2019

Detection of saliva using Seratec Amylase Paper

BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

DETECTION OF SALIVA USING SERATEC AMYLASE PAPER

by

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B.S., University of Massachusetts, Boston, 2013

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

2019

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ACKNOWLEDGMENTS

I would like to thank Professors Amy Brodeur and Robin Cotton, for their patience and assistance during this project. I would also like to thank Beth Goodspeed and Dr. Gabriela Roca for their input and willingness to help. I could not have completed this project without the valuable guidance from all of you.

To my family, especially David, and my parents, thank you for your patience and encouragement throughout this process. It's been a lot of work and your love and support have been extremely appreciated.

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ABSTRACT

Biological fluids, like saliva, are commonly encountered in forensic casework. The ability to locate and identify the type of biological fluid on a piece of evidence can lead to further testing including DNA extraction and analysis. Saliva stains are often found on a variety of surfaces in the presence of additional bodily fluids. Many of these stains cannot be readily seen, which makes detection difficult. A study utilizing mapping with Seratec® Amylase Paper and the use of an alternative light source (ALS) for better visibility and detection was conducted to test the effectiveness of this medium. Five different types of stains were prepared, including saliva, saliva and blood, saliva and semen, saliva and urine, and saliva, blood, semen, and urine. The stains were deposited onto four different textile types, including cotton, denim, fleece, and spandex. The results indicated that the presence of other body fluids may adversely affect the detection of saliva using Seratec® Amylase Paper.

In order to effectively test whether the Amylase Paper itself inhibited DNA extraction and quantification, only half of each saliva stain was mapped with the paper. This left half of the stain untouched, and available for a comparative DNA study. The same saliva donor, donor C, was used for the entirety of the DNA study, and stains were extracted from the cotton and fleece textiles. A Harris micro-punch was used to collect identical 3mm samples from the portion of the stain in contact with the Amylase Paper and the portion of the same stain not in contact with the Amylase Paper. In addition, samples of the Amylase

Paper that had not been used for previous testing were tested to see if the internal positive control (IPC) was affected by the paper itself. The results of the DNA extraction and quantification showed no inhibition in the samples in contact with the Amylase Paper, the samples not in contact with the Amylase Paper, nor from the Amylase Paper itself.

These results show that Seratec® Amylase Paper can identify saliva in most mixed samples including blood, semen, and urine. In addition, the application of the Amylase Paper does not inhibit or prevent the subsequent extraction or quantification of DNA, and allows for samples in contact with Amylase Paper to be used for DNA testing downstream. Seratec® Amylase Paper is an effective screening method in forensic casework when the presence of saliva is suspected and can be used even when DNA testing is anticipated in the future.

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LIST OF ABBREVIATIONS

ALS	Alternative light source
Cm	Centimeter
C_t	Cycle threshold
dL	Deciliter
DNA	Deoxyribonucleic acid
g	Gram
In	Inch
IPC	Internal positive control
IU	International Units
L	Liter
mL	Milliliter
mm	Millimeter
mRNA	Messenger ribonucleic acid
mIU	Milli-International Units per liter
ng	Nanogram
nm	Nanometers
qPCR	Quantitative polymerase chain reaction
µL	Microliter
UV	Ultraviolet
Vis	Visible

1. INTRODUCTION

The discovery of biological fluids at a crime scene can help link a victim or suspect to the scene and may help reconstruct the crime itself. Saliva is an important biological fluid that is frequently found on evidence such as cigarette butts and water bottles, and it is often deposited during of a violent crime and/or sexual assault.[1] Saliva is often difficult to visualize, and depending on the circumstances of the crime, identification of where it is found may be important.[1] In these cases, it often up to the victim to accurately relay the details of the crime in order to locate potential biological fluid evidence.

Saliva is identified primarily through the presence of alpha-amylase. Alpha-amylase is an enzyme that is found in many biological fluids such as breast milk and perspiration, but it is most abundant in saliva.[1] Alpha-amylase is secreted through the salivary gland and the pancreas, then it is absorbed into the bloodstream via the digestive system before being eliminated through urine and perspiration.[2] Alpha-amylase levels vary between individuals and the levels even vary in the same individual throughout the day based on many factors such as diet, hydration level, and the individual's health.[3] Alpha-amylase's main purpose is to break down starch into glucose.[4] It is this activity that is exploited in many preliminary screening techniques for saliva.

Amylase levels fluctuate throughout the day and have been observed to rise after eating to aid in the digestion process.[5] It was also reported in a study by Sahu et al. that stress levels raise salivary alpha-amylase levels in individuals, and there is a 15-28% increase in these levels in males.[5]

Amylase is not only found in humans, but also in plants, animals, and some bacteria.[1,2,6] Epithelial cells are found in many places, including the inside of the cheek. This puts the cells in frequent contact with saliva, which then enables the collection of epithelial cells when a stain is collected and identified.[7] Saliva can be a DNA source, as the mean number of epithelial cells in saliva is roughly 4.3×10^5 per 1mL.[8] In addition, saliva has some advantages over other biological fluids for DNA analysis as it is frequently deposited and easily collected from subjects.

1.1 Forensic Detection and Identification of Saliva

The forensic detection of saliva begins with a visual examination of the evidence and includes screening with the naked eye and with an alternate light source (ALS). Saliva is often difficult to locate because it is clear and lacks significant solid components.[1] When it dries, a saliva stain may be visible as a white stain. If visualization in white light is impossible, an ALS might be necessary, as saliva will faintly fluoresce under certain ALS settings.[1] Due to the fact that many biological fluids and chemicals also fluoresce, additional testing is important for correct identification.[9]

Other preliminary testing methods for saliva utilize the enzymatic activity of alpha-amylase. Seratec® Amylase Paper (Seratec®, Göttingen, Germany) is a commercially available filter paper that is embedded with starch. The paper is moistened and pressed onto a surface where the presence of saliva is suspected.[10] After a ten minute incubation period, a potassium iodide solution is applied to the paper and if alpha-amylase is present, a yellow area will develop on the paper.[10,11] This color change occurs because alpha-

amylase breaks down the starch in the paper and the potassium iodide only dyes the area of the paper where starch is present.

Another amylase paper product is the Phadebas® Forensic Press Test (Magle Life Sciences, Cambridge, MA). This test also relies on the ability of alpha-amylase to break down starch. One side of the paper is pre-treated with water insoluble starch microspheres attached to a blue dye. When alpha-amylase is present, it digests the starch complex and releases the dye.[12] When this occurs, the dye is mobilized by the amylase and it diffuses through the paper until the color is visible on the non-reagent side of the paper.

The Phadebas® Tube test (Magle Life Sciences, Cambridge, MA) utilizes the same water insoluble starch complex as the previously mentioned Press Test. Instead of the paper, tablets are crushed and mixed with deionized water, then a sample that was extracted in deionized water is added in a tube. If alpha-amylase is present, a blue color will appear. This indicates that the starch bound to the tablet has been digested and the blue dye has been released into solution.[12]

Radial diffusion is a gel based test that also involves the digestion of starch. The gel is prepared using agarose and a soluble starch. When the gel sets, several wells are created in the gel and sample extracts are deposited into the wells. Next, the gel is incubated at 37°C overnight, allowing the sample to diffuse radially into the gel matrix. If amylase is present in a sample, a diffusion ring can be visualized following iodine staining around the sample well where the starch has been digested by the amylase in the sample.[13]

SALIgAE® (Abacus Diagnostics, West Hills, CA) is an easy to use colorimetric test. The sample is extracted in deionized water, centrifuged, and incubated for 30 minutes.

The sample is then added to the SALIgAE® solution, and the development of a yellow color indicates that saliva is present. If the solution remains clear, then there is no detectable saliva present in the sample. The exact mechanism of this test is unknown because it is proprietary information protected by the manufacturer's patent.[14]

UV-Vis Spectroscopy is a type of absorption spectroscopy that can be used as a presumptive screening method for the identification of bodily fluids. Some biological fluids can absorb or emit fluorescence when illuminated with UV light.[15] Saliva emits fluorescence under UV-Vis Spectroscopy, which allows it to be identified on a piece of evidence. The primary peak of emission spectra for saliva is 345 – 355nm with excitation at 282nm.[16] This fluorescence does not identify the type of bodily fluid present, but rather indicates that a biological sample is present.

IR Spectroscopy analyzes infrared light and how it interacts with a molecule.[17] This includes the absorption, reflection and emission of light. The vibration of the molecule is measured which allows for the identification of certain functional groups.[17] Because bodily fluids have unique IR spectrums, the identification of saliva is possible, but more research is necessary to determine the specific spectra for each bodily fluid.[17]

Raman Spectroscopy is a non-destructive technique that also examines the fundamental vibrations of molecules.[17] Raman does not involve the absorption of light, but rather the scattering of light, which is why it is ideal for liquid samples. Raman spectral signatures can be developed for bodily fluids, including saliva. This procedure involves the use of statistical techniques and mathematical methods of multivariate analysis.[17]

Lateral flow chromatographic immunoassays are easy to use tests that rely on the reaction of antibodies and antigens to identify the presence components associated with certain biological fluids; therefore, they don't involve the amylase and starch reaction. One of these tests, the Seratec® Amylase Test (Seratec®, Göttingen, Germany), is a plastic cartridge that utilizes immobilized and mobilized murine antibodies.[18] The test cartridge contains a membrane that facilitates a capillary action that moves the sample extract from the sample area to the test area, and finally, to the control area. The sample extract is deposited onto the sample area, which contains mobile gold-labeled anti-alpha-amylase monoclonal murine antibodies. If amylase is present in the sample it will bind with the mobile murine antibodies and the antigen-antibody complex will travel to the test area. The test area contains immobilized monoclonal anti-alpha-amylase murine antibodies, which bind with the antigen-antibody complex, forming an antigen sandwich complex and then a red line, indicating a positive result. The mobile sandwich complex then continues to the control area where immobilized polyclonal goat anti-murine antibodies are present. If the capillary action is working correctly, a red line will form at the control line as well because the buffer in the sample will carry the mobilized gold-labeled murine anti-bodies from the sample area to the control area which will react with the anti-murine antibodies to form a red line. This is a confirmatory test for alpha-amylase, though not necessarily saliva.[18] The RSID™ Saliva Kit (Independent Forensics, Lombard, IL) is another chromatographic immunoassay test. Instead of murine antibodies the test utilizes dual monoclonal antibodies that are specific to human salivary alpha-amylase antigen.[19]

DNA is an important tool in forensic investigations, but while it may identify the individual who the sample came from, it cannot determine the type of bodily fluid.[20] The comparison of specific genes and mRNA expression levels can help identify different bodily fluids. A targeted multiplexed next generation mRNA sequencing assay has been developed with 33 targets, including 6 for saliva.[20] This method has been shown to have greater specificity, improved testing time and decreased sample consumption than traditional methods.[20]

After saliva has been identified in a biological stain, the next step is usually DNA analysis. DNA can be successfully extracted from saliva and has been found to yield complete profiles.[7,9,10,21] DNA testing is usually performed after presumptive and confirmatory testing of a sample. This means that ensuring that enough sample is present after screening is an important issue. Most presumptive and confirmatory testing is destructive, so the sample cannot be reused. Previous tests have been conducted using the Phadebas® Forensic Press Test to determine if the Phadebas® paper could be used on a suspected saliva stain, then whether DNA could be recovered from both the stain and paper afterwards. Herman et al. determined that recovery of DNA and even the ability to generate a DNA profile after the Phadebas® test was performed was possible.[21] In cases where samples size is limited, preliminary testing methods that are non-destructive are important because they allow the sample to be preserved for repeat or additional tests.

DNA concentration can be determined using the Quantifiler® Duo quantification kit (Applied Biosystems, Foster City, CA) and the 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). The Quantifiler® Duo quantification kit measures male

DNA, human DNA, and an internal positive control (IPC). The IPC is a synthetic DNA profile that is amplified simultaneously with the target DNA contained in the samples to ensure that the procedure is running as expected. Primers and TaqMan® MGB probes are also utilized to label the sample a reporter dye.[22] The 7500 Sequence Detection System utilizes three phases of amplification during the PCR process. The first is an exponential or geometric phase where the PCR product is amplified significantly. The second phase is a linear phase where the amplification slows as the PCR products decrease in supply. Plateau is the final phase, which occurs when PCR stops. The cycle threshold (C_t) value is generated using fluorescent dyes from the Taqman® probes. The C_t value is determined based on the starting template copy number and the efficiency of the DNA amplification during the PCR process by measuring when the fluorescent signals increase beyond the threshold setting.[22]

1.2 Purpose

Three questions are addressed in this study: 1.) Does Seratec® Amylase Paper detect alpha-amylase in saliva stains that have been deposited on different textile surfaces that vary in absorbency, thickness, color, and elasticity?; 2.) Does Seratec® Amylase Paper detect alpha-amylase on these different textile materials when saliva is mixed with other biological fluids?; and 3.) Does Seratec® Amylase Paper inhibit the recovery of DNA in saliva samples that have come into direct contact with the paper?

The textiles included in this study were dark blue denim, gray spandex, light blue cotton and blue and brown patterned fleece. Neat saliva stains were deposited on these

fabrics, as were 1:1 mixtures of saliva and semen, saliva and blood, and saliva and urine. Also, a four part mixture, 1:1:1:1, of saliva, semen, blood and urine was deposited on all of the textiles. The recovery of DNA included an extraction of stains in contact with the paper, those stains not in contact with the paper, and the actual paper itself, followed by quantification of the recoverable amount of DNA. The protocol followed for all procedures was from published literature and/or manufacturer's instructions, and the level of sensitivity and specificity of the reagents is considered on each part of the study.

2. MATERIALS AND METHODS

Saliva was collected from five anonymous donors, four female and one male, referred to as A, B, C, D, and E. The donors were instructed not to eat, drink, or smoke for one hour prior to sample collection. The samples were then frozen until ready for use. Samples of semen and blood were obtained from additional, separate donors and stored frozen. The urine sample was collected from another donor and used fresh, and was not frozen. All frozen samples were defrosted completely and vortexed before use. In addition, three pairs of underwear were obtained from a single female participant following oral sex with her male partner. The underwear samples were worn post-oral sex for 6, 8 and 12 hours and the samples were designated as F, G and H, respectively. Sample F was a pink pair of women's underwear with a white crotch panel, Sample G was a purple pair of women's underwear with a purple crotch panel, and Sample H was a dark and light striped pair of women's underwear with a light purple crotch panel.

Saliva was obtained from another donor, with the same guidelines, and used fresh. A 20µL volume of saliva was deposited onto 100% cotton fabric. A volume of 20µL deionized water was also deposited onto the same cotton fabric swatch, approximately 3 inches from the saliva stain. The saliva stain was used for a positive control, and the deionized water stain was used for a negative control. The Seratec® Amylase Paper test was performed in accordance of the manufacturer's recommendations, and a facemask was worn during sample preparation and testing to prevent any contamination.

2.1. Procedural Study of Effectiveness

A study was conducted prior to testing to determine the most efficient procedure for yielding the most distinctive positive result for the Seratec® Amylase Paper test. For this test, filter paper was used as the substrate, and neat saliva samples were used. A pipette was used to deposit a 20µL stain of saliva onto filter paper circles that measured approximately 4.33 inches in diameter; this process was repeated 23 times on separate pieces of filter paper to make identical stains. The Seratec® Amylase Paper kit came with 25 sealed A4 size Amylase Paper sheets, 8.27 x 11.69 inches in size, a 50mL glass bottle with Iodine Stock Solution (Potassium Iodide), and user instructions. The Seratec® validation study for the Amylase Paper recommends holding the Amylase Paper on the test area for 10-15 seconds, and using 50µL of a 1:15 dilution of Potassium Iodide solution to water, for 88in^2 of Amylase Paper.[10,11] An incubation time of 10 minutes was observed, and the results were recorded within 5 minutes as recommended by the manufacturer.

The Potassium Iodide dilution, number of water sprays onto the Amylase Paper, and the duration of time that the Amylase Paper was pressed onto the filter paper were all variables in this effectiveness study. The iodine solution was mixed with deionized water in different dilutions, including 1:10, 1:15 and 1:20. A spray bottle was filled with deionized water, and the number of sprays to wet the Amylase Paper was tested at 1, 2, 3 and 4 sprays, measuring approximately 1mL, 2mL, 3mL and 4mL, respectively. Finally, the paper and the stain contact time was also tested, at 15 seconds and 30 seconds.

2.2. Preparation of Stains

Fabric swatches from four different textile types were prepared and each was cut into fifteen 3” x 6” swatches using a template. The four fabric types were selected based on their different components such as absorbance, thickness, color, and material. The first fabric type was a dark gray spandex material, made up of 88% polyester and 12% spandex. The second type of fabric selected was a thin light blue bed sheet that was 100% cotton. The third fabric was a dark blue denim, comprised of 99% cotton and 1% spandex. The final fabric used in this study was a blue fleece material with a dark brown bear pattern, made of 100% polyester. Five neat 20µL saliva stains, one from each of the saliva donors, were placed on each of the four fabric types. This was repeated two more times to ensure triplicate testing was possible. The stains were then put aside and left to dry at room temperature.

The blood mixture stains were prepared by combining 120µL of saliva from donor A with 120µL of blood and vortexing. A pipette was used to deposit 20µL of the mixture on the left side of the first cotton swatch. This stain and orientation was repeated for the second and third cotton swatches, the three denim swatches, and the three fleece swatches. The process of preparing the blood and saliva mixture was then repeated for the four remaining donors. The stains were arranged in a row from donor A to donor E in advancing order. The stains were put aside and left to dry at room temperature. The same procedure completed for the blood and saliva mixture was then used to prepare the semen and blood mixture stains and the urine and blood mixture stains.

The mixture of saliva from donor A, blood, semen and urine was prepared by pipetting 60µL of each biological fluid into a 2ml tube, and vortexing. The stains were then deposited onto the left side of each fabric swatch. The same process was repeated for donors B-E, and the stains were arranged in a row in ascending order. The swatch was then labeled with the stain number with marker, or if the swatch was too dark, with marker on tape. The stains were put aside and left to dry. All stains, when dry, were packaged based on stain type with a Kimwipe® (Kimberly-Clark, Roswell, GA) in between each fabric swatch. The packages were labeled and stored in the laboratory.

Figure 1. Preparation of stains. Five blood and saliva mixture stains on twelve fabric swatches (top to bottom): three spandex, three fleece, three cotton, cotton, and three denim.

2.3. Visual Examination of Stains

All 60 swatches and the three underwear samples were examined in white light prior to testing. If the stains were not visible in white light, they were then examined under

an ALS with an orange filter at 415 nm – 500 nm. The stains were then outlined with a wax crayon, so the stain's parameters were visible.

2.4. Seratec® Amylase Paper Testing

The Seratec® Amylase Paper was cut into sections measuring 3 inches x 6 inches to correlate with the size of the textile swatches. Cotton swatch number 1 with the saliva stains from donors A-E was laid out onto bench paper placed on a clean laboratory bench. A spray bottle was filled with deionized water and the Amylase Paper was sprayed with 3-4 sprays until completely saturated. The paper was placed on the bottom half of the cotton swatch which included the bottom half of the saliva stains. A flat plastic clipboard was then placed on top of the paper and cotton swatch, and a weight was placed on top of the plastic clipboard for a total of 15 seconds. Once the 15 seconds had elapsed, the weight, clipboard and cotton swatch were moved aside, and the Amylase Paper was placed in a glass dish with a 3.5 inches x 7.25 inches flat glass base. A timer was set for 10 minutes and the Amylase Paper was left untouched during this time. During this time, an iodine working solution was prepared as 1 part iodine and 15 parts deionized water. The working solution was prepared in an amber glass bottle and stirred with a metal stir bar.

At the completion of 10 minutes, 10ml of the iodine working solution was measured in a small graduated cylinder and poured over the Amylase Paper, making sure that the entire sheet was adequately covered with the solution. The results were recorded within 10 minutes to avoid any fading, and photos were taken in a white light box. A positive result was a color change to a light yellow, while the negative area remained a brownish-purple

from the iodine staining. This process was repeated for all 60 textile swatches and the three underwear samples.

2.5. Zygem DNA Extraction

Proper protective equipment was worn in the DNA laboratory, including a lab coat, gloves, googles and a mouth covering. The extraction area and all equipment were cleaned with a solution of 10% bleach followed by 70% ethanol. A 3mm Harris Micro-Punch® (Ted Pella, Redding, CA) was used to uniformly cut twelve samples. These samples included three saliva stains on cotton taken from the side in contact with the Amylase Paper, three saliva stains on fleece from the side in contact with the Amylase Paper, three saliva stains on cotton from the side not in contact with the Amylase Paper, three saliva stains on fleece from the side not in contact with the Amylase Paper, and three samples of unused Amylase Paper. The twelve samples were then placed individually into 0.2mL tubes, with 87µL of water, vortexed, and left to extract for one hour in 87µL of deionized water. A reagent blank of just 87µL of deionized water was also prepared.

After the hour long extraction, two 20µL aliquots of each sample, including the reagent blank, were taken and distributed into 0.2mL tubes. Next, 69µL of deionized water was added to the samples. Finally, 10µL of Buffer Blue was added to each sample, followed by 1µL of Zygem (MicroGEM International, Southampton, UK), adding up to 100µL of total volume. The samples were then vortexed and placed in the thermal cycler in the DNA extraction lab. The samples were incubated at 75°C for 15 minutes to activate the Zygem protease activity, which extracts DNA through cell lysis, and which is possible

due to a mixture of enzymes.[23] Then the samples were incubated for 5 minutes at 95°C to inactivate the activity of the protease. The samples were then removed and centrifuged.

2.6. qPCR of Samples

The samples were prepared with the Quantifiler® Duo quantification kit and run on the ABI 7500. The qPCR master mix was prepared using 378µL of Quantifiler Duo Primer Mix and 450µL of Quantifiler PCR Reaction Mix. After the master mix was vortexed, 23µL was added to each reaction well. All samples were then vortexed, and 2µL of each sample was added to the appropriate reaction well. In addition, 2µL of standard was added to the positive control, and 2µL of the Master Mix was added to the negative control. The samples were covered and then vortexed.

The samples were quantified using the ABI Prism 7500 computer and software. The samples were inserted into the reaction plate, and the location of each was noted in the computer software. Detectors for Duo Male DNA, Duo Human DNA and Duo IPC were set up.

2.7. C_t Values and Validated Curve

The raw data was collected and evaluated with the aid of an external calibration curve. The calibration curve was generated in a study by Grgicak et al. using a dilution series.[24] This process allows for the quantity of DNA to be calculated from the C_t value. Two equations were derived from the validated curve that calculated the C_t value for the

human DNA concentration and male DNA concentration. The human DNA concentration was calculated using Equation 1:

$$\text{Human DNA ng/µL} = 10^{\left(\frac{Ct-29.55006)}{-3.38784}\right)}$$

The male DNA concentration was calculated using Equation 2:

$$\text{Male DNA ng/µL} = 10^{\left(\frac{(ct-30.62442)}{-3.41224}\right)}$$

3. RESULTS AND DISCUSSION

3.1. Procedural Study of Effectiveness

Prior to any testing, a comparison study was performed to ensure that the best conditions were used to produce the most distinctive positive versus negative color results. A positive result was described in two different ways by the manufacturer, as a vibrant yellow spot or creamy white area surrounded by an area of blueish black, and a negative result was reported as showing no white area or area of vibrant yellow.[10,11] Therefore, it was important to run several tests to observe the color changes and potential differences in order to accurately identify positive and negative responses. Since air bubbles may be caught under the Amylase Paper and could cause an otherwise negative area to appear white, care was taken to prevent this occurrence.

Three variables were tested to make sure that the optimal test procedures were being performed to produce the best color contrast between negative and positive results. Also, it was important to distinguish between the creamy white and vibrant yellow color descriptions listed in the manufacturer's directions and validation study, respectively. The variables included the water to Potassium Iodide solution ratio, the number of sprays used to moisten the Amylase Paper, and the length of time the Amylase Paper was pressed against the sample.

The number of sprays was tested first to ensure that the test size, a 3 inch by 6 inch area, would be sufficiently moistened before contact with a sample when sprayed with a spray bottle. The manufacturer's instructions did not specify the volume of water to use but directed the user to "moisten" the paper.[10] The volume of water in each spray was

approximately 1mL, and in order to ensure that testing conditions remained uniform throughout the study, 1, 2, 3, and 4 sprays were tested on areas of the Amylase Paper cut to 3 inches by 6 inches.

Each spray type was tested three times to determine the optimal number of sprays in order to entirely moisten the 3 inches by 6 inches paper. One spray was only enough to moisten the center of the paper, approximately 1 inch wide, leaving an inch dry on either side and a half inch dry at the top and bottom in most cases. This area was not saturated throughout and could not be considered "moistened". Two sprays left the paper moist in the center, but the dry area was longer and wider than the one produced by one spray. Three sprays left most of the paper saturated throughout, but the edges and corners remained completely dry. Finally, four sprays were tested, and it left the entire paper, including the edges and corners, completely saturated.

The Potassium Iodine solution to water dilution ratios for this study were 1:10, 1:15, and 1:30. The dilutions were tested using four sprays of water to moisten the paper based on the results listed above. The dilutions were each tested using both iterations of the final variable - transfer time between the sample and the Amylase Paper - at 15 seconds and 30 seconds. The 1:10 dilution produced a distinct purplish-brown background "negative" color, and gave positive results that were a yellow-white color and easy to identify. There was no difference in color between the 15 second and 30 second transfer time. The results of the 1:15 dilution were almost identical to the 1:10 results. The color contrast was enough to easily see the difference between the negative and positive areas. There was also no difference in the two transfer times at this dilution. The 1:30 dilution did

not produce a good contrast between the negative and positive color changes. The purplish-brown "negative" background color was much more dilute, making the white-yellow "positive" area very difficult to identify. Once again, there was no difference in the negative and positive results due to the 15 second and 30 second transfer times.

Based on these results, the final procedure used for subsequent testing was as follows: four sprays of water were used to ensure that the entire 3 inches by 6 inches paper was saturated before the transfer began. Transfer time was set to 15 seconds to shorten the overall testing time since there was no difference in color between the 15 second and 30 second transfer time. The Potassium Iodide solution dilution of 1:15 was used because there was no distinct difference in the results between the 1:10 dilution and the 1:15 dilution and the 1:15 dilution was the manufacturer's recommendation.

3.2. Visual Examination of Stains in White Light

The first step in a forensic investigation is a visual examination of the evidence and crime scene. This includes looking for possible stains that may be of biological origin. Biological fluids are important in forensic investigations because they can corroborate a witness or victim's retelling of the incident by linking what happened to specific sources. In addition, it can link suspects to the crime through DNA profiling, which is done after the biological fluid is located and identified.

The first type of visual identification is performed using white light, with no additional equipment. Many stains are visible in these conditions, which allows for them to be easily sampled and tested. All 60 fabric swatches, each with five stains from five

donors, were analyzed under white light to see if the stains were visible. This was necessary before testing the stains with the Amylase Paper because half of the stain needed to remain untouched by the paper. This was only possible by identifying the top and bottom of the stain. If the stain was readily visible, then marking the stain was not necessary.

3.2.1. Saliva Stain Results

The five saliva stains were visible on several of the fabric swatches in white light. The most visible stains were on the absorbent fleece fabric, with all five saliva stains located on swatch one, four stains visible on swatch two, and three on swatch three. This may have been due to the light blue fabric, and the texture of the fleece which would break up the stain and add dimension, and therefore visibility. The fleece was absorbent, but the threads were thick, which may have left more of the stain visible. The least visible saliva stains were on the denim swatches, with only one stain visualized on swatch one, zero stains visualized on swatch two, and one on swatch three. This may be due to the flat, dense, less-absorbent material of denim. The dark color of the denim may also have masked the saliva stains. The results of saliva stain visibility for all swatches in white light are displayed in Table 1.

3.2.2. Saliva and Semen Mixture Stain Results

The most visible saliva and semen mixture stains were on the spandex fabric swatches, with all stains for those fabric swatches visible. The spandex material was least absorbent, and a dark gray color, which may have made the pure saliva samples, made up

of 99.5% water, difficult to see.[25] Because of this, saliva may be more difficult to see under white light than other bodily fluids such as semen.[1] In this case the semen's solid components may have rested on the top of the spandex material to result in a crusty white stain. In addition, all the semen and saliva mixture stains were visible on the cotton swatches. The least visible semen and saliva mixture stains were on the denim fabric swatch, just as with the neat saliva sample.

3.2.3. Saliva and Blood Mixture Stain Results

All five of the saliva and blood mixture stains were visible on all four of the fabric stains. In all cases, the visibility was due to the reddish-brown color of the mixture, which resulted from the heme component of blood.[26] The color of the blood masked the color of the saliva, which was especially useful on darker substrates such as the denim that may have otherwise affected the saliva's visibility.

3.2.4. Saliva and Urine Mixture Stain Results

The saliva and urine stains were only visible on the fleece fabric swatches. Four of the stains were visible on swatch one, three on swatch two, and just one stain was visible on swatch three. The stains were not visible on the denim, cotton, or spandex fabric swatches. The fleece's long threads and light blue color may have facilitated the urine and saliva mixture stain's visibility. Urine is difficult to see in white light because like saliva it is made up of a lot of water, generally 91-96%.[27]

3.2.5. Blood, Semen, Saliva and Urine Mixture Stain Results

The blood, semen, saliva and urine mixture stains were all visible on all four of the fabric types. This was also due to the reddish-brown color attributed to the blood's heme. The fleece fabric was harder to visualize the stains on due to the brown pattern on the fabric, but the stain was still visible in all cases. All results for the blood and saliva mixture stains, and the blood, semen, saliva and urine stains, are recorded in Table 1.

Table 1. Visibility of Saliva Stains and Saliva Mixture Stains in White Light. When the stains were visible, considered a positive result, it is indicated by (+) and when a stain was not visible, considered a negative result, it is indicated by (-).

	Donor	**A**			**B**			**C**			**D**			**E**		
Stain	**Swatch**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Saliva	**Cotton**	-	-	-	-	-	+	+	+	+	+	+	+	-	+	+
	Denim	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-
	Fleece	+	+	+	+	-	-	+	+	+	+	+	+	+	+	-
	Spandex	-	-	-	-	-	-	+	+	+	+	+	-	-	-	+
Saliva/ Semen	**Cotton**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Denim	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Fleece	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+
	Spandex	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Saliva/ Blood	**Cotton**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Denim	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Fleece	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Spandex	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Saliva/ Urine	**Cotton**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Denim	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Fleece	-	+	-	+	+	-	+	+	+	+	+	-	+	+	-
	Spandex	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Saliva/Semen/ Blood/Urine	**Cotton**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Denim	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Fleece	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Spandex	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

3.2.6. Post-oral Sex Sample Results

Finally, the three post-oral sex underwear samples were examined in white light. Some white staining was visible on samples G and H, which were worn 8 hours and 12 hours after separate occurrences of oral sex, respectively. Sample G had some staining on the crotch panel that was yellowish-brown in color. Sample H had some whitish staining in the crotch panel. Sample F did not have any staining visible, but an area of the white crotch panel of the underwear was stiff; this sample was worn for the least amount of time, 6 hours. Vaginal fluid ranges in color from clear to milky white and is made up of liquid, cells and bacteria.[28] This light color, mixed with clear saliva, may be diluted even further, which could make it more difficult to see. In addition, the light color of Sample F's crotch panel may have provided less contrast between the stain and the fabric. These results are listed in Table 3, along with the results from the ALS examination.

3.3 Visual Examination of Stains Using ALS

3.3.1. Saliva Stain Results

The saliva stains that were not visible with white light were examined under an ALS with an orange barrier filter to locate the dimensions of the stains in preparation for testing with Amylase Paper. Since the first cotton swatch had five visible stains, only swatches two and three were examined. In both cases, white stains with thin borders were observed at 455nm. The saliva stains on denim were also examined under an ALS, using a range of 415 nm – 515 nm, and no stains fluoresced on swatches one or two. The stain on swatch three did fluoresce under an ALS. All three spandex swatches were examined under an

ALS at 455nm. Three stains on swatch one fluoresced, including one that was invisible in white light. Four stains fluoresced on swatch two, including two that were invisible in white light. Three stains fluoresced on swatch three, including one that was invisible in white light. All five stains fluoresced at 455nm for all three cotton swatches [Figure 2].

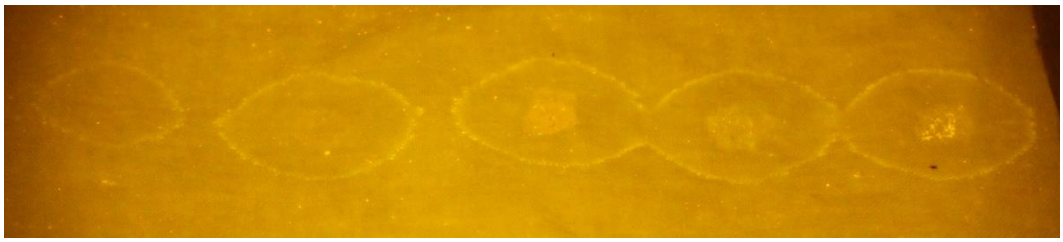

Figure 2. Saliva Stains Under ALS on Cotton Swatch One. Saliva stains from donors A-E (left to right) under 455nm with an orange barrier filter.

The saliva stains on the darker fabrics, denim and spandex, were the most difficult stains to see under an ALS. In comparison, the stains on the light cotton swatches were all visible. Darker substrates have the ability to mask bodily fluids even under the ALS because darker colors can absorb light released as fluorescence.[16] In addition, some saliva samples may have different levels of water, due to the donor's hydration level. When the body's water content is reduced, so is salivatory flow.[16] If the water content of the saliva is increased, then visibility may be decreased. Similarly, if some donors are dehydrated, they may have less water content and more of the other saliva components that may be visible under ALS.

3.3.2. Saliva and Semen Mixture Stain Results

All of the semen and saliva mixture samples were visible under an ALS on all the fabric types. The stains fluoresced at 475nm with an orange filter. The stains fluoresced

brighter than the saliva stains, and were more consistent in distribution, with fluorescence throughout the stain rather than a thin border as seen in the saliva stains [Figure 3]. Dry semen has a strong photoluminescence quality and a typical emission spectrum region of 400nm-700nm,[16] which is consistent with the stains in this study. The broad excitation spectrum of semen may have been the reason why the stains were all detected under an ALS. These results were similar to those found in a study by Vandenberg and van Oorschot, in which semen stains were difficult, but not impossible to visualize on absorbent fabrics.[9]

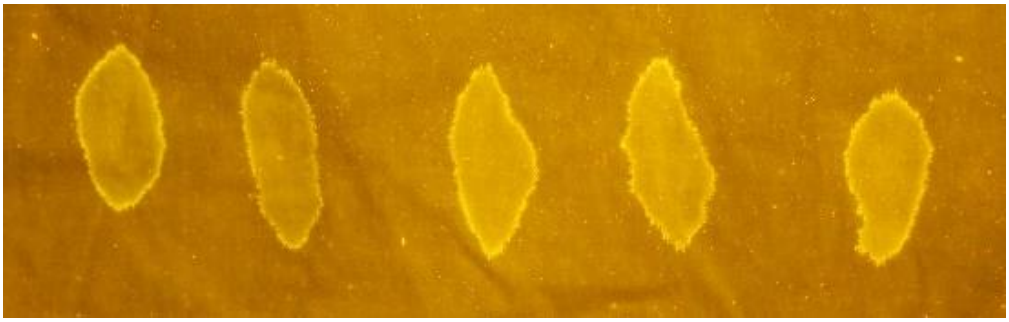

Figure 3. Semen and Saliva Mixture Stains on Cotton Swatch One. The stains A-E (left to right) seen under 475nm with an orange barrier filter.

3.3.3. Saliva and Blood Mixture Results

The blood and saliva mixture stains were visible under an ALS for each donor, and for each fabric type. Blood is darker than most body fluids, thus it absorbs light and doesn't fluoresce at 455nm. The stains appeared dark with a thin line in the center which fluoresced. An ALS has the ability to provide contrast between dark substrates and blood stains,[29] which may have helped the visibility of the stains on darker fabric such as the denim. The thin line which appeared to fluoresce may have been components of the saliva stain.

3.3.4. Saliva and Urine Mixture Stain Results

The urine and saliva mixture stains fluoresced between 455nm and 475nm on all fabrics except for fleece. The stains did not fluoresce on eight out of the fifteen fabric swatches. There were no fluorescence issues with the saliva on the other fabrics, so the particular saliva donors did not affect the results. The urine donor did not affect the results either, because the same urine donor was used throughout the study. Therefore, the fleece fabric was the variable that affected the fluorescence in this part of the study. The pattern on the fleece, and the absorbent nature of the fabric may have led to limited visibility under the ALS. Urine is mostly water, like saliva, and it is also pale yellow in color due to the pigment urochrome and is often masked by the substrate.[1]

3.3.5. Blood, Semen, Saliva and Urine Mixture Stain Results

The four part mixture of blood, semen, saliva and urine was visible under an ALS between 415nm and 515nm for all four fabric types. This was once again due to the color of the blood, which absorbed light and contrasted with the background color of the substrate; no fluorescence was observed through the blood. The type of fabric and its color and absorbency did not affect the visibility of the mixture stains. The only issue with this type of stain is that the dark color of the blood masks the other biological fluids in the stain. All results for the ALS visibility study are shown in Table 2.

Table 2. Visibility of Saliva Stains and Saliva Mixture Stains Under ALS between 415nm and 515nm with an orange barrier filter. When the stains were visible, considered a positive result, it is indicated by (+) and when a stain was not visible, considered a negative result, it is indicated by (-).

	Donor	**A**			**B**			**C**			**D**			**E**		
Stain	**Swatch**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Saliva	**Cotton**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Denim	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-
	Fleece	+	-	-	+	+	-	-	+	+	+	+	+	-	-	-
	Spandex	-	-	-	-	-	-	+	+	+	+	+	-	-	-	+
Saliva/ Semen	**Cotton**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Denim	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Fleece	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Spandex	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Saliva/ Blood	**Cotton**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Denim	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Fleece	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Spandex	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Saliva/ Urine	**Cotton**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Denim	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Fleece	+	-	+	+	-	+	-	-	+	-	+	+	-	-	-
	Spandex	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Saliva/Semen/ Blood/Urine	**Cotton**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Denim	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Fleece	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Spandex	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

3.3.6. Post-oral Sex Sample Results

All three oral sex underwear samples were examined under an ALS for fluorescent properties. Sample F, which did not have any staining visible under white light, did have a visible stain on the inside and outside of the underwear's crotch panel. The stain was yellowish-white in color and covered an area of approximately 10cm x 3cm. The stain fluoresced at 475nm with an orange barrier filter. A fluorescent stain was also found on Sample G, both on the inside and outside of the underwear's crotch panel. The stain was whitish-yellow, and bigger and brighter than the stain visualized on Sample G. It was

observed at 455nm using an orange filter and covered an area of about 12cm x 5cm [Figure 4]. No stains fluoresced under an ALS when Sample H was examined. A range of 445nm – 515nm using an orange filter and a range of 515nm – 555nm using a red filter was tested, and still no fluorescence was visible.

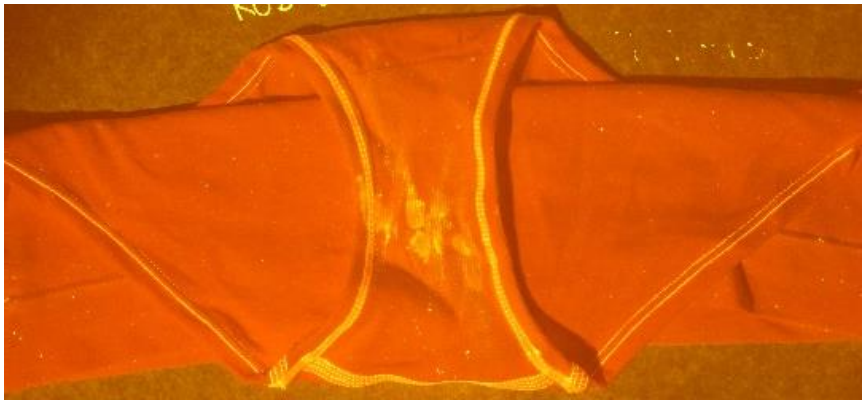

Figure 4. ALS Examination of Post-Oral Sex Sample G. Examined under 455nm with an orange barrier filter.

The oral sex samples were comprised of saliva and vaginal fluid. Vaginal fluid, like saliva, is naturally fluorescent and mostly made up of water.[30] Sample F had a visible stain under ALS, as did Sample G. Both samples had crotch panels that were lighter in color, which may have led to the visibility of the stains. In comparison, Sample H had the darkest crotch panel, which may have masked any fluorescence. The samples were not deposited on the same days, so the concentration of saliva components may have also varied. Vaginal fluid concentration and discharge levels may also affect the results. Many women have fluctuating levels of vaginal discharge throughout the day as well as the stage of their menstrual cycle,[31] which may have been a contributing factor to the degree of fluorescence observed.

Table 3. Visibility of Post Oral Sex Stains Under White Light and ALS between 415nm and 515nm with an orange barrier filter. When the stains were visible, considered a positive result, it is indicated by (+) and when a stain was not visible, considered a negative result, it is indicated by (-).

Sample	Time Post Oral Sex (hours)	White Light Visibility	ALS Visibility
F	6	-	+
G	8	+	+
H	12	+	-

Biological fluids are difficult to visualize on absorbent and darker colored substrates. Even if a stain is not observed under an ALS, the presence of a biological fluid cannot be ruled out.[32] Seidl et al. reported that various biological fluids cannot be distinguished from one another because the stains appear different on different fabrics,[33] which was also found to be the case in this study. The exceptions were the blood mixture stains, because the dark color in blood masked the lighter color of the other biological fluids. Blood doesn't fluoresce and it was easy to determine the difference.

3.4. Seratec® Amylase Paper Results

3.4.1. Saliva Stain Results

Out of the 60 stains deposited on four fabric types, tested in triplicate, there were 57 positive identifications of amylase using the Seratec® Amylase Paper [Table 4]. Of the three cotton swatches, and 15 stains, 14 tested positively for the presence of amylase using the Seratec® Amylase Paper. For the denim fabric swatches, 14 out of 15 stains tested positively. Of the fleece fabric swatches, 14 stains out of 15 tested positive using the Amylase Paper, and all of the 15 stains on the spandex fabric swatches tested positive. Therefore, 95% of the known positive saliva stains were detected. None of the negative

results were stains from the same donor, as they were from donors A, C and E for the cotton, denim and fleece fabrics, respectively.

Table 4. Seratec® Amylase Paper Results for Saliva Stains. A positive result (+) indicates that a yellowish spot appeared against a purple-brown background. A negative (-) indicates that no yellowish spot was seen on the testing paper.

Donor	**A**			**B**			**C**			**D**			**E**		
Fabric Type	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Cotton	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Denim	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
Fleece	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
Spandex	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

The negative results were almost equally split between the fabric types, which suggested that the type of fabric and its absorbency had no effect on the Seratec® Amylase Paper tests. Some negative results may be expected due to different situational circumstances surrounding the testing conditions, or human error. Similar studies have shown similar positive test rates. A study by Venneman et al. resulted in a positive test result rate between 91.07 – 96.43%.[34] This test utilized a red starch paper that identified amylase due to the digestion of starch, a similar process to the Seratec® Amylase Paper test. The mechanism also involved a press test and the range in positive result rates was due to a difference in the test incubation period.

The specific activity of amylase decreases over time, which may have affected some of the test results. A study by Tsutsumi et al. showed that amylase activity decreased to 26% after just 24 hours, then an additional 3% from 1 – 28 days.[4] The Seratec® Amylase Paper test identifies amylase based on its activity, which could have decreased in some of

the samples. The lower detection limit of the Amylase Paper was found to be 125 mIU/mL.[9] Since testing was not completed on the samples immediately after their collection, the starting level of amylase activity and the level in the dried stains at the time of testing cannot be compared.

The level of amylase differs among individuals, so it is assumed that different donors may have had higher starting levels. The samples were all handled the same way after collection, so there was no difference in temperature between them, which has also been reported to affect the level of amylase activity in saliva.[35]

3.4.2. Saliva and Semen Mixture Results

Out of the 60 saliva and semen mixed stains deposited on four fabric types tested in triplicate, there were 57 positive identifications of amylase using the Seratec® Amylase Paper [Table 5]. Of the 15 stains on cotton, 13 tested positively for the presence of amylase using the Seratec® Amylase Paper. For the denim fabric swatches, 14 out of 15 stains tested positively. Of the fleece fabric swatches, all 15 stains tested positive using the Amylase Paper. Similarly, all of the 15 stains on the spandex fabric swatches tested positive as well. This test produced a 95% rate of positive identification for the saliva and semen mixture stains. Some of the negative results were stains from the same donor. Donor C had two negative results, for the cotton and the denim swatches.

Table 5. Seratec® Amylase Paper Results for Saliva and Semen Mixture Stains. A positive result (+) indicates that a yellowish spot appeared against a purple-brown background. A negative (-) indicates that no yellowish spot was seen on the testing paper.

Donor	**A**			**B**			**C**			**D**			**E**		
Fabric Type	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Cotton	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+
Denim	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
Fleece	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Spandex	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Results for the neat saliva stains and the saliva and semen mixture stains were similar; both had positive results for 57 of 60 stains. There was at least one negative result for the cotton swatch and one for the denim swatch. The difference was that the saliva and semen mixture stains had two negatives for the cotton fabric, and the saliva stains had only one. Also, the saliva stains had one negative result on the fleece fabric, but the semen and saliva mixture stain had all positives for the fleece fabric.

The 1:1 semen and saliva mixtures did not show more negative results than were seen in the neat saliva study. The semen samples all came from a single ejaculate collected from one donor, so any variant in the semen affecting the results can be disregarded.

Several studies have been conducted on the cross-reactivity of semen for the Phadebas® test, and the results were found to be similar.[36,37] The mechanism of the Phadebas® test is very similar to the one used in the Seratec® Amylase Paper Test. This means that the level of amylase activity is integral to a positive result. In studies performed using 1:1 semen and saliva concentrations, no interference was found in the results.[36,37] In a study by Wornes et al., it was determined that Phadebas® paper is specific for the activity of amylase, but not saliva itself.[36] This is also the case for the Seratec® Amylase Paper.

A study conducted by Kipps and Whitehead determined the amount of amylase in semen based on an assay by plate diffusion technique.[3] The level was found to be 95 IU/L, which was much lower than the amylase concentration found in saliva, which was determined to be 350,000 IU/L. This is a large difference, but the detection limit of amylase in the Seratec® Amylase Paper is reported to be 125 mIU/mL.[10] Combining the two bodily fluids may increase the amylase levels, and not interfere with the amylase activity, but rather, enhance it.

Another reason semen does not likely inhibit the amylase activity in saliva may be due to the pH of both biological fluids. The pH of alpha-amylase in the body is 7.0, while the pH of semen is 7.2 – 7.8,[5] which is not that far off from the pH of the alpha-amylase. Mixing two biological fluids with similar pH values should not significantly affect the overall pH of the mixture. Therefore, the alpha-amylase activity would still be active, and would be detectable on starch based tests like the Seratec® Amylase Paper Test and the Phadebas® Forensic PressTest.

3.4.3. Saliva and Blood Mixture Stain Results

Out of the 60 saliva and blood mixed stains deposited on four fabric types tested in triplicate, there were 55 positive identifications of Amylase using the Seratec® Amylase Paper [Table 6]. Of the cotton swatches containing 15 stains, 14 tested positively for the presence of amylase using the Seratec® Amylase Paper. For the denim fabric swatches, 12 out of 15 stains tested positively. Of the fleece fabric swatches, all 15 stains out of 15, tested positive using the Amylase Paper [Figure 5]. For the spandex fabric swatches, 14 of

the 15 stains tested positive. This test produced a 92% rate of positive identification for the saliva and blood mixture stains. Some of the negative results were stains from the same donor, as donor E had three negative results on a cotton swatch, denim swatch, and spandex swatch.

Table 6. Seratec® Amylase Paper Results on Saliva and Blood Mixture Stains. A positive result (+) indicates that a yellowish spot appeared against a purple-brown background. A negative (-) indicates that no yellowish spot was seen on the testing paper.

Donor	**A**			**B**			**C**			**D**			**E**		
Fabric Type	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Cotton	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Denim	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+
Fleece	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Spandex	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-

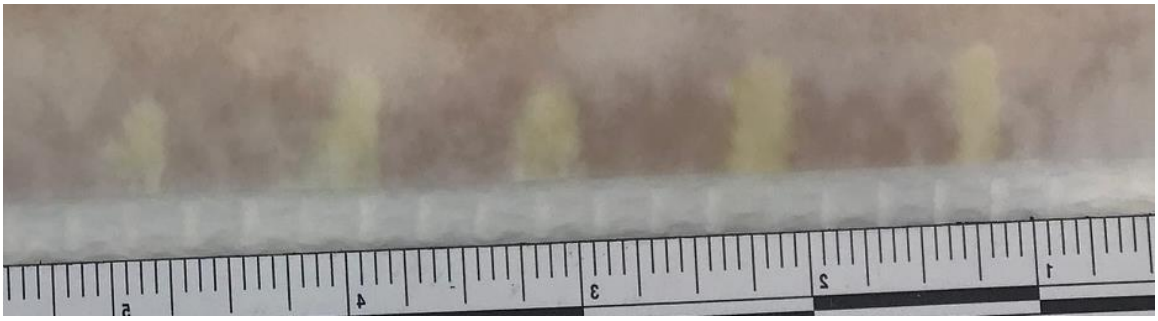

Figure 5. Seratec® Amylase Paper Results for Saliva and Blood Mixture Stains on Fleece Fabric. The five stains on the fleece swatch all tested positive for the presence of saliva. The positive results are indicated by a yellowish color on the paper, surrounded by a purple-brown background.

The blood and saliva mixture stains had fewer positive identifications than the neat saliva stains, or the saliva and semen mixture stains. The fabrics on which the stains were deposited did have similar results. All three stain types on the fleece fabric resulted in positive results, while cotton and denim each had a few negative results. The main

difference was that spandex had one negative result with the saliva and blood mixture, but the results of the saliva, and saliva and semen mixture stains were all positive.

Several studies have found that amylase activity is decreased when saliva is mixed with blood.[36,37] Amylase concentration levels in whole blood have been measured at an average of 160 IU/L. This is compared with an amylase concentration of 350,000 IU/L in saliva and 95 IU/L in semen. Higher levels of amylase in the blood, up to three times, have been attributed to pancreatitis, due to an overactive pancreatic gland.[38] However, since the blood level is higher than the amylase found in semen, and semen and saliva mixtures have no interference in Seratec® Amylase Paper testing, another component of blood could be the cause of the additional negative results.

One difference in blood versus saliva is the protein level. Blood has a much higher protein level than saliva. Blood's protein concentration is between 6 – 8 g/dL, while saliva's is between 0.14 – 0.64 g/dL.[5] This difference in protein concentration could decrease the activity of the amylase in the saliva when blood is mixed with saliva. Tsutsumi tested the levels of amylase in saliva prior to blood contamination, and then at different concentrations of blood and saliva for comparison.[5] The starting level of amylase in the saliva sample was 42.2 IU/mg and when blood was added to a ratio of 1:11, saliva to blood, the amylase activity was measured much lower at 8.12 IU/mg. When a 1:1.1 ratio of saliva to blood was observed, the amylase activity dropped even further to only 0.07 IU/mg.[5] This suggests that when blood is added to saliva, it interferes in some way with the amylase activity. However, since the lower detection limit of amylase is 125 mIU/mL for the Seratec® Amylase Paper, the test should still work for most 1:1 mixtures.[10]

Another component of blood that may affect the activity of amylase and the ability of Seratec® Amylase Paper to detect amylase in its presence is pH. The pH of blood is very specific and controlled with a small range of just 7.35 – 7.45.[40] The pH optimal for alpha amylase activity is 7.0,[41] which is only slightly less. The pH level of blood does not differ greatly to the pH level of blood, so it is unlikely the ability to detect amylase is affected. However, a difference in pH has been shown to cause false negatives in a previous study by Tsutsumi et al.[5]

3.4.4. Saliva and Urine Mixture Stain Results

Out of the 60 saliva and urine mixed stains deposited on four fabric types tested in triplicate, there were 50 positive identifications of amylase using the Seratec® Amylase Paper [Table 7]. Of the 15 stains on cotton, 13 tested positively for the presence of amylase using the Seratec® Amylase Paper. For the denim fabric swatches, 7 out of 15 stains tested positively. Of the fleece fabric swatches, all 15 stains out of 15 tested positive using the Amylase Paper. For the spandex fabric swatches, 15 of the 15 stains tested positive. This test produced an 83% rate of positive identification for the saliva and urine mixture stains. Some of the negative results were stains from the same donor, as donor A had four negative results across the different fabrics.

Table 7. Seratec® Amylase Paper Results on Saliva and Urine Mixture Stains. A positive result (+) indicates that a yellowish spot appeared against a purple-brown background. A negative (-) indicates that no yellowish spot was seen on the testing paper.

Donor	**A**			**B**			**C**			**D**			**E**		
Fabric Type	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Cotton	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+
Denim	-	-	+	-	-	-	-	-	+	-	+	+	+	+	+
Fleece	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Spandex	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

The saliva and urine mixture stains had more negative results than the previous tests on saliva and semen, and saliva and blood. The most negative results came from the denim swatches. The previous tests have negative results on the denim swatches as well, but not as many as here. The fleece and spandex tests were all positive, as was the case for the saliva and semen stains. This indicates that the stain type, as well as the fabric type, may have led to the inability to positively identify the amylase in the saliva and urine mixture.

The increase in negative results could indicate that the addition of urine affects the Seratec® Amylase Paper Test results by blocking the detection of amylase. Because amylase is secreted into the bloodstream via the digestive system, one of the ways it is eliminated is through urine.[2] Due to this, some levels of amylase are expected in urine, however high levels may signify something more serious, including renal disease, pancreatitis, mumps and abdominal disorders.[42]

Urine has been shown to inhibit the results of colorimetric tests, which indicates a decrease in amylase activity, as shown in a study by Barni et al. where urine was tested alone for the kinetic behavior of amylase.[2] In this case it was determined that while the activity was there, and on par with the activity seen in pure saliva samples, it took much

longer for the activity to occur. This may have affected the Seratec® Amylase Paper Test results because they are very time sensitive and only a ten minute window is recommended after the test is performed. In addition, the test paper is only in contact with the sample for 15 seconds, which may not have been long enough for five of the urine and saliva mixture samples to activate their amylase activity. Although a test was performed to see if a 30 second contact time period would increase accuracy, this was not done for the mixture stains, just the pure saliva samples.

A study by Ojala et al. investigated the effectiveness of Phadebas® starch tablets on urine and saliva mixtures. The result was that not all mixture samples produced positive results reliably, and it was likely because urine dilutes saliva and adds proteins to the mixture. This ultimately dilutes and inhibits the amylase activity of the urine and saliva, although it is still detectable in many cases. It is important to note that Ojala et al. used different donors for each urine sample, which this study did not. This may add to additional variation and inability to reproduce results.[43]

The dilution effect of urine is due to its high water content, which is highly dependent on the donor's hydration level. The high water level may add additional time to the activation of the amylase activity. Although saliva is also mostly water, it has a much higher amylase concentration than urine.[4] The pH levels of urine and saliva are also very different, with saliva at 7.0 and urine with a range of 4.6-8.0.[44] The levels could potentially be identical, which would be ideal for amylase activity, or very different, which could inhibit it.

3.4.5 Blood, Semen, Saliva and Urine Mixture Stain Results

Out of the 60 blood, semen, saliva, and urine mixed stains deposited on four fabric types, tested in triplicate, there were 50 positive identifications of amylase using the Seratec® Amylase Paper [Table 8]. Of the 15 stains on cotton, 12 tested positively for the presence of amylase using the Seratec® Amylase Paper. For the denim fabric swatches, 10 out of 15 stains tested positively. Of the fleece fabric swatches, 14 stains out of 15 tested positive using the Amylase Paper. Similarly, 14 of the 15 stains on the spandex fabric swatches tested positive. This test produced an 83% rate of positive identification for the blood, semen, saliva and urine mixture stains. Some of the negative results were stains from the same donor. Donor B had four negative results split among the cotton, denim and spandex swatches. Donor A had all positive results for all 12 swatches.

Table 8. Seratec® Amylase Paper Results for Blood, Semen Saliva and Urine Mixture Stains. A positive result (+) indicates that a yellowish spot appeared against a purple-brown background. A negative (-) indicates that no yellowish spot was seen on the testing paper.

Donor	**A**			**B**			**C**			**D**			**E**		
Fabric Type	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Cotton	+	+	+	-	+	+	+	+	+	-	+	+	+	+	-
Denim	+	+	+	+	-	-	-	+	-	+	+	-	+	+	+
Fleece	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Spandex	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+

The blood, semen, saliva and urine mixture had 10 negative results, which was the same as the saliva and urine mixture results. The denim swatches, again, had the highest number of negative results, and therefore an inability to reliably identify amylase in the mixture sample. This sample also had three negative results for the stains on the cotton

swatches, which is the highest number for this type of fabric. This indicates that the type of stain may affect the results more than the type of fabric in this case.

The same rate of positive results, 83%, was obtained when the blood and saliva mixture samples were tested. This suggests that blood inhibits amylase activity, either completely or for enough time that it is not able to register on the Seratec® Amylase Paper. This may be due to the pH level of blood which is slightly higher than saliva, and also the protein level of blood which is also higher. Both may inactivate or delay amylase activity. In addition, urine may also inhibit amylase activity due to pH level. A mixture with both of these components could increase the chance of false negatives.

3.4.6. Post-oral Sex Sample Results

The three oral sex samples were tested with Seratec® Amylase Paper. Sample F, the pink pair of underwear worn for 6 hours after oral sex, tested positive for amylase. Sample G, the purple pair of underwear worn for 8 hours, also tested positive for amylase. The last sample, Sample H, the purple striped underwear worn for 12 hours, tested negatively for amylase activity [Table 9].

Table 9. Seratec® Amylase Paper Results for post-Oral Sex Samples. A positive result (+) indicates that a yellowish spot appeared against a purple-brown background. A negative (-) indicates that no yellowish spot was seen on the testing paper.

Sample	Time Post Oral Sex (hours)	Result
F	6	+
G	8	+
H	12	-

The post-oral sex underwear samples were not controlled, and the stain components were essentially unknown. It was assumed that the samples were part vaginal fluid and part saliva. The amount of each was also unknown, and it is likely to have varied between samples. This could have led to some variability in the results, as only two of the three samples were positive. The samples were also from the same donors, but on different days so the starting amylase levels in the saliva may have varied.

Amylase levels in pure vaginal swabs were tested in a study by Wornes et al. using the Phadebas® Forensic PressTest. All vaginal swab samples came back negative for amylase activity.[36] Another study by Tsutsumi et al. found similar results with Phadebas® test, which indicates that vaginal fluid inhibits amylase activity in mixtures.[5] Since the Phadebas® test and Seratec® Amylase Paper Test both detect amylase activity, it is not surprising that sample H did not result in a positive test.

Vaginal fluid levels vary among and within individuals throughout the day, week and month.[30] If the vaginal fluid in the saliva and vaginal fluid mixture did inhibit the result in sample H, it could be due to the pH level in vaginal fluid, typically 3.8-4.5, which is much lower than saliva's pH of 7.0.[45] A difference in pH has been shown to affect results of amylase tests in the study by Tsutsumi et al. and a similar effect is seen in blood and saliva mixtures.[5]

The main difference between the three underwear samples was how long they were reported to have been worn after oral intercourse, which also could have affected the saliva samples. These samples mimic case samples since they were not developed under controlled laboratory protocols. The length of time the three pairs of underwear were worn

following oral sex was reported by the anonymous donor. The two positive samples, F and G, were from underwear worn for 6 and 8 hours, respectively. Sample H, which was negative, was from underwear worn for 12 hours, which would keep the sample moist and at a higher temperature than room temperature for a longer period. In a previous study by Schneyer, it was shown that an increase in temperature can cause a decrease in an enzymatic activity.[35] This may explain why sample H produced a negative test result in comparison to samples F and G. The exact composition of saliva to vaginal fluid was also uncontrolled and unknown in all three cases so it is possible that there was more vaginal fluid present in sample H, and less saliva. This could potentially dilute the amylase and render it undetectable.

3.5. qPCR of Samples

3.5.1. qPCR of Saliva Samples

DNA concentrations were calculated for selected cotton and fleece saliva samples, and also for the reagent blank, positive control, and negative control. This included three saliva stains on cotton in contact with the Seratec® Amylase Paper (CC), three saliva stains on cotton not in contact with the Seratec® Amylase Paper (CN), three saliva stains on fleece in contact with the Seratec® Amylase Paper (FC), and three saliva stains on fleece not in contact with the Seratec® Amylase Paper (FN). All 12 samples were run in duplicate for 24 total samples, and all were from donor C. The positive control, negative control, and reagent blank showed no contamination, and were within the laboratory's acceptable range for IPC and human DNA and male DNA values (for the positive control).

The qPCR results correctly indicated that the DNA was from a female. Since the samples were all from one female donor this was expected. The human DNA quantities were calculated using Equation 1. The rest of the DNA quantities for the 24 saliva samples contained sufficient DNA to yield a DNA profile if the next step in DNA analysis was taken. The mean value of each sample type was calculated, as was the standard deviation [Figure 6].

The mean value for the CC sample was 6.27 ng/ul. The mean value for the CN sample was 2.19 ng/ul. The mean value for the FC sample was 5.43 ng/ul, and 2.62 ng/ul for the FN sample. The results indicate that the samples in contact with the Seratec® Amylase Paper had higher DNA concentrations, while the samples not in contact had lower concentrations. The CC sample average was higher than the FC sample average, but CC range overlaps the FC range. However, CC was not significantly higher than FC, nor was FN significantly higher than CN. The standard deviation for CC encompasses FC, making them statistically similar.

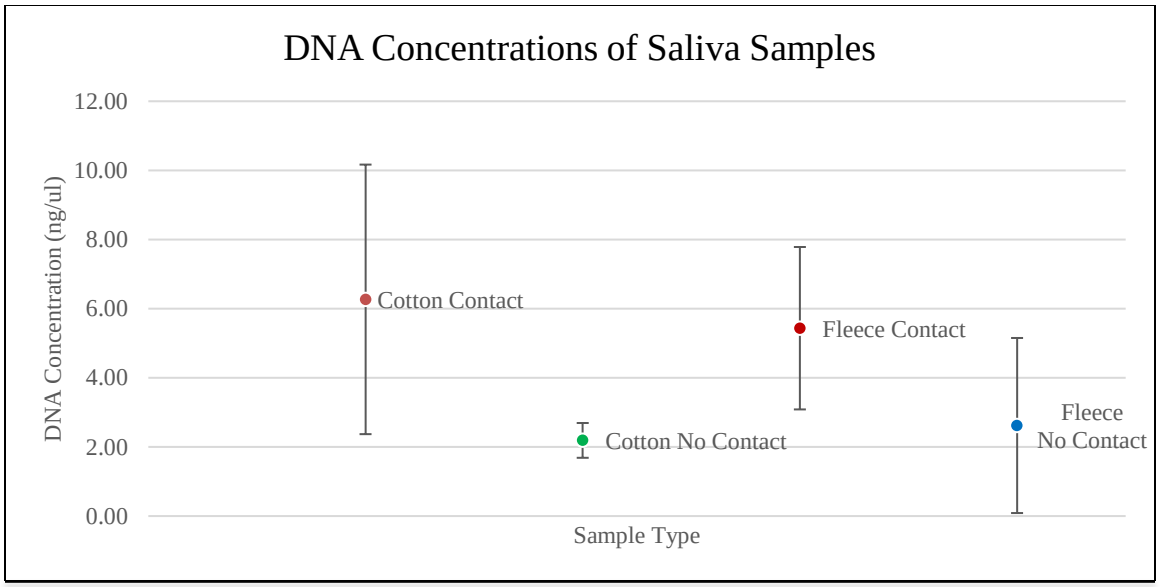

Figure 6. DNA concentrations for saliva samples. DNA concentration averages for CC, CN, FC, and FN saliva samples, including one set of negative and positive standard deviation bars.

The type of fabric did not affect the DNA qPCR results, as the results for cotton and fleece were quite similar for the contact and no contact samples. The main difference was between the no contact and contact samples, for both fabrics. Since the same sample donor was used, and the samples were extracted and prepared at the same time and under the same conditions, it is possible that undergoing the Seratec® Amylase Paper process improved the DNA detection for these samples, although it is unclear why this would occur. The same donor was used for all samples, which would rule out other common sources of qPCR inhibition such as heritable inhibitors, environmental factors or conditions like oral hygeine.[22]

The CC and FC samples had higher DNA concentrations even though they came into contact with the Seratec® Amylase Paper, which theoretically could have inhibited the sample chemically, or physically removed some of the sample during contact. It was found

that neither of these issues were present, due to the DNA concentration and IPC values, which were close to the laboratory's acceptable range of 29.3608 to 30.9117 [Table 10].

Sample	Average	St Dev
CC IPC	29.18136	±0.158772
CN IPC	29.16756	±0.041021
FC IPC	29.20323	±0.054611
FN IPC	29.20953	±0.023235

Table 10. The IPC Values for Saliva Samples. The average IPC values for CC, CN, FC, and FN values, including standard deviation.

In a previous study by Hedman et al. DNA was recovered from saliva stains after contact with the Phadebas® Forensic PressTest, which shares a similar protocol to the Seratec® Amylase Paper test. This would also indicate that the action of pressing the test paper onto the stain does not decrease the DNA concentration of the stain. Hedman et al. were able to recover entire DNA profiles from non-dilute and dilute samples.

3.5.2. qPCR of Seratec® Amylase Paper

Three samples of the Seratec® Amylase Paper were extracted and quantified via qPCR, in duplicate. The paper was removed directly from the manufacturer's test kit and had never been used. The results were all negative for human and male DNA. The average IPC C_t value was 29.24 for all six samples, with a standard deviation of ± 0.06. The range of the six IPC values was 29.17 to 29.29. The acceptable IPC C_t value range used by this laboratory is 29.3608 to 30.9117. The paper IPC values have lower C_t values than the

standard IPC values, which indicates the cycle threshold was reached sooner than expected and that no PCR inhibition from the Seratec® Amylase Paper was detected.

4. CONCLUSIONS AND FUTURE DIRECTIONS

Saliva is an important body fluid in forensic investigations. Saliva is found on many items of evidence commonly found at a crime scene, such as drinking vessels, cigarettes, and face masks.[3,6] DNA testing can be performed on saliva once it is identified due to the presence of epithelial cells.[6] Its presence can place a suspect at the scene of crime, including a physical or sexual assault, and corroborate a victim's version of events.[3]

In this study, three components were investigated. The first was whether Seratec® Amylase Paper could detect amylase when saliva was mixed with other body fluids like semen, blood, urine and vaginal fluid. Next, was whether Seratec® Amylase Paper could detect amylase on different fabrics, including cotton, denim, fleece and spandex in saliva stains and saliva mixture stains. The final portion of this study was whether DNA testing could be performed on a saliva stain after it was tested and in contact with Seratec® Amylase Paper.

When the different body fluids were tested with Seratec® Amylase Paper it was found that most still produced a positive result. The mixtures were half saliva, and half another body fluid type. The most positive results came from the neat saliva stains and the saliva and semen mixture stains. They both had 57 positives out of 60 tests, which led to a 95% accuracy rating. This was similar to the results found in other studies of this type.[37,38]

The saliva and urine mixture, however, had a lot of negative results with the Seratec® Amylase Paper. It had only 50 positive identifications out of 60 stains, leading to an 83% accuracy. This rate of accuracy was identical to the saliva, blood, semen, and urine mixture

stains which also had 50 positive tests out of 60. This may suggest that urine affected the results of the test and interfered with the detection of amylase.

The oral sex samples had a 66% rate of positive results. However, due to the small sample size and uncontrolled nature of the samples, it could not be definitively determined. Future studies could determine whether downstream testing with other preliminary tests to detect the other body fluids could be performed. A similar study was performed by Herman et al. using Phadebas® paper and the AP Spot Test for acid phosphatase, which detects semen. It was found that semen can be preliminarily identified with this test, even after the stain was tested using Seratec® Amylase Paper.[18] This could be repeated with other tests such as phenolphthalein for the presence of blood, and the DMAC test for urine detection.

The effectiveness of Seratec® Amylase Paper on different fabrics was tested using cotton, denim, spandex and fleece. For all body fluid stains, neat saliva and the mixtures, the denim fabric had the most negative results. This may have been due to the material itself and the less absorbent nature of the denim, which was thin and most liquid samples did not absorb into the fabric. The more absorbent fabrics like fleece and cotton had more positive results in comparison. There was little difference in the type of stain on the denim and Seratec® Amylase Paper's ability to detect amylase. Future studies could test different fabric types or surfaces, such as linen or ceramic tile. This has been investigated in previous studies but not in mixtures of body fluids.[3,6] Dilutions of saliva could also be tested on these surfaces for effectiveness.

The final portion of this study investigated whether DNA could be recovered from stains that had come into contact with Seratec® Amylase Paper. To determine this, stains

in contact with the paper, and stains not in contact with the paper were tested. In addition, unused Seratec® Amylase Paper was tested for the presence of any inhibitors. It was found that the stains that had come into contact with Seratec® Amylase Paper had a slightly higher concentration of DNA than stains that had not been in contact with the paper. The IPC values of the paper only extraction were slightly below the laboratory's accepted range, thus the PCR process was very efficient, and did not indicate any inhibition.

One future suggestion for DNA-related Seratec® Amylase Paper testing could investigate the ability to pick up DNA profiles from the test paper after it has been used. A study by Hedman et al. looked at the ability to get DNA from Phadebas® paper after it had been in contact with a stain, and a similar test could be used for the Seratec® Amylase Paper. In addition, different fabrics could be tested after contact with Seratec® Amylase Paper, not just fleece and cotton. Finally, other extraction methods could be investigated to see if the DNA concentrations are higher, or the IPC levels were in range.

In conclusion, the Seratec® Amylase Paper test is an effective method for presumptively determining the presence of saliva in mixtures with other body fluids, although some false negatives were obtained. The test may not be as accurate in detecting amylase in mixtures of three or more fluids, or in the presence of urine. The Seratec® Amylase Paper test is also effective on most fabrics but may have difficulty with certain fabrics like denim. Finally, DNA can be successfully recovered from biological stains that have been in contact with Seratec® Amylase Paper, but further testing is necessary to optimize the methods.

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