alpha value, the null hypothesis was not rejected. Since the p-value of 0.00243 was

smaller than the alpha value of 0.05, the null hypothesis was rejected, meaning that at least one group was significantly different than the other groups.

Table 6. ANOVA test results. The relative percentages of the four elution time groups were tested.

	Degrees of Freedom	Sum of Squares	Mean Squares	F-Value	P-Value
Group	3	23.20	7.732	5.8	0.00243
Residuals	36	1.333			

In order to determine which of the groups was or were different, Tukey's test of multiple pairwise comparisons was performed, also utilizing R software and using an alpha value of 0.05 (Table 7). A difference that is not statistically significant is indicated in two ways: a p-value greater than 0.05 and a confidence interval containing 0. The results show that there was a statistically significant difference between the 1 minute initial elution and 30 minutes initial elution categories, as well as between the 1 minute initial elution and 2 hours elution categories. There could have been a significant difference between the 1 minute initial elution and 1 hour initial elution categories if the sample size had been larger; that there was no significant difference is likely due to sampling error.

Table 7. Tukey's test results. Each pair of the four elution time categories was tested.

Elution Time Category Pair	Difference in Means	Confidence Interval Lower Bound	Confidence Interval Upper Bound	P-Value
1 min / 30 min	2.04	0.65	3.43	0.00
1 min / 1 hr	1.23	-0.16	2.62	0.10
1 min / 2 hr	1.62	0.23	3.01	0.02
30 min / 1 hr	-0.81	-2.20	0.58	0.41
30 min / 2 hr	-0.42	-1.81	0.97	0.85
1 hr / 2 hr	0.39	-1.00	1.78	0.87

3.2 Spermatozoa Staining In-Tube Results

The experimentally determined final procedure for in-tube spermatozoa staining resulted in microscope slides that allowed for the visualization of spermatozoa with minimal background staining. However, the number of spermatozoa on each slide was greatly reduced compared to slides prepared using the traditional staining method. This can be seen in the contrast between Parts A and C of Figure 3; Part A shows the normal distribution of an abundance of spermatozoa, while Part C shows a small clump of spermatozoa. Similarly, Part B shows a reduced number of spermatozoa (as well as the background staining caused by H&E). Part D shows a large conglomeration of Xmas Tree Stain B and spermatozoa, which resulted from fixing the entire remaining volume of spermatozoa pellet onto the microscope slide.

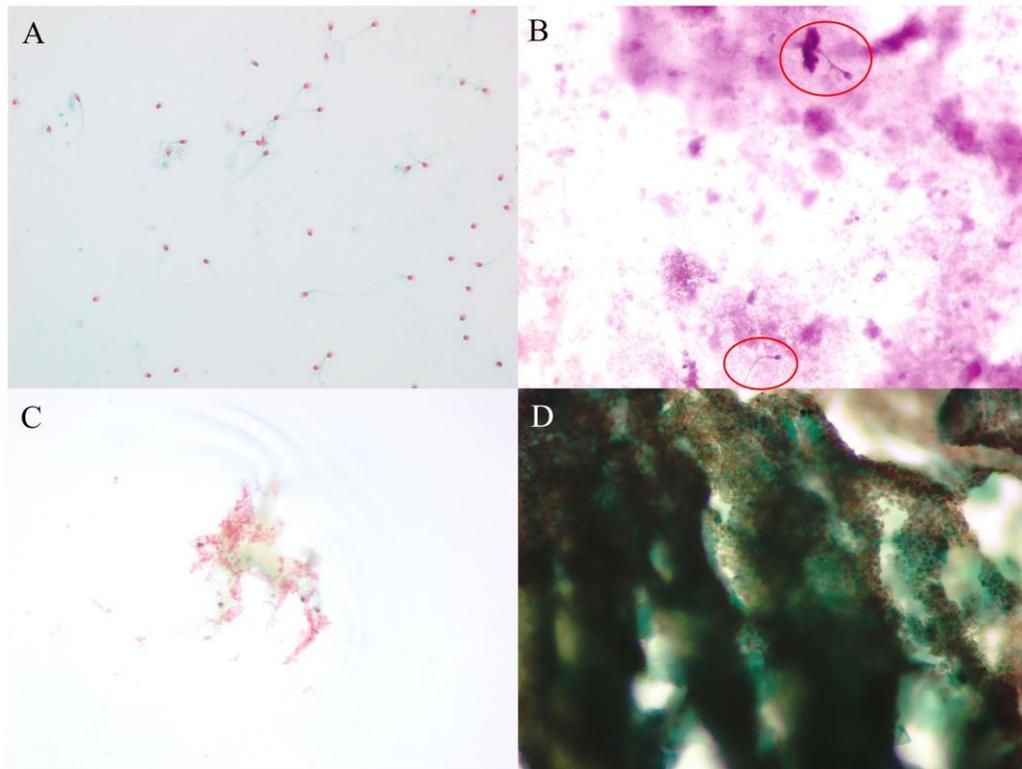


Figure 3. Microscope slides (400X) of semen samples. A) Traditional staining method with KPIC, 1:10 semen B) In-tube staining method with H&E, 1:50 semen, with the 2 spermatozoa circled C) In-tube staining method with KPIC, 3 μ L of pellet, 1:10 semen D) In-tube staining method with KPIC, entire remaining pellet, 1:10 semen

3.3 AP Spot Test and Post-Elution DNA on Swabs Results

3.3.1 Semen-Only Swabs

AP Spot reagent did not seem to affect the appearance of the microscope slides, including the spermatozoa and background staining. However, for the neat, 1:25, and 1:100 sample types, the semen replicates that were tested with AP Spot tended to have lower spermatozoa scores than the control samples, which were not tested with AP Spot (Table 8). The four control p30 samples and all the experimental and control replicates tested positive for semen using the ABACard® p30 immunoassay. The experimental neat

semen samples had a pink background color from the AP Spot reagent, but the test and control lines were still distinguishable.

Table 8. Semen-only swabs: spermatozoa scores. Experimental samples were tested with AP Spot reagent and control samples were not. R=replicate

Experimental		Control	
Sample	Spermatozoa Score	Replicate	Spermatozoa Score
Neat R#1	2+/3+	Neat R#1	3+
Neat R#2	<1+	Neat R#2	2+
Neat R#3	1+/2+	Neat R#3	3+
Neat R#4	1+/2+	Neat R#4	3+
1:25 R#1	<1+	1:25 R#1	1+
1:25 R#2	<1+	1:25 R#2	1+
1:25 R#3	1+	1:25 R#3	2+
1:25 R#4	<1+	1:25 R#4	1+
1:100 R#1	0	1:100 R#1	1+
1:100 R#2	<1+	1:100 R#2	1+/2+
1:100 R#3	1+	1:100 R#3	1+/2+
1:100 R#4	<1+	1:100 R#4	2+
1:250 R#1	<1+	1:250 R#1	0
1:250 R#2	0	1:250 R#2	0
1:250 R#3	<1+	1:250 R#3	0
1:250 R#4	0	1:250 R#4	1+

Quantities of DNA (small autosomal values) were measured by qPCR for all of the samples (except for the experimental 1:100 replicate #2 pellet) and the ratio between the quantity of DNA from the swab and pellet was calculated for each swab/pellet pair (Table 9).

Table 9. Semen-only swabs: DNA quantities of post-elution swabs and pellets. Experimental samples were tested with AP Spot reagent and control samples were not. R=replicate Exp=experimental Ctrl=control

Sample	Quantity (ng/ μ L)	Swab Quantity / Pellet Quantity
Exp neat swab R#1	6.3101	13.4
Exp neat pellet R#1	0.4704	
Exp neat swab R#2	8.4870	17.5
Exp neat pellet R#2	0.4843	
Exp 1:25 swab R#1	0.2879	7.0
Exp 1:25 pellet R#1	0.0410	
Exp 1:25 swab R#2	0.1992	4.5
Exp 1:25 pellet R#2	0.0446	
Exp 1:100 swab R#1	0.0083	1.9
Exp 1:100 pellet R#1	0.0044	
Exp 1:100 swab R#2	0.0066	n/a
Exp 1:100 pellet R#2	0	
Exp 1:250 swab R#1	0.0349	6.2
Exp 1:250 pellet R#1	0.0056	
Exp 1:250 swab R#2	0.0104	3.2
Exp 1:250 pellet R#2	0.0033	
Ctrl neat swab R#1	3.770	13.2
Ctrl neat pellet R#1	0.2864	
Ctrl neat swab R#2	4.3130	44.4
Ctrl neat pellet R#2	0.0971	
Ctrl 1:25 swab R#1	0.1436	2.8
Ctrl 1:25 pellet R#1	0.0508	
Ctrl 1:25 swab R#2	0.1426	2.7
Ctrl 1:25 pellet R#2	0.0536	
Ctrl 1:100 swab R#1	0.0247	1.0
Ctrl 1:100 pellet R#1	0.0237	
Ctrl 1:100 swab R#2	0.1609	2.1
Ctrl 1:100 pellet R#2	0.0748	
Ctrl 1:250 swab R#1	0.0198	3.6
Ctrl 1:250 pellet R#1	0.0056	
Ctrl 1:250 swab R#2	0.0037	2.7
Ctrl 1:250 pellet R#2	0.0014	

Full DNA profiles were obtained from all of the replicates amplified (the swab samples only). The average peak height was calculated for each profile (Table 10). The average peak height ratio was calculated for each profile using the heterozygous loci (Table 11).

Table 10. Semen-only swabs: average profile peak heights. Experimental samples were tested with AP Spot reagent and control samples were not. R=replicate

Experimental		Control	
Sample	Average profile peak height	Replicate	Average profile peak height
Neat R#1	7328	Neat R#1	4836
Neat R#2	4029	Neat R#2	4403
1:25 R#1	4759	1:25 R#1	5372
1:25 R#2	6379	1:25 R#2	10,726
1:100 R#1	645	1:100 R#1	3213
1:100 R#2	1076	1:100 R#2	7304
1:250 R#1	2882	1:250 R#1	1406
1:250 R#2	1583	1:250 R#2	529

Table 11. Semen-only swabs: average profile peak height ratios. Experimental samples were tested with AP Spot reagent and control samples were not. R=replicate

Experimental		Control	
Sample	Average profile peak height ratio	Replicate	Average profile peak height ratio
Neat R#1	0.871	Neat R#1	0.901
Neat R#2	0.878	Neat R#2	0.909
1:25 R#1	0.888	1:25 R#1	0.878
1:25 R#2	0.906	1:25 R#2	0.885
1:100 R#1	0.748	1:100 R#1	0.904
1:100 R#2	0.821	1:100 R#2	0.839
1:250 R#1	0.897	1:250 R#1	0.832
1:250 R#2	0.880	1:250 R#2	0.726

3.3.2 Semen-Saliva Swabs

As in the case of the semen-only swabs, AP Spot did not seem to affect the appearance of the microscope slides prepared from the semen-saliva swabs. Spermatozoa scores did not seem to vary greatly between the experimental and control samples (Table 12).

Table 12. Semen-saliva swabs: spermatozoa scores. Experimental samples were tested with AP Spot reagent and control samples were not. R=replicate

Experimental		Control	
Sample	Spermatozoa Score	Sample	Spermatozoa Score
Neat R#1	2+	Neat R#1	2+
Neat R#2	2+	Neat R#2	3+
1:25 R#1	<1+	1:25 R#1	1+/2+
1:25 R#2	<1+	1:25 R#2	<1+
1:100 R#1	<1+	1:100 R#1	1+
1:100 R#2	<1+	1:100 R#2	0
1:250 R#1	0	1:250 R#1	0
1:250 R#2	0	1:250 R#2	<1+

All of the experimental and control samples tested positive for semen using the ABACard® p30 immunoassay, although the second replicates of the experimental 1:100 and 1:250 sample types had faint bands. The experimental neat semen replicates had a pink background color, but the test and control bands were still visible. All of the experimental and control samples tested positive for saliva using the RSID™-Saliva cards except for the second replicate of the experimental 1:250 sample type. The control and experimental replicates tended to have faint bands.

Quantities of DNA (small autosomal values) were obtained via qPCR for all the replicates and the ratio between the quantity of DNA from the swab and pellet was calculated for each swab/pellet pair (Table 13).

Table 13. Semen-saliva swabs: DNA quantities of post-elution swabs and pellets. Experimental samples were tested with AP Spot reagent and control samples were not. R=replicate Exp = experimental Ctrl=control Ep = epithelial cell S=spermatozoa

Sample	Quantity (ng/ μ L)	Swab Quantity / Pellet Quantity
Exp neat swab R#1 Ep	0.2585	1.0
Exp neat pellet R#1 Ep	0.2717	
Exp neat swab R#2 Ep	1.8032	2.3
Exp neat pellet R#2 Ep	0.7921	
Exp 1:25 swab R#1 Ep	0.0086	1.7
Exp 1:25 pellet R#1 Ep	0.0052	
Exp 1:25 swab R#2 Ep	0.0152	2.3
Exp 1:25 pellet R#2 Ep	0.0067	
Exp 1:100 swab R#1 Ep	0.1292	16.1
Exp 1:100 pellet R#1 Ep	0.0080	
Exp 1:100 swab R#2 Ep	0.0161	4.9
Exp 1:100 pellet R#2 Ep	0.0033	
Exp 1:250 swab R#1 Ep	0.0017	4.7
Exp 1:250 pellet R#1 Ep	0.0004	
Exp 1:250 swab R#2 Ep	0.0006	1.6
Exp 1:250 pellet R#2 Ep	0.0003	
Exp neat swab R#1 S	1.1462	0.5
Exp neat pellet R#1 S	2.1424	
Exp neat swab R#2 S	5.3618	1.7
Exp neat pellet R#1 S	3.1629	
Exp 1:25 swab R#1 S	0.0241	8.4
Exp 1:25 pellet R#1 S	0.0029	
Exp 1:25 swab R#2 S	0.0128	1.1
Exp 1:25 pellet R#2 S	0.0115	
Exp 1:100 swab R#1 S	0.1889	5.9
Exp 1:100 pellet R#1 S	0.0319	
Exp 1:100 swab R#2 S	0.0242	1.5
Exp 1:100 pellet R#2 S	0.0163	
Exp 1:250 swab R#1 S	0.0007	0.6
Exp 1:250 pellet R#1 S	0.0013	

Sample	Quantity (ng/ μ L)	Swab Quantity / Pellet Quantity
Exp 1:250 swab R#2 S	0.0005	1.8
Exp 1:250 pellet R#2 S	0.0003	
Ctrl neat swab R#1 Ep	0.0202	0.7
Ctrl neat pellet R#1 Ep	0.0292	
Ctrl neat swab R#2 Ep	1.2895	4.5
Ctrl neat pellet R#2 Ep	0.2884	
Ctrl 1:25 swab R#1 Ep	0.0757	3.7
Ctrl 1:25 pellet R#1 Ep	0.0206	
Ctrl 1:25 swab R#2 Ep	0.0093	3.2
Ctrl 1:25 pellet R#2 Ep	0.0029	
Ctrl 1:100 swab R#1 Ep	0.0002	0.2
Ctrl 1:100 pellet R#1 Ep	0.0012	
Ctrl 1:100 swab R#2 Ep	0.0003	3.1
Ctrl 1:100 pellet R#2 Ep	0.0001	
Ctrl 1:250 swab R#1 Ep	0.0013	0.7
Ctrl 1:250 pellet R#1 Ep	0.0017	
Ctrl 1:250 swab R#2 Ep	0.0013	0.9
Ctrl 1:250 pellet R#2 Ep	0.0014	
Ctrl neat swab R#1 S	0.0632	0.8
Ctrl neat pellet R#1 S	0.0751	
Ctrl neat swab R#2 S	2.4188	4.9
Ctrl neat pellet R#1 S	0.4898	
Ctrl 1:25 swab R#1 S	0.1408	8.6
Ctrl 1:25 pellet R#1 S	0.0163	
Ctrl 1:25 swab R#2 S	0.0055	4.0
Ctrl 1:25 pellet R#2 S	0.0014	
Ctrl 1:100 swab R#1 S	0.0746	10.4
Ctrl 1:100 pellet R#1 S	0.0072	
Ctrl 1:100 swab R#2 S	0.0005	0.6
Ctrl 1:100 pellet R#2 S	0.0007	
Ctrl 1:250 swab R#1 S	0.0084	1.5
Ctrl 1:250 pellet R#1 S	0.0056	
Ctrl 1:250 swab R#2 S	0.0027	1.3
Ctrl 1:250 pellet R#2 S	0.0021	

DNA profiles were generated from most of the semen-saliva swab replicates; some were full profiles and others only partial (Table 14, Figures 4-5). All of the epithelial cell fraction replicate profiles contained peaks from the spermatozoa fraction, i.e., they were mixed profiles, presumably due to an imperfect separation via differential extraction (Figure 6). A few of the spermatozoa fraction replicate profiles contained peaks from the epithelial cell fraction. The average peak height was calculated for each profile, including shared alleles and omitting alleles known to belong to the other donor (Table 15). The average peak height ratio was calculated for each profile using the heterozygous loci (Table 16).

Table 14. Semen-saliva swabs: DNA profile completeness. Experimental samples were tested with AP Spot reagent and control samples were not. A total of 23 loci was considered for the spermatozoa fraction profiles (the Amelogenin locus was not considered) and a total of 21 for the epithelial cell fraction profiles (loci Y-indel and DYS391 were not considered because they are male specific loci). *Via generation of a profile from the saliva donor alone (data not shown), it was determined that certain alleles were shared at particular loci between the semen and saliva donors. Therefore, when an epithelial cell fraction profile “lacks epithelial fraction exclusive alleles,” it means that the profile contains shared alleles that are presumably due to the spermatozoa fraction. R=replicate Ep=epithelial cell S=spermatozoa

Experimental		Control	
Sample	Profile completeness	Sample	Profile completeness
Neat R#1 Ep	Partial	Neat R#1 Ep	Partial
Neat R#2 Ep	Partial	Neat R#2 Ep	Full
Neat R#1 S	Full	Neat R#1 S	Full
Neat R#2 S	Full	Neat R#2 S	Full
1:25 R#1 Ep	Partial	1:25 R#1 Ep	Full
1:25 R#2 Ep	Partial	1:25 R#2 Ep	Partial
1:25 R#1 S	Full	1:25 R#1 S	Full
1:25 R#2 S	Full	1:25 R#2 S	Partial
1:100 R#1 Ep	Lacks epithelial fraction exclusive alleles*	1:100 R#1 Ep	Lacks epithelial fraction exclusive alleles*
1:100 R#2 Ep	Partial	1:100 R#2 Ep	No peaks
1:100 R#1 S	Full	1:100 R#1 S	Full
1:100 R#2 S	Full	1:100 R#2 S	No peaks
1:250 R#1 Ep	Lacks epithelial fraction exclusive alleles*	1:250 R#1 Ep	Lacks epithelial fraction exclusive alleles*
1:250 R#2 Ep	No peaks	1:250 R#2 Ep	Lacks epithelial fraction exclusive alleles*
1:250 R#1 S	Partial	1:250 R#1 S	Partial
1:250 R#2 S	No peaks	1:250 R#2 S	Partial

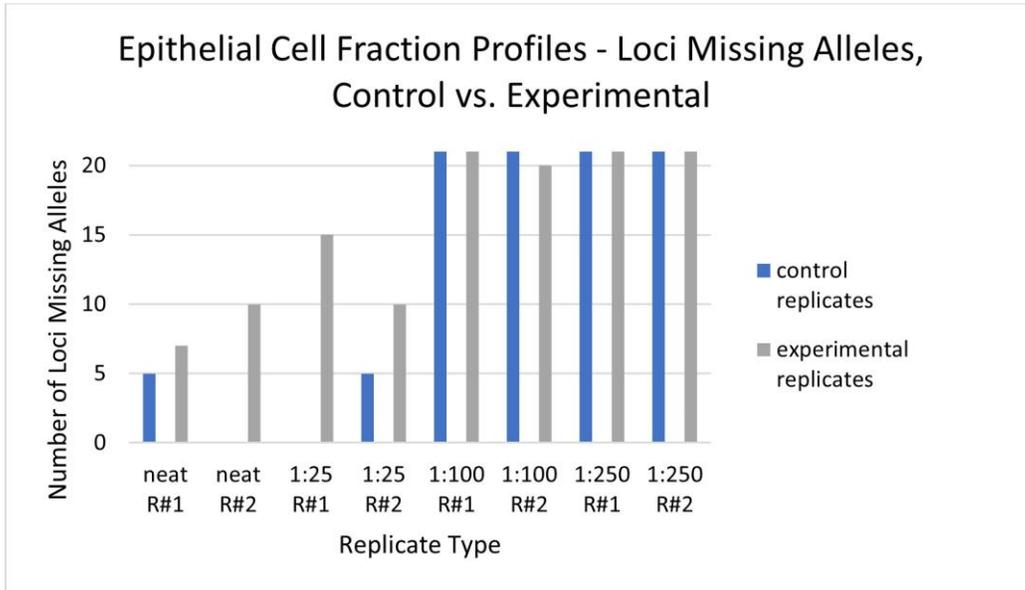


Figure 4. Semen-saliva swabs: epithelial cell fraction profiles – number of loci with missing alleles.

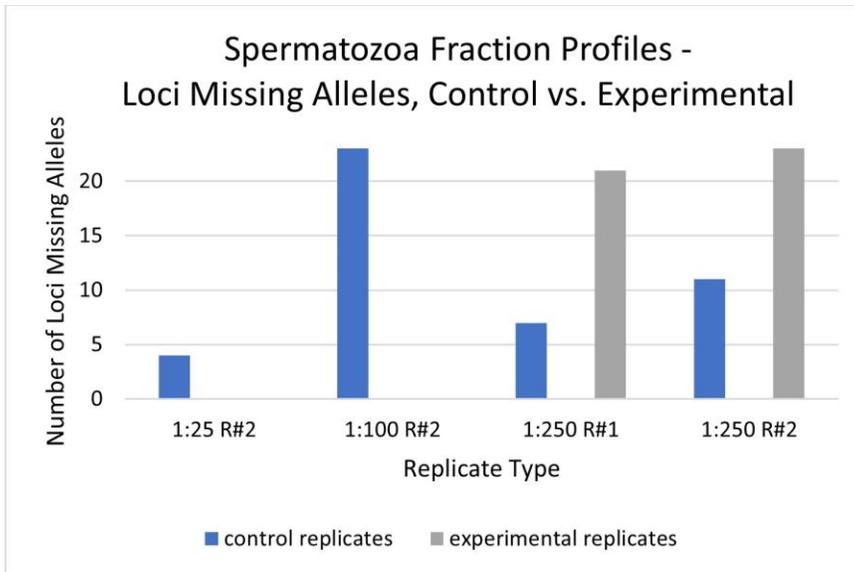


Figure 5. Semen-saliva swabs: spermatozoa fraction profiles – number of loci with missing alleles.

Table 15. Semen-saliva swabs: average profile peak heights. Experimental samples were tested with AP Spot reagent and control samples were not. R=replicate Ep=epithelial cell S=spermatozoa

Experimental		Control	
Sample	Average profile peak height	Sample	Average profile peak height
Neat R#1 Ep	803	Neat R#1 Ep	428
Neat R#2 Ep	1203	Neat R#2 Ep	850
Neat R#1 S	1322	Neat R#1 S	1792
Neat R#2 S	2327	Neat R#2 S	2193
1:25 R#1 Ep	259	1:25 R#1 Ep	757
1:25 R#2 Ep	285	1:25 R#2 Ep	351
1:25 R#1 S	684	1:25 R#1 S	2479
1:25 R#2 S	530	1:25 R#2 S	238
1:100 R#1 Ep	756	1:100 R#1 Ep	300
1:100 R#2 Ep	n/a (no peaks)	1:100 R#2 Ep	n/a (no peaks)
1:100 R#1 S	1272	1:100 R#1 S	1659
1:100 R#2 S	923	1:100 R#2 S	n/a (no peaks)
1:250 R#1 Ep	149	1:250 R#1 Ep	145
1:250 R#2 Ep	n/a (no peaks)	1:250 R#2 Ep	152
1:250 R#1 S	114	1:250 R#1 S	303
1:250 R#2 S	n/a (no peaks)	1:250 R#2 S	222

Table 16. Semen-saliva swabs: average profile peak height ratios. Peak height ratios were calculated for full and partial profiles containing at least 2 heterozygous loci. Data is limited to experimental-control pairs for which peak height ratios could be calculated, therefore some replicates/replicate types are not included. Experimental samples were tested with AP Spot reagent and control samples were not. R=replicate Ep=epithelial cell S=spermatozoa

Experimental		Control	
Sample	Average profile peak height ratio	Sample	Average profile peak height ratio
Neat R#1 Ep	0.295	Neat R#1 Ep	0.332
Neat R#2 Ep	0.227	Neat R#2 Ep	0.538
Neat R#1 S	0.842	Neat R#1 S	0.874
Neat R#2 S	0.879	Neat R#2 S	0.864
1:25 R#1 Ep	0.591	1:25 R#1 Ep	0.443
1:25 R#2 Ep	0.596	1:25 R#2 Ep	0.512
1:25 R#1 S	0.723	1:25 R#1 S	0.869
1:25 R#2 S	0.776	1:25 R#2 S	0.772
1:100 R#1 S	0.867	1:100 R#1 S	0.824
1:100 R#2 S	0.792	1:100 R#2 S	n/a

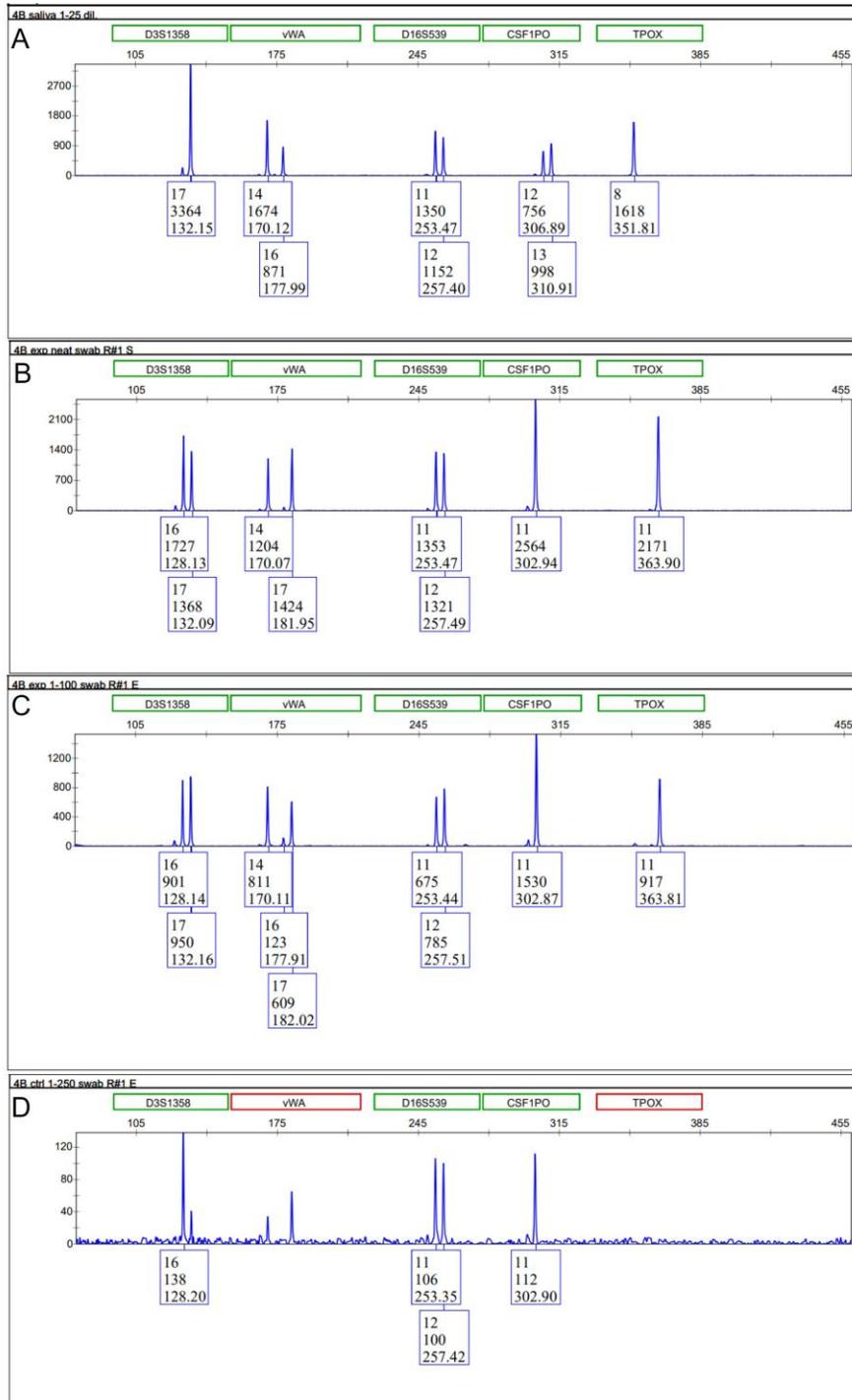


Figure 6. Electropherogram blue panel comparison. A) Saliva donor profile used as a comparison, obtained via extraction of saliva alone. B) Semen donor profile, i.e. spermatozoa fraction, obtained via differential extraction. C, D) Saliva donor profile, i.e. epithelial cell fraction, obtained via differential extraction. None of the epithelial cell fraction exclusive alleles (vWA 16, CSF1PO 12, 13 or TPOX 8) are present. In panel C, the vWA allele 16 is in stutter position so it cannot be determined if it is a true allele or stutter.

4. DISCUSSION

4.1 Elution Time and Number of Centrifugations

Relative percentage of spermatozoa eluted for each replicate was used as a measure of comparison between replicates, rather than the number of spermatozoa eluted, because it was not possible to prepare samples with equal numbers of spermatozoa present by pipetting equivalent volumes of semen (29). It was necessary to consider separately the sum of spermatozoa observed from each replicate, because the total number of spermatozoa present on each quarter swab varied considerably.

Since there was no statistically significant difference between the 30 minute initial elution and longer time categories, it can be inferred that there is no benefit to an elution time longer than 30 minutes. Adopting a time limit of 30 minutes would, especially in the case of labs that currently require 2 hours or longer, greatly reduce the length of time needed to process SAKs and is therefore recommended based on these results. Furthermore, based on the data from the 2 hour elution, a second centrifugation had no major effect on spermatozoa elution and can be eliminated from lab protocol.

4.2 Spermatozoa Staining In-Tube

The experimental procedure for staining spermatozoa eluate while in the tube was considered ineffective due to the reduced number of spermatozoa present on the microscope slides. This was likely due to the staining reagents causing the spermatozoa to clump in the tube and remain there unless the entire volume was removed.

4.3 AP Spot Test and Post-Elution DNA on Swabs

4.3.1 Semen-Only Swabs

The AP Spot reagent did not appear to affect the microscope slides prepared from the semen-only swabs. Even though the experimental replicates tended to have lower spermatozoa scores than the control replicates, it is not possible to conclude that AP Spot is responsible for fewer spermatozoa having been pipetted onto the microscope slides because of the inherent variation in the number of spermatozoa transferred when pipetting semen. AP Spot reagent did not have an effect on p30 immunoassay card testing for any of the semen dilutions, which concurs with previous findings in this laboratory that AP Spot does not interfere with p30 testing of neat semen (23). It also appeared that AP Spot reagent did not decrease the number of alleles recovered, the average profile peak height or the average profile peak height ratio (PHR). When comparing each experimental replicate type to its corresponding control replicate type, it can be seen that there is no trend of the control samples having a higher average peak height or average PHR than the experimental samples. This is in agreement with this lab's previous finding that AP Spot does not affect DNA profiles generated from neat semen (23), and opposes Lewis' finding that AP reagent lowers average peak heights (3). It may be conjectured that Lewis' AP reagent, which used Fast Black salt as opposed to Fast Blue, reacted differently than the AP Spot Test reagent used in this lab. Also, Lewis' results only consider average peak heights for all dilutions overall and do not include data of average peak heights of specific semen dilutions; perhaps the more dilute semen dilutions (up to 1 in 3000) skewed the results, as semen dilutions above 1 in 500 may not produce full profiles (30).

The DNA quantities obtained from qPCR show a trend of more DNA remaining on the eluted semen-only swab than contained in the pellet. This is in accordance with previous studies which found that the swab contains a large percentage of DNA after extraction (24–26) and suggests that the eluted swab and pellet should be recombined prior to DNA extraction to ensure the maximum amount of DNA possible is recovered.

4.3.2 Semen-Saliva Swabs

The results of the AP Spot experiment with semen-saliva swabs were, overall, similar to those of the semen-only swabs. It seems that saliva does not have a negative interaction with AP Spot reagent or inhibit AP activity, which is in agreement with previous research that generated a DNA profile from a Phadebas[®] sheet containing semen and saliva that was treated with AP test solution (31).

Unlike the semen-only swabs, many of the semen-saliva swabs generated partial profiles, but this was mainly among the epithelial cell fraction profiles, as it seems the differential extraction did not provide a complete separation. Allele dropout among the semen-saliva profiles was greater for the experimental replicates than the control ones for the neat and 1:25 categories, but not for the 1:100 and 1:250 sample types. This absence of an overall trend makes it difficult to determine if AP Spot reagent had an effect on allele dropout.

The quantities of DNA obtained and the profiles generated from the swabs indicate that, while the amount of DNA present on the eluted swab was not consistently greater than

the amount of DNA in the pellet, the post-eluted swab still represents a significant source of DNA.

4.4 Further Directions

4.4.1 Elution Time

While it has been established that there is a statistically significant difference between an initial elution time of 1 minute and that of 30 minutes, the time point at which this difference occurs may be less than 30 minutes. It would be worthwhile investigating if 15 minutes initial elution, for instance, is statistically different from 30 minutes.

It also could be valuable to test elution time when swabs were prepared longer than 24-72 hours before testing because SAK swabs may not be processed in a forensic laboratory for weeks or months after collection.

5. CONCLUSIONS

Based on the results of this study, a number of recommendations can be made to improve the efficiency of SAK swab testing. Firstly, semen-containing swabs may be eluted for 30 minutes, followed by a single centrifugation, because a longer elution time is not shown to increase the percentage of spermatozoa eluted. Secondly, the same swab cutting tested with AP Spot reagent may be used for preparation of a microscope slide, p30 (and RSID™-Saliva) immunoassay card testing, and DNA analysis, since no negative effects on downstream testing were observed when AP Spot reagent was used. Thirdly, the eluted swab should be combined with the pellet for DNA analysis, as the post-eluted swab represents a significant source of DNA.

LIST OF JOURNAL ABBREVIATIONS

Can Soc Forensic Sci J	Canadian Society of Forensic Science Journal
Forensic Sci Int	Forensic Science International
J Forensic Sci	Journal of Forensic Sciences
Sci Justice	Science & Justice

BIBLIOGRAPHY

1. National best practices for sexual assault kits: a multidisciplinary approach. 2017. <https://www.ojp.gov/pdffiles1/nij/250384.pdf> Accessed 17 November 2021.
2. Sensabaugh GF. The quantitative acid phosphatase test. A statistical analysis of endogenous and postcoital acid phosphatase levels in the vagina. *J Forensic Sci* 1979;24(2):10841J. <https://doi.org/10.1520/JFS10841J>.
3. Lewis J, Baird A, McAlister C, Siemieniuk A, Blackmore L, McCabe B, et al. Improved detection of semen by use of direct acid phosphatase testing. *Sci Justice* 2013;53(4):385–94. <https://doi.org/10.1016/j.scijus.2013.04.009>.
4. Li R. *Forensic biology*. 2nd edn. Boca Raton: CRC Press, Taylor & Francis Group, 2015; 259-63,281-4.
5. Serological Research Institute. AP spot test safety data sheet. Richmond, CA: 2015.
6. Jones EL. The identification of semen and other body fluids. In: Saferstein R, editor. *Forensic Science Handbook*. Englewood Cliffs, NJ: Prentice-Hall, 2005;329–99.
7. Desroches AN, Buckle JL, Fournay RM. Forensic biology evidence screening past and present. *Can Soc Forensic Sci J*. 2009;42(2):101–20. <https://doi.org/10.1080/00085030.2009.10757600>.
8. Bancroft JD, Layton C, Suvarna SK. The hematoxylin and eosin. *Bancroft's theory and practice of histological techniques*. Elsevier, 2018;126–38.
9. Allery J-P, Telmon N, Mieusset R, Blanc A, Rougé D. Cytological detection of spermatozoa: comparison of three staining methods. *J Forensic Sci* 2001;46(2):14970J. <https://doi.org/10.1520/JFS14970J>.
10. Sensabaugh G. Isolation and characterization of a semen-specific protein from human seminal plasma: a potential new marker for semen identification. *J Forensic Sci* 1978;23(1). <https://doi.org/10.1520/JFS10659J>.
11. Virginia department of forensic science manuals and procedures forensic biology procedure manual screening and collection for DNA analysis. 2021. <https://www.dfs.virginia.gov/wp-content/uploads/2021/07/210-D2003-FB-PM-Screening-and-Collection-for-DNA-Analysis.pdf> Accessed 27 October 2021.

12. Tennessee bureau of investigation forensic services division forensic biology policy and procedures manual. 2021. https://downloads.tbi.tn.gov/forensic-services/forensic-biology/FB_SOP-001.pdf Accessed 27 October 2021.
13. Indiana state police forensic biology section test methods. 2021. https://www.in.gov/isp/labs/files/Biology_Casework_Test_Method_09-01-2021.pdf Accessed 27 October 2021.
14. NYC office of chief medical examiner forensic biology serology procedures manual. 2019. https://www1.nyc.gov/assets/ocme/downloads/pdf/technical-manuals/forensic-biology-technical-manuals/cell_separation_and_christmas_tree_staining_090319.pdf Accessed 27 October 2021.
15. Boston police department crime laboratory - the criminalistics technical manual - extraction preparation of sexual assault swabs or cuttings. 2021.
16. Kim J-Y, Kim MI, Lee HH, Kim HL, Lee E-J, Lee Y-H, et al. Application of hematoxylin reagent for sperm cell separation in sexual crime evidence. *Forensic Sci Int* 2020;307:110114. <https://doi.org/10.1016/j.forsciint.2019.110114>.
17. Dima-Simonin N, Grange F, Brandt-Casadevall C. PCR-based forensic testing of DNA from stained cytological smears. *J Forensic Sci* 1997;42(3):14157J. <https://doi.org/10.1520/JFS14157J>.
18. Simons JL, Vintiner SK. Effects of histological staining on the analysis of human DNA from archived slides. *J Forensic Sci* 2011;56:S223–8. <https://doi.org/10.1111/j.1556-4029.2010.01595.x>.
19. Counterterrorism and Forensic Science Research Unit - Laboratory Division - Federal Bureau of Investigation. Automating the forensic analysis of nuclear DNA: the FBI's research and development initiative. Quantico, VA: 2004. https://archives.fbi.gov/archives/about-us/lab/forensic-science-communications/fsc/oct2004/research/2004_10_research04.htm Accessed 11 November 2021.
20. ASTM International. Standard practice for receiving, documenting, storing, and retrieving evidence in a forensic science laboratory. West Conshohocken, PA: 2017.
21. ASTM International. Standard specifications for preparation of laboratory analysis requests in sexual assault investigations. West Conshohocken, PA: 2015.
22. Scientific Working Group on DNA Analysis Methods. Recommendations for the efficient DNA processing of sexual assault evidence kits. 2016.

23. Brown LT. Migration patterns of seminal fluid components and spermatozoa in semen stains exposed to water and blood [Master's thesis]. Boston, MA: Boston University; 2016.
24. Adamowicz MS, Stasulli DM, Sobestanovich EM, Bille TW. Evaluation of methods to improve the extraction and recovery of DNA from cotton swabs for forensic analysis. *PLoS ONE* 2014;9(12):e116351. <https://doi.org/10.1371/journal.pone.0116351>.
25. Taveira CN. Optimizing cell elution conditions for a novel enzymatic DNA extraction technique for spermatozoa on cotton swabs [Master's thesis]. Boston, MA: Boston University; 2015.
26. Nicholas EL. Comparison of results using temperature controlled differential extraction and differential extraction using the Qiagen EZ1® Advanced [Master's thesis]. Boston, MA: Boston University; 2021.
27. ABACard® p30 Test For The Forensic Identification of Semen Technical Information Sheet. West Hills, CA: 2017.
28. Rapid Stain Identification of Human Saliva (RSID™-Saliva) Technical Information and Protocol Sheet. Lombard, Il: 2016.
29. Lombardi C. Efficacy of slide fixative methods for examining sperm [Master's thesis]. Boston, MA: Boston University; 2019.
30. Allard JE, Baird A, Davidson G, Jones S, Lewis J, McKenna L, et al. A comparison of methods used in the UK and Ireland for the extraction and detection of semen on swabs and cloth samples. *Sci Justice* 2007;47(4):160–7. <https://doi.org/10.1016/j.scijus.2007.09.010>.
31. Herman Y, Feine I, Gafny R. Acid phosphatase test on Phadebas® sheets — An optimized method for presumptive saliva and semen detection. *Forensic Sci Int* 2018;288:218–22. <https://doi.org/10.1016/j.forsciint.2018.04.047>.

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