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Targeted-Ion Mass Spectrometry for the Identification of Forensically Relevant Biological Fluids and Samples from Sexual Assault Evidence

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Abstract

Forensic practitioners have long sought efficient and reliable means for identifying those samples that are best suited for successful genetic profiling. Traditional serological screening methodologies rely upon enzyme activity and antibody-based serological tests. These tests can be consumptive, laborious and costly while reliance on antibody-based serological testing can be prone to error. Positive results resulting from non-target biological fluids, the potential for cross- reactivity and non-specific binding events yield merely presumptive results. This has led forensic biologists to omit serological testing, at least in the case of sexual assault kit samples, in favor of Y-Screen assays. While these Y-Screen approaches achieve rapid screening of samples for the presence of a detectible male DNA, they do not provide any serological information and therefore lack critical investigative/biological context.

A more sensitive and accurate technology for the confirmatory identification of biological fluids would greatly bolster the weight of serological evidence presented in court and assist with more informed sample prioritization. A particularly promising approach combines high- specificity protein biomarkers with a target-ion mass spectrometry. Applying absolute quantitation of protein targets in the biomarker panel will enable forensic practitioners to make fuller use of serological information in their decision making on downstream analyses in order to improve the successful analysis of challenging sexual assault samples.

This research demonstrated the prevalence of false-positive results associated with antibody-based serological methods, developed and validated a multiplex targeted-ion mass spectrometry-based assay for the identification of six forensically relevant biological fluids, demonstrated improved sensitivity and specificity of mass-spectrometry based body fluid identification as compared to traditional techniques, developed a modified assay for seminal fluid compatible with sexual assault kit evidence and evaluated the relationship between quantitative levels of target seminal fluid peptides and the ability to generate Y-STR profiles. These results provide the forensic and criminal justice communities with a powerful tool to aid in the criminal investigation of violent crimes.

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by

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CHAPTER 1: INTRODUCTION

1 Introduction

Forensic serology is the study of blood, saliva, semen and other bodily fluids in relation to legal matters [1]. Biological fluid detection and identification provides important contextual information to a forensic investigation. While genetic testing can help to establish from whom DNA may have come, only serological testing can provide an indication of the body fluid or tissue from which a DNA profile may have originated [2]. There exist myriad examples of how the unambiguous identification of biological fluids can be critical to an investigation. Take for example the analysis of a victim's clothing from an alleged sexual assault. The identification of biological stains such as semen through forensic serological techniques may render more probable the inference that a sexual act occurred while a match between the DNA profile generated from that stain and a suspect's reference profile helps to establish a link between the suspect and victim. Both pieces of information can be presented in court to corroborate allegations of sexual assault by a given suspect. The ability to obtain the most probative value from a biological stain in a criminal investigation, therefore, requires both the development of an interpretable DNA profile and the identification of the biological substance from which the profile originated.

1.1 Past and Current Serological Techniques

Current forensic tools for the identification of biological fluids are based on the same fundamental methods that have been employed for much of the history of forensic science. Namely, these are chemical reactions involving components of a body fluid; detection of enzymatic activity (typically through colorimetric reactions) characteristic of a body fluid; immunological binding to antigens that are characteristic of a body fluid or, in the case of semen, direct visualization of spermatozoa by microscopy. All of these techniques have value to forensic investigations; however, they also suffer from a variety of substantial test-specific limitations. These tests as they apply to the identification of blood, semen, saliva, urine, vaginal fluid and feces will be outlined below as will their advantages and limitations.

1.1.1 Presumptive Detection of Blood

Bloodstains are commonly submitted for analysis as part of criminal investigations. Blood as a matrix is composed of a watery, protein rich fluid called plasma and a cellular component comprised of erythrocytes, leukocytes and thrombocytes. Erythrocytes are responsible for the transport of oxygen throughout the body and mainly consist of the metalloprotein hemoglobin. Given its high abundance in blood, the hemoglobin protein has long served as the primary target for most blood detection reactions. Normal adult hemoglobin consists of four globular polypeptide chains (2α chains and 2β chains), each

of which is tightly associated with a non-protein heme group, an iron ion held in a porphyrin ring.

Currently, the most common form of presumptive tests for suspected bloodstains are chemical color reaction based assays as exemplified by the phenolphthalein or Kastle-Meyer test. This reaction takes advantage of the peroxidase-like activity of heme. The iron in heme readily transitions between the ferrous and ferric state. This movement of electrons helps catalyze a reaction with a peroxide group (most commonly in the form of hydrogen peroxide) to create free radical species. These free radicals then react with an indicator compound (*e.g.*, phenolphthalin), which becomes oxidized to phenolphthalein (**Figure 1**) generating a bright pink color indicative of a positive result (**Figure 2**) [3]. Additional chemical color tests for blood that follow the same reaction mechanism employ tetramethylbenzidine (used commercially in the Hemastix® test), orthotolidine, leucomalachite green and luminal. These assays, while extremely sensitive, are not specific. Strong chemical oxidants (such as hypochlorite ions in bleach and household cleaners or metal salts like copper and nickel salts) can directly oxidize an indicator compound to produce a color change in the absence of heme. Additionally, there are many plant tissues *(i.e.,* potato, tomato, red onion, horseradish) that possess peroxidase-like activity that will also catalyze this reaction [4]. Additionally, strong reductants, when present, may cause false negative results [3].

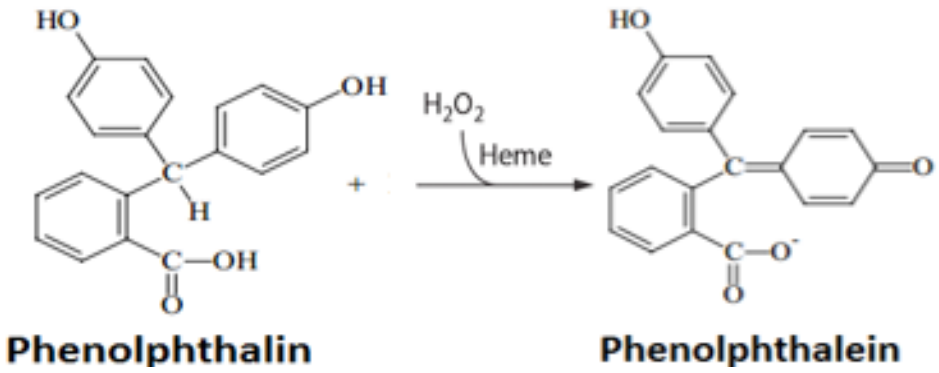


Figure 1: Chemical reaction of underlying the phenolphthalein assay (Kastle-Meyer reaction) for the presumptive detection of blood. Phenolphthalin (colorless) is oxidized in the presence of hydrogen peroxide and heme to produce phenolphthalein which appears pink.

Figure 2: Photograph of phenolphthalein assay results. The swab on the left is an example of a negative reaction. The swab on the right provides an example of a positive reaction.

Microcrystal assays have also been historically used for bloodstain detection. These tests apply chemicals to suspected bloodstains to form characteristic heme crystals. In 1853, the Teichmann crystal assay was developed. In this assay, glacial acetic acid and salts (potassium bromide, potassium chloride and potassium iodide) were used to react with

hematin to form characteristic brown rhomboid crystals [3]. In 1912, the Takayama method in which heme was combined with sodium hydroxide, saturated glucose, pyridine and water was published. This method produced characteristic feathery reddish/purple crystals, comprised of pyridine ferroprotoporphyrin ring structures that could be readily viewed under polarized light [1]. While more specific than the catalytic color reactions for blood, these microcrystalline tests produced positive results with heme containing molecules other than hemoglobin, such as bacterial catalases and peroxidases [5]. Additionally, in cases where dilute or only trace quantities of evidence are available, crystal assays were found to lack the sensitivity required for the reliable detection of blood in many forensic contexts.

Immunological reactions are also commercially available for blood detection. These include Hexagon OBTI (Human Gesellschaft fur Biochemica), HemDirect (Seratec®), ABAcard HemaTrace® (Abacus Diagnostics) and RSID™ Blood (Independent Forensics). The first three detect hemoglobin in blood while RSID™ Blood targets glycophorin A on the surface of red blood cells. Glycophorin A is a transmembrane protein responsible for cell-cell binding interactions [6]. All four of these methods are immunochromatographic assays. Therefore, they function in a similar manner and are subject to similar limitations.

In an immunochromatographic assay, labeled (*e.g.*, colloidal gold conjugated) mobile-phase antibodies specific to a protein antigen (*i.e.*, a biomarker) of interest are localized to a sample well within an immunochromatographic assay cartridge. When an

aqueous sample extract is added, the target antigen binds with these antibodies and the complex diffuses down a nitrocellulose membrane. Immobilized at a test site in the membrane are additional antibodies for a second epitope on the target protein. This binds to the antigen-antibody complex and produces a line to indicate a positive result based on the accumulation of labeled antibodies at the test site. An internal control consisting of immobilized anti-immunoglobulin antibodies that are specific to the antibodies contained on the dye-labeled mobile-phase particles form the sample well is also built into the assay. The internal control antibodies are patterned into an immobilized control line further down the test strip (**Figure 3**). Test results are only considered valid when the line in the control zone can be observed. A positive result therefore appears as at least two colored lines, one at the test zone and one at the control zone, whereas a negative test result appears as a single line at the control zone (**Figure 4**). The specificity of some of these assays for hemoglobin and glycophorin A is such that they are capable of distinguishing between human blood (and that of higher order primates) and the blood of other nonhuman species. Therefore, they can be used not only for presumptive blood detection but also for presumptive species categorization.

While sensitive, fast and easy to use, these assays can be costly and suffer from a lack of body fluid specificity. Target protein biomarkers present at lower concentrations in other biological fluids can also generate a positive reaction [7]. For example, the sensitivity of the ABAcard HemaTrace® can be as low as 0.07 μg/mL of hemoglobin, making the assay more sensitive than chemical color reactions for blood. As a result, however, the test has been shown to produce positive results with seminal fluid stains, and

oral, vaginal, anal, and rectal swabs. At this level of sensitivity, it is thought that the assay is detecting very low amounts of hemoglobin in these non-target fluids; thereby generating false positive reactions in regard to the biological fluid being targeted [3]. Unpredictable cross-reactivity with non-target molecules having similar conformational epitopes is also possible as are non-specific binding events due to extremes of pH or other sample-specific chemical compounds. Environmental contaminants also have the possibility of interfering with antibody binding [7]. Degraded samples will not work with these assays due to loss of conformational integrity of the target protein. Additionally, at high concentrations of target antigen, these tests suffer from a phenomena called the high-dose hook effect, leading to false negative reactions [8].

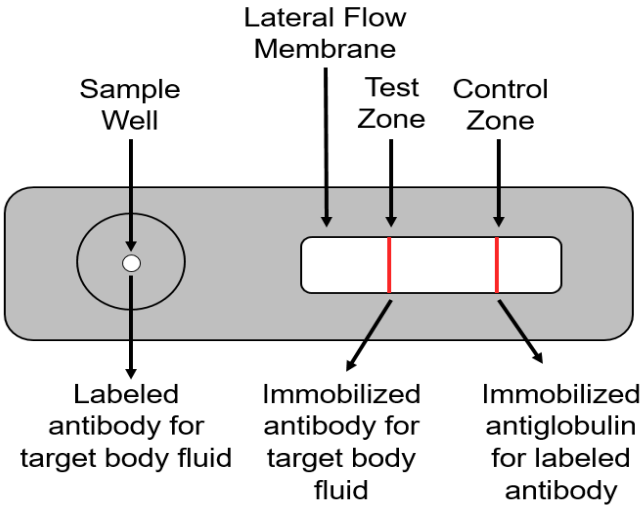


Figure 3: Lateral Flow Immunochromatographic Test Strip Design

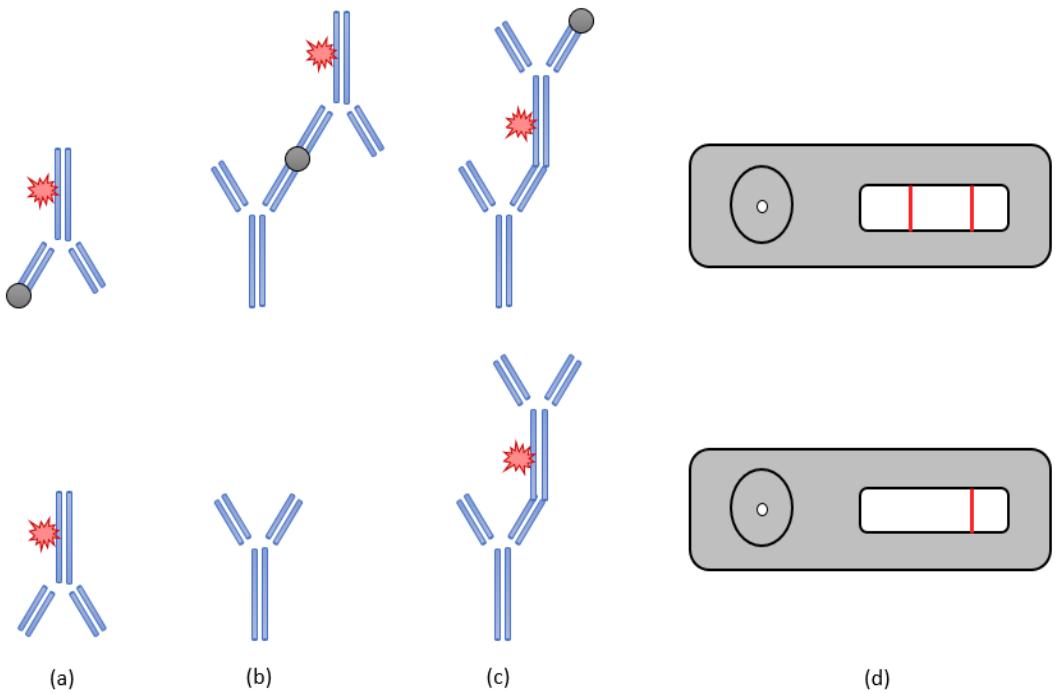


Figure 4: Immunochromatographic Assay depicting a positive (top) and negative (bottom) reaction. (a) In the sample well the target antigen (hemoglobin or glycophorin A in the case of blood assays) present in the sample binds to the mobile phase antibodies conjugated to colloidal gold particles. (b) At the test zone, the colloidal gold conjugated antigen-antibody complex to binds to immobilized target antigen antibodies to form a labeled antibody antigen antibody sandwich. If the sample is positive, colloidal gold conjugated antigen-antibody complex will start to accumulate in these sandwich formations forming a visible colored line at the test zone. If the sample is negative, the antigen-free colloidal gold conjugated antibodies will flow past the test zone. (c) At the control zone, the antigen-free colloidal gold conjugated antibodies from the sample well will bind to immobilized anti-immunoglobulin antibodies forming a visible colored line at the control zone. (d) A positive test result has a line at both the test and control zones. A negative test has no line at the test zone and a line at the control zone.

More historical methods of species identification also employed antigen-antibody interactions but were based on the visualization of a precipitation reaction following immunodiffusion. The most basic example of this approach was the ring assay in which a sample extract, containing target antigens if positive, is layered on top of an antisera solution without mixing. Both antigen and antibody will diffuse (double immunodiffusion)

toward each other. In a positive reaction, a white precipitate forms at the interface of the two layers. Another example of a double immunodiffusion assay is the Ouchterlony assay. This assay is performed by punching a series of wells into an agarose gel matrix. Antisera is loaded into one well and sample extracts possibly containing target antigens are loaded into surrounding wells. The gel is then incubated to allow for the diffusion of both antigen and antibody through the gel matrix. When target antigens are present, a precipitate line will form between the sample well and the well containing antisera (**Figure 5**). In lieu of passive diffusion, an electric field can be applied to the gel such as during cross-over electrophoresis. This serves to drive antigens across an agar gel resulting in enhanced sensitivity. As with the ring and Ouchterlony assays, a positive reaction is denoted by formation of a visible precipitate.

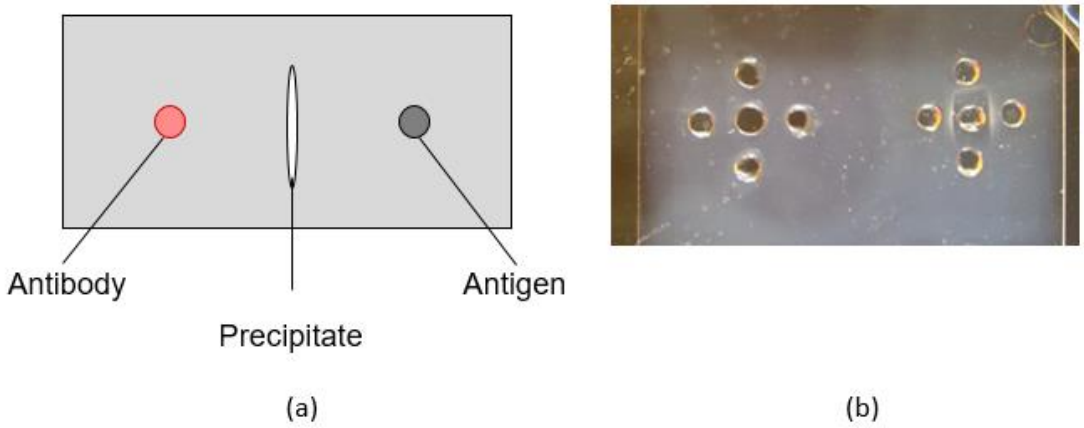


Figure 5: (a) Diagram of the Ouchterlony assay. (b) Two rosettes punched into an agarose gel. In the central well of each rosette, antisera is loaded. In the rosette to the right, the top well was loaded with a positive control; the bottom well was loaded with a negative control and the two side wells were loaded with sample extract. Both samples and the positive control produced a positive reaction as indicated by the visible white precipitate line. The negative control is negative as it lacks a visible precipitate between it and the antisera well.

1.1.2 Presumptive and Confirmatory Detection of Semen

Semen is comprised of a cellular component, spermatozoa (sperm cells), leukocytes and epithelial cells, as well as a fluid portion. During spermatogenesis, spermatozoa are formed in seminiferous tubules in the testes. Spermatozoa are then transported to the epididymis where they mature, a process that takes approximately three months. During ejaculation, sperm cells leave the epididymis through the vas deferens where they can ultimately join with secretions from glandular tissues. An average ejaculation produces 2-5 mL of semen containing 10^7 to 10^8 spermatozoa per milliliter. Sperm cells account for only 1-5% of the total ejaculate volume [9].

Human spermatozoa are comprised of three regions, the head, the midpiece, and the tail. The head contains the nucleus which contains a single set of chromosomes from the male. The acrosomal cap surrounds the very tip of the head. The acrosomal cap contains lytic enzymes that aid in the digestion of the outer membrane of the ovum, allowing for sperm penetration. The midsection connects the head to the tail. This is where the mitochondria that generate ATP to provide energy for tail movement are localized. The tail itself contains microfilaments that contract to provide forward motion.

Seminal plasma is a complex fluid mixture made up of contributions from the seminal vesicle, prostate and bulbourethral glands. Pre-ejaculatory fluid consists almost entirely of secretions from the bulbourethral gland. This can contain traces of acid phosphatase and prostate specific antigen. Less frequently, spermatozoa are observed and these are thought to be due to carryover from a prior ejaculation. The bulbourethral gland

secretes mainly galactose used for energy and mucus production. Secretions from the prostate gland account for approximately 30% of the ejaculate and include the enzyme acid phosphatase, prostate specific antigen, citric acid, proteolytic enzymes and zinc. Both acid phosphatase and prostate specific antigen are protein markers that are typically targeted for the presumptive detection of seminal fluid. The seminal vesical contributes 65-75% of the overall semen volume and secretes semenogelin proteins, flavins, fructose and prostaglandins [10, 11]. Flavins are notable for their contribution to semen's ability to fluoresce under ultraviolet light – a property which is used to search for possible seminal stains. Semenogelin I and II serve as additional targets for the immunochromatographic detection of seminal fluid.

Chemical reaction-based assays such as the Barberio and Florence crystal tests have also been used historically for the presumptive detection of seminal fluid. Due to their lack of specificity and reproducibility however, they have been replaced with tests targeted to enzyme activity and antibody-based detection of protein antigens. As previously stated, prostatic fluid secretions include the enzyme acid phosphatase which has long served as a presumptive marker for the detection of seminal fluid. Prostatic acid phosphatase cleaves phosphate from substrates such as α-naphthol phosphate. The resulting α-naphthol undergoes an azo coupling reaction to form a pink/purple colored product indicative of a positive reaction (**Figure 6**) [12]. As this reaction requires enzymatic activity, loss of this activity over time due to such factors as microbial-associated protein degradation, exposure to extremes of temperature and/or pH or inhibitory chemical agents can limit the sensitivity and the time period during which this assay is useful.

While generally sensitive, this test is presumptive in nature as positive results can also be generated by bacteria present in vaginal secretions as well as by endogenous acid phosphatase produced by cervical epithelial cells. False positive results can also be obtained with a variety of food products [13]. Several other acid phosphatase isoenzymes have also been identified in human tissues aside from prostatic acid phosphatase. These include erythroid acid phosphatase, lysosomal acid phosphatase, macrophage acid phosphatase, and testicular acid phosphatase [3]. Elevated concentrations of acid phosphatase in serum is seen (and used diagnostically) in patients with prostate cancer.

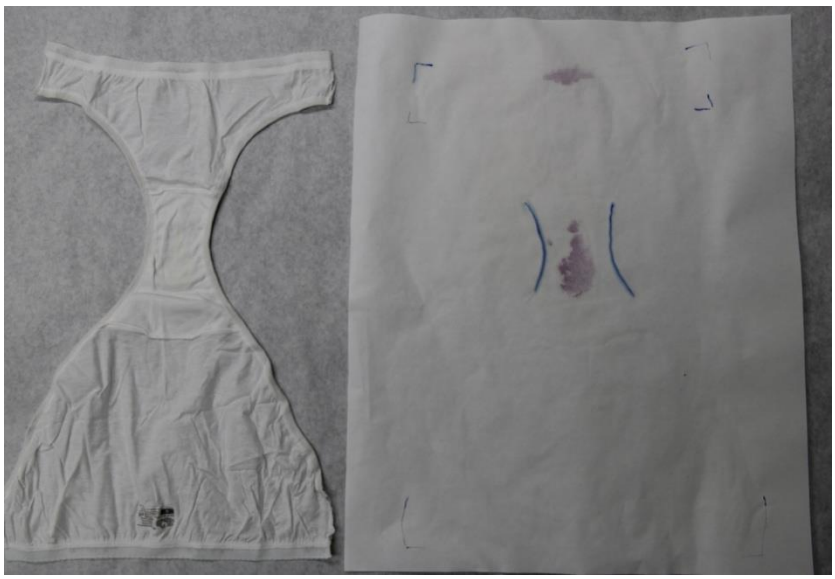


Figure 6: Acid phosphatase overlay used to detect seminal fluid stains on undergarments such as those submitted in cases of alleged sexual assault. The purple/pink color indicates the presumptive presence of acid phosphatase.

Historically, the Ouchterlony assay as well as enzyme-linked immunosorbent assays (ELISA) were used to detect seminal fluid protein markers such as prostate specific antigen. During an ELISA assay for prostate specific antigen (PSA) which is also known as p30, an anti-PSA antibody was bound to the bottom of wells on a polystyrene plate. Sample extracts to be tested for the possible presence of PSA were then added to the wells. Samples containing PSA allowed formation of a complex between the anti-PSA antibody and the PSA antigen. A second anti-PSA antibody for a different epitope on the target antigen was then added. This resulted in the formation of an antibody-antigen-antibody sandwich in wells containing extracts from positive samples. An enzyme-conjugated anti-immunoglobulin antibody targeted to that second antibody in the sandwich was then added to each assay well. This was designed to bind to the sandwich complex in wells with extracts that were positive for PSA. The wells were then washed to remove any unbound anti-immunoglobulin antibodies. Detection of any enzyme-conjugated anti-immunoglobulin antibodies that remained bound to the sandwich complex (typically by addition of an appropriate substrate for the conjugated enzyme) indicated a presumptive positive result (**Figure 7**).

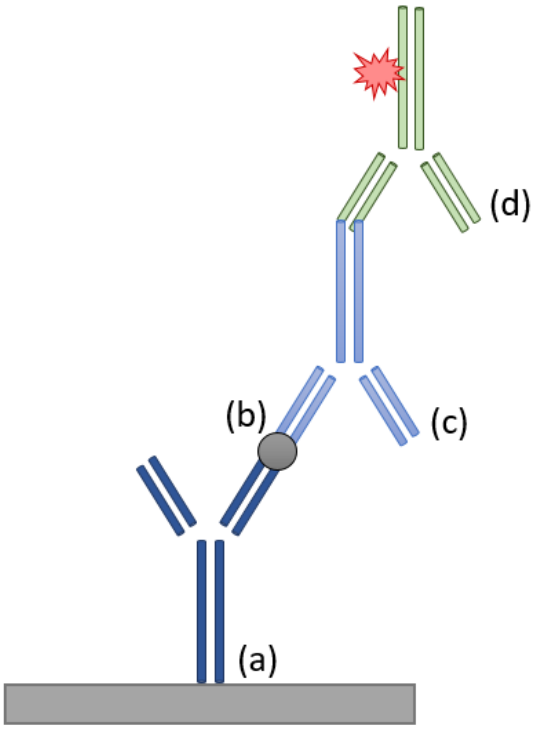


Figure 7: ELISA assay for prostate specific antigen (PSA). (a) anti-PSA antibody immobilized to the bottom of a well on a polystyrene plate. (b) PSA antigen from a positive sample extract is added. (c) A second anti-PSA antibody for a different epitope is added forming an antibody-antigen-antibody sandwich. (d) enzyme-conjugated anti-immunoglobulin antibodies which are expected to bind only in positive wells allows for detection of positive results.

In recent years, however, these techniques have been replaced by alternative antigen-antibody assays based on immunochromatographic cartridges. The ABA p30 (Abacus Diagnostics), PSA SemiQuant® (Seratec), and RSID™ Semen (Independent Forensics) are three examples of commercially available assays for seminal fluid. The ABA p30 and PSA SemiQuant® cartridges target p30/PSA while the RSID™ Semen targets semenogelin, a protein secreted by the seminal vesicle. Contrary to its name, PSA is not specific to the male prostate. It can also be found in female vaginal secretions [14],

amniotic fluid [15], breast milk [16], and urine [17]. False positive results have also been observed with semen-free vaginal (**Figure 8**) and postmortem rectal swabs [18]. Similarly, semenogelin has been identified in kidney, colon and tracheal tissues as well as the sera of lung cancer patients [3]. Moreover, non-specific binding events have been readily observed to occur in the presence of organic acids.

(A)

(B)

Figure 8: Examples of putative false positive reactions from semen-free vaginal swabs targeting semenogelin (A) and prostate specific antigen (B). Each set of four cartridges includes one positive control and three test samples. Test lines indicating the presumptive presence of seminal fluid proteins in semen-free vaginal swabs ranged from faint to moderately strong.

Cellular components from a suspected seminal stain can be identified by microscopy. Visual detection of human spermatozoa by a trained analyst is considered to be confirmation of the presence of semen. Staining techniques such as the Christmas tree stain (nuclear fast red and picroindigocarmine) or Sperm HyLiter™ (Independent Forensics) are commonly used to facilitate the visualization of sperm cells (**Figures 9-10**). Sperm HyLiter™ incorporates a fluorochrome-conjugated antibody into the staining process. This antibody is targeted to a nuclear membrane protein in spermatozoa [19]. The greatest advantage to microscopic visualization techniques for the identification of semen is its confirmatory nature. However, the staining process and visualization of samples can be time consuming. Sperm HyLiter™ can reduce the search time needed to locate sperm but it produces poor spermatozoa morphology with degraded samples. Additionally, these techniques are not applicable to cases involving males who are vasectomized or suffer from aspermia.

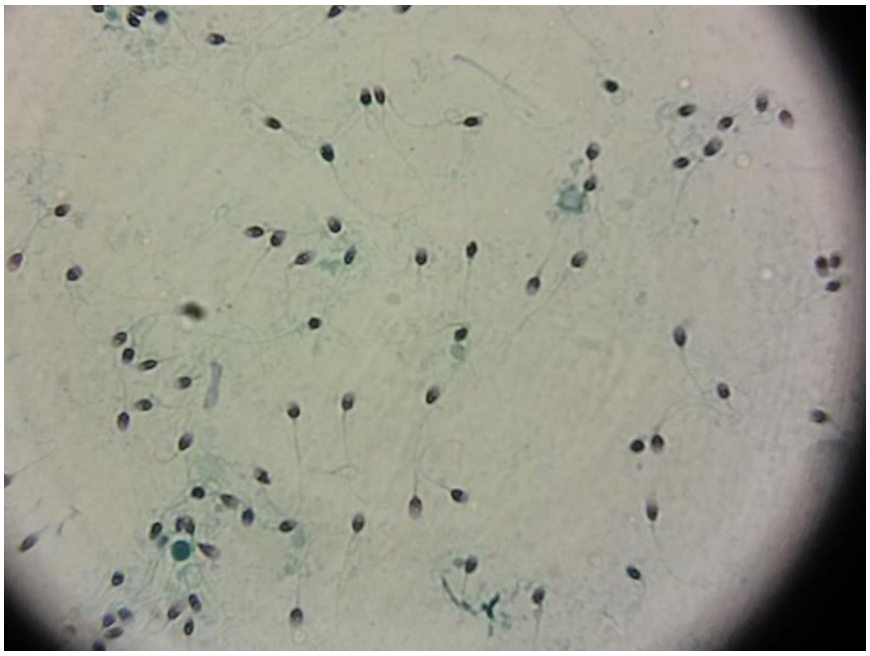


Figure 9: Spermatozoa visualized via light microscopy following the addition of Christmas tree staining (nuclear fast red and picroindigocarmine).

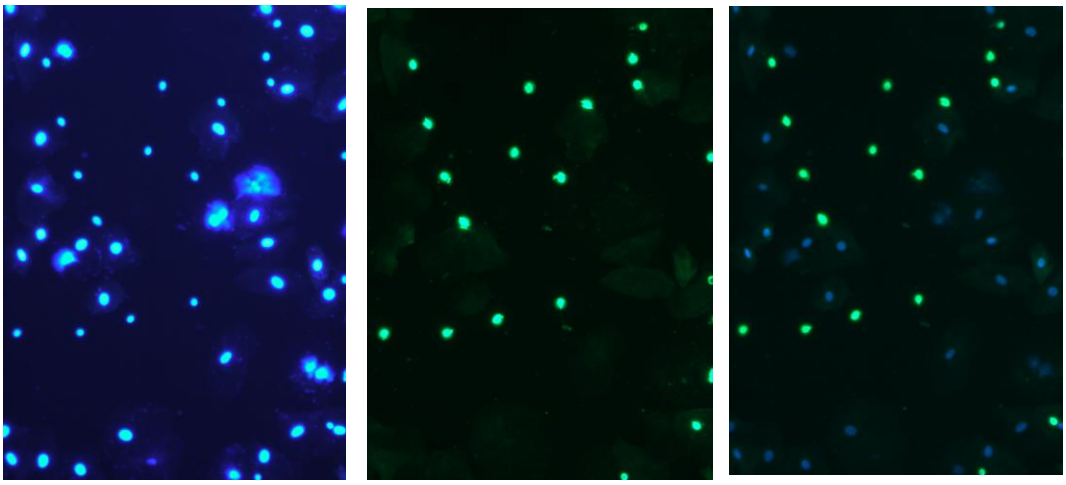


Figure 10: Spermatozoa visualized via Sperm HyLiter staining in combination with fluorescent microscopy (sperm and epithelial cells visualized under the DAPI filter (LEFT), sperm cells visualized under the FITC filter (CENTER) and sperm and epithelial cells visualized with an overlay (RIGHT)).

1.1.3 Presumptive Detection of Saliva

Saliva is composed primarily of water but also contains electrolytes, buffers, glycoproteins, antibodies and enzymes. Approximately 1.0-1.5 L of saliva is produced daily by both serous and mucous acini cells, the basic secretory units of the salivary glands. The three major salivary glands, the parotid, submaxillary and sublingual, produce approximately 90% of saliva while the remaining 10% is produced by the minor salivary glands. Saliva serves many roles in the body; it acts as a lubricant and binder to protect the esophagus from masticated food, solubilizes dry food so that it can be tasted, flushes the oral cavity of food and debris for oral hygiene and initiates starch digestion. The enzyme α-amylase is the component of saliva responsible for cleaving the glycosidic bonds of polysaccharide carbohydrates; thereby breaking them down into smaller oligo- and monosaccharide molecules. Due to its abundance in salivary secretions, the detection of α-amylase serves as the basis for the presumptive presence of saliva.

Several types of amylases have been characterized that differ based on their mechanism of hydrolysis. The faster acting α-amylases require calcium as a cofactor and act at random locations along a polysaccharide carbohydrate chain producing glucose and maltose molecules. Mostly found in mammals, two isoforms of α-amylase exist – salivary and pancreatic amylase. Encoded by the *Amy1* and *Amy2* loci respectively, the amino acid sequences of these isoforms are highly homologous and therefore difficult to distinguish from each other. Found mostly in plants, fungal and bacterial sources, β-amylase moves from the non-reducing end of polysaccharide carbohydrate catalyzing the hydrolysis of

every other α-1,4 glycosidic bond to yield maltose molecules [20]. A third isoform, γ-amylase, hydrolyzes α-1,6 glycosidic linkages and unlike the other isoforms of amylase, will continue to function in acidic environments [21].

A common presumptive test for saliva is an enzyme activity-based test for amylase called the starch-iodine radial diffusion test. For this assay, starch is incorporated into a gel matrix. Suspected saliva stains are then allowed to incubate within wells in this matrix. Through passive diffusion, the amylase in saliva will cleave starch molecules into oligosaccharides within the gel matrix surrounding the well. Iodine is then used to stain the gel as iodine reacts strongly with amylose in starch to form a dark blue complex. Any clear areas around wells of the gel indicate a lack of starch and therefore the presence of amylase activity. The size of the clear "halo" around the well can be correlated to the amount of amylase activity in a sample (**Figure 11**). This test, however, is not specific to α-amylase as it will also react with β-amylase, which as previously indicated, is present in plant and bacterial sources. In addition, small amounts of amylase enzyme present in body fluids other than saliva, *e.g.*, breast milk [22], sweat, tears, semen [23], vaginal fluid and feces [24] are also capable of yielding positive results..

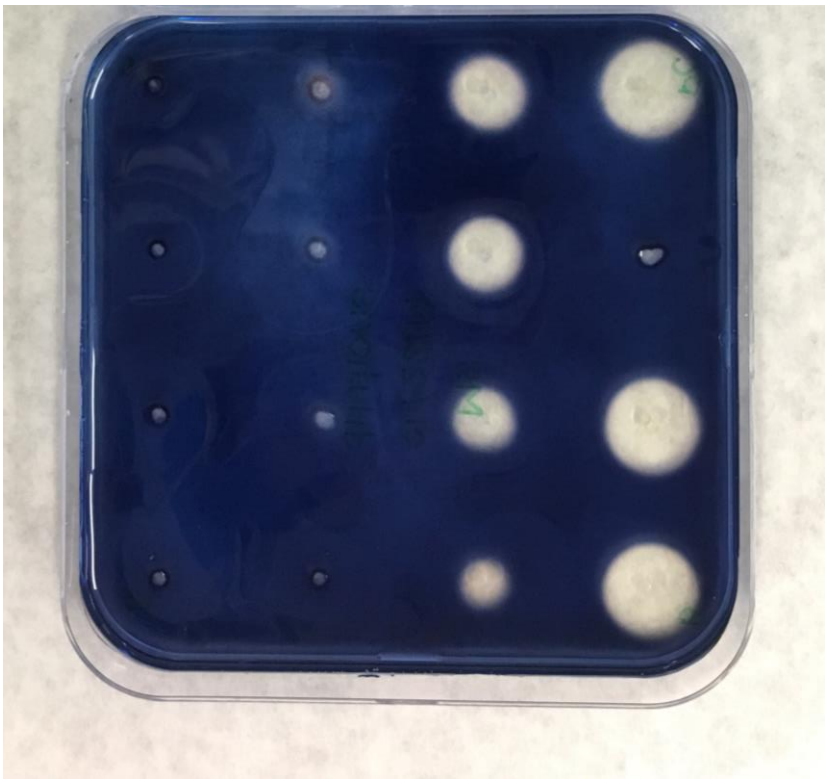


Figure 11: Starch radial diffusion test. The clear wells indicate the presence of amylase. The larger the radius of the clear area around the well, the greater the concentration of amylase activity.

Additional assays that test for amylase activity include the Phadebas® test (Magel Life Sciences) and the SALIgAE® test (Abacus Diagnostics) [25]. These are colorimetric assays that utilize insoluble dye-labeled amylase substrates. When a suspected saliva stain is assayed, amylase activity will cleave the dye-labeled amylase substrates, forming smaller soluble saccharide molecules. This allows for dye solubilization and thus color development indicating the presence of amylase activity (**Figure 12**). These assays are

again considered presumptive as they are not specific to human salivary α-amylase and have the potential to react with the variety of substances and non-saliva body fluids listed above which also contain amylase.

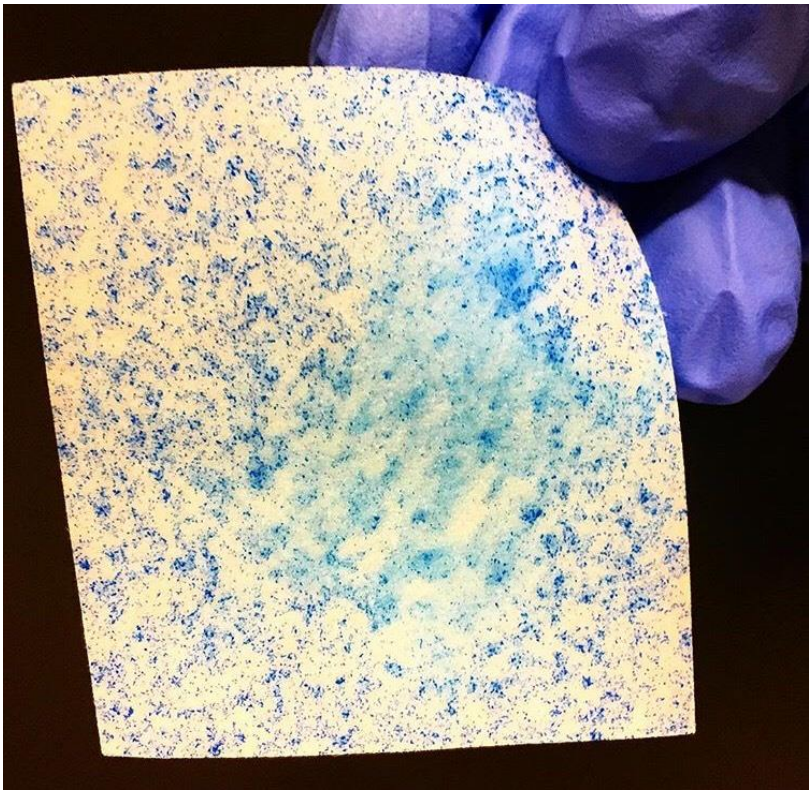


Figure 12: The smear of pale blue in the center of the substrate indicates the presumptive presence of saliva using the Phadebas® test.

Commercially available immunochromatographic assays for saliva include the RSID™ Saliva (Independent Forensics) test [26]. This assay employs monoclonal anti-human salivary α-amylase antibodies conjugated to colloidal gold particles (typically) within the sample well. A second monoclonal anti-human salivary α-amylase antibody is

immobilized at the test zone of the cartridge. Positive results from non-saliva samples reported in the literature for this assay include breast milk, urine, feces, semen and rat saliva [23, 26, 27]. ELISA as well as the Ouchterlony assays, both described previously, can also be used to detect human salivary α-amylase as well as statherin which is another saliva biomarker protein [28, 29]. As with any antibody-based assay, unpredictable cross-reactivity with non-target molecules having similar conformational epitopes is a concern as are non-specific binding events due to extremes of pH or other sample-specific chemical compounds which may lead to false positive reactions.

1.1.4 Presumptive Detection of Urine and Fecal Matter

Urine is composed primarily of water but also contains organic molecules, ions, leukocytes and epithelial cells. The kidneys are responsible for the formation of urine which removes the waste products of cellular metabolism. Urea, an end-product of protein degradation, is one of the most abundant waste components of urine. Creatinine, a product of muscle metabolism, is another major waste product found in urine. A number of chemical reaction-based assays as well as enzyme-activity based assays have been developed to test for the presence or both urea and creatinine.

Urea can be detected with the Nessler's reagent. In this assay, urease is used to catalyze the hydrolysis of urea to liberate ammonia and carbon dioxide. The production of ammonia is detected with Nessler's reagent (potassium hydroxide, mercuric iodide and potassium iodide) through the formation of an orange/brown precipitate. Ammonia formation following urease application has also historically been detected using

bromthymol blue, an acid base indicator that turns blue in the presence of urine. Manganese and silver nitrates, which turn black in the presence of urine, have also been used. Alternatively, para-dimethylaminocinnamaldehyde (DMAC) has been used to directly detect urea based on the formation of a pink/red color in its presence. However, none of these methods described are specific to urine. Other bodily fluids namely vaginal secretions, semen, saliva, and sweat can all produce positive reactions [30]. Historical use of microscopic crystal assays for the detection of urea and converted urea nitrate crystals can also be found in the literature [5].

Creatinine can be detected using a colorimetric reaction called the Jaffe color test. During this test, the addition of sodium hydroxide and picric acid are used to convert creatinine to creatinine picrate which forms a yellow/orange precipitate. Recently, a test cartridge called Uritrace (Abacus Diagnostics) has become commercially available for the detection of creatinine. This test also employs a colorimetric mechanism of action. The Salkowski test was another historically used colorimetric reaction in which sodium nitroprusside reacted with creatinine upon heating to form a blue product. As with other urine assays, these reactions suffered from specificity limitations. While found in higher concentrations in urine, creatinine is not specific to urine. During muscle cell metabolism creatinine is formed through the metabolism of phosphocreatine through an intermediary and creatine released into the blood. From there it is filtered by the kidneys into urine for excretion. As a result, it can be detected in blood as well as semen. Additionally, the amount of creatinine present in the urine is directly proportional to an individual's muscle mass resulting in high interindividual variability in detection sensitivity.

RSID™ Urine (Independent Forensics) targets the most abundant protein in urine, uromodulin or Tamm-Horsfall glycoprotein. Tamm-Horsfall glycoprotein is synthesized in the epithelial cells of the loop of Henle (*ansa nephroni*) and secreted into the lumen. Historically, an ELISA assay was used to detect Tamm-Horsfall glycoprotein. As with most of the other currently employed serological tests mentioned above, however, these assays are presumptive in nature as urine from non-human species as well as synthetic urine and a number of other commercial products have been found to produce positive reactions (**Figure 13**).

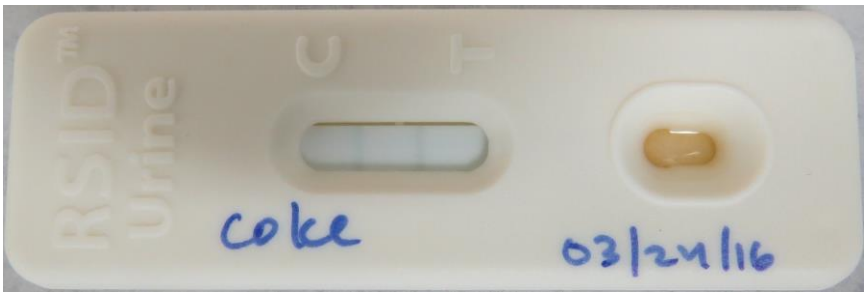


Figure 13: False positive result using an RSID TM Urine assay following the addition of Coca Cola in the absence of urine.

Fecal material is comprised mostly of undigested food, water and bacteria. The test for fecal matter, known as the Edelman test, identifies the presence of urobilinogen, a product of bilirubin reduction formed in the intestines. Mercuric chloride has historically been used to oxidize urobilinogen to urobilin which in turn forms a zinc-urobilin complex in the presence of alcoholic zinc chloride. This chelated complex appears candy apple green when viewed under UV light (**Figure 14**). This test cannot distinguish between

human and other mammalian fecal material. Additionally, as urobilin is also present in urine (albeit at lower concentrations), positive results can also be obtained with urine samples. Therefore, this test is presumptive in nature. Additionally, visualization of the chelated complex with UV light can be obscured by the presence of fats [3].

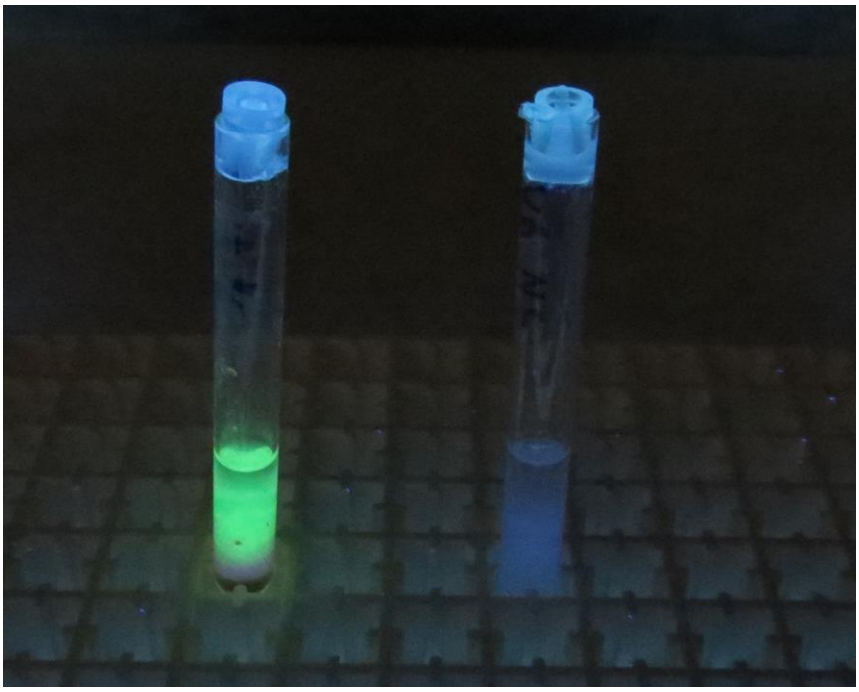


Figure 14: A positive result (LEFT) and negative result (RIGHT) using the urobilinogen test for fecal matter.

1.1.5 Vaginal Secretions and Menstrual Fluid

Currently, there are no tests routinely used or that are commercially available for the reliable detection of vaginal fluid in forensic laboratories. Historically, Lugol's staining of glycogenated epithelial cells of the vaginal wall was thought to provide an indication of the presence of vaginal fluid. The iodine molecules in the Lugol's stain fit

into the helical structures that make up the glycogen molecule forming a dark brown complex. However, this is no longer considered to be specific for vaginal cells as it is difficult to differentiate vaginal and buccal epithelial cells with this stain [31]. Glycogenated epithelial cells are also found in the lining of the anus, pharynx, esophagus, urethra and glans penis [3]. Additionally, the amount of glycogenated cells in the vagina varies with hormonal changes. High levels of estrogen support higher concentrations of glycogenated cells, but these levels drop with menstruation, in pre-pubescent and post-menopausal women. Fluctuations in the levels of glycogenated cells have also been observed during pregnancy and in association with the use of hormonal contraceptives.

The Dane's staining method has also been evaluated as a means of differentiating glycogenated epithelial cells originating from the vaginal versus the oral/buccal cavity. When applied, the Dane's stain (a mixture of hemalum, phloxine, Alcian blue, and orange G) generally stains buccal cells orange/pink with red nuclei (although this showed considerable variability within and between individuals) and stains vaginal cells bright orange with orange nuclei. The Dane's stain will also stain epithelial cells from skin (cells which often lack nuclei) red and orange [3]. While pure samples of vaginal, epithelial, and buccal cells can be readily differentiated with the Dane's stain, mixtures of these cell types could not be reliably distinguished. As a result, this histological staining approach has limited applicability when working with forensic samples.

During menstruation, blood and the degenerated lining of the endometrium from the uterus are sloughed off and eliminated from the body. Blood loss is controlled through

a balance of blood coagulation and clot dissolution that allows for removal of tissue fragments from the uterus. During clot dissolution, cross-linked fibrin is cleaved by the enzyme plasmin, producing a degradation product, *D-dimer*, in the process. An ELISA assay can be used to detect the *D-dimer*. Additionally, Seratec PMB is a recently developed immunochromatographic multiplex assay that allows for the simultaneous detection of human hemoglobin and *D-dimer* for the differentiation between peripheral blood and menstrual fluid [32]. While *D-dimer* is present in peripheral blood, it is found at much lower concentrations which are generally below the detection limits of these assays. However, postmortem blood contains higher levels of *D-dimer* and thus can produce a positive result in the absence of menstrual blood.

Historically, lactate dehydrogenase (LDH) detection was also used for the forensic identification of menstrual fluid. LDH plays a major role in glycolysis. Five LDH isoenzymes can be found in blood, each composed of four subunits with various combinations of subunit A and subunit B. For example, LDH1 is composed of four identical B subunits while LDH5 is composed of four identical A subunits (**Figure 15**). Each of the five isoforms can be differentiated based on differences in mobility using electrophoresis [33]. Typically, LDH1, LDH2 and LDH3 are predominantly observed in peripheral blood while LDH4 and LDH5 are predominantly observed in menstrual fluid. As LDH levels can fluctuate and can be found in other tissues, the forensic utility of this test for menstrual fluid is considered to be limited at best [34].

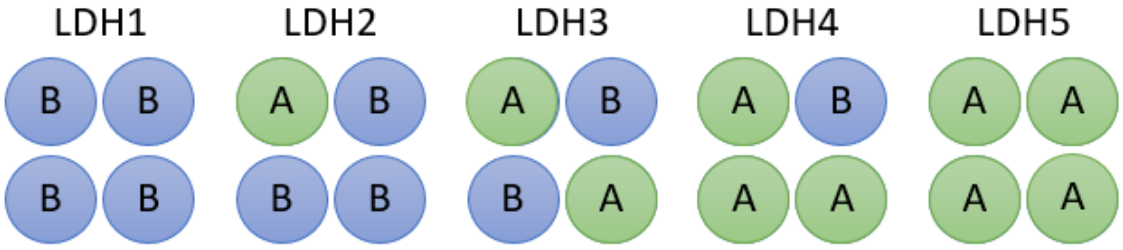


Figure 15: Five isoforms of lactate dehydrogenase (LDH) found in blood. Each isoenzyme is composed of four subunits. Isoenzymes differ from one another based on the specific combinations of the A and B subunits.

1.2 Future Serological Techniques

Given the substantial limitations associated with current serological techniques, several novel approaches to identifying biological fluids have been explored in recent years. These include the use of epigenetic modifications, messenger RNA markers, micro RNA expression patterns, and high-specificity protein biomarkers. Of these, epigenetics has also been used in an effort to predict age signatures from biological fluids. All of these emerging techniques aim to improve the sensitivity and specificity of forensic body fluid identification while allowing for rapid sample analysis and easy adoption by analysts in a case-working environment.

1.2.1 DNA Methylation Assays for Body Fluid Identification

Epigenetics is the study of potentially transmissible modifications to DNA that are typically associated with changes in DNA methylation that leads to changes in gene expression. Methylation of cytosine residues, typically located at CpG islands in promoter regions upstream of genes, enable gene silencing [35]. Conversely, unmethylated/

undermethylated promotor regions of genes allow for the transcription of genetic information. Methyltransferase is responsible for *in vivo* cytosine methylation by transferring a methyl group from S-adenosylmethionine to cytosine at the carbon-5 position. Tissue-specific patterns of DNA methylation have shown promise as a means of body fluid identification.

This technique relies on detecting these tissue-associated differences in methylation pattern. For example, several regions have been found that are consistently hypomethylated in cells from seminal fluid as compared to other biological fluids. Typically, bisulphate conversion is used to convert un-methylated cytosine residues to uracil via hydrolytic deamination (**Figure 16**). Methylated cytosine residue specific PCR primers can then be used to amplify targeted regions of interest. Alternatively, methylation specific restriction enzymes can be used to cleave DNA at unmethylated sites, leaving methylated DNA intact. The polymerase chain reaction can then be used to amplify intact, methylated DNA while the cleaved, unmethylated regions are not copied. Another technique for isolating methylated DNA is methyl-DNA immunoprecipitation. Antimethylcytosine antibodies are used to bind methylated cytosines on sheared DNA which can then be isolated via immunoprecipitation.

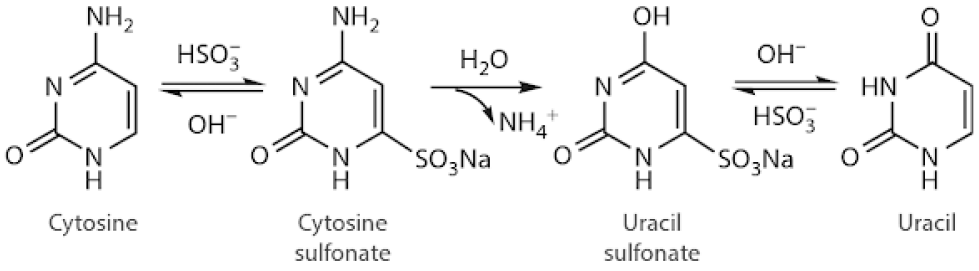


Figure 16: Bisulphate conversion of 5-methylcytosine.

Blood (based on the *FOX03* and *EFS* genes) [36], saliva (based on the *SLC12A8* and *BCAS4* genes), semen (based on the *DACT1* and *C12orf12* genes) [37] and skin cells have all been successfully identified using methylation-based tissue identification [35]. More recently, tissue specific differentially methylated regions have also been identified for vaginal secretions (based on the *LOC404266* and *HOXD9* genes) and menstrual fluid (based on the *LC26A10* and *LTBP3* genes), allowing for its differentiation from peripheral blood [32]. Positive results have also been generated for casework-type samples for semen and bloodstains that had been aged for up to 20 months. A commercial assay for the detection of seminal fluid, Nucleix DSI-Semen kit, has been developed and validated [38, 39]. Multiplex testing has recently been published but not yet commercialized [40, 41].

A potential advantage of this assay is that it can easily be incorporated into the existing DNA workflow for most operational forensic laboratories. Additionally, the multiplexed analysis of different body fluids in one assay is possible and would eliminate the need for multiple separate tests to be performed on the same sample for each biological fluid of interest. This would save time and sample as well as eliminate the need for analysts

to be trained and to stay proficient in multiple analytical techniques. Further research, however, is still needed in order to assess how methylation patterns change in response to certain factors such as environmental stimuli, aging and disease as well as to evaluate the degree of inter-individual epigenetic variation [42] that exists with human populations.

While epigenetic modifications associated with DNA from seminal fluid has been shown to be robust and reliable, the pattern of varying degrees of methylation in other bodily fluids makes interpretation of results, especially in mixed fluid samples, complex. Furthermore, while techniques such as DNA methylation microarrays and genome bisulfite sequencing allow for the detection of enough multiplexed targets to be forensically informative, these approached require large quantities of high-quality DNA. This may not be feasible for many forensic samples that are often present only in trace amounts or have been subjected to environmental degradation. Alternative techniques such as methylation quantitative PCR and bisulphate pyrosequencing are more amenable for lower quality/quantity input but these approaches are more limited in terms of their multiplexing capabilities [43, 44].

As mentioned previously, methylation patterns have been found to be susceptible to change due to the natural aging process. To detect methylation, targeted bisulfite conversion detected with a SNaPshot assay or pyrosequencing has been the method of choice. The bisulfite conversion deaminates non-methylated cytosine and converts it to uracil (PCR amplification converts this to thymine) while methylated cytosine is unaffected. The SNaPshot assay can be used to detect single base differences by

incorporation of terminating dideoxynucleotide triphosphate bases. Extension products can then be analyzed using capillary electrophoresis (**Figure 17**). The percent of methylation can then be estimated by dividing C/G intensity (unconverted methylated DNA) by C/G plus T/A (bisulfite converted unmethylated DNA) intensities. Review of online databases of genome-wide methylation profiling (most commonly from Illumina's Human Methylation Bead Chip technology) has been used to identify candidate target methylation sites [45].

To build age prediction models, samples of a particular biological fluid are collected from individuals spanning gender, ethnicities and chronological age. Multivariate linear regression coefficients and significance of correlation between chronological age and DNA methylation ratios can then be used to identify promising targets for age prediction [45]. These accumulated methylation changes associated with age may be tissue specific. Studies have demonstrated that different sites are better correlated with age in specific tissues. Therefore, multiple sites within a specific tissue which correlate with the aging process for that particular tissue type will need to be identified.

For example, in one study methylation of a CpG site, *PRMT2*, showed no correlation with age in saliva or semen samples. However, this epigenetic marker was found to be age associated in blood samples [35]. In another study, two epigenetic markers in the *TTC7B* gene and one additional epigenetic marker in the NOX4 gene showed a high correlation between predicted and chronological age in semen samples [45]. An additional

six age-associated CpG markers on the *SST*, *CNGA3*, *KLF14*, *TSSK6*, *TBR1* and *SLC12A5* genes have been identified in saliva [46]. Multiple other markers have been identified in blood samples from individuals of varying age and ethnicity that correlate to age prediction – typically with an estimate of error of between 3 to 6 years [47].

While this work looks promising, the identification and application of DNA specific methylation patterns as a predictive tool for age estimation is still in the early stage of development. Additional areas of research have been focusing on combining epigenetic analysis and next generation sequencing approaches for body fluid identification. It is thought that this may provide additional supporting evidence for predictive age signature applications [48].

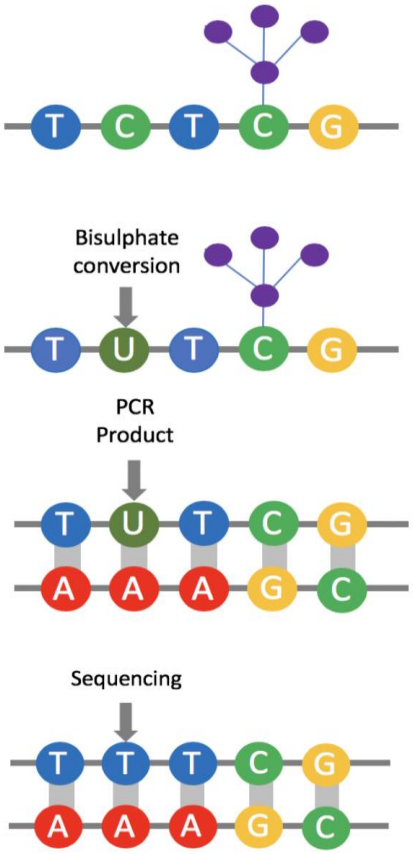


Figure 17: Illustration of bisulfite sequencing of cytosine methylation where unmethylated cytosine is converted to uracil. PCR and sequencing then ultimately convert cytosine to thymine; thereby indicating the site was unmethylated.

1.2.2 RNA Based Assays for Body Fluid Identification

Messenger ribonucleic acid (mRNA) is the product of transcription that conveys genetic information from DNA to the ribosome for translation where it specifies the amino acid sequences of the protein products of gene expression. Just as tissue specific differentially methylated regions of DNA were identified for the epigenetic identification

of biological fluids, tissue specific gene expression has been targeted for mRNA-based assays. These assays seek to detect mRNA transcripts that are exclusive to certain cell and tissue types.

A major advantage to mRNA body fluid analysis is that it uses standard techniques and instrumentation already widely implemented in forensic biology laboratories. The two most common techniques used for mRNA body fluid analysis are reverse transcription followed by end-point PCR and capillary electrophoresis as well as reverse transcription coupled with quantitative PCR (qPCR). During reverse transcription, a complementary DNA (cDNA) is produced from an mRNA template by reverse transcriptase. Primers for established body fluid-specific gene transcripts are incorporated during a multiplex or several singleplex PCR reaction(s) and the resulting amplicons are analyzed via capillary electrophoresis. The expression of multiple mRNA markers is used to deduce the biological source of a particular sample [49]. Alternatively, cDNA can be amplified via quantitative PCR. During qPCR, pre-designed target-specific primers and TaqMan (or similar) probes can be used to quantify gene expression [50-52]. Reverse transcription coupled with end-point PCR and capillary electrophoresis or qPCR are sensitive techniques for quantifying mRNA expression in samples, however, the degree of multiplexing is limited. As a result, only a few mRNA biomarkers can be assayed in a single reaction.

To identify novel body fluid specific markers and better understand the gene expression variation between forensically relevant body fluids, whole transcriptome analysis was needed. DNA microarrays has been useful in these discovery projects. In

one approach, oligonucleotide probes are attached to or synthesized on the solid surface of a chip. These are then hybridized to cDNA or RNA strands of interest. Fluorophores can be used to detect probe-target hybridization events [53, 54]. Subsequent analyses of gene-expression profiles in populations of study subjects have identified multiple tissue-specific mRNA markers for those body fluids most frequently encountered in forensics.

For example, HBA1 (alpha 1 hemoglobin), HBB (beta hemoglobin), SPTB (beta spectrin) and PBGD (porphobilinogen deaminase) are a few of the mRNA markers reported to be "specific" to peripheral blood. Similarly, HTN3 (histatin 3), MUC7 (mucin 7) and STATH (statherin) are mRNA markers reported to be "specific" to saliva. The KLK3 (prostate-specific antigen), PRM1&2 (protamines 1 & 2), and SMG1 (semenogelin 1) transcripts have been proposed as specific mRNA markers for seminal fluid; HBD1 (beta defensin 1) and MUC4 (mucin 4) transcripts have been proposed as -"specific" markers for vaginal secretions; and the MMP7 and 11 (matrix metalloproteinase 7 and 11) transcripts have been proposed as "specific" markers menstrual fluid. A large number of studies in the published literature have assessed the potential utility of these mRNA markers [50, 55-59]. In addition to the tissue-specific gene transcripts used for body fluid identification, a number of consistently expressed housekeeping genes have also been proposed for use as internal controls [52]. Recently, a commercial product, ParaDNA® Body Fluid ID System, has been developed. This portable device targets mRNA markers for seminal fluid, sperm cells, vaginal fluid, saliva, blood and menstrual fluid [60].

While these assays and commercially available tests are more specific than current testing methodologies, they suffer from the general stability limitations of any work involving mRNA targets. Degradation due to endogenous ribonucleases frequently effect mRNA stability in biological samples. Additionally, crime scene samples are often exposed to ultraviolet light, moisture and wide temperature ranges – all of which can lead to mRNA degradation in biological fluid samples. However, body fluids have been successfully detected using mRNA markers in aged samples that have been maintained under appropriate storage conditions.

In an effort to address the stability concerns associated with larger mRNA transcripts, microRNAs have been evaluated as an alternative type of RNA biomarker. MicroRNAs, which regulate gene expression, are much smaller and more abundant than mRNAs. While this makes them more stable targets, the expression of microRNAs is more ubiquitous. Thus, although broad expression patterns characteristic for forensically relevant fluids can be identified, the interpretation of body fluid mixtures continues to poses a formidable challenge [61, 62].

1.2.3 Proteomic Based Assays for Body Fluid Identification

A particularly promising approach for the serological identification of biological fluids combines high-specificity protein biomarkers with mass spectrometry. This allows for the direct identification of target proteins (even in partially degraded samples); true confirmatory identification and greatly enhanced sensitivity.

A proteome is the full complement of proteins present in an organism while proteomics is the study of these expressed proteins. Protein biomarkers have attracted significant interest in recent years due in large part to the strides that have been made in the tools available to identify and characterize them. It is now possible to rigorously map entire proteomes with high reproducibility. Techniques such as differential 2-D gel electrophoresis or multidimensional protein identification technology (MudPIT) [63, 64] have made it possible to identify a vast number of candidate protein biomarkers [65-68]. Once potentially useful candidate protein biomarkers have been identified, mass-spectrometry-based targeted-ion assays can facilitate the unambiguous detection and quantitation of even low abundance biomarker protein targets against a background of other non-target molecules in complex biological matrices [63, 69, 70].

This has resulted in a wealth of new opportunities to develop protein-based assays for both medical and forensic applications. Most forensic approaches for stain identification follow a "bottom-up" shotgun approach to biomarker detection and identification. In this approach, a complex biological sample is first enzymatically cleaved and small peptides (~15 amino acids in length) are the fractionated by liquid chromatography followed by identification of protein targets of interest using tandem mass spectrometry [71].

One of the significant advantages of a protein biomarker approach is the diversity of potential targets that are made possible due to post-translational modification in different tissues. Another key advantage is the stability of many proteins under conditions that lead

to degradation of other biological macromolecules. Proteins are among the most long-lasting of all biological molecules having been routinely isolated from even ancient biological material [72] and post-mortem tissue [73]. Even when degradation begins to occur, simple modification of detection methodologies focusing on the detection of fragmented proteins still allows for reliable detection.

1.3 Research Objectives

This dissertation research was designed to develop and assess the potential utility of a targeted-ion Triple Quadrupole Mass Spectrometry in Multiple Reaction Monitoring mode (QQQ-MRM) approach for the identification of biomarker targets specific to forensically relevant biological fluids. The application of this technology, especially for the front-end processing of sexual assault evidence, has been a central focus of this research. To achieve this, selected biomarkers from previous studies were incorporated into a multiplex QQQ-MRM method for the simultaneous detection of up to six biological fluids. The subsequent developmental validation of this QQQ-MRM assay provided forensic analysts with a high level of confidence in the accuracy of the results obtained.

This multiplex assay was then used as a foundation for the development and optimization of a monoplex QQQ-MRM assay for semen, with the goal of using this for the high-throughput analysis of sexual assault samples. This was achieved by eliminating from the larger multiplex assay all biomarker peptides not specific to semen. Doing so maximized the dwell time efficiency of the instrument; thereby increasing the assay's detection sensitivity for seminal fluid targets. Assay specificity was assessed by analyzing

a series of mixtures consisting of saliva, semen, vaginal secretions, urine, peripheral and menstrual fluid. The extent to which the optimized monoplex QQQ-MRM assay for semen can extend the post-coital interval for the detection of seminal fluid in cervico-vaginal samples was then assessed relative to the results obtained with the immunochromatographic assays currently employed by forensic laboratories.

The qualitative monoplex assay for seminal fluid detection was then further modified to develop an absolute quantitation QQQ-MRM assay for seminal fluid. By establishing the limit of detection (LOD) and quantitation (LOQ) for the assay, the relationship between quantitative levels of target seminal fluid peptides and the ability to generate male-targeted Y-chromosome short tandem repeat (Y-STR) haplotypes from vaginal swabs collected at various post-coital intervals was assessed. The quantitative monoplex assay was also used to estimate of the rate of authentic false positive results associated with immunochromatographic assays that target the same proteins quantitated by the QQQ-MRM method. The successful completion of these objectives has important implications for the successful prosecution of the perpetrators of sexual assault as well as the effective defense of those who are wrongly accused.

1.4 Hypotheses

The overarching hypothesis that was tested in the course of this dissertation research is that a targeted-ion mass spectrometry based proteomic assay would provide for the sensitive and specific identification of biological fluid specific protein targets

(especially as compared to currently employed immunochromatographic based serological assays). The specific hypotheses that were at the core this research therefore are:

1. The use of a mass-spectrometry based assay for seminal fluid will surpass the sensitivity levels of the antibody-based assays employed by most forensic laboratories.
2. The accuracy, reliability and enhanced sensitivity of the proposed assay will extend the post-coital interval during which it is reasonable to collect sexual assault samples that are likely to yield useful results.
3. A quantitative mass-spectrometry based seminal fluid assay will make it possible to assess the likelihood of obtaining a useful Y-STR haplotype based on the quantity of seminal fluid biomarkers present on the post-coital swab (*i.e.*, the ability to use protein quantities in the same way as DNA quantitation data is currently used to screen samples for downstream DNA profiling).
4. The accuracy and enhanced sensitivity of a QQQ-MRM assay for semen proteins can be used to independently assess the rate of actual false positive results (*i.e.*, non-specific binding events) associated with the antibody-based lateral flow tests.

1.5 Dissertation Structure

Within each chapter of this dissertation an introduction will establish the necessary background content and justification for the given set of experiments. A description of the experimental methods will be provided and a summary of all pertinent results will be

detailed. Each chapter discusses the significant research findings that were made including any caveats relevant to adoption by forensic practitioners in an operational environment.

Chapter 2 focuses on highlighting one of the inherent limitations associated with the most commonly employed serological method in forensic laboratories. Namely, this is the well-documented lack of specificity that has been encountered with the use of immunochromatographic assays. Chapter 3 focuses on the developmental validation of an MRM method for the concurrent identification of six biological fluids of forensic interest. Chapter 4 assesses the feasibility of a mass spectrometry-based body fluid assay for the analysis of sexual assault samples by narrowing the scope of the assay to seminal fluid in order to maximize assay sensitivity. Chapter 5 focuses on how a quantitative (versus qualitative) method for seminal fluid detection can be used to establish true false positive rates for common immunochromatographic assays that target prostate specific antigen and semenogelin proteins. Chapter 5 also focuses on the use of semen protein quantitation by mass spectrometry for the reliable prioritization of samples for downstream genetic testing.

CHAPTER 2: FALSE POSITIVE IMMUNOCHROMATOGRAPHIC TEST RESULTS ASSOCIATED WITH ORGANIC ACIDS

2. Introduction

The ability to detect biological fluids recovered from a crime scene can provide useful information for the investigation of a crime. Specifically, this information may be used to prioritize testing of items of evidence, direct downstream testing methodologies for the development of genetic profiles, or to provide investigators contextual information paramount to the criminal investigation. In some instances, being able to detect a biological fluid on an evidentiary sample, even in the absence of genetic analysis, may be sufficient to influence the outcome of an investigation and in some instances a court case. It is therefore critical for forensic practitioners to be able to provide information on the detection (or lack thereof) of a biological fluid as well as to be aware of the limitations associated with the applied methodology. It is for this reason, that an increased emphasis has been placed on developing serological tests that optimize sensitivity and specificity while minimizing sample processing time so as to reduce the potential for backlogs in the testing of crime scene evidence. One of the most common serological methods employed currently in crime laboratories for the identification of biological fluids is the use of immunochromatographic assays.

Laminar flow immunochromatographic assays are commercially available from multiple manufacturers and are commonly used in forensic practice to detect the presence of bodily fluids including, blood, semen, saliva, and urine [1, 7, 26, 74-85]. These assays employ labeled antibodies specific to a protein or other small molecular target considered to be characteristic of (but not necessarily unique to) a given bodily fluid. When an extract of a test sample is introduced to the sample well, the target antigen forms a complex with mobile phase antibodies that are typically conjugated to colloidal gold particles. The antigen-antibody complexes that form migrate down a lateral flow membrane. Immobilized at a test site on the membrane are additional antibodies for different epitopes on the same target protein that bind the complex forming an antibody-antigen-antibody sandwich. Accumulation of the labeled antibodies from the sample well at the test zone facilitates visual detection of target antigen. Labeled antibodies not captured at the test zone continue to migrate to a control zone where they are captured by immobilized anti-immunoglobulin antibodies to form another visible line. This second visible line indicates the test performed as designed. Sometimes, depending on manufacturer, an additional control line is added making it possible to estimate the quantity of target protein in a sample. While immunochromatographic assays represent a sensitive and efficient method for forensic serological testing, the limitations associated with these assays must be fully understood so as not to mislead investigators or the trier of fact.

Given their reliance on antibody binding reactions, these assays suffer from similar types of limitations regardless of manufacturer. Depending upon the body fluid specificity of the proteins used for any particular assay, there may be other non-target biological fluids

that contain equal or lower concentrations of the target biomarker that are still capable of producing positive reactions [7, 14, 15, 18, 26, 73, 86]. Additionally, while not as well documented in the literature, chemically induced non-specific protein aggregation as well as cross-reaction to structurally similar non-target antigens can both lead to false positive reactions [18, 87]. One product's user manual highlights the potential for non-specific binding events by suggesting test results may be influenced by acidic pH in combination with the presence of organic acids [88]. This study aimed to evaluate how pH and the presence of organic acids may influence false positive results of multiple immunochromatographic assays designed to target blood, semen, saliva and urine.

Immunochromatographic assays from multiple different manufacturers were evaluated. Specifically, the ABAcard® p30 and ABAcard® HemaTrace® (Abacus Diagnostics®); RSID™-Urine, RSID™-Semen, RSID™-Blood, and RSID™-Saliva (Independent Forensics); and PSA Semiquant, HemDirect, and Amylase Test (Seratec®) were evaluated. Citric acid and lactic acid were selected based on widespread use of these organic acids in commercial products. Sample pH was adjusted to determine the degree of pH dependence of false positive results with organic acids. Manufacturer-specific buffers were utilized to evaluate their efficiency in mitigating false positive results. Common household and commercial products that contain organic acids were also analyzed.

2.1 Methods

2.1.1 Chemicals and Reagents

Citric acid (anhydrous) and lactic acid (85% pure) were obtained from Sigma Aldrich (Allentown, PA). Hydrochloric acid (HCl) (36.5-38%) and 10N sodium hydroxide (NaOH) were purchased from BDH Analytical Chemicals (Poole, United Kingdom). Deionized water was obtained in house. All pH measurements were made using a Mettler Toledo FiveGo pH/mV meter (Washington Crossing, PA).

RSID™-Saliva, RSID™-Semen, RSID™-Urine, and RSID™- Blood kits were purchased from Independent Forensics (Hillside, IL). ABAcard® p30 and ABAcard® HemaTrace kits were purchased from Abacus Diagnostics (West Hills, CA). SERATEC® PSA Semiquant, SERATEC® HemDirect Hemoglobin Test, and the SERATEC® Amylase Test kits were purchased from Seratec® (Goettingen, Germany). See **Table 1** for more specific information pertaining to selected immunochromatographic assays evaluated in this study. All reagents were stored according to manufacturer's guidelines. Household products and beverages that were evaluated for their potential to produce false positive results included 1% cow's milk, orange juice, white wine, apple juice, Monster Energy drink, Windex®, Febreze, white vinegar, and Pine-Sol). These were purchased from local retail outlets or voluntarily donated by laboratory staff.

Table 1. Overview of Immunochromatographic Assays Evaluated.

Manufacturer	Test	Target Antigen	Dye
Seratec®	PSA Semi-quant	Prostate Specific Antigen	Colloidal Gold
	HemDirect	Hemoglobin	Colloidal Gold
	Amylase	α-amylase	Colloidal Gold
Abacus Diagnostics, Inc.	ABAcard® p30	Prostate Specific Antigen	Possible Colloidal Gold, Colloidal Silver, Carbon, Latex, Dye, Enzyme
	ABAcard® HemaTrace®	Hemoglobin	Possible Colloidal Gold, Colloidal Silver, Carbon, Latex, Dye, Enzyme
Independent Forensics	RSID™-Semen	Semenogelin	Colloidal Gold
	RSID™-Blood	Glycophorin A	Colloidal Gold
	RSID™-Saliva	Salivary Amylase	Colloidal Gold
	RSID™-Urine	Tamm Horsfall Glycoprotein (Uromodulin)	Blue Latex Bead

2.1.2 Solution Preparation

A 0.3M citric acid solution was prepared by adding 5.76 grams of citric acid anhydrous to 100 mL of deionized water. A serial dilution of the 0.3M citric acid stock solution was used to prepare the following series of two-fold dilutions: 0.15M, 0.075M, 0.0375M, 0.0187M, and 0.0093M. A 0.3M lactic acid solution was prepared using 2.62 mL 85% pure lactic acid added to 97.38 mL of deionized water. All stock solutions were

adjusted to a pH of 2 to 12 while dilutions were adjusted to pH 4 using HCl and NaOH solutions. Neat citric acid and lactic acid solutions were determined to have a pH of 1.74 and 2.19, respectively.

2.1.3 Citric Acid and Lactic Acid pH Series Studies

The citric and lactic acid stock and dilutions were tested on all immunochromatographic assays which included RSID™ Saliva, RSID™ Semen, RSID™ Urine, and RSID™ Blood kits; ABAcard® p30 and ABAcard® HemaTrace kits; PSA Semiquant, HemDirect Hemoglobin Test, and the Amylase Test kits following the manufacturer's recommendations. Negative results on all assays were confirmed after 10 minutes. The dilutions that produced the last observable false positive result at both pH extremes were performed in triplicate.

2.1.4 Deionized Water Study

To determine the potential effect of pH separate from that of organic acids, deionized water was adjusted to a pH of 2 to 9. The pH values that generated a false positive reaction with the citric acid stock solution (assay specific) and the acidified water (pH 2) were assessed across all immunochromatographic assays.

2.1.5 Kit-Specific Buffer Study

The citric acid stock solution (pH 4) was diluted 1:1 in kit specific assay buffer. The resulting buffer-diluted organic acid solutions were then re-tested on the corresponding immunochromatographic assay.

2.1.6 Common Beverages and Household Products

Household products or beverages were purchased from retail outlets or voluntarily donated by laboratory staff. All liquids were analyzed neat following individual manufacturer guidelines. If a positive or invalid result was generated, the product was diluted 1:1 with kit specific buffer and then re-analyzed.

2.2 Results

2.2.1 Citric Acid and Lactic Acid pH Series

The range of false positive results observed using the 0.3M citric acid stock solution varied both by assay and manufacturer (**Table 2**). The Seratec® Amylase Test and RSID™ Urine assay generated false positive results over the widest pH range (between pH 1.74 – 11 and pH 3 – 12 respectively). For most other assays, solutions with extreme pH values (pH 2 and pH 12) consistently produced invalid results. The ABAcard® HemaTrace® generated the fewest false positive results (*i.e.*, only pH 4 produced a false positive result). Across all kits, the greatest frequency of false positive results was observed between pH 4

and pH 10. Each test resulted in reproducible false positive responses when tested in triplicate.

Table 2. 0.3M Citric Acid Results for all Lateral Flow Assays Evaluated Over a pH Range of 1.74 to 12.

	Assay	1.74	2	3	4	5	6	7	8	9	10	11	12
Abacus® Diagnosti	ABAcard® p30	NT	INV	INV	+++	+	+	+	+	+	+++	NEG	INV
	ABAcard® HemaTrace®	NT	INV	INV	+++	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
Independent Forensics	RSID™ Semen	NT	INV	+++	+	+	+	+	+	+	+++	NEG	INV
	RSID™ Blood	NT	INV	NEG	NEG	NEG	NEG	+++	+	+	+++	NEG	INV
	RSID™ Saliva	NT	INV	+++	+	+	+	+	+	+	+++	NEG	INV
	RSID™ Urine	NT	INV	+++	+	+	+	+	+	+	+	+	+++
SERATEC®	PSA Semiquant	NT	INV	+++	+	+	+	+	+	+	+++	NEG	NEG
	HemDirect	NT	INV	NEG	+++	+	+	+	+	+++	NEG	NEG	INV
	Amylase	+++	+	+	+	+	+	+	+	+	+	+++	+++

Note: Not tested (NT); Positive (+); Positive confirmed in triplicate (+++); Negative (NEG); Invalid (INV).

The pH ranges that generated false positive results for each assay differed between citric and lactic acid (**Table 3**). Despite having readily generated false positive results with citric acid solutions, neither the ABAcard® Hematrace® nor the SERATEC® HemDirect assays produced any false positive responses in the presence of lactic acid. In contrast, the Independent Forensics assays produced generally concordant results with 0.3M lactic acid (pH 4 to 11) as compared to citric acid. The ABAcard® p30 and SERATEC® PSA Semiquant assays produced false positive results with lactic acid only at acidic pH values

(pH 2 to 4) despite producing false positive results at both acidic and basic pH values in the presence of citric acid. False positive results demonstrated repeatability when testing was performed in triplicate.

Table 3. 0.3M Lactic Acid Results for all Lateral Flow Assays Evaluated Over a pH Range of 2.19 to 12.

	Assay	2.19	2	3	4	5	6	7	8	9	10	11	12
Abacus® Diagnosti	ABAcard® p30	INV	+++	+	+++	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	ABAcard® HemaTrace©	INV	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
Independent Forensics	RSID™ Semen	NT	INV	+++	+	+	+	+	+	+	+	+++	NEG
	RSID™ Blood	NT	INV	+++	+	+	+	+	+	+	+	+++	NEG
	RSID™ Saliva	NT	INV	+++	+	+	+	+	+	+	+	+++	NEG
	RSID™ Urine	INV	NEG	+++	+	+	+	+	+	+	+	+++	NEG
Seratec®	PSA Semiquant	INV	+++	+	+++	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	HemDirect	INV	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	Amylase	+++	+	+	+	+	+	+	+	+	+	+++	NEG

Note: Not tested (NT); Positive (+); Positive confirmed in triplicate (+++); Negative (NEG); Invalid (INV).

2.2.2. Citric Acid Dilution Series

A molarity of 0.3 was selected for the citric acid stock solution as this represents the most concentrated molarity found in natural products (citric juices). A citric acid dilution series was also assessed for the potential to produce false positive results (**Table 4**). Both the ABAcard® p30 and RSID™ Urine produced false positive results across the entire dilution range evaluated. All other assays, with the exception of the SERATEC®

HemDirect, produced false positive results down to a 0.0375M or 0.0187M solution. Of particular note were the results obtained for the RSID™ Blood assay. Originally, this lateral flow test generated a negative result with the 0.3M citric acid stock solution. However, when diluted, the citric acid then produced in false positive results down to a citric acid dilution of 0.0375M.

Table 4. 0.3M Citric Acid Dilution Series Results for all Lateral Flow Assays Evaluated.

	Assay	0.3M	0.15M	0.075M	0.0375M	0.0187M	0.0093M
Abacus® Diagnostics	ABAcard® p30	+	+	+	+	+	+++
	ABAcard® HemaTrace®	+	+	+	+	+/+/NEG	NEG
Independent Forensics	RSID™ Semen	+	+	+	+++	INV	NT
	RSID™ Blood	NEG	+	+	+++	INV	NT
	RSID™ Saliva	+	+	+	+++	INV	NT
	RSID™ Urine	+	+	+	+	+	+++
Seratec®	PSA Semiquant	+	+	+	+	+++	NEG
	HemDirect	+	+	+++	NEG	NT	NT
	Amylase	+	+	+	+	+++	NEG

Note: Not tested (NT); Positive (+); Positive confirmed in triplicate (+++); Negative (NEG); Invalid (INV).

2.2.3 Deionized Water Study and Kit-Specific Buffer Studies

To assess the potential effect of pH in the absence of organic acids on non-specific binding events of lateral flow assays, pH adjusted water samples were also assayed. Acidified deionized water (pH 2) and median citric acid false positive pH values (kit specific) were tested. No immunochromatographic assays produced false positive results for pH adjusted water in the absence of an organic acid (**Table 5**).

The recommended best practice for testing liquid samples using immunochromatographic assays is dilution of questioned samples with kit-specific buffered solutions and use of manufacturer-validated testing protocols. In all instances, with the exception of the SERATEC® HemDirect and SERATEC® Amylase Test assays, dilution of the 0.3M citric acid solution (pH 4) with kit specific buffers was insufficient in mitigating false positive events (**Table 5**).

Table 5. Immunochromatographic Assay Results with Deionized Water at Various pH Values and with Kit Specific Buffer/Citric Acid Solutions.

	Assay	Median H_2O Result (pH of water)	Acidified H_2O Result (pH of water)	Kit Specific Buffer Dilution Result (1:1 Dilution of 0.3M citric acid)
Abacus® Diagnostics	ABAcard® p30	NEG (7)	NEG (2)	+
	ABAcard® HemaTrace®	NEG (4)	NEG (2)	+
Independent Forensics	RSID™ Semen	NEG (7)	INV (2)	+
	RSID™ Blood	NEG (9)	INV (2)	+
	RSID™ Saliva	NEG (7)	INV (2)	+
	RSID™ Urine	NEG (7)	INV (2)	+
Seratec®	PSA Semiquant	NEG (7)	NEG (2)	+
	HemDirect	NEG (7)	NEG (2)	NEG
	Amylase	NEG (7)	NEG (2)	NEG

Note: Deionized water at various pH levels in the absence of organic acids were tested on all immunochromatographic assays (first two results columns). Results with each assay for 0.3M Citric Acid (pH 4) diluted 1:1 with kit specific buffer are illustrated in the third results column. In all but two instances, the presence of kit specific buffer failed to mitigate false positive results due to non-specific binding events. Positive (+); Negative (NEG).

2.2.4 Common Beverages and Household Products

Common commercial products containing organic acids were selected for evaluation. Neat liquid samples were assayed first. In the event that a positive or invalid test result was observed, a 1:1 dilution with kit specific buffer was also tested. All immunochromatographic assays were found to generate a false positive result with at least

one commercial product (**Table 6**). Apple juice produced the most false positive results across all assays. Neat white wine and white vinegar generated the highest rate of invalid test results. Dilution of these samples in kit specific buffer produced both negative and false positive results depending on the assay. The ABAcard® HemaTrace® assay continued to produce the fewest false positive results, only generating a positive reaction with white wine and buffer diluted white vinegar. In over half of all instances, dilution of commercial products with kit specific buffer failed to mitigate false positive results due to non-specific binding events.

Table 6. Results for immunochromatographic assays when tested with common commercial products containing various organic acids (neat and diluted 1:1 with kit specific buffer).

	Assay	Apple Juice		Orange Juice		1% Milk		White Wine		White Vinegar		Pine-Sol		Windex®		Febreze		Monster Energy Drink	
		Neat	Dilution	Neat	Dilution	Neat	Dilution	Neat	Dilution	Neat	Dilution	Neat	Dilution	Neat	Dilution	Neat	Dilution	Neat	Dilution
Abacus® Diagnostics	ABAcard® p30	+	+	+	+	NEG	NT	+	+	NEG	NT	NEG	NT	NEG	NT	NEG	NT	+	+
	ABAcard® HemaTrace	NEG	NT	NEG	NT	NEG	NT	+	NEG	INV	+	NEG	NT	NEG	NT	NEG	NT	NEG	NT
Independent Forensics	RSID™ Semen	+	+	INV	+	+	NEG	INV	+	INV	+	NEG	NT	NEG	NT	+	NEG	INV	+
	RSID™ Blood	+	NEG	INV	NEG	NEG	NT	INV	+	INV	+	NEG	NT	NEG	NT	+	NEG	INV	NEG
	RSID™ Saliva	+	+	INV	+	NEG	NT	INV	+	INV	+	NEG	NT	NEG	NT	+	NEG	+	+
	RSID™ Urine	+	NEG	+	NEG	NEG	NT	INV	NEG	INV	INV	NEG	NT	NEG	NT	NEG	NT	NEG	NT
Seratec®	PSA Semiquant	+	NEG	+	NEG	NEG	NT	+	NEG	INV	+	NEG	NT	NEG	NT	NEG	NT	+	NEG
	HemDirect	NEG	NT	+	+	INV	NEG	NEG	NT	+	+	+	+	NEG	NT	NEG	NT	NEG	NT
	Amylase	+	NEG	+	NEG	NEG	NT	+	+	INV	NEG	NEG	NT	NEG	NT	NEG	NT	+	+

Note: Not tested (NT); Positive (+); Negative (NEG); Invalid (INV).

2.3 Discussion

A lack of specificity was observed for all immunochromatographic assays evaluated, regardless of target fluid or manufacturer. Findings from this study suggest it is possible to obtain false positive results due to non-specific binding in the presence of organic acids over a wide pH range. Therefore, the effects of organic acids do not appear to be strongly associated with pH as has been previously suggested. Rather, these results may depend in some cases on the strength of the organic acid being tested (12). Moreover, the addition of kit specific buffer often fails to negate these unwanted results. While simple and sensitive, the possibility of false positive results due to non-specific binding within immunochromatographic cartridges should be taken into consideration when reporting results and conveying the potential significance of results to the trier of fact. Doing so should help to prevent overstatement of the strength of the results obtained with these assays (13, 26).

Assay sample well antibodies are bound to microparticles (*e.g,* colloidal gold) through physical interactions involving non-covalent bonds which can be further strengthened through the use of chemical linkers (27, 28, 29, 30, 31). The addition of an organic acid may disrupt these bonds resulting in microparticles with reactive sites. As these microparticles migrate past immobilized antibodies on the lateral flow strip, it is reasonable to anticipate that these reactive sites could facilitate the aggregation of the microparticles in the absence of the target protein. Regardless of mechanism, however, it should be emphasized that the findings from this study demonstrate how lateral flow

immunochromatographic tests can be greatly affected by non-targeted fluids, resulting in a false positive reaction that cannot be visually distinguished from that of a true positive result. Therefore, these data underscore the presumptive nature of immunochromatographic assays for forensic body fluid detection.

CHAPTER 3: DEVELOPMENTAL VALIDATION OF A MULTIPLEX PROTEOMIC ASSAY FOR THE IDENTIFICATION OF FORENSICALLY RELEVANT BIOLOGICAL FLUIDS

3. Introduction

Current forensic methodologies for the identification of biological fluids still apply many of the same analytical techniques that have been used historically for a century or more; namely chemical reactions, color reactions produced as a result of enzymatic activity, immunological reactions, or, in the case of semen, direct visualization of spermatozoa by microscopy. All of these techniques, however, suffer from not insignificant test-specific limitations. Many of these tests are laborious, consumptive of evidentiary material (especially when multiple tests in series are required) and necessitate that analysts be proficient with a methodologically diverse range of laboratory techniques. In addition, most existing serological assays suffer from low selectivity, limited specificity and even when successful, produce only presumptive results, as highlighted in the previous chapter. For some body fluids (*e.g.*, vaginal fluid, menstrual fluid and nasal mucus), reliable serological assays do not currently exist – at least in the commercial space.

Due in large part to the limitations associated with existing methods of biological stain identification, several novel approaches to serological testing are being explored. A preference for multiplex analysis that can simultaneously identify multiple body fluids without the need for additional testing has been a long-standing desire by the community of forensic practitioners. At the same time, the throughput demands faced by many forensic laboratories necessitate that any novel procedures for body fluid identification be compatible with current the protocols and overall workflow for DNA analysis. The potential to bring greater standardization and automation to forensic serological testing is akin to the type of progress that has been achieved over the past few decades in DNA profiling.

This chapter reports the development and validation of a targeted proteomic method for the simultaneous identification of forensically relevant biological fluids – namely peripheral blood, semen, saliva, urine and vaginal/menstrual fluid. This method makes use of Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) in Multiple Reaction Monitoring (MRM) mode. This allows for the selective detection of a large number of peptides derived from body fluid-specific proteins [63, 89, 90].

Previously, 2-dimensional HPLC has been used to identify candidate body fluid specific protein biomarkers. Following enzymatic cleavage, target peptides of specific amino acid sequences were selected for use in a target ion mass spectrometry. In the present work, the most abundant and highly specific candidate peptide biomarkers for each biological fluid of interest were evaluated via LC-MS/MS analyses to confirm their body

fluid specificity in a sample population of at least fifty individuals. Following analytical optimization and selection of product ion transitions for each target, a final multiplex MRM method was designed to simultaneously and unambiguously identify six biological fluids of interest. This targeted ion assay underwent rigorous developmental validation and its forensic utility was demonstrated using simulated casework samples covering a wide variety of sample types consistent with those encountered in an operational forensic environment.

3.1 Methods

3.1.1 Chemicals and Reagents

HemogloBind™ was purchased from Biotech Support Group. Ammonium Bicarbonate (ABC), Dithiothreitol (DTT), and Iodoacetamide (IAA), and 2,2,2-Trifluoroethanol (TFE) were purchased from Sigma-Aldrich (St. Louis, MO). Mass Spectrometry grade Trypsin Gold was sourced from Promega (Madison, WI). LCMS grade water, acetonitrile, methanol, and acetone were purchased from Honeywell/Burdick and Jackson (Muskegon, Michigan). All sample preparation was carried out in Eppendorf LoBind Proteion microcentrifuge tubes. Absolute Quantification (AQUA) C-terminus labelled peptides were custom synthesized by New England Peptide (Gardner, MA) and delivered as lyophilized 2 nmol aliquots. Intact myelin basic and aprotinin stock solutions (1 mg/mL) were purchased from New England Peptide (Gardner, MA) for use as internal positive controls.

3.1.2 Body Fluid Collection

Body fluids were collected in accordance with procedures approved by the University of Denver's Institutional Review Board for Research Involving Human Subjects. Peripheral blood was collected via venipuncture into blood tubes containing EDTA. Semen and urine were both separately collected by having donors deposit fresh samples directly into sterile plastic specimen cups. Saliva was obtained by having participants place Sarstedt Salivette™ saliva collection sponges into their mouths for 3-4 minutes. Sponges were then centrifuged for 2 min at 1500 RPM at 4°C to recover saliva. Semen-free vaginal secretions and menstrual fluid were collected from participants who had abstained from sexual contact for a minimum of 12 days. Vaginal secretions were collected using a Softcup, which was placed in the vagina for a minimum of 1 hour then removed and the collected fluids placed into a sterile specimen cup. The surface of the Softcup was irrigated with 1mL ultrapure water and transferred into a 15 mL conical for agitation by vortexing. Menstrual fluid was collected using a DivaCup® which was placed in the vagina for a minimum of 1 hour during the first or second day of menstruation and then removed and placed into a sterile specimen cup. The surface of the DivaCup® was irrigated with 1mL ultrapure water and the resulting fluids were transferred into a 15 mL conical for agitation by vortexing. Following collection and processing, all samples were aliquoted into 1.5mL microcentrifuge tubes and stored at -80ºC. In general, and unless otherwise indicated, 50 µl of blood or 125 µl of all other biological matrices were used for the proteomic identification of target proteins.

3.1.3 Casework-Type Samples

The applicability of a mass-spectrometry based body fluid assay to samples encountered in a forensic context was assessed using a series of casework-type samples. Specifically, the ability of the biomarkers to be detected in body fluid samples recovered from a variety of substrates including cotton, denim, leather, metal, glass, plastic, sanitary napkins and Styrofoam™ were tested. Similarly, the impact of exposure to environmental contaminants/insults was also assessed. For these assays, aliquots of bodily fluids applied to sterile cotton tipped applicators that had previously been dipped in such agents as 10% bleach, neat bleach, soil, detergent, spermicidal lubricants, chewing tobacco, and soda were used. Swabs designed to simulate sexual assault type evidence were also assessed. These included oral swabs, rectal swabs, vaginal swabs, penile swabs and finger swabs. Finally, a series of aged body fluids stored at room temperature for a period of 2-7 years were analyzed.

3.1.4 Protein Extraction, Quantification, and Digestion

Dried stains were resolubilized by soaking in 400µL of diH_2O for 30 minutes with frequent agitation by vortexing. This was designed to facilitate the separation of biological material from the substrate. Sample substrates were then transferred into clean spin baskets and centrifuged at 14,000 RPM for 10 minutes. Fluid samples were centrifuged at 14,000 RPM for 10 minutes. If samples appeared to contain excessive quantities of hemolyzed red blood cells, 400 µL of HemogloBind™ was added to selectively remove cell-free

hemoglobin. Samples were vortexed for 30 seconds and mixed via inversion for 15 minutes prior to two centrifugation steps of 7,000 RPM for 2 minutes each. For samples containing suspected denaturants (*i.e.*, urea, detergents, etc.), precipitation using 1.2 mL of acetone was carried out. Samples were vortexed, stored at -20 °C for 30 minutes then centrifuged in a refrigerated microcentrifuge at 12,000 RPM for 10 minutes at 4°C. An additional 600 µL of cold acetone was added to pelleted material and samples were stored at -20 °C for 15 minutes prior to centrifugation at 12,000 RPM for 10 minutes in a refrigerated microcentrifuge at 4°C. To resolubilize the pelleted protein, 150 µL of 50 mM ABC was added and samples which were placed in a thermomixer set at 30 °C and 850 RPM for 15 minutes. Samples underwent a final centrifugation step in a refrigerated microcentrifuge at 4°C at 12,000 RPM for 10 minutes. The resulting supernatant was then transferred to a clean 1.5mL microcentrifuge tube for analysis. Following sample preparation protocols, total protein concentration was determined using a modified bicinchoninic acid assay (Micro BCA Protein Assay, Thermo Scientific Pierce) using bovine serum albumin as a known standard.

Following protein quantification, 20 µg of total protein was transferred to a 1.5mL low retention microcentrifuge tube and lyophilized in a vacuum evaporator with the addition of 16 pmol bovine myelin basic and aprotinin internal digestion controls. Dried protein samples were reconstituted in 30 µL of denaturant buffer (50% TFE in 50 mM ABC with 5 mM DTT) and incubated at 60 °C with shaking (850 RPM) for 1 hour. The resuspended proteins were then alkylated by the addition of 1.5 µL of 200 mM

Iodoacetamide (IAA) and shaken in the dark for 30 minutes at room temperature. Denaturant was diluted with 250 µL of 50 mM ABC and digested overnight at 37°C using trypsin at a 50:1 protein/enzyme mass ratio. Digested samples were then lyophilized in a vacuum evaporator and resuspended in 3% acetonitrile and 0.1% formic acid to a final concentration of 0.5 µg/µl. Following digestion and purification, 10 µL labelled peptide master mix, consisting of 0.4 pmol/µl AQUA peptide stocks in 30% acetonitrile with 0.1% formic acid, were added to each sample.

3.1.5 LC-MS/MS Analysis

All samples were analyzed by LC-MS/MS on an Agilent Technologies HPLC-chip/MS system coupled to an Agilent 6430 Quadrupole Mass Spectrometer operating in positive dynamic Multiple Reaction Monitoring (dMRM) mode. Chromatographic separation was carried using a high capacity chip containing a 150mm 300 Å C18 analytical column with a 160 nL enrichment column. Columns were equilibrated in 0.1% formic acid in water. Run conditions employed “Buffer A” (0.1% formic acid in water) and “Buffer B” (90% acetonitrile, 10% water, 0.1% formic acid). An initial 30-minute run employed a gradient of 3% Buffer B to 35% Buffer B over 24 minutes. This was followed by 5 minutes at 90% Buffer B to flush the column and then reequilibration at 3% mobile phase A. A volume of sample containing 1 µg of total protein was injected with a flow rate of 400 nL/min. This assay targets a total of 26 individual precursor ions consisting of 6 peripheral blood peptides, 5 saliva peptides, 6 seminal fluid peptides, 2 urine peptides and 7 vaginal/menstrual fluid peptides. Data were acquired through Mass Hunter software

(Agilent Technologies v.B.04.01). Skyline software (v.3.1.0 MacCross Lab Software, University of Washington, USA) was used for the *in silico* selection of peptide transitions, optimization of collision energies as well as data analyses. Target peptide detection was assessed as "positive" when all transition ions were detected at established ion and retention time ratios for "natural/native" and "heavy" labeled internal standards were met and peak morphology was of sufficient quality with a signal to noise ratio of at least 1:3. At least one peptide of a biological fluid must be unambiguously identified in order to positively identify the corresponding biological fluid.

3.2 Results

3.2.1 Confirmation of Body Fluid-Specific Targets

This study leveraged a database of preexisting targeted-ion data generated using Quadrupole Time-of-Flight (QTOF) mass spectrometry. These data were generated in the course of previously published biomarker validation studies by the Danielson research group [90]. In an effort to ensure specificity, peptides and transitions were evaluated to identify those that had a unique fragmentation pattern, were abundant, efficiently ionized and had a mass to charge ratio greater than that of the tryptic peptide. Peptides with interfering signals or those with a low response were eliminated from the list. Selection based on these criteria was intended to yield an assay that would be both specific and sensitive. A comprehensive list of target biomarker peptides and transitions for the

detection of each of six human body fluids (*i.e.*, urine, semen, saliva, vaginal/menstrual fluid, and peripheral blood) is provided in **Tables 7-11**.

Table 7. Urine Biomarker, Peptide and Transition List.

Fluid	BioMarker	Peptide Sequence	Charge State	m/z	Targeted Ions
Urine	Uromodulin	TLDEYWR	2	491.7	
					[y5] - 768.3311
					[y4] - 653.3042
		STEYGEGYA[Cys(CAM)]DTDLR	2	868.9	
					[y11] - 1256.5212
					[y10] - 1199.4997
					[y9] - 1070.4571

Table 8. Seminal Fluid Biomarker, Peptide and Transition List.

Fluid	BioMarker	Peptide Sequence	Charge State	m/z	Targeted Ions
Seminal Fluid	Prostatic Acid Phosphatase	ELSELSLLSLYGIHK	3	568	
					[y8] - 930.5407
					[y7] - 817.4567
					[y6] - 730.4246
		FQELESETLK	2	612.3	
					[y8] - 948.4884
					[y7] - 819.4458
					[y6] - 706.3618
	Prostate Specific Antigen (PSA)	LSEPAELTDAVK	2	636.8	
					[y10] - 1072.5521
					[y9] - 943.5095
					[y7] - 775.4196
		IVGGWE[Cys(CAM)]EK	2	539.3	
					[y8] - 964.4193
					[y7] - 865.3509
	Semenogelin-2	DIFTTQDELLVYNK	2	849.9	
					[y11] - 1323.6791
					[y10] - 1222.6314
		DVSQSSISFQIEK	2	734.4	[y8] - 993.5251
					[y9] - 1038.5466
					[y8] - 951.5146
					[y6] - 751.3985

Table 9. Saliva Biomarker, Peptide and Transition List.

Fluid	BioMarker	Peptide Sequence	Charge State	m/z	Targeted Ions
Saliva	Statherin	FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF	3	1215.2	
					[y13] - 1687.7751
					[y8] - 1074.4891
					[b11] - 1229.5626
	Submaxillary Gland Androgen Regulated Protein	IPPPPPAPYGPGIFPPPPPQP	3	710.7	
					[y7] - 729.3930
					[b10] - 987.5298
					[b12] - 1141.6041
		GPYPPGPLAPPQPFGPGFVPPPPPPPYGPGR	3	1034.5	
					[b9] - 850.4458
					[y12] - 1228.6473
					[b12] - 1172.6099
	Amylase	LSGLLDLALGK	2	550.3	
					[y10] - 986.6
					[y9] - 899.6
					[y7] - 729.5
		IAEYMNHLIDIGVAGFR	3	640.3	
					[y9] - 947.5
					[y16] - 903.5
					[y15] - 867.9

Table 10. Vaginal/Menstrual Fluid Biomarker, Peptide and Transition List.

Fluid	BioMarker	Peptide Sequence	Charge State	m/z	Targeted Ions
Vaginal/ Menstrual Fluid*	Cornulin	ISPQIQLSGQTEQTQK	2	893.5	
					[y11] - 1247.6226
					[y10] - 1119.5640
					[y9] - 1006.4800
		GQNRPGVQTQGQATGSAWVSSYDR	3	850.7	
					[y11] - 1228.5593
					[y10] - 1127.5116
					[b10] - 1066.5388
	Matrigel-induced Gene C4 protein	DGVTGPGFTLSGSC[Cys(CAM)]C[Cys(CAM)]QGSR	3	971.9	
					[y11] - 1212.5
					[y10] - 1111.5
					[y9] - 998.4
		GC[Cys(CAM)]VQDEFC[Cys(CAM)]TR	3	636.3	
					[y8] - 1054.5
					[y7] - 955.4
					[y6] - 827.3
	Suprabasin	ALDGINSGITHAGR	3	461.2	
					[y9] - 912.5
					[y8] - 798.4
					[y12] - 599.3
					[y11] - 541.8
	Neutrophil Gelatinase-Associated Lipocalin	SYPGLTSYLVR	2	628.3	
					[y9] - 1005.5728
					[y8] - 908.5200
					[y6] - 738.4145
		WYVVGLAGNAILR	2	716.4	
					[y11] - 1082.6681
					[y10] - 983.5996
					[y9] - 884.5312

Table 11. Peripheral Blood Biomarker, Peptide and Transition List.

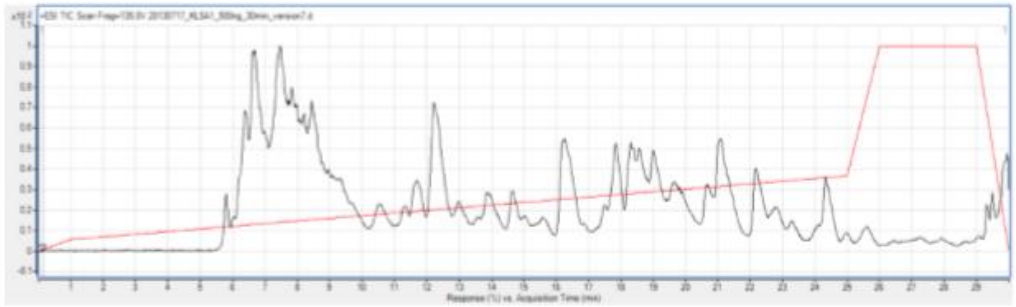
Fluid	Protein	Peptide Sequence	Charge State	m/z	Targeted Ions
Peripheral Blood	Alpha-1 Antitrypsin	LSITGTYDLK	2	555.8	
					[y9] - 997.5
					[y8] - 910.5
					[y7] - 797.4
					[y6] - 696.4
		SVLGQLGITK	2	508.3	
					[y7] - 875.5098
					[y6] - 761.4668
					[y5] - 662.3984
	Hemopexin	NFPSPVDAAFR	2	610.8	
					[y9] - 959.4945
					[y8] - 862.4417
					[y7] - 775.4097
		GGYTLVSGYPK	2	571.3	
					[y8] - 864.4825
					[y7] - 763.4349
					[y6] - 650.3508
	Hemoglobin subunit beta	GTFATLSELH[Cys(CAM)]DK	2	739.9	
					[y9] - 1102.5197
					[y8] - 1001.4721
					[y7] - 888.3880
		SAVTALWGK	2	466.8	
					[y7] - 774.4509
					[y6] - 675.3824
					[y5] - 574.3348

3.2.2 Development of a Multiplex Proteomic Assay for Body Fluid Identification

Optimized transitions for each target peptide as well as collision energy voltages were initiated with *in silico* predictions using the Skyline Proteomics Environment Software. To confirm optimized transition selection, the *in silico* settings were compared to fragmentation spectra obtained experimentally through LC-MS/MS analysis of five single-source reference samples for each target body fluid. Peak shape, abundance and retention time were monitored in order to confirm the unambiguous detection of each precursor-product ion pair. This information was used to evaluate the reliability with which

transitions were detected. Synthetic peptides were used for the optimization of collision energy voltage so as to avoid any possible matrix effects from the biological fluids of interest at this stage of the validation process. Targeted-ion inclusion lists were compiled for each biological fluid. Using saliva as an example, **Figure 18** shows the total ion chromatogram (A) and the MRM TIC for the target semenogelin-II peptides (B).

(A)



(B)

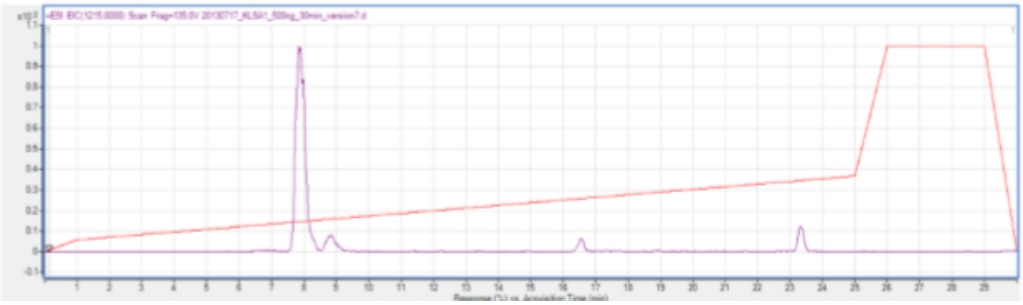


Figure 18: (A) Total ion chromatogram of a reference saliva sample with the percent organic phase used in the gradient overlaid in red. (B) MRM ion chromatogram of saliva with the percent organic phase used in the gradient overlaid in red.

Biological fluids, analyzed in duplicate, were then compared to synthetic peptide reference standards to ensure consistency in ion ratios and retention time. These measures were used to empirically verify that the assays actually identified the biomarker amino acid

sequences they were designed to detect. While ion ratios were consistent between synthetic peptides and biological fluids, slight shifts in retention times were noted in a small number of samples. This phenomenon is not uncommon when working with nano flow systems. To normalize for this, internal reference standards purchased from New England Peptide (Gardner, MA) were used. These standards are synthesized with amino acid sequences that are identical to target biomarker sequences but with the incorporation of stable "heavy" isotope labels. The stable isotope label behaves the same during HPLC separation but produces a mass shift, which allows the standard and natural peptide from a sample to be simultaneously monitored on the LC-MS/MS system (**Figure 19**).

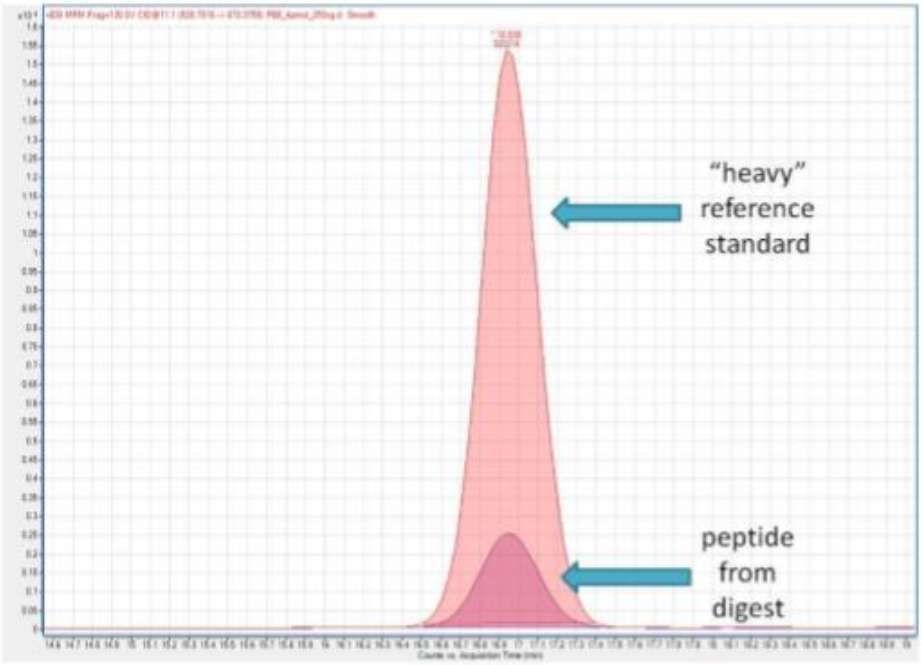


Figure 19: Co-elution of an Absolute Quantitation (AQUA) peptide reference standard and a natural peptide generated from a sample digest.

Because forensic casework-type samples are typically of unknown composition and/or may contain environmental contaminants or other compounds with inhibitory properties that are co-extracted from evidentiary substrates, internal positive controls (IPCs) are often incorporated into forensic biological chemistries. As it is reasonable to assume that forensic samples may also contain enzyme inhibitors that may adversely impact the efficiency of the proteolytic digestion of protein targets required for LC-MS/MS identification, an IPC consisting of a trypsin-cleavable protein was incorporated into this assay. Ideal IPC candidates have highly specific peptide sequences and would not likely be found in casework-type samples. Ultimately, bovine myelin basic protein from bovine brain was selected as an appropriate IPC. This globular protein was added to samples and digested with trypsin alongside target proteins from biological fluids of interest. The selected digest target (DTGILDSLGR) was evaluated *in silico* against the UniProt/Swiss-Prot database to ensure that the sequences did not match any biomarker of interest as well as any other protein sequence found in humans. The ratio between cleavage products resulting from sample digestion and that of a non-radioactive "heavy" carbon isotope labeled peptide of the same sequence was monitored during sample injections.

MRM is the ideal protein analysis detection mode because the mass spectrometer is able to handle a large number of transitions per run without compromising sensitivity. A single MRM method capable of simultaneously detecting all biological fluids was developed after optimization and assessment of individual MRM methods for each fluid of interest. The final multiplexed MRM assay included a total of 26 peptides and 88

transitions. A comprehensive list of all selected peptide sequences, the m/z of their precursor and product ions and optimized collision energies can be found in **Appendix I**.

3.2.3 Multiplex Validation

A series of developmental validation studies were conducted based on multiple forensic community guidelines as applicable including the 2012 Scientific Working Group on DNA Analysis Methods (SWGDAM) Validation Guidelines for DNA Analysis Methods. These were designed to meet Standard 8.2 of the FBI's "Quality Assurance Standards for Forensic DNA Testing Laboratories". While these guidelines were developed for the validation of DNA and RNA associated methods, they are in large part applicable to the validation of novel target ion mass-spectrometry methods for serological analyses. In addition, the 2013 Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology were also consulted and applicable guidelines followed to facilitate the design of a rigorous set of validation studies.

3.2.3.1 Carry Over Study

The reliability of the developmental validation studies requires clean injections of each sample such that the components of one injection do not interfere with subsequent injections. In order to assess run-to-run carryover, proteins were extracted from neat body fluids with the most abundant and hydrophobic markers (*i.e.*, hemoglobin beta found in peripheral blood and amylase found in saliva, respectively). These were injected at

maximum column capacity (*i.e.*, up to 1µg of total protein). Each "maximum protein" assay was followed by a series of blank injections consisting of 3% acetonitrile, 0.1% formic acid to monitor for sample carry over. No sample carryover was observed with blood digests at the maximum loading capacity of the column (1µg). However, carryover of amylase in saliva, which is both abundant and hydrophobic, was detected in the blank that followed injection of 1µg of saliva digest (**Figure 20**). Carryover was not detected in the subsequent injection (*i.e.*, the second blank sample). Based on these results, a blank sample was interspersed between all test samples for the remainder of the validation studies.

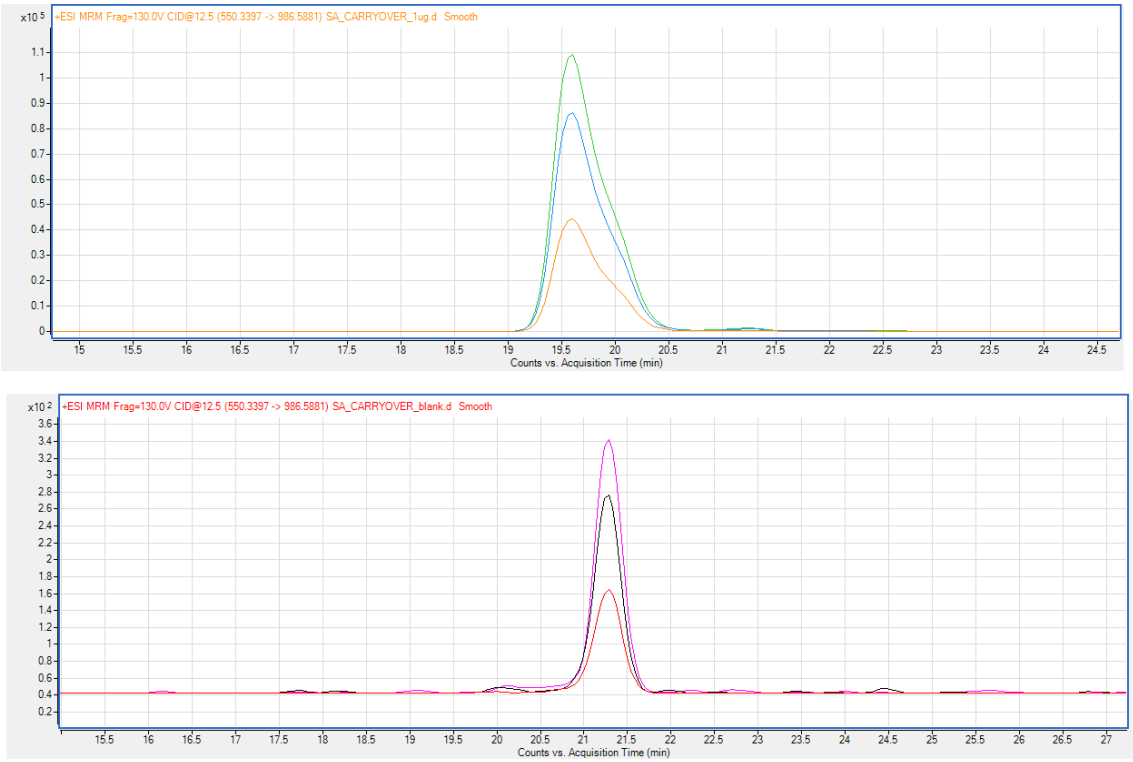


Figure 20: (TOP) Amylase peptide marker LSGLLDLALGK with 1ng saliva on column. Note that the y-axis scale is in units of 105. (BOTTOM) Detection of trace amylase peptide marker LSGLLDLALGK carryover in a subsequent blank injection on the LC-QQQ. Note that the y-axis scale is in units of 102.

3.2.3.2 Stability

Stability studies assessed the stability of targets and standards over extended time periods on the instrument autosampler at room temperature (approximately 20 ºC). Pooled samples from 10 donors were created for each biological fluid of interest. Samples prepared in triplicate were analyzed for autosampler stability at time 0. These samples were then left on the autosampler and reinjected for an additional 3 days. All peptide targets for all proteins of interest demonstrated autosampler stability up to 3 days as assessed on the basis of peak height intensity (**Figure 21**).

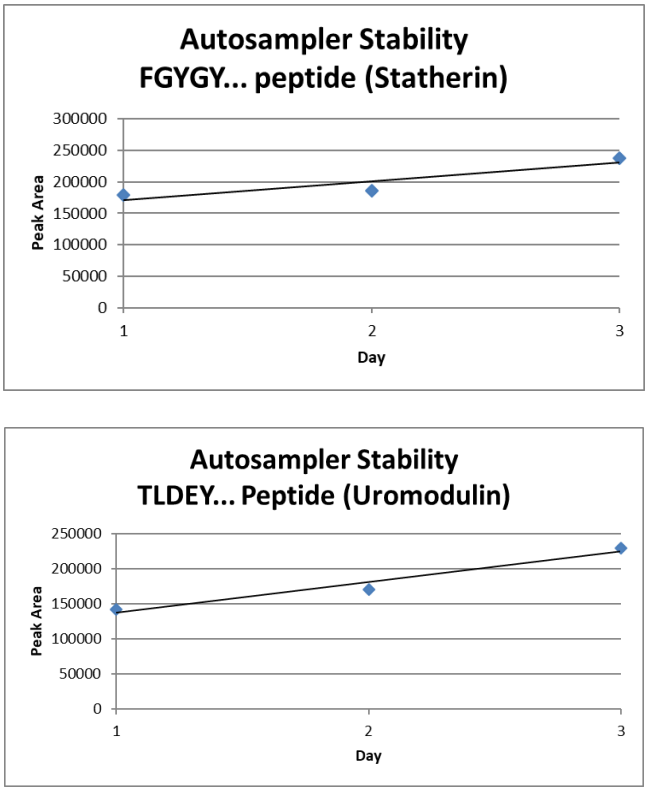


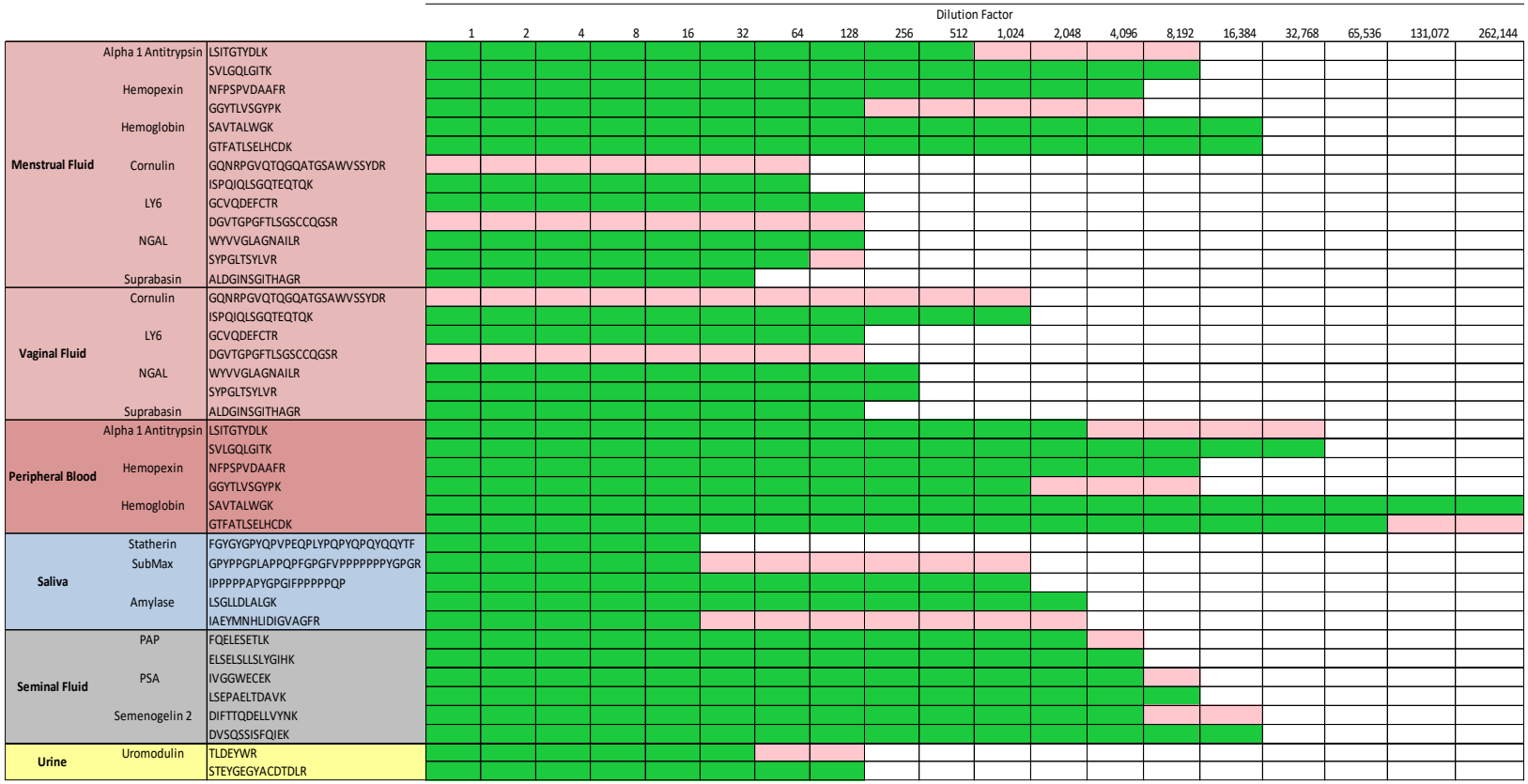
Figure 21: Autosampler stability data as measured by peak area of target peptides as a function of days on autosampler. Two of the least abundant peptides based on sensitivity studies are show for illustrative purposes. Note the apparent increase in peptide peak area is most likely attributable to the progressive evaporation of the samples occurring over the 3-day period.

3.2.3.3 Sensitivity

Sensitivity studies were carried out to determine the range of body fluid quantities that produced reliable results. For these studies, pooled samples from 10 human subjects were diluted with 50 mM ABC. A series of 2-fold dilutions ranging from 1:1 to 1:262,144 were prepared in triplicate for each fluid. Results from these sensitivity studies are presented in **Table 12**. The limits of detection for each target biological fluid varied in that

peripheral blood peptide targets were the most sensitive (detection limit 1:262,144 or 0.0001μL of target fluid for hemoglobin), the urine peptide targets were least sensitive (detection limit 1:128 or 0.3906μL of target fluid for uromodulin), and other fluids were of intermediate sensitivity; semen (detection limit 1:16,384 or 0.0031μL of target fluid for semenogelin II), saliva (detection limit 1:2,048 or 0.0244μL of target fluid for amylase), and vaginal fluid (detection limit 1:1,024 or 0.0488μL of target fluid for cornulin).

Table 12: Sensitivity Limits for Individual Biological Matrices.



Note: Peptides that were identified in the majority of prep replicates are shown in dark green. Red indicates where a paired peptide for a target protein dropped out prior to the secondary peptide target for the protein. White indicates the failure to detect a given peptide in the majority or all of prep replicates.

3.2.3.4 Repeatability and Reproducibility

To assess repeatability and reproducibility, equal-volume samples of a single body fluid from 10 human subjects were pooled. From these stocks, a total of 18 replicates for each fluid were prepared such that two separate analysts could extract and analyze three samples from each fluid per day over a period of three days. Sample extraction repeatability and reproducibility was assessed by evaluating the variation in calculated average BCA protein quantitative values. The calculated percent coefficient of variation (%CV) values for each body fluid are indicated in **Tables 13-18**. All targets fell within the maximum allowable %CV of 25%, which is a common industry benchmark for extraction repeatability and reproducibility. The only exception to this was associated with the urine targets where greater sample-to-sample variation was expected here to the additional precipitation step that was included in the sample preparation protocol to reduce the negative impact of urea on the samples assay.

Table 13. Extraction Reproducibility for Peripheral Blood

Day	Sample Name	Prep Date	Concentration (µg/mL)
1	PB HM 1.1	5/20/2014	14956.8
	PB HM 1.2	5/20/2014	14597.7
	PB HM 1.3	5/20/2014	13045.2
	PB KL 1.1	5/20/2014	19776.3
	PB KL 1.2	5/20/2014	19970.5
	PB KL 1.3	5/20/2014	18028.0
2	PB HM 2.1	5/21/2014	19962.2
	PB HM 2.2	5/21/2014	17290.2
	PB HM 2.3	5/21/2014	17785.2
	PB KL 2.1	5/21/2014	17567.9
	PB KL 2.2	5/21/2014	19646.1
	PB KL 2.3	5/21/2014	19994.3
3	PB HM 3.1	5/22/2014	17493.9
	PB HM 3.2	5/22/2014	18880.3
	PB HM 3.3	5/22/2014	20135.6
	PB KL 3.1	6/22/2014	18196.7
	PB KL 3.2	6/22/2014	20626.5
	PB KL 3.3	6/22/2014	19180.4
STATISTICS		AVERAGE	18174.1
		MIN	13045.2
		MAX	20626.5
		STDEV	2129.29
		%CV	11.72

Table 14. Extraction Reproducibility for Urine (Precipitated)

Day	Sample Name	Prep Date	Concentration (µg/mL)
1	UR HM 1.1	5/20/2014	1468.4
	UR HM 1.2	5/20/2014	1352.5
	UR HM 1.3	5/20/2014	1574.7
	UR KL 1.1	5/20/2014	1542.1
	UR KL 1.2	5/20/2014	1533.7
	UR KL 1.3	5/20/2014	1380.8
2	UR HM 2.1	5/21/2014	2339.2
	UR HM 2.2	5/21/2014	2443.3
	UR HM 2.3	5/21/2014	2280.2
	UR KL 2.1	5/21/2014	2458.8
	UR KL 2.2	5/21/2014	2336.1
	UR KL 2.3	5/21/2014	2458.2
3	UR HM 3.1	5/22/2014	2785.3
	UR HM 3.2	5/22/2014	2541.9
	UR HM 3.3	5/22/2014	2168.3
	UR KL 3.1	6/22/2014	2389.3
	UR KL 3.2	6/22/2014	2724.8
	UR KL 3.3	6/22/2014	1735.9
STATISTICS		AVERAGE	2084.1
		MIN	1352.5
		MAX	2785.3
		STDEV	495.55
		%CV	23.77

Table 15. Extraction Reproducibility for Saliva

Day	Sample Name	Prep Date	Concentration (µg/mL)
1	SA HM 1.1	6/17/2014	841.4
	SA HM 1.2	6/17/2014	814.0
	SA HM 1.3	6/17/2014	821.0
	SA KL 1.1	6/17/2014	856.5
	SA KL 1.2	6/17/2014	847.2
	SA KL 1.3	6/17/2014	833.7
2	SA HM 2.1	6/18/2014	843.8
	SA HM 2.2	6/18/2014	815.0
	SA HM 2.3	6/18/2014	808.5
	SA KL 2.1	6/18/2014	820.0
	SA KL 2.2	6/18/2014	844.3
	SA KL 2.3	6/18/2014	869.0
3	SA HM 3.1	6/19/2014	853.9
	SA HM 3.2	6/19/2014	832.1
	SA HM 3.3	6/19/2014	826.2
	SA KL 3.1	6/19/2014	837.3
	SA KL 3.2	6/19/2014	861.6
	SA KL 3.3	6/19/2014	859
STATISTICS		AVERAGE	838.1
		MIN	808.5
		MAX	869
		STDEV	17.96
		%CV	2.14

Table 16. Extraction Reproducibility for Seminal Fluid

Day	Sample Name	Prep Date	Concentration (µg/mL)
1	SE HM 1.1	6/30/2014	29071.0
	SE HM 1.2	6/30/2014	32419.9
	SE HM 1.3	6/30/2014	30793.9
	SE KL 1.1	6/30/2014	28036.1
	SE KL 1.2	6/30/2014	32299.6
	SE KL 1.3	6/30/2014	27349.3
2	SE HM 2.1	7/1/2014	35244.3
	SE HM 2.2	7/1/2014	29658.7
	SE HM 2.3	7/1/2014	28308.0
	SE KL 2.1	7/1/2014	36520.3
	SE KL 2.2	7/1/2014	23027.4
	SE KL 2.3	7/1/2014	32292.7
3	SE HM 3.1	7/2/2014	29543.8
	SE HM 3.2	7/2/2014	28485.0
	SE HM 3.3	7/2/2014	24971.0
	SE KL 3.1	7/2/2014	27410.4
	SE KL 3.2	7/2/2014	35218.8
	SE KL 3.3	7/2/2014	25495.4
STATISTICS		AVERAGE	29230.3
		MIN	23027.4
		MAX	35244.3
		STDEV	3354.96
		%CV	11.47

Table 17. Extraction Reproducibility for Vaginal Fluid

Day	Sample Name	Prep Date	Concentration (µg/mL)
1	VF HM 1.1	6/24/2014	2836.3
	VF HM 1.2	6/24/2014	2906.0
	VF HM 1.3	6/24/2014	2882.9
	VF KL 1.1	6/24/2014	2825.1
	VF KL 1.2	6/24/2014	2849.0
	VF KL 1.3	6/24/2014	2817.3
2	VF HM 2.1	6/25/2014	3045.8
	VF HM 2.2	6/25/2014	2994.4
	VF HM 2.3	6/25/2014	2901.8
	VF KL 2.1	6/25/2014	2871.7
	VF KL 2.2	6/25/2014	2886.3
	VF KL 2.3	6/25/2014	2902.2
3	VF HM 3.1	6/26/2014	2825.7
	VF HM 3.2	6/26/2014	2802.3
	VF HM 3.3	6/26/2014	2969.5
	VF KL 3.1	6/26/2014	2876.3
	VF KL 3.2	6/26/2014	2972.9
	VF KL 3.3	6/26/2014	2905.9
STATISTICS		AVERAGE	2892.8
		MIN	2802.3
		MAX	3045.8
		STDEV	66.63
		%CV	2.30

Table 18. Extraction Reproducibility for Menstrual Fluid

Day	Sample Name	Prep Date	Concentration (µg/mL)
1	MB HM 1.1	6/30/2014	1013.8
	MB HM 1.2	6/30/2014	1143.6
	MB HM 1.3	6/30/2014	1161.8
	MB KL 1.1	6/30/2014	1212.7
	MB KL 1.2	6/30/2014	1347.4
	MB KL 1.3	6/30/2014	1395.0
2	MB HM 2.1	7/1/2014	966.1
	MB HM 2.2	7/1/2014	997.8
	MB HM 2.3	7/1/2014	875.6
	MB KL 2.1	7/1/2014	1181.0
	MB KL 2.2	7/1/2014	1360.0
	MB KL 2.3	7/1/2014	1198.1
3	MB HM 3.1	7/2/2014	1354.8
	MB HM 3.2	7/2/2014	1358.6
	MB HM 3.3	7/2/2014	1325.5
	MB KL 3.1	7/2/2014	1062.1
	MB KL 3.2	7/2/2014	917.4
	MB KL 3.3	7/2/2014	1492.0
STATISTICS		AVERAGE	1188.3
		MIN	875.6
		MAX	1492.0
		STDEV	185.01
		%CV	15.56

The %CV values for each body fluid for the overall analytical method was assessed by evaluating the variation in normalized peak area (response ratio of tryptic peptide compared to labeled internal standard) and retention times (**Table 19**). As was observed with the measured repeatability and reproducibility of the extraction protocol, the majority of overall analytical method repeatability and reproducibility were within maximum allowable ranges, with the notable exception of urine. Elevated peak area %CV values for hemoglobin peptides (SAVTALWGK and GTFATLSELHCDK) and semenogelin-II peptide (DIFTTQDELLVYNK) were present in high abundance leading to suboptimal peak morphology and integration with high protein input samples. As a result, greater %CVs were observed for the area ratios of "natural" and "heavy" labeled peptides. This was readily ameliorated through sample dilution. Conversely, the ALDGINSGITHAGR peptide for suprabasin exhibited elevated peak area %CV values for natural ion response ratios as this peptide was present at low quantities which approached the lower limit of detection. The difficulty of detection at the lower limit of the assay unavoidably leads to greater %CV as it does with any other analytical assays.

Table 19. Repeatability (Analyst 1 and 2) and Reproducibility (Overall) of the Analytical Method

			Normalized Peak Area			Retention Time		
			Analyst 1	Analyst 2	Overall	Analyst 1	Analyst 2	Overall
			%CV	%CV	%CV	%CV	%CV	%CV
Menstrual Fluid	Alpha 1 Antitrypsin	LSITGTYDLK	8.30	7.70	8.30	0.61	0.38	0.50
		SVLGQLGITK	10.10	5.70	8.30	0.48	0.39	0.43
	Hemopexin	NFPSPVDAAFR	3.00	7.90	8.40	0.79	0.61	0.71
		GGYTLVSGYPK	23.50	20.45	21.80	0.88	0.59	0.76
	Hemoglobin	SAVTALWGK	3.30	4.20	7.20	0.80	0.45	0.64
		GTFATLSELHCDK	28.90	39.90	34.60	1.37	0.87	1.14
	Cornulin	ISPQIQLSGQTEQTQK	21.98	20.80	21.30	0.82	0.51	0.68
	LY6	GCVQDEFCTR	13.50	12.50	13.00	0.90	0.44	0.73
	NGAL	WYVVGLAGNAILR	26.50	24.40	26.70	0.33	0.28	0.33
		SYPGLTSYLVR	7.00	6.80	7.10	0.49	0.36	0.43
	Suprabasin	ALDGINSGITHAGR	28.60	21.08	25.70	1.09	0.55	0.86
Peripheral Blood	Alpha 1 Antitrypsin	LSITGTYDLK	16.40	17.30	16.70	0.59	0.94	0.78
		SVLGQLGITK	15.30	17.20	16.10	1.00	0.62	0.83
	Hemopexin	NFPSPVDAAFR	13.10	10.70	11.80	1.08	0.79	0.94
		GGYTLVSGYPK	10.50	8.80	9.70	0.90	0.95	0.95
	Hemoglobin	SAVTALWGK	16.20	27.10	39.90	0.88	1.12	1.01
		GTFATLSELHCDK	44..2	23.20	39.40	0.79	1.68	1.00
Vaginal Fluid	Cornulin	GQNRPGVQTQGQATGSAWVSSYDR	7.80	3.20	89.60	0.94	0.49	0.75
		ISPQIQLSGQTEQTQK	7.40	7.50	7.40	0.59	0.43	0.53
	LY6	GCVQDEFCTR	7.50	3.50	8.50	1.45	0.44	1.07
	NGAL	WYVVGLAGNAILR	6.20	4.30	5.30	0.24	0.23	0.23
		SYPGLTSYLVR	4.30	3.00	3.70	0.62	0.88	0.75
	Suprabasin	ALDGINSGITHAGR	12.20	12.10	12.60	1.04	0.54	0.82
Saliva	Statherin	FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF	6.10	5.00	5.60	0.19	0.13	0.16
	SubMax	GPYPPGPLAPPQPFGPGFVPPPPPPPYGPGR	3.70	2.60	3.40	0.25	0.18	0.22
		IPPPPPAPYGPGIFPPPPPQP	6.70	7.50	7.10	0.36	0.48	0.46
	Amylase	LSGLLDLALGK	6.80	7.80	7.20	0.28	0.32	0.30
		IAEYMNHLIDIGVAGFR	3.10	6.70	8.00	0.25	0.23	0.24
Seminal Fluid	PAP	ELSELSLLSLYGIHK	25.40	20.20	22.70	0.21	0.22	0.22
	PSA	IVGGWECEK	24.10	17.30	20.70	1.37	1.15	1.31
		LSEPAELTDAVK	21.10	16.30	18.60	0.54	0.34	0.47
	Semenogelin 2	DIFTTQDELLVYNK	34.50	31.30	32.70	0.35	0.32	0.34
		DVSQSSISFQIEK	24.30	18.70	21.50	0.55	0.38	0.49
Urine	Uromodulin	TLDEYWR	16.90	45.30	46.50	1.37	1.02	1.23
		STEYGEGYACDTDLR	63.80	81.80	79.10	1.15	0.97	1.06

3.2.3.5 Species Specificity

Both *in silico* and empirical methods were used to assess assay species specificity. The amino acid sequences of all target peptides were screened against the SWISS-PROT database containing 550,116 district proteins from 13,257 species. Additionally,

conceptual amino acid translations of all DNA sequences in GenBank and NCBI RefSeq were searched using the PSI-BLAST algorithm to search for position specific matches. Results of these database searches are provided in **Tables 20-24**. There is some shared homology with higher order primates as expected given the close evolutionary relatedness of these species to modern humans. Overall, however, there are a subset of select peptides for each fluid that are human-specific with no know shared expression in non-human primates. This fact has been taken into consideration in the design of interpretation guidelines generated for this multiplex assay.

Table 20: Species Specificity of Urine Biomarker Peptides Based on *in silico* Searches

Fluid	BioMarker (Accession #)	Peptide Sequence	Non-Human Organisms with Shared Sequence
Urine	Uromodulin (P07911)	TLDEYWR	Higher order primate (Sumatran orangutan)
		STEYGEGYACDTDLR	Higher order primate (Sumatran orangutan)

Table 21. Species Specificity of Seminal Fluid Biomarker Peptides based on *in silico* Searches

Fluid	BioMarker (Accession #)	Peptide Sequence	Non-Human Organisms with Shared Sequence
Seminal Fluid	Prostatic Acid Phosphatase (P15309)	ELSELSLLSLYGIHK	Mouse and Rat
	Prostate Specific Antigen (PSA) (P07288)	LSEPAELTDAVK	none
		IVGGWECEK	Primate (Rhesus Monkey and Crab-Eating Macaque)
	Semenogelin-2 (Q02383)	DIFTTQDELLVYNK	Primate
		DVSQSSISFQIEK	none

Table 22. Species Specificity of Saliva Biomarker Peptides Based on *in silico* Searches

Fluid	Biomarker (Accession #)	Peptide Sequence	Non-Human Organisms with Shared Sequence
Saliva	Statherin (P02808)	FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF	none
	Submaxillary Gland Androgen Regulated Protein 3B (P02814)	IPPPPPAPYGPGIFPPPPPQP	none
		GPYPPGPLAPPQPFGPGFVPPPPPPPYGPGR	none
	Amylase (P04745)	LSGLLDLALGK	none
		IAEYMNHLIDIGVAGFR	none

Table 23. Species Specificity of Vaginal/Menstrual Fluid Biomarker Peptides Based on *in silico* Searches

Fluid	BioMarker (Accession #)	Peptide Sequence	Non-Human Organisms with Shared Sequence
Vaginal / Menstrual Fluids	Cornulin (Q9UBG3)	ISPQIQLSGQTEQTQK	none
		GQNRPGVQTQGQATGSAWVSSYDR	none
		GCVQDEFCTR	none
	Suprabasin (Q6UWP8)	ALDGINSGITHAGR	none
	Neutrophil Gelatinase-Associated Lipocalin (P80188)	SYPGLTSYLVR	none
		WYVVGLAGNAILR	none

Table 24. Species Specificity of Peripheral Blood Biomarker Peptides Based on *in silico* Searches

Fluid	Biomarker (Accession #)	Peptide Sequence	Non-Human Organisms with Shared Sequence
Peripheral Blood	Alpha-1 Antitrypsin (P01009)	LSITGTYDLK	Primate
		SVLGQLGITK	none
	Hemopexin (P02790)	NFPSPVDAAFR	Higher order primate (Sumatran orangutan)
		GGYTLVSGYPK	Higher order primate (Sumatran orangutan)
	Hemoglobin Subunit Beta (P68871)	GTFATLSELHCDK	Some Mammal Genera
		SAVTALWGK	Some Mammal Genera

While *in silico* database searches represent a near exhaustive approach to assessing species specificity, additional non-human samples were tested to empirically demonstrate the human specificity of target biomarker peptides in this panel. As hemoglobin is the target biomarker that is most widely conserved across non-human mammalian species, blood samples from a variety of mammals including domestic pets (dogs and cats) and species commonly hunted in the US (bear, turkey, deer, and coyote) were tested. No peaks corresponding to human proteins were detected in any tested samples (**Table 25 and Figure 22**).

Table 25. Species Specificity of Peripheral Blood Biomarker Peptides based on empirical testing using the QQQ-MRM Assay and non-human blood.

			Dog 1	Dog 2	Cat 1	Cat 2	Deer 1	Deer 2	Bear 1	Bear 2	Otter	Turkey	Coyote
Peripheral Blood	Alpha 1 Antitrypsin	LSITGTYDLK	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
		SVLGQLGITK	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Hemopexin	NFPSPVDAAFR	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
		GGYTLVSGYPK	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Hemoglobin	SAVTALWGK	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
		GTFATLSELHCDK	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

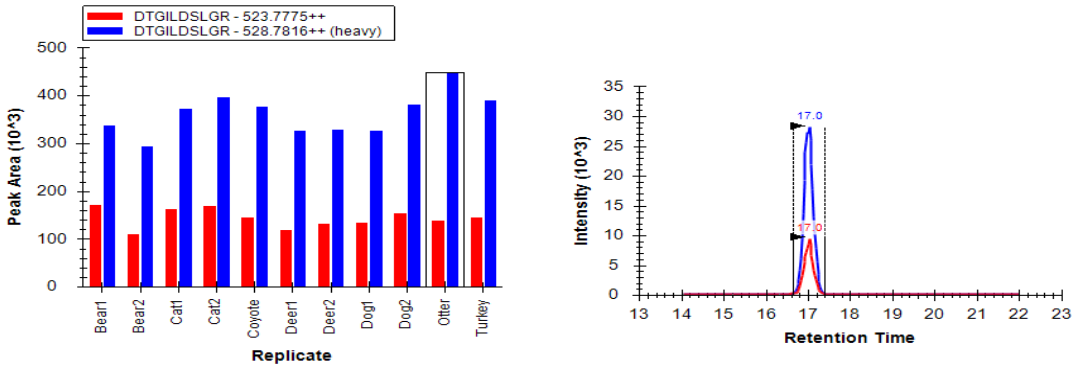


Figure 22. Results from the myelin basic protein internal positive control used with the non-human blood samples. Successful digestion of extracted proteins is indicated by the presence of digested myelin basic protein (red) and corresponding AQUA peptide standard (blue).

3.2.3.6 Mixture Study

Mixtures prepared at a 1:1 ratio (25 μL total volume) were generated in triplicate for all possible pairings of the six targeted biological fluids. In total, 45 mixed samples were tested. As previously indicated, successful identification of a biological fluid required the unambiguous identification of one or more protein biomarker targets. In 42 of the 45 samples tested in this study, the assay accurately characterized the fluids present in the mixture and did not detect any additional targets that were not present (*i.e.*, no false positive results were obtained) (**Tables 26-31**). In the remaining three mixture samples, the assay failed to identify uromodulin when mixed with semen, menstrual fluid or peripheral blood (**Table 31**). As described under the sensitivity section of this paper, the assay's sensitivity for the detection of uromodulin was the lowest (*i.e.*, the least sensitive) of all markers targeted by the assay while semen and blood (menstrual and peripheral) contain the biomarker peptides found in greatest abundance. Since mixed body fluid samples were analyzed on the basis of the total protein concentration of the initial extract, the inability to detect the body fluid with a low amount of protein when mixed with a body fluid of high protein content is not necessarily unexpected. This is at least in part due to dilution of lower abundance protein target as well as potential ion suppression by the high-abundance protein biomarker.

Table 26. Peripheral Blood Detection in Mixed Body Fluids

		Menstrual Fluid	Vaginal Fluid	Saliva	Semen	Urine
Peripheral Blood	Alpha 1 Antitrypsin					
	Hemopexin					
	Hemoglobin					
Sufficient Peripheral Blood Markers for Identification		**YES**	**YES**	**YES**	**YES**	**YES**

Table 27. Saliva Detection in Mixed Body Fluids

		Menstrual Fluid	Peripheral Blood	Vaginal Fluid	Seminal Fluid	Urine
Saliva	Statherin	ND	ND	ND	ND	
	SubMax					
	Amylase					
Sufficient Saliva Markers for Identification		**YES**	**YES**	**YES**	**YES**	**YES**

Table 28. Seminal Fluid Detection in Mixed Body Fluids

		Menstrual Fluid	Peripheral Blood	Vaginal Fluid	Saliva	Urine
Semen	PAP					
	PSA					
	Semenogelin 2					
Sufficient Semen Markers for Identification		**YES**	**YES**	**YES**	**YES**	**YES**

Table 29. Vaginal Fluid Detection in Mixed Body Fluids

		Menstrual Fluid	Peripheral Blood	Saliva	Semen	Urine
Vaginal Fluid	Cornulin				ND	
	LY6		ND			
	NGAL					
	Suprabasin		ND		ND	
Sufficient Vaginal Fluid Markers for Identification		**YES**	**YES**	**YES**	**YES**	**YES**

Table 30. Menstrual Fluid Detection in Mixed Body Fluids

		Peripheral Blood	Vaginal Fluid	Saliva	Semen	Urine
Menstrual Fluid	Alpha 1 Antitrypsin					
	Hemopexin					
	Hemoglobin					
	Cornulin		ND	ND	ND	
	LY6					
	NGAL	ND				
	Suprabasin					
Sufficient Menstrual Fluid Markers for Identification		**YES**	**YES**	**YES**	**YES**	**YES**

Table 31. Urine Detection in Mixed Body Fluids

		Menstrual Fluid	Peripheral Blood	Vaginal Fluid	Saliva	Semen
Urine	Uromodulin	ND	ND			ND
Sufficient Urine Markers for Identification		**NO**	**NO**	**YES**	**YES**	**NO**

3.2.3.7 Casework Samples

Simulated casework samples were prepared to assess performance of the multiplexed body fluid identification assay over a broad range of sample conditions. Over 100 samples were prepared which included single-source body fluids, mixed body fluids and sexual assault type stains recovered from a variety of substrates (*e.g.*, cotton, denim, leather, synthetic fibers, latex and glass). The potential impact of environmental contaminants and potential inhibitory substance (*e.g.*, spermicides, personal lubricants, detergent, soil, acids, leather, indigo dye, bleach and tobacco juice) were assessed. To explore the impact of degradation, samples were subjected to a variety of environmental insults (*e.g.*, aging and known proteolytic enzymes). All casework-type samples were prepared and tested in triplicate.

As shown in **Table 32**, MRM analysis was able to unambiguously identify individual protein components for almost all simulated casework samples. However, mixing neat laundry detergent or 10% bleach with samples resulted in a failure to detect any blood-specific proteins. As is the case with genetic analysis, the development of additional front-end sample preparation protocols may enable successful processing of these samples (**Figure 23**).

Table 32. QQQ-MRM Detection of Body Fluid Biomarkers in Forensic Casework-Type Samples.

		Fluid Confirmation				
		Vaginal Fluid	Peripheral Blood	Saliva	Semen	Urine
Vaginal Fluid Case Samples	10 µL on Cotton		ND	ND	ND	ND
	Finger Swab		ND	ND	ND	ND
	Penile Swab		ND	ND	ND	ND
Menstrual Blood	10 µL on Cotton			ND	ND	ND
	10 µL on Denim			ND	ND	ND
	10 µL on Pad			ND	ND	ND
	5 µL on Rectal Swab			ND	ND	ND
	50 µL Dried on Spermicide Condom collected with 2% SDS swab			ND	ND	ND
	5 µL plus 5 µL Lubricant Dried on Swab			ND	ND	ND
Peripheral Blood	10 µL on Swab Containing Soil	ND		ND	ND	ND
	10 µL on Swab with 10 µL 10% Bleach	ND		ND	ND	ND
	10 µL on Leather	ND		ND	ND	ND
	10 µL on swab plus 50 µL Detergent	ND		ND	ND	ND
	10 µL on Denim	ND		ND	ND	ND
	10 µL on Cotton	ND		ND	ND	ND
Saliva Case Samples	Gum	ND	ND		ND	ND
	50 µL dried on Glass Bottle collected with 2% SDS swab	ND	ND		ND	ND
	10 µL on Cotton	ND	ND		ND	ND
	10 µL on Condom collected with 2% SDS swab	ND	ND		ND	ND
	10 µL Chewing Tobacco Spit Dried on Swab	ND	ND		ND	ND
Semen Case Samples	50 µL dried on Condom collected with 2% SDS swab	ND	ND	ND		ND
	10 µL on Cotton	ND	ND	ND		ND
	10 µL on Denim	ND	ND	ND		ND
	10 µL on Oral Swab	ND	ND	ND		ND
	10 µL on Rectal Swab	ND	ND	ND		ND
	50 µL dried on Spermicide Condom collected with 2% SDS swab	ND	ND	ND		ND
	5 µL with 5 µL Lubricant on Swab	ND	ND	ND		ND
Urine Case Samples	10 µL on Cotton	ND	ND	ND	ND	
	10 µL plus 50 µL Soda on Swab	ND	ND	ND	ND	
	100 µL Dried on Ceramic Cup colleted with 2% SDS swab	ND	ND	ND	ND	
	100 µL Dried on Styrofoam Cup colleted with 2% SDS swab	ND	ND	ND	ND	

Note: Dark Green indicates all peptide targets were present. Light green indicates at least 1 target peptide was present and at least 1 target peptide was not detected. Red indicates no target peptides were detected.

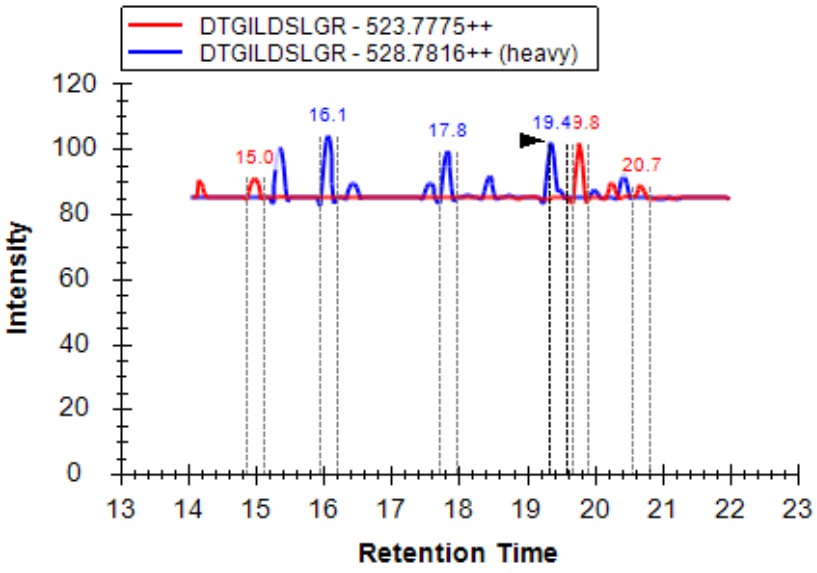


Figure 23: Results obtained for the myelin basic protein internal positive control. Target peptides representing bovine myelin basic protein were undetectable in this sample consisting of peripheral blood mixed with detergent. This indicates that the digestion of peripheral blood proteins that is required to produce the target peptides had failed.

Given the frequency with which partially degraded samples are encountered by forensic practitioners, protein degradation was also evaluated. The results obtained were consistent with those expected, given the published literature which supports the greater stability of proteins over time as compared to nucleic acids. A series of saliva, peripheral blood, semen and urine samples which had been aged at room temperature from 2 to 7 years were analyzed. As illustrated in **Tables 33-36**, the MRM assay provided for the confident identification of all body fluids based on the presence of at least one and often multiple target biomarkers in all aged samples tested.

Table 33. QQQ-MRM Detection of Peripheral Blood Biomarkers Aged Bloodstains

			Blood				
			7 years	5 years	4 years	2 years	2 years
Peripheral Blood	Biomarkers	Alpha 1 Antitrypsin					
		Hemopexin					
		Hemoglobin					

Table 34. QQQ-MRM Detection of Saliva Biomarkers Aged Stains

			Saliva		
			3 Years	3 Years	3 Years
Saliva	Biomarkers	Statherin	ND	ND	ND
		SubMax			
		Amylase			ND

Table 35. QQQ-MRM Detection of Seminal Fluid Biomarkers Aged Stains

			Semen						
			5 years	5 years	4 years	4 years	2 years	2 years	2 years
Semen	Biomarkers	Acid Phosphatase		ND	ND		ND	ND	ND
		Prostate Specific Antigen		ND	ND		ND		ND
		Semenogelin 2							

Table 36. QQQ-MRM Detection of Urine Biomarkers Aged Stains

			Urine		
			3 years	3 years	3 years
Urine	Biomarkers	Uromodulin			

3.3 Discussion

Highly tissue-specific target protein biomarkers for biological fluids of forensic interest – namely peripheral blood, semen, saliva, urine, and vaginal/menstrual fluids have been identified. Additionally, the assay targeting these markers has undergone extensive developmental validation including an assessment of aged samples, environmental impact, species specificity, stability, sensitivity, reproducibility/repeatability, and mixture analysis. Overall, it has been demonstrated that a multiplex targeted ion mass spectrometry-based assay allows for the serological identification of body fluids most commonly encountered in forensic casework. With sufficient gains in sensitivity and specificity, serological identification utilizing protein mass spectrometry analysis offers significant advantages compared to the existing immunological and biochemical tests currently employed by forensic serologists.

The deliverable to the forensic community following the research presented in this chapter includes a functional and developmentally-validated multiplex human body fluid identification assay. This assay has the potential to significantly improve the accuracy and sensitivity of serological testing of forensically relevant biological fluids. While the multiplex design of this assay eliminates the need to perform separate tests on an unknown stain, however, it requires longer analytical run times and may be unnecessarily comprehensive for routine screening of targeted forensic workflows. The analysis of items of items of evidence from sexual assault kits for example are typically only screened for semen (and possibly saliva). The creation of a targeted assay for this specific purpose

would allow for faster analytical run times and greater sensitivity for fluids relevant to targeted workflows.

The remaining two chapters of this dissertation will therefore focus on the application of a QQQ-MRM assay that has been optimized for analysis of sexual assault kit evidence capable of detecting seminal fluid markers. This approach would enable forensic analysts to obtain a confirmatory identification of semen in extended post-coital samples. This approach would also allow for the confirmation of semen in samples where there was insufficient DNA to obtain an interpretable profile (e.g., vasectomy, lack of ejaculation or minimal sexual contact). In short, the conversion of this multiplex assay to a fit-for-purpose monoplex assay for the analysis of sexual assault kit evidence would make it possible to obtain probative results from samples that might otherwise have yielded inconclusive or no results at all, providing the forensic and criminal justice communities with a powerful tool to aid the investigation and prosecution of sexual assault.

CHAPTER 4: IMPROVING SEMINAL FLUID DETECTION SENSITIVITY IN EXTENDED POST-COITAL INTERVALS BY QQQ MASS SPECTROMETRY

4. Introduction

In 2017, the FBI Crime Statistics reveled that an estimated 135,755 rapes were reported to US law enforcement agencies [91]. After several consecutive years of increases, the overall number of violent crimes reported according to the FBI figures decreased in 2017, however, the number of reported aggravated assaults and rapes continued to increase by 1.0 and 2.5 percent respectively over 2016 numbers. Approximately 18% of women in the US have been raped in their lifetime [92]. This includes an estimated 1.8 million adolescent victims [93]. While the timely recovery of physical evidence is critical to sexual assault investigations, many sexual assault victims delay reporting the incident to authorities for three to four days after an attack. This is especially true of child victims, where disclosure of sexual abuse and rape may be delayed even longer [76].

The timely recovery of physical evidence of a potential sexual assault is vital. As the post-coital interval is extended, the potential for successful identification of probative evidence such as seminal fluid and/or DNA diminishes rapidly. For this reason, the length of time after a sexual assault (*i.e.*, the post-coital interval) can influence the potential for subsequent forensic testing to yield probative results; the priority assigned to testing a

sexual assault evidence kit (especially in the case of backlog reduction efforts); and even the decision of whether or not an effort to collect physical evidence of sexual assault will be made at all.

In sexual assault cases, the detection of seminal markers in the vagina or cervix constitutes important physical evidence of sexual contact. Accordingly, numerous studies have evaluated the persistence of semen in the post-coital interval. Semen, in these studies, is usually identified by the presence of spermatozoa although the persistence of biochemical markers of seminal fluid (*e.g.*, choline, acid phosphatase, PSA/p30 and semenogelin) have also been investigated. Difficulty in detecting seminal fluid markers, sperm and/or DNA past a post-coital interval of 5-7 days, however, has been widely reported in the literature [94, 95]. Aside from vasectomized and azoospermic males, semen loss due to vaginal lavage, drainage and degradation can all impede the ability to detect spermatozoa and/or obtain interpretable DNA typing results. Similarly, these factors limit the ability to detect evidence of sexual contact through the use of serological assays that indicate the potential presence of seminal fluid.

There are widely varying estimates of how long into the post-coital interval the cellular and biochemical components of semen can be detected. The literature on sperm detection in the vagina and cervix exemplifies this. Estimates of the time period within which sperm can be recovered from the vaginal cavity of healthy females range from 30 minutes to 19 days post coitus [96-105]. The majority of authors, however, report finding spermatozoa up to 3 days post coitus in the vagina and up to 7 days post coitus in the cervix.

Most reports suggesting a 17-19 day post-coital interval for sperm recovery cite two articles published in 1891 and 1977; both of which relied on volunteer self-reporting of findings that were regarded as "possibly correct" [106].

Serological detection of seminal fluid is typically based on antibody-antigen interactions (*i.e.*, immunochromatography). PSA/p30 or semenogelin are commonly used as the target protein biomarkers. Validation studies using commercial assays suggest that spermatozoa persist longer than seminal fluid protein markers. In one study, post-coital vaginal swabs failed to produce positive results for semenogelin or PSA just 3 and 33 hours after intercourse, respectively [81]. Even when nylon flocked swabs were used to maximize sample release, semenogelin and PSA/p30 were reliably detected only up to 12 hours post-coitus. In rare cases, positive results were obtained up to 60 hours after intercourse. What is important, however, is that in 50% of samples that were negative for semenogelin and PSA, partial male DNA profiles were still generated [107].

The ability to generate interpretable male DNA profiles at various post-coital intervals has also been widely investigated. While DNA profiling can help to establish the identity of a male contributor, sexual assault samples often contain an excess of epithelial cells from a female victim. This can hinder, or entirely preclude, the detection of the male fraction of a mixture when autosomal STRs are used [108]. In such cases, amplification of male-targeted Y-STR loci is used. Though Y-STR haplotyping allows for the selective isolation of a male profile, the results have a much lower power of discrimination than profiling using autosomal markers. In general, however, complete Y-STR haplotypes can

be obtained from vaginal/cervical swabs up to approximately 3-4 days post coitus. After that, partial profiles continue to be detected up to approximately 5-6 days post-coitus [109-111].

The apparent rapid loss of protein indicators of seminal fluid is somewhat unexpected given that proteins typically remain stable under conditions that lead to the degradation of other biomolecules. In fact, as previously mentioned proteins are among the most long-lasting of all biological molecules having been routinely isolated from even ancient biological material [72, 112]. In a forensically applicable study, protein levels remained relatively constant even in post-mortem brain tissue [73]. This suggests that the difficulty of detecting seminal fluid proteins in the post-coital interval may be due more to the sensitivity limits of conventional immunochromatographic assay systems than to the loss of the actual target proteins. Antibody-based tests are also subject to both false positive and false negative results – the former being due either to the presence of the target seminal fluid antigen in non-target body fluids (*e.g.*, female ejaculate [14], breast milk [16], and urine [17])(*i.e.*, a true positive for the target biomarker but a false indication of seminal fluid) or non-specific binding events such as those triggered by organic acids as indicated in chapter 2 (*i.e.*, a true false positive result). Even when successful, however, these tests provide only a presumptive indication that seminal fluid may be present.

A more sensitive and specific technology for the confirmatory identification of seminal fluid – one that could match the sensitivity of DNA testing methodologies or even identify seminal plasma in vaginal fluid several days after an alleged sexual assault in cases

where there is insufficient DNA to obtain an interpretable profile – would provide critical physical evidence of sexual contact. With improved sensitivity, forensic examiners would have the potential to extend the post-coital interval for sample collection with an improved likelihood of successfully obtaining an interpretable DNA profile.

Selected reaction monitoring (SRM) has a long history of use in the fields of toxicology and pharmacokinetics [113]. SRM allows for the specific monitoring of a targeted analyte in a complex mixture. Typically, a triple quadrupole-based mass spectrometer is employed to achieve this. The first mass analyzing quadrupole of the triple quadrupole system allows for the selective passage of a target parent ion by specifying a narrow mass window. This parent ion is then fragmented in the second quadrupole, while the third quadrupole scans for a desired fragment ion. The identification of both a parent and fragment ion (*i.e.*, a transition) provides for high-confidence peptide identification. In contrast to SRM strategies, Multiple Reaction Monitoring (MRM) approaches scan for several different parent and multiple fragment ions within one run. MRM allows for greater productivity over SRM but generally this is achieved at the cost of sensitivity [114]. As the number of transitions monitored per assay increases, the dwell time (*i.e.*, the time the instrument takes to cycle through the separation and detection of each transition) for each targeted ion decreases. Therefore, the more ions targeted, the less time the instrument spends detecting and measuring any one ion. This leads unavoidably to an overall decrease in sensitivity.

A well-established technology for the unambiguous detection of proteins in complex biological fluids is triple quadrupole mass spectrometry utilizing multiple reaction monitoring (QQQ-MRM). These studies outlined in this chapter have applied a QQQ-MRM approach to the detection of seminal fluid in cervico-vaginal swabs collected at extended post-coital intervals of ≥5 days). The results of this testing were compared to existing antibody-based methods to assess the relative utility of a QQQ-MRM approach in the analysis of sexual assault samples.

Narrowing the scope of the multiplex body fluid proteomic assay detailed in Chapter 3, the creation of a seminal fluid-specific monoplex assay is expected to enhance the sensitivity of the method beyond what has been achieved to date. Moreover, the enhanced sensitivity should allow for the detection of seminal fluid protein markers in samples well past the post-coital interval that is attainable with the immunochromatographic assays currently used by forensic labs. The research outlined in this chapter therefore aims to:

(1) Develop and optimize a monoplex QQQ-MRM assay for seminal fluid using single- and mixed-stain swabs that are representative of sexual assault samples.

(2) Rigorously assess the extended post-coital time limit for which seminal fluid biomarkers can be confirmed in sexual assault type samples.

The successful completion of these aims will facilitate the analysis of challenging sexual assault evidence and extend the critical widow within which sexual assault kits can be used

to collect samples from the victim with a reliable expectation of obtaining probative test results.

4.1 Methods

4.1.1 Chemicals and Reagents

Dithiothreitol (DTT), and Iodoacetamide (IAA), and 2,2,2-Trifluoroethanol (TFE) were purchased from Sigma-Aldrich (St. Louis, MO). Mass Spec grade Trypsin gold was sourced from Promega (Madison, WI). LCMS grade water acetonitrile, methanol, and acetone were purchased from Honeywell/Burdick and Jackson (Muskegon, Michigan). All sample preparation was carried out in Eppendorf LoBind Protein microcentrifuge tubes. Absolute Quantification (AQUA) C-terminus labelled peptides were custom synthesized by New England Peptide (Gardner, MA) and delivered as lyophilized 2 nmol aliquots. Intact myelin basic and aprotinin stock solutions (1 mg/mL) were purchased from New England Peptide (Gardner, MA) for use as internal positive controls.

4.1.2 Body Fluid Collection

Body fluids were collected in accordance with procedures approved by the University of Denver's Institutional Review Board for Research Involving Human Subjects as previously described in Chapter 3. Following collection and processing, all samples (peripheral blood, menstrual blood, vaginal secretions, semen, urine and saliva) were aliquoted into 1.5mL microcentrifuge tubes and stored at -80ºC. In general, and unless

otherwise indicated, 50 µl of blood or 125 µl of all other biological matrices were used for the identification of target protein biomarkers.

4.1.3 LC-MS/MS Analysis

Optimal parent-fragment ion pairs for high-specificity biomarkers had already been identified for semen, saliva, urine, peripheral blood and vaginal/menstrual fluid as described previously in Chapter 3. In the original multiplex assay, up to three proteins/fluid were selected. Generally, two to three optimal peptides were selected (as parent ions) for each protein. Similarly, two to three fragment ions were selected per parent ion. This redundancy allowed for greater productivity and selectivity in the multiplex assays. As mentioned previously, however, this comes at the cost of sensitivity when using SRM assays.

The primary objective of the work described in this chapter, therefore, was to develop and optimize a monoplex QQQ-MRM assay for seminal fluid. This was achieved by importing the existing multiplex method but eliminating all biomarker peptides not specific to seminal fluid. What remained were the parent-fragment ion pairs for prostatic acid phosphatase, prostate specific antigen and semenogelin. Iterations of this method were developed in which parent and fragment ion pairs were sequentially eliminated until an SRM method with one peptide for each seminal fluid protein biomarker with one fragment remained. This was done with the objective of maximizing the dwell time efficiency of the instrument; thereby maximizing detection sensitivity. Each of the resulting monoplex assay methods was assessed for sensitivity by analyzing vaginal swabs spiked with known

quantities of semen. Neat pooled seminal fluid from 10 male donors was diluted at the following ratios: 1:800, 1:4,000, and 1:20,000 and digested. After digestion, 125µL aliquots were lyophilized and reconstituted for analysis by LC-MS/MS. Each sample was analyzed under each of the iterations of the seminal fluid assay.

Assay specificity was also assessed by analyzing a series of 25 replicate two-, three- and four-component mixtures consisting of saliva, semen, vaginal secretions, urine, peripheral and/or menstrual blood. The method found to be the most specific for seminal fluid with the greatest sensitivity was used for the remainder of the study.

The second part of the optimization process was to evaluate injection quantity. With a multiplex assay, it is difficult to establish a set injection quantity, given the greatly varied amounts of targeted protein per matrix. For example, the amount of hemoglobin in a given volume of blood is not comparable to the amount of submaxillary gland androgen-regulated protein 3B in the same volume of saliva. With one matrix and one sample type from sexual assault kits (vaginal, oral and rectal swabs), it is easier to evaluate how much protein can be injected without overloading the column. Neat semen (25µL) was added to pooled vaginal secretions and quantified for total protein content. The following amounts of total protein were targeted for digestion: 50 µg, 75 µg, 100 µg, 150 µg and 200 µg. These amounts were loaded onto 96-well plates for digestion and sample clean-up which was performed on the AssayMAP Bravo Platform. All samples were reconstituted in 2% acetonitrile/0.1% formic acid solution to a 1µg/µL concentration and a 10µL aliquot was injected on the column.

4.1.4 Post-Coital Interval Assessment

The second objective of the research reported in this chapter was to determine the extent to which an optimized monoplex QQQ-MRM assay for semen could be used to extend the post-coital interval within which the presence of seminal fluid can be reliably detected in cervico-vaginal samples. Typically, these studies employ a self-collection swabbing method at various time points after sexual intercourse. However, this introduces a great amount of variability into the data set thereby compromising the precision of results both amongst and within the sample sets for the individuals participating in the study.

As it has already been documented in the literature (at least for spermatozoa) that seminal persistence is greater at the cervix, this study used cervical swabs obtained by a trained sexual assault nurse examiner and collected with a speculum to better represent samples that would be generated as part of an authentic sexual assault examination. Two swabs at a time were collected from female volunteers after separate acts of sexual intercourse at multiple time points (2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, and 9 days) using sterile nylon flocked applicators. In order to eliminate variability due to the combined effect of multiple acts of intercourse, volunteers were asked to abstain from intercourse for 12 days prior to the sexual act that was to be followed by sample collection. In order to prevent loss of seminal fluid markers from the sampling process itself, only one set of swabs was collected after each act of sexual intercourse. All swabs were air dried, packaged in sterile paper envelopes and stored at -20°C until extracted for analysis. At least two separate collections per post-coital time interval were assessed.

4.2 Results

4.2.1 Development of the Monoplex QQQ-MRM Assay for Seminal Fluid

The existing six-body fluid multiplex assay was imported and all biomarker peptides that were not specific to seminal fluid were eliminated. What remained were parent-fragment ion pairs for prostatic acid phosphatase, prostate specific antigen and semenogelin I and II. Iterations of this method were developed in which parent and fragment ion pairs were sequentially eliminated in order to maximize the dwell time efficiency of the instrument; thereby maximizing detection sensitivity while maintaining assay specificity. Additional biomarkers, not part of the original six-body fluid multiplex assay, were also incorporated into the methods to further optimize the specificity and sensitivity. These markers, many of which were high quality target biomarkers, had originally been eliminated due to the fact that they generated assay interference because they coeluted with protein biomarker targets from other biological fluids that the assay was looking for at approximately the same retention time. Since the detection of these other body fluids was no longer part of the seminal fluid assay, the concerns associated with coeluting species were eliminated and the potential utility of these biomarker targets could be reevaluated. Targets incorporated into the various iterations of the monoplex seminal fluid assay as well as their respective specificities can be found in **Table 37**.

Table 37. Peptide Targets Evaluated for the Development of the Monplex QQQ-MRM Seminal Fluid Assay.

Protein	Peptide Abbreviation	Retention Time (min)	Non-Human Presence	Co-Expressed In
Prostatic Acid Phosphatase	FVTL*	11.3	Mouse, Cow	Pancreas, Colon, Breast Tissue
	FQEL	6.3	None Reported	
	ELSE	14.3	Mouse, Rat	
Prostate Specific Antigen	FLRP*	10.1	None Reported	Brain, Colon Tissue
	LSEP	7.6	None Reported	
Semenogelin-1	DIFS*	12.3	None Reported	Kidney, Eye, Trachea Tissue
	QITI*	3.5	None Reported	
Semenogelin-2	GSISIQTEEK*	3.6	Primates	Kidney, Trachea Tissue
	GSIS…HGK*	4.6	Primates	
	DVSQ	8.6	None Reported	
Epididymal Secretory Protein	DCGS*	4.3	None Reported	None Reported
	SGIN*	3.0	Pig, Mouse, Dog	

Note: Those peptides abbreviated with a "*" were not part of the original 6 fluid multiplex assay and reincorporated for evaluation with the seminal fluid assays.

The most abundant peptides per protein were selected based on preliminary qualitative studies. A scheduled and unscheduled method incorporating all target peptides identified in Table 37 was first compared. A scheduled method utilizes retention time windows in order to target specific transitions at a precise retention time. This is an alternate strategy for decreasing dwell time (**Figure 24**). Four additional paired down scheduled methods were also assessed.

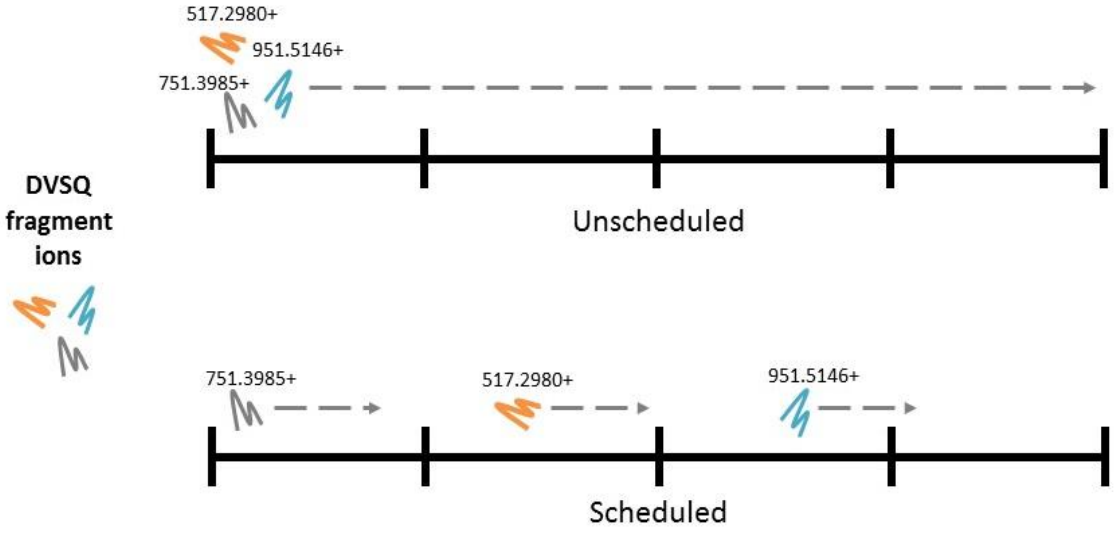


Figure 24. Scheduled (bottom) versus unscheduled (top) methods of analysis on an LC-MS/MS. During an unscheduled method, the instrument filters and scans for all targets throughout the entirety of the run. During a scheduled method, detection windows are set based on retention times for each fragment, allowing the instrument to scan and filter for those target analytes only during specified periods of time during the run, decreasing dwell time of the method and therefore increasing overall sensitivity.

When comparing results from the unscheduled and scheduled methods, significant improvements in sensitivity were observed with the scheduled method (**Figure 25**). An ANOVA with a 95% confidence interval was performed on normalized peak areas for each peptide across all 6 methods evaluated (1 unscheduled and 5 scheduled) to determine statistical significance. Significant differences between methods were observed for all but the FQEL peptide of the prostatic acid phosphatase protein (ELSE: F_s=33.833; df=2,6; P=5.40e-4, IVGG: F_s=28.338; df=2,6; P=8.77e-4, LSEP: F_s=23.207, df=4,10; P=4.79e-5, DIFS: F_s=9.625; df=5,12; P=0.0007, LPSE: F_s=51.291; df=3,8; P=1.438e-5, DVSQ: F_s=6.736; df=5,12; P=0.0032, DIFT: F_s=5.693; df=3,8; P=0.0219; FQEL: F_s=2.947;

df=1,4; P=0.1611). A post-hoc Tukey test with a 95% confidence interval showed that all scheduled methods evaluated produced significantly greater peak areas and intensities for all peptides as compared to the unscheduled method while no statistical differences of mean peak areas were observed between any scheduled methods assessed in which parent and fragment ion pairs were eliminated with the exception of the LPSE peptide for SgI (**Appendix II**). Given that scheduled methods were employed and that there was no coelution of targets in the method, this was an expected outcome.

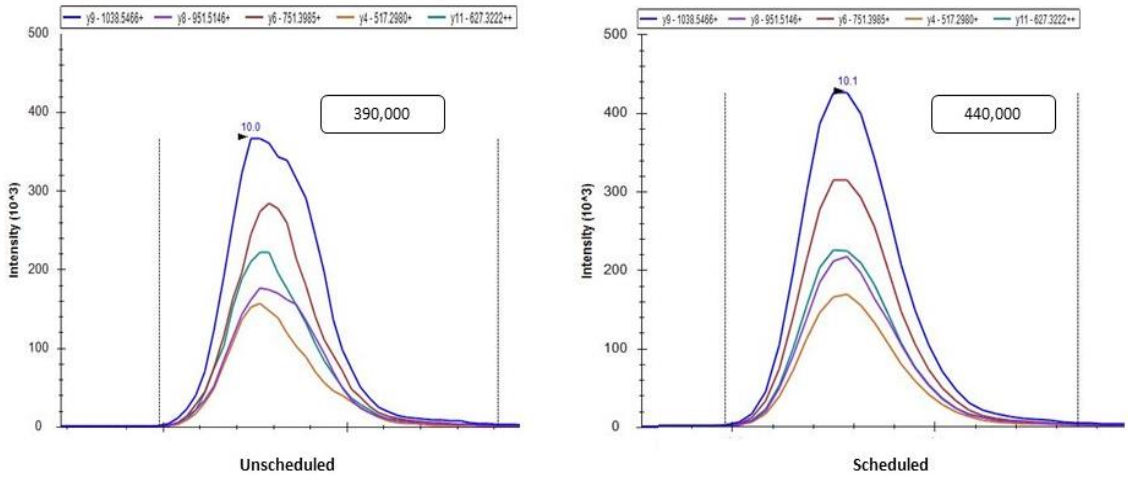
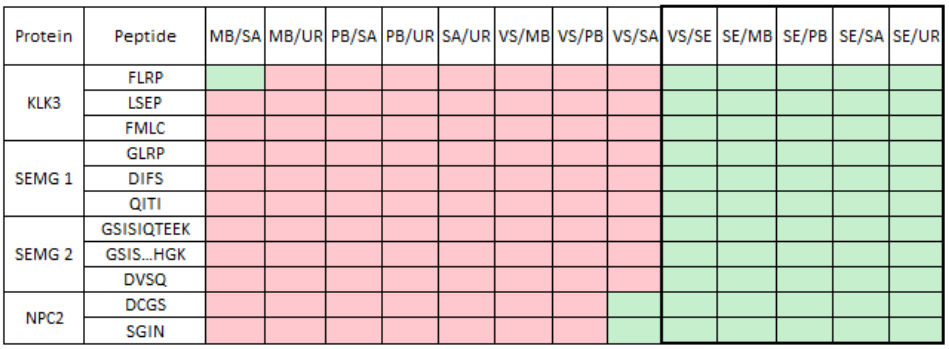
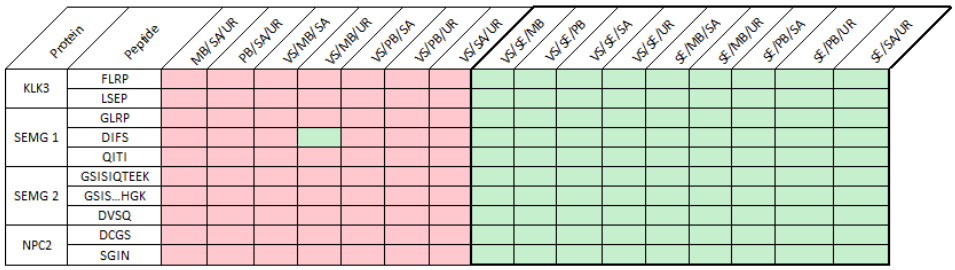


Figure 25. Chromatographic comparison of the unscheduled and scheduled methods for the Semenogelin II peptide DVSQ. The peak intensities are labeled in the upper right hand corner of each chromatogram.

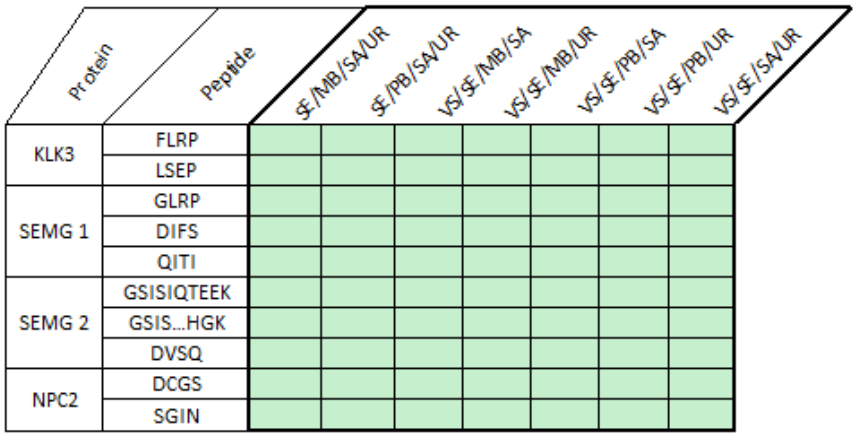
Assay specificity was assessed by analyzing a series of at least 25 replicate two-, three- and four-component mixtures consisting of saliva, semen, urine, peripheral blood and vaginal/menstrual fluids (**Figure 26**). Since no significant difference in sensitivity was observed amongst scheduled methods, specificity only needed to be assessed for the scheduled method incorporating all peptide targets in order to confirm individual target specificity.



(A)



(B)



(C)

Figure 26. Two component (A), three component (B) and four component (C) mixtures containing combinations of menstrual blood (MB), peripheral blood (PB), saliva (SA), vaginal secretions (VS), urine (UR), and semen (SE). Green boxes indicate the presence of a peptide at a detectible level and red boxes indicate the absence of a peptide at a detectible level. Bolded boarders indicate where positive results were expected based on mixture composition.

Prostatic acid phosphatase had multiple peptides that failed to be detected in samples that contained semen (samples not shown). At the same time, these peptides were detected in samples that did not contain semen. In all instances where prostatic acid phosphatase was detected in a mixture that did not contain seminal fluid, vaginal secretions were present in the mixture. This is consistent with the published literature which indicates the presence of acid phosphatase in vaginal secretions albeit at lower concentrations than seminal fluid. Epididymal secretory protein was also identified in two samples containing semen-free vaginal fluid. Given the lack of observed specificity of these two biomarkers, all peptides for both prostatic acid phosphatase and epididymal secretory protein were eliminated from the final seminal fluid assay. The final method, therefore, that was found

to be most specific for seminal fluid and which had the greatest sensitivity is included in **Appendix III**. This method was used for the remainder of the study.

A sensitivity comparison of the monoplex assay to the original multiplex assay for all six biological fluids demonstrated a gain in sensitivity of nearly one order of magnitude. The original multiplex was able to detect a pooled sample of seminal fluid at a dilution of 1:16,384 while the optimized monoplex assay for seminal fluid was able to detect seminal fluid at a dilution of 1:131,072 (**Figure 27**).

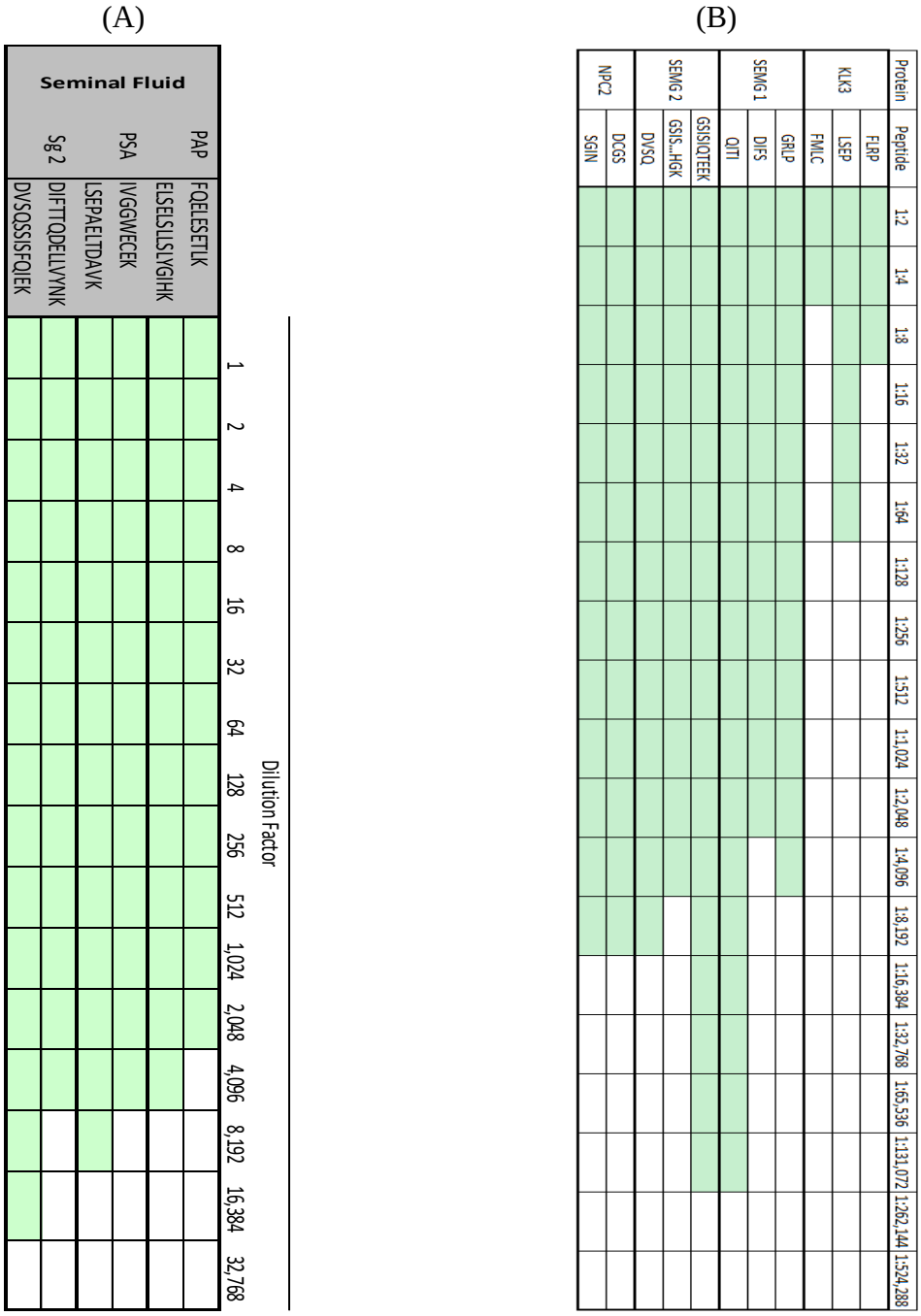


Figure 27. (A) Sensitivity limits of seminal fluid for the original multiplex assay for six forensically relevant biological fluids. (B). Sensitivity limits for semen using the optimized monoplex assay for seminal fluid representing a three-fold increase in sensitivity obtained for seminal fluid.

An evaluation was made to determine the total amount of protein can be loaded on the sample preparation robotic platform (Agilent AssayMAP Bravo) for injection onto the LC-MS/MS instrument. To achieve this, peak areas for targeted peptides were assessed for overall abundance. A decrease in peak abundance with an increase in total protein targeted for digestion indicates C18 cartridge overload on the robotic platform. The C18 cartridges on the robotic platform use a hydrophobic sorbent phase to retain peptide fragments. As more protein is loaded onto the sorbent phase, preferential binding of hydrophobic peptides and concurrent loss of hydrophilic peptides will occur. As the more abundant seminal fluid peptides in the final assay are hydrophilic, this would result in decreased assay sensitivity. Based on the results of these experiments the optimum protein loading quantity was determined to be 100 µg based on observations made of all peak areas for protein targets (**Figure 28**).

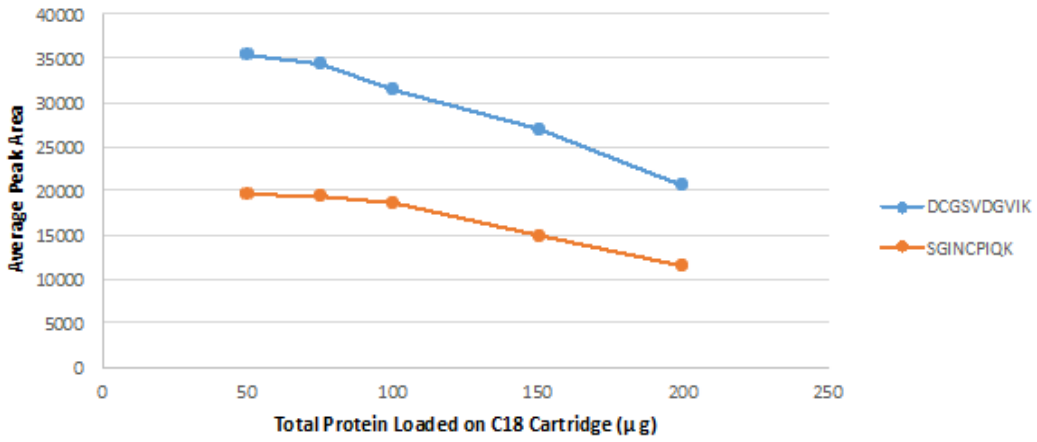


Figure 28. Peptide abundance as measured by average peak area compared to total protein loading amount on the C18 cartridge. As the peptides for epididymal secretory protein are the most hydrophilic, they were used to assess when preferential binding on the C18 cartridge occurred.

4.2.3. Assessment of the Sensitivity of the Monoplex QQQ-MRM Assay for Seminal Fluid Using Authentic Post-Coital Cervico-Vaginal Samples

The extent to which the optimized monoplex QQQ-MRM assay for semen could extend the post-coital interval during which the presence of seminal fluid could be reliably detected in cervico-vaginal samples was assessed. A small cutting from each swab was collected and placed in 500 µl of Universal Buffer (Independent Forensics). This solution contains buffer and salts (Tris, NaCl, KCl) for physiological stability, a chelating agent (EDTA) for stability, detergents and surfactants (Triton X-100 and Tween 20) for extraction efficiency and solubility maintenance, protein (BSA) for reducing non-specific adsorption and loss and a preservative (sodium azide). Following a 30-minute incubation at room temperature with agitation, manufacturer recommended volumes were tested using ABAcard® p30 (Abacus Diagnositcs), RSID™-Semen (Independent Forensics), and PSA Semiquant (Seratec). Consistent with the published literature, the ability to generate positive results for seminal fluid (with one exception) using immunochromatographic assays were lost at just 48 hours post-coitus (**Table 38**). Analysis of authentic post-coital cervico-vaginal swabs using the QQQ-MRM monoplex assay for seminal fluid, however, was demonstrated to greatly extend the post-coital interval of detection for seminal fluid. Semen in the same paired samples was detected up to 8 days post-coitus using the QQQ-MRM assay, not only far exceeding the sensitivity of commercial antibody-based methods but matching and exceeding that reported for DNA-based approaches (**Table 39**).

Table 38. Immunochromatographic Results for Seminal Fluid using the RSID™ Semen (semenogelin) and the ABAcard® p30 and PSA Semiquant (PSA/p30) Assays Kits.

Sample ID	Days Post-Coital	RSID	ABAcard	Seratec
PC1	2	+	+	+
PC2	2	+	+	+
PC3	2	+		
PC4	3			
PC5	3			
PC6	4	+		
PC7	4			
PC8	5			
PC9	5			
PC10	6			
PC11	6			
PC12	6			
PC13	7			
PC14	7			
PC15	8			
PC16	8			
PC17	9			
PC18	9			
		Control 1:100 diluted SE		

Table 39. QQQ-MRM Assay Results for Seminal Fluid Protein Targets PSA/p30 and Semenogelin I and II.

		QQQ-MRM Results		
Sample ID	Days Post-Coital	PSA LSEP...	SEMG1 QITI...	SEMG2 GSIS...
PC1	2			
PC 2	2			
PC 3	2	(-)		
PC 4	3	(-)		
PC 5	3	(-)		
PC 6	4	(-)		
PC 7	4	(-)	(-)	(-)
PC 8	5	(-)	(-)	(-)
PC 9	5	(-)	(-)	(-)
PC 10	6	(-)		
PC 11	6	(-)	(-)	(-)
PC 12	6	(-)		
PC 13	7	(-)	(-)	(-)
PC 14	7	(-)	(-)	(-)
PC 15	8	(-)	(-)	
PC 16	8	(-)	(-)	(-)
PC 17	9	(-)	(-)	(-)
PC 18	9	(-)	(-)	(-)

4.3 Discussion

For these experiments, a monoplex (single body fluid) QQQ-MRM assay was developed to enhance the sensitivity of seminal fluid detection beyond that of both the multiplex method and contemporary immunochromatographic assays. By eliminating from the multiplex, proteins not specific to seminal fluid and adding additional seminal fluid proteins that had not previously been used, the resulting monoplex assay maximized instrument dwell time efficiency and thus detection sensitivity. The sensitivity limit for this new seminal fluid specific assay was such that a 1 to 131,072 dilution of seminal fluid could be confidently detected. Application of the optimized assay to two-, three- and four-component mixtures of semen, vaginal and menstrual fluids, saliva, urine, and peripheral blood showed it to be highly sensitive and specific for human seminal fluid. Analysis of authentic post-coital cervico-vaginal swabs demonstrated that the enhanced sensitivity of the QQQ-MRM assay far exceeded that of commercial antibody-based methods as illustrated by the detection of semen in authentic vaginal swabs collected up to 8 days post coitus. With a level of sensitivity that is equal to or greater than that of Y-STR DNA analysis, comes the need to better understand how quantitative levels of semen peptides might correlate with recoverable male DNA. A "peptide cutoff/threshold level" for example may aid forensic analysts in assessing the likelihood of obtaining an interpretable male DNA profile from the remainder of the sample extract. Similarly, such quantitative thresholds could be used for paired analyses of seminal fluid-free vaginal swabs (*i.e.*,

QQQ-MRM vs. immunochromatography) to better estimate the actual rate of false positives in widely used serological tests.

CHAPTER 5: QUANTITATIVE SEMINAL FLUID DETECTION BY MASS SPECTROMETRY AS AN INDICATOR OF MALE DNA PROFILING SUCCESS AT EXTENDED POST-COITAL INTERVALS

5. Introduction

Despite their prevalence, sexual assault kit (SAK) samples can often be among the more challenging samples handled by forensic laboratories. Using standard autosomal short tandem repeat (STR) DNA profiling, an abundance of female DNA on intimate swabs can mask the presence of trace quantities of male DNA. While this can be overcome by using male-targeted Y- STR profiling/haplotyping, the statistical weight of a Y-STR match is typically a tiny fraction of that calculated for an autosomal match. PCR inhibitors from bacteria, blood, fecal matter and/or other chemical compounds may be present which impede DNA amplification. Finally, SAK samples encompass wide variation with regard to the age and quality of the biological material. These factors alone – or in combination – can make it difficult to generate an informative male DNA profile or haplotype using either autosomal or Y-STR chemistries. In fact, many SAK samples fail to produce any detectable male DNA at all [115]. As a result, forensic practitioners have long relied on traditional serological screening as a means of identifying those samples that are the best candidates for successful DNA profiling.

Traditional workflows for the processing of SAK samples have relied upon enzyme activity and antibody-binding based serological tests for the detection of seminal fluid and/or saliva as well as microscopy for the detection of spermatozoa. The laborious nature of performing multiple serological assays to screen evidence and the uncertainty associated with what are typically presumptive results, however, have contributed to increased popularity of Y-screen assays as an alternative workflow for SAK samples.

Currently, many forensic laboratories use one of two Y-screen workflows. In the first approach, all samplesundergo differential extraction to enrich for sperm cells followed by male DNA quantitation to select samples for advancement to genetic profiling. In the second approach, rapid lysis of a test cutting is followed by male DNA quantitation to prioritize samples for differential extraction and genetic profiling. While both approaches achieve rapid screening for the presence of a detectible male contributor, they require that either laborious differential extraction be used for all samples or multiple cuttings be extracted/quantified for each item. More critically, though, neither method provides investigators with any serological information. The resulting lack of critical investigative/biological context, opens the door to alternative explanations for the presence of the male DNA (*e.g.*, secondary/indirect transfer of trace DNA from skin cells or cell-free DNA sources [116-118]). In these increasingly common types of cases involving trace DNA profiles, the ability to detect semen provides both investigators and the trier of fact with critical context for evaluating what are often the contradictory claims of the victim and the defendant.

Traditional serological assays, however, lack comparable sensitivity to that of Y-screen workflows meaning that many SAK samples that yield interpretable DNA profiles would not likely yield useful serological information even if they were tested. In addition to the sensitivity limitations associated with degradation, dilution and visual interpretation of immunochromatographic assays for PSA or Sg I/II, false positive indications of seminal fluid may also arise due to the presence of the target antigen in biological fluids other than semen, cross-reactivity or other non-specific antibody binding events. This underscores the presumptive nature of these assays. The PSA glycoprotein, for example, is a serine protease [119] secreted by the prostate that cleaves semenogelin [120]. This is responsible for the liquefaction of seminal fluid. PSA is present in seminal fluid at concentrations that range from 0.2 to 5.5 mg/mL [85, 121]. While PSA concentrations are highest in seminal fluid, however, it is also present in vaginal fluid (originating from the periurethral gland that is homologous to the prostate [14]), albeit at what are typically lower levels [16, 121-125]. Saliva, serum, breast milk and amniotic fluid also contain low levels of PSA (**Table 40**). Based on these reported concentrations, however, only breast milk and amniotic fluid may contain sufficient PSA concentrations to produce a positive test result using lateral flow assays designed for seminal fluid detection. Similarly, the Sg I/II proteins originate mostly from the seminal vesicle and are the main component of semen coagulum [126]. While Sg I/II concentrations are highest in seminal fluid (10 to 20 mg/mL) [126], it too is not semen specific. Transcripts for Sg I have been found in the gastrointestinal tract including tissues of the throat and skeletal muscle while transcripts for Sg II have been found in kidney tissue. Based on the reported concentrations of PSA and Sg in other fluids,

however, it is unclear as to whether false positive results on lateral flow assays with non-target body fluids are been due to trace but detectible levels of these proteins or due to non-specific antibody binding events akin to those demonstrated in Chapter 2 of this dissertation. Regardless, the need for an enhanced approach to screening SAK samples which simultaneously provides both a reliable means of selecting/prioritizing samples for DNA profiling as well as reliable serological information has been demonstrated.

Table 40. PSA Concentrations in Biological Matrices other than Seminal Fluid.

Biological Material	Reported PSA Concentration (ng/mL)
Vaginal fluid	0.01-1.25
Saliva	**0.04-0.34**
Female Serum	0.02-0.16
Male Serum	**<4.0**
Male Serum from patient with Prostate Cancer	Up to 200+
Breast Milk	**0.5-100**
Amniotic Fluid	Maximum 8.98

It has already been demonstrated that a QQQ-MRM assay for the detection of seminal fluid provides enhanced detection sensitivity and accuracy relative to immunochromatography. This approach (which need not consume cellular DNA) allows the detection of seminal fluid in authentic vaginal swabs past the reported post-coital interval for Y-STR DNA typing. Thus, the overarching goal of this research was to glean

additional practitioner-relevant information through a quantitative analysis of sexual assault samples collected across a wide post-coital interval.

To achieve this, an already robust qualitative QQQ-MRM assay for seminal fluid was converted into a quantitative assay. Absolute quantitation was achieved through the use of intact protein and isotopically labeled synthetic peptide internal standards [127] for multiple peptides from the same protein [128]. Then, by comparing quantitative protein data with genetic data from Y-STR testing of the same samples, it was possible to assess the degree of correlation between the detection of a given quantity of targeted seminal fluid proteins and the success rates for obtaining a male Y-STR profile. Additional studies focused on assessing the rate and potential impact of true false positive immunochromatographic results with casework-type samples. A true false positive result is defined as a false positive due to non-specific antibody interactions rather than a positive result arising as a result of target protein expression in a non-target tissue (i.e., the detection of a seminal fluid biomarker protein expressed in a body fluid other than seminal fluid). These goals were achieved through the successful completion of the following three core research objectives:

(1) A quantitative QQQ-MRM assay was developed and optimized using synthetic PSA and Sg I/II proteins to establish a standard curve which was then used to quantitate these proteins in forensic-type samples.

(2) The correlation between peptide quantitative values for target seminal fluid peptides and the ability to generate Y-STR profiles from vaginal swabs collected at various post-coital intervals was assessed.

(3) The rate of false positive results associated with immunochromatographic tests of semen-fee vaginal swabs was determined to assess whether target proteins in the sample were actually present above the assay's sensitivity threshold.

5.1 Methods

5.1.1 Development of an Absolute Quantitative QQQ-MRM Assay for Seminal Fluid

Known concentrations of PSA/p30 and Sg were added to vaginal secretions and digested with trypsin. Synthetic isotope-labeled peptides retain the chemical and chromatographic properties of natural peptides but have a mass shift due to the introduction of a stable "heavy" isotope. These "heavy" peptides were added to samples at a fixed concentration and the ratio of the target peptide recovered from a standard to the synthetic labeled peptides was plotted against the known concentration to generate a linear standard curve. The response of a natural peptide in a test sample was normalized to the "heavy" standard in order to calculate its concentration from the standard curve [129, 130].

Non-matrix curves for each peptide were used to select protein standard and labeled peptide concentrations for the in-matrix curve. A fit-for-purpose analytical method assessment was then performed in order to assess the performance of the assay. This included evaluating the linearity/calibration model, limit of detection (LOD), and limit of

quantitation (LOQ). A straight fit line using weighted linear regression with inverse concentration-squared weights was used to evaluate the working range. Acceptable criteria for these parameters included correlation coefficient >0.98. LOD was assessed using three blank pooled vaginal matrix samples analyzed over three runs concurrently with fortified serial dilutions of the lowest standard. The LOD was defined as the lowest concentration yielding an average signal:noise ratio greater than 3. The precision and accuracy of the LOQ was evaluated across a three-day reproducibility study from three separate sources of blank vaginal swabs. An acceptable LOQ level was defined as a %CV within 20% of the calculated mean and within 20% of the target (0.5 fmol/µl).

5.1.2 Assessment of the Relationship between Quantitative Levels of Target Seminal Fluid Peptides and the Generation of Y-STR Profiles from Post-Coital Vaginal Swabs

Self-collected vaginal swabs were collected at various post-coital intervals (2 swabs per sample) from study participants who completed a survey indicating the time since their last known act of barrier-free sexual intercourse. Fifty (50) self-collected post coital vaginal samples were tested.

Each self-collected post coital vaginal swab was solubilized in 1mL of deionized water for 30 minutes at room temperature with periodic vortexing. Swabs were then placed into spin basket inserts and centrifuged at 1200 RPM for 10 minutes to pellet cellular material. After centrifugation, the swab cutting and spin baskets were removed. The supernatant was transferred to a clean 1.5mL microcentrifuge tube and cuttings were placed back into pelleted material and retained. For the QQQ-MRM analyses, 100 µl of extract

(*i.e.*, the supernatant) was used. To ensure quantitative concordance, swabs were analyzed in duplicate by quantitative QQQ-MRM and the values averaged to determine the concentrations of target seminal fluid proteins.

The pelleted material and cuttings underwent DNA extraction utilizing an AutoMate Express Robotic Extraction platform (Thermo Fisher Scientific) and the PrepFiler Express chemistry (Thermo Fisher Scientific). All samples were eluted in a final volume of 100 µl. All DNA extracts were quantified by Quantifiler® Trio and typed using Yfiler® Plus chemistries (Thermo Fisher Scientific) on a 3500 Genetic Analyzer. The resulting data were analyzed with GeneMapper IDX Software. The analytical thresholds (AT) applied for profile interpretation were based on previous validation studies that independently evaluated the S/N characteristics for each dye channel. For a 15 second injection the AT values used were blue: 40 RFU; green: 55 RFU; yellow: 50 RFU; purple: 50 RFU; and red: 50 RFU. Seminal fluid protein content was compared to the percent of Y-STR loci in order to determine the seminal fluid peptide concentration at which Y-STR typing consistently failed to yield interpretable results.

5.1.3 Estimation of the Rate of Authentic False Positive Results Associated with Immunochromatographic Assays for Seminal Fluid

Self-collected vaginal swabs (2 swabs/sample) were collected from ≥50 participants who were not engaging in barrier-free vaginal intercourse and who indicated that it has been at least 1 month since the last known act of condomless sexual intercourse.

A matrix blank (pooled vaginal fluid) and positive semen control was analyzed with each batch of samples.

Each full swab was solubilized in 1mL Universal Buffer (Independent Forensics) for 30 minutes at room temperature with periodic vortexing. Swabs were then placed into spin basket inserts and centrifuged at 1200 RPM for 10 minutes to pellet the cellular material. Following centrifugation, the cutting and baskets were removed and discarded. The supernatant was transferred to a clean 1.5mL microcentrifuge tube and pelleted material was retained. The samples were analyzed per the manufacturer's instructions for the RSID Semen (Independent Forensics), PSA SemiQuant® (Seratec), and ABAcard p30 (Abacus Diagnostics) immunochromatographic assays; 100 µl extract was placed in the sample window of the cassette for RSID Semen and 200 µl extract was placed in the sample window of the cassette for ABACard p30 and PSA SemiQuant®. The remaining supernatant was prepared for analysis by the QQQ-MRM method.

As the purpose of this objective was to evaluate the rate of true false positive reactions obtained with the immunochromatographic assays being analyzed, normal testing procedures that closely followed those recommended by manufacturers were desired. It is for that reason that swabs were solubilized in Universal Buffer instead of water as per internal standard operating procedures for QQQ-MRM sample preparation. This required the addition of an initial solid phase extraction for the 100 µl sample extract prior to digestion and introduction to the LC system in order to prevent the introduction of detergents to the LC column.

Any samples producing positive results on any of the immunochromatographic assay were evaluated to determine whether the target protein was actually present at levels above the reported sensitivity limits of the lateral flow tests. If the mass spectrometry results indicate a target protein concentration below the sensitivity limits, the result will be considered a false positive event (pelleted material from swabs was also saved to confirm the absence of sperm cells using Sperm HyLiter). For any samples with positive QQQ-MRM results (*i.e.*, a target peptide concentration above the LOQ) were analyzed in duplicate and the values averaged to determine the concentrations of target seminal fluid proteins and to ensure quantitative concordance between measurements.

5.2 Results

5.2.1 Development of an Absolute Quantitative QQQ-MRM Assay for Seminal Fluid

The ratio of the synthetic isotope-labeled "heavy" *vs.* the "natural" peptide was plotted against known peptide concentrations to generate a linear standard curve for absolute quantitation. Similarly, with any case-type sample tested in a forensic context, the response of the tryptic "natural" peptide *vs.* the "heavy" standard can be used to calculate its concentration from the standard curve.

Initially, neat or non-matrix curves for each peptide were generated in order to establish an analytic measurement range (AMR) for the analytical assay. Calibrators were generated in 2% acetonitrile with 0.1% formic acid at the following levels: 0.5 fmol/µL, 1 fmol/µL, 5 fmol/µL, 10 fmol/µL, 25 fmol/µL, 50 fmol/µL, and 100 fmol/µL with the

isotopically labelled internal standards added at 25 fmol/µL. Linear calibration curves with a weighting factor of 1/2 were generated for each peptide, producing a correlation coefficients of 0.99 or greater.

In-matrix curves (*i.e.*, in vaginal fluid) were then assessed to ensure the reliability of the analytical method. This performance check assessed the calibration model, LOD, and LOQ over the course of three days to verify reproducibility and performance.

The calibration model was assessed from three separate calibration curves generated over three separate days. The intercept and linearity/R^2 were assessed for each peptide within the scope of the analytical method. All compounds performed adequately with R^2 values of >0.99 across all test batches (**Table 41 and Figure 29**).

Table 41. Assessment of the Analytical Calibration Model.

Calibration Model Assessment		
Compound	R2	Intercept
Semenogelin QITIPSQEQEHSQK	0.99	0.00
Semenogelin GSISIQTEEQIHGK	0.99	0.01
PSA LSEPAELTDAVK	0.99	0.00

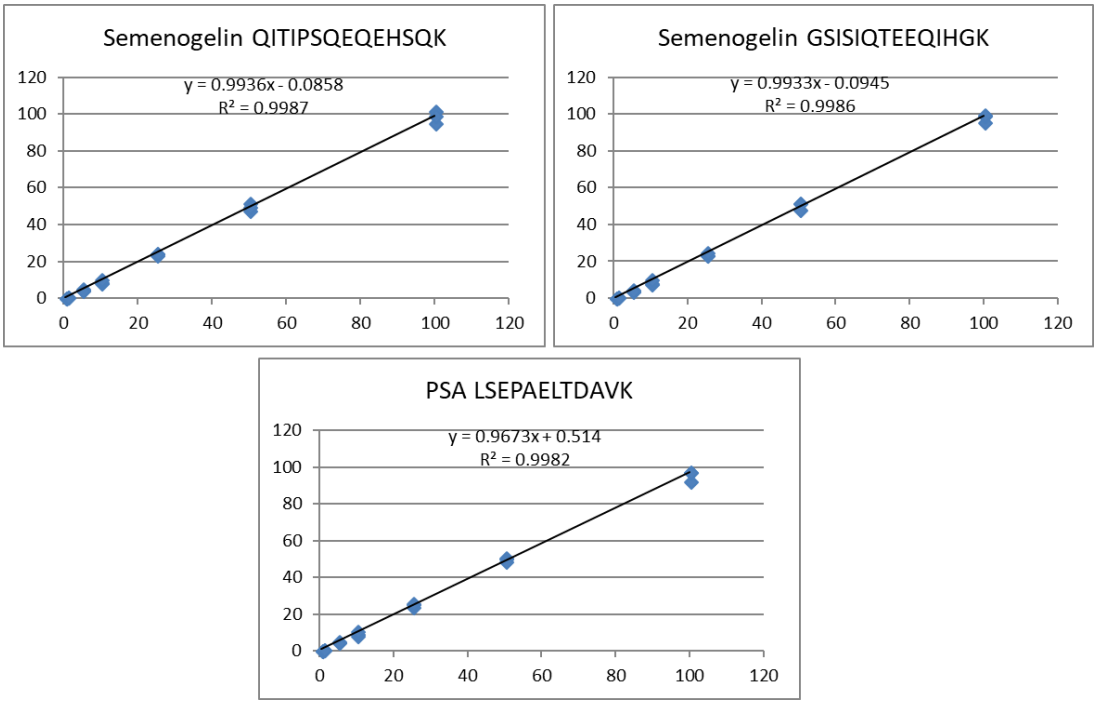


Figure 29. Linear calibration models for all peptides within the scope of the analytical method.

The LOQ was assessed by analyzing three separate sources of blank matrices in triplicate over three days. Each of these values was quantified against a calibration curve prepared on that same day in order to determine the method bias and precision. Method bias, which was measured as the % difference from the target concentration (0.5 fmol/ µL), was below 15% for all target analytes. Similarly, precision variation was below 15% showing acceptable LOQ reproducibility (**Table 42**). Representative chromatograms for each target are shown in **Figure 30**. The final quantitative QQQ-MRM seminal fluid assay method parameters are detailed in **Appendix III**.

Table 42: Limit of quantitation assessment

Limit of Quantitation Assessment

Compound	Bias (% Difference)	Precision (%CV)
Semenogelin QITIPSQEQEHSQK	12.9	7.7
Semenogelin GSISIQTEEQIHGK	3.0	4.7
PSA LSEPAELTDAVK	1.3	14.7

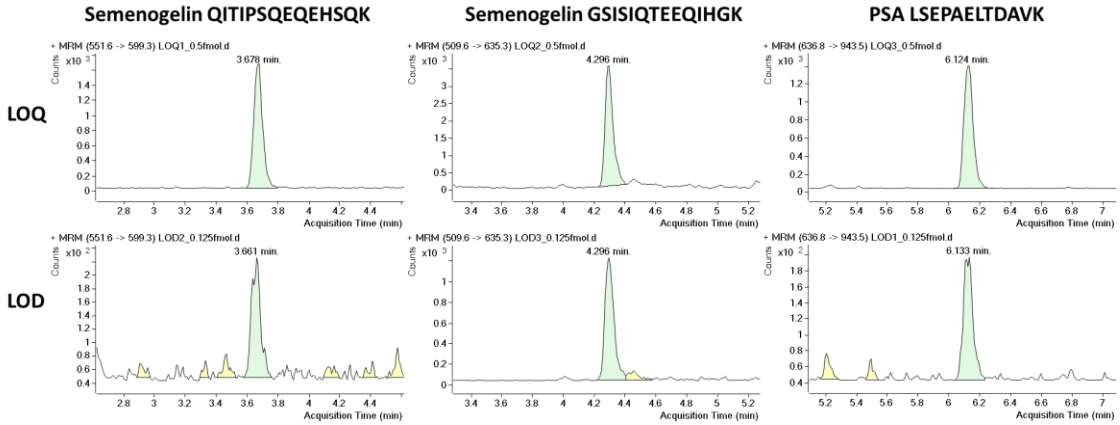


Figure 30. Chromatogram for each target analyte at the limit of detection and quantitation.

5.2.2 Assessment of the Relationship between Quantitative Levels of Target Seminal Fluid Peptides and the Generation of Y-STR Profiles from Post-Coital Vaginal Swabs

Seminal fluid protein concentrations were compared to the percent of Y-STR loci detected (out of a total of 27 loci) to determine whether there was a consistent relationship between seminal fluid peptide concentrations and Y-STR haplotyping success (**Table 43**).

Out of the 50 samples analyzed, there was full concordance in all but 2 instances between the QQQ-MRM target peptide concentrations and the ability to produce interpretable Y-STR profiles when targeting the semenogelin I peptide target (QITI peptide). The PSA/p30 peptide target (LSEP peptide) did not perform as well as the semenogelin peptide targets. In these two instances (samples number 22 and 25) partial DNA profiles were produced with 67% and 52% of donor alleles detected above the applied analytical thresholds respectively. In these two samples however, no target seminal fluid peptides were detected. In all other instances, when no Y-STR donor alleles were detected, no target peptides were detected either. Conversely, when full and partial Y-STR profiles were produced the QITI peptide was detected above the LOQ and could be reliably quantified. In three instances, Y-STR profiles were obtained within a defined "uninformative range" (between 2 to 5 alleles produced). In these three instances, mixed results were obtained for both semenogelin targets. In two instances both the QITI and GSIS peptides were detected above the LOQ and in 1 instance they were not detected.

Table 43. Relationship Between Target Protein Quantity and Y-STR Profiling Success in Post-Coital Vaginal Swabs.

Sample ID	Days Post Coitus	Y Filer Plus (15s)	% Y Profile	QITI Protein Concentration (fmol/ul)	GSIS Protein Concentration (fmol/ul)	LSEP Protein Concentration (fmol/ul)
1	1	Full (26 alleles)	100	11.257	1.67	(-)
2	1	Full (26 alleles)	100	3.582	0.6244	(-)
3	1	Full (27 alleles)	100	25.8335	9.7626	(-)
4	1	Full (27 alleles)	100	33.0449	10.617	(-)
5	1	Full (27 alleles)	100	424.9819	35.5791	0.9348
6	1	Full (27 alleles)	100	1416.9186	199.8537	2.2256
7	1	Full (27 alleles)	100	1.4199	0.9801	0.6485
8	1	Full (27 alleles)	100	2.4021	1.8503	1.4456
9	1	Full (27 alleles)	100	11.7173	7.525	5.0878
10	1	Full (26 alleles)	100	1.0639	0.8321	1.6974
11	1	Full (26 alleles)	100	0.8404	0.7073	1.4372
12	2	Full (27 alleles)	100	1.0763	0.8259	0.5832
13	2	Full (27 alleles)	100	132.1065	36.4736	(-)
14	Unknown	Full (26 alleles)	100	1.587	0.8126	(-)
15	2	Full (27 alleles)	100	88.8538	19.1607	(-)
16	3	Full (27 alleles)	100	8.5282	4.5612	(-)
17	3	Full (27 alleles)	100	5.2404	2.8667	(-)
18	3	Full (26 alleles)	100	1.9207	(-)	(-)
19	3	Full (26 alleles)	100	1.399	(-)	(-)
20	2	Partial (22 alleles)	81	0.9687	0.8106	0.6412
21	3	Partial (19 alleles)	70	0.854	1.5641	(-)
22	2	Partial (18 alleles)	67	(-)	(-)	(-)
23	3	Partial (17 alleles)	63	1.0265	0.9836	0.7347
24	3	Partial (17 alleles)	63	0.9473	0.7499	0.5549
25	2	Partial (14 alleles)	52	(-)	(-)	(-)
26	3	Partial (6 alleles)	22	1.098	2.8809	(-)
27	4	Partial (5 alleles)	18	1.5929	1.4179	(-)
28	5	Partial (4 alleles)	15	(-)	(-)	(-)
29	2	Partial (2 alleles)	8	0.5471	0.5202	(-)
30	4	1 Allele Detected	4	(-)	(-)	(-)
31	4	1 Allele Detected	4	(-)	(-)	(-)
32	4	1 Allele Detected	4	(-)	(-)	(-)
33	7	1 Allele Detected	4	(-)	(-)	(-)
34	7	1 Allele Detected	4	(-)	(-)	(-)
35	3	No Alleles Detected	0	(-)	(-)	(-)
36	3	No Alleles Detected	0	(-)	(-)	(-)
37	3	No Alleles Detected	0	(-)	(-)	(-)
38	4	No Alleles Detected	0	(-)	(-)	(-)
39	4	No Alleles Detected	0	(-)	(-)	(-)
40	4	No Alleles Detected	0	(-)	(-)	(-)
41	4	No Alleles Detected	0	(-)	(-)	(-)
42	4	No Alleles Detected	0	(-)	(-)	(-)
43	4	No Alleles Detected	0	(-)	(-)	(-)
44	5	No Alleles Detected	0	(-)	(-)	(-)
45	5	No Alleles Detected	0	(-)	(-)	(-)
46	5	No Alleles Detected	0	(-)	(-)	(-)
47	6	No Alleles Detected	0	(-)	(-)	(-)
48	6	No Alleles Detected	0	(-)	(-)	(-)
49	8	No Alleles Detected	0	(-)	(-)	(-)
50	8	No Alleles Detected	0	(-)	(-)	(-)

Note: Days post coitus was self-reported in the sample questionnaire that accompanied collection packets. Percent Y-STR Profile was calculated by dividing the number of

observed donor alleles detected by the number of donor alleles expected. Green boxes indicate a positive result (for YSTR results, this is represented by the detection of 6 or more donor alleles; for the QQQ-MRM results, this is represented by the quantitative value of a target peptide). Red boxes indicate the absence of a peptide at a detectible level on the QQQ-MRM method and 1 or fewer donor alleles detected. Yellow boxes denote partial Y-STR profiles falling within the uninformative range of 2 to 5 donor alleles detected.

5.2.3 Estimation of the Rate of Authentic False Positive Results Associated with Immunochromatographic Assays for Seminal Fluid

Originally, this research proposed to analyze 50 negative vaginal swabs, however, in order to confirm these findings, an additional batch of 50 negative vaginal swabs (for a total of 100 samples) was tested. Out of the 100 samples analyzed, 17 produced false positive results for ABAcard p30 and PSA Semiquant while 6 produced false positive results for RSID Semen resulting in a 17% and 6% false positive rate respectively. Interestingly, in no instance, did a sample produce a false positive result on all three immunochromatographic assays. It was also not always the case that a sample which produced a false positive on one assay targeting PSA (ABAcard p30 or PSA Semiquant) would necessarily produce a false positive result on the other assay targeting PSA.

Subsequent analyses of these presumed false positive samples by mass spectrometry did not detect the presence of the protein targets for any of the immunochromatographic assays that were evaluated. This renders more probable the inference that prostate specific antigen and semenogelin proteins were either not present in these samples or were present at such low levels that one would not expect to detect them by immunochromatography (**Tables 44-45; Figure 31**).

Additionally, all cellular components of the 26 samples that produced positive immunochromatographic results were confirmed to be sperm free utilizing Sperm HyLiter and fluorescent microscopy. This, coupled with the quantitative QQQ-MRM results indicates that the positive immunochromatographic results were likely to be true false positive non-specific binding events rather than an unexpected positive result due to the presence of the target proteins at low levels in these particular samples. These data underscore the presumptive nature of immunochromatographic assay results and should alert forensic practitioners to the fact that the rate of true false positive results is not insubstantial.

Table 44. Batch 1 of "seminal fluid free" vaginal swabs.

	Immunochromatographic Assay Results			QQQ-MRM Results			Sperm
Sample #	RSID™ Semen	ABAcard® p30	PSA Semiquant	PSA	SEMG1	SEMG2	HyLiter® Results
1	-	-	-	-	-	-	NT
2	-	-	-	-	-	-	NT
3	-	-	-	-	-	-	NT
4	-	-	-	-	-	-	NT
5	-	-	-	-	-	-	NT
6	-	-	-	-	-	-	NT
7	-	-	-	-	-	-	NT
8	-	-	-	-	-	-	NT
9	-	+	+	-	-	-	-
10	-	+	+	-	-	-	-
11	-	+	+	-	-	-	-
12	-	+	+	-	-	-	-
13	-	+	+	-	-	-	-
14	-	-	-	-	-	-	NT
15	-	-	-	-	-	-	NT
16	-	-	-	-	-	-	NT
17	-	-	-	-	-	-	NT
18	-	-	-	-	-	-	NT
19	-	-	-	-	-	-	NT
20	-	-	-	-	-	-	NT
21	-	-	-	-	-	-	NT
22	-	-	+	-	-	-	-
23	-	-	-	-	-	-	NT
24	-	-	-	-	-	-	NT
25	-	-	-	-	-	-	NT

	Immunochromatographic Assay Results			QQQ-MRM Results			Sperm
Sample #	RSID™ Semen	ABAcard® p30	PSA Semiquant	PSA	SEMG1	SEMG2	HyLiter® Results
26	+	-	+	-	-	-	-
27	+	-	+	-	-	-	-
28	-	-	-	-	-	-	NT
29	-	-	-	-	-	-	NT
30	-		-	-	-	-	NT
31	+	-	-	-	-	-	-
32	-	+	+	-	-	-	-
33	-	-	+	-	-	-	-
34	-	-	-	-	-	-	NT
35	-	-	-	-	-	-	NT
36	-	-	-	-	-	-	NT
37	-	-	+	-	-	-	-
38	-	-	-	-	-	-	NT
39	-	-	-	-	-	-	NT
40	-	-	+	-	-	-	-
41	-	-	-	-	-	-	NT
42	-	-	+	-	-	-	-
43	-	-	-	-	-	-	NT
44	-	-	-	-	-	-	NT
45	-	-	+	-	-	-	-
46	-	-	-	-	-	-	NT
47	-	-	-	-	-	-	NT
48	-	-	-	-	-	-	NT
49	-	-	-	-	-	-	NT
50	-	-	-	-	-	-	NT
Matrix Blank	-	-	-	-	-	-	NT
Semen Control	+	+	+	+	+	+	+

Note: Green boxes indicate a positive test result (for the immunochromatographic assays means a line both at the control and the test zones; for the QQQ-MRM assay, this means a target peptide quantity above the LOQ). Red boxes indicate the absence of a peptide at a detectible level on the QQQ-MRM assay and a negative test result on the immunochromatographic assays. Gray boxes indicate the sample was not tested.

Table 45. Batch 2 of "seminal fluid free" vaginal swabs.

	Immunochromatographic Assay Results			QQQ-MRM Results			Sperm
Sample #	RSID™ Semen	ABAcard® p30	PSA Semiquant	PSA	SEMG1	SEMG2	HyLiter® Results
1	-	-	-	-	-	-	NT
2	-	-	-	-	-	-	NT
3	-	-	-	-	-	-	NT
4	-	+	+	-	-	-	-
5	-	+	-	-	-	-	-
6	-	+	-	-	-	-	-
7	-	+	-	-	-	-	-
8	-	+	+	-	-	-	-
9	-	+	-	-	-	-	-
10	+	-	+	-	-	-	-
11	-	+	-	-	-	-	-
12	-	+	-	-	-	-	-
13	-	-	-	-	-	-	NT
14	+	+	-	-	-	-	-
15	-	+	-	-	-	-	-
16	-	-	-	-	-	-	NT
17	-	-	-	-	-	-	NT
18	-	-	-	-	-	-	NT
19	-	-	-	-	-	-	NT
20	-	-	-	-	-	-	NT
21	-	-	-	-	-	-	NT
22	-	-	-	-	-	-	NT
23	-	-	-	-	-	-	NT
24	-	-	-	-	-	-	NT
25	-	-	-	-	-	-	NT

	Immunochromatographic Assay Results			QQQ-MRM Results			Sperm
Sample #	RSID™ Semen	ABAcard® p30	PSA Semiquant	PSA	SEMG1	SEMG2	HyLiter® Results
26	-	-	-	-	-	-	NT
27	-	-	-	-	-	-	NT
28	-	-	-	-	-	-	NT
29	-	-	-	-	-	-	NT
30	-	-	-	-	-	-	NT
31	-	-	-	-	-	-	NT
32	-	-	-	-	-	-	NT
33	-	-	-	-	-	-	NT
34	-	-	-	-	-	-	NT
35	-	-	-	-	-	-	NT
36	-	-	-	-	-	-	NT
37	-	-	-	-	-	-	NT
38	-	-	-	-	-	-	NT
39	+	+	-	-	-	-	-
40	-	-	-	-	-	-	NT
41	-	-	-	-	-	-	NT
42	-	-	-	-	-	-	NT
43	-	-	-	-	-	-	NT
44	-	-	-	-	-	-	NT
45	-	-	-	-	-	-	NT
46	-	-	-	-	-	-	NT
47	-	-	-	-	-	-	NT
48	-	-	-	-	-	-	NT
49	-	-	-	-	-	-	NT
50	-	-	-	-	-	-	NT
Matrix Blank	-	-	-	-	-	-	NT
Semen Control	+	+	+	+	+	+	+

Note: Green boxes indicate a positive test result (for the immunochromatographic assays means a line both at the control and the test zones; for the QQQ-MRM assay, this means a target peptide quantity above the LOQ). Red boxes indicate the absence of a peptide at a detectible level on the QQQ-MRM assay and a negative test result on the immunochromatographic assays. Gray boxes indicate the sample was not tested

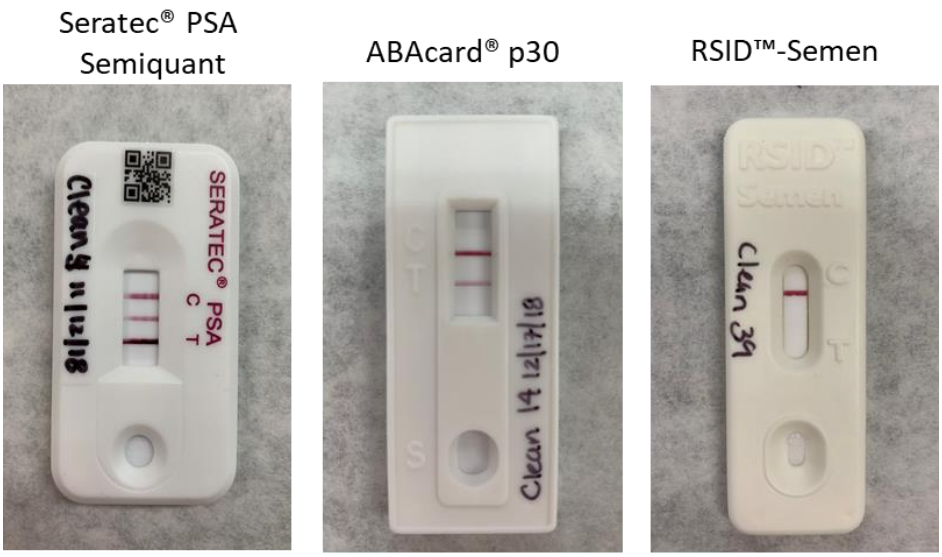


Figure 31. Sample images of false positive results obtained following testing of semen-free vaginal swabs using multiple commercial immunochromatographic assays designed to target PSA (Seratec Semiquant and ABAcard) or Sg (RSID Semen) in seminal fluid illustrating the range of strong to weak false positive results obtained.

5.3 Discussion

The data outlined in this chapter illustrate how the use of high-sensitivity targeted-ion mass spectrometry can be used to not only address the limitations of existing methods for semen detection but also to establish a framework for the use of quantitative information on seminal fluid proteins in forensic testing. This will aid the development of more informed confirmatory interpretation guidelines for protein-based seminal fluid identification; inform forensic analysts about the probability of successful downstream genetic analysis; and address the inherent limitations of the serological approaches currently employed in case-working laboratories to detect the potential presence of seminal fluid in sexual assault-type evidentiary samples. *In toto*, this assay will provide the forensic community with powerful information to aid in the investigation of sexual assault.

5.4. Future Directions and Impact on the Criminal Justice System

Past work comparing the proteomes of five body fluids commonly encountered in a case-working context resulted in the identification of multiple candidate high-specificity biomarkers for the confident identification of human body fluids. The current studies have further expanded this body of knowledge. A triple Quadrupole Multiple Reaction Monitoring (QQQ-MRM) assay for the simultaneous confirmatory detection of protein biomarkers in six human body fluids was produced and developmentally validated. This multiplex QQQ-MRM assay will provide analysts with high confidence in the body fluid identification results obtained for a given stain. This is made possible by the use of not just one protein biomarker but rather on the presence of multiple proteins which in turn are based on multiple precursor and product ion pairs. Studies on casework-type samples have demonstrated the reliable performance of the assay; even with aged/weathered or otherwise chemically compromised samples. It was further demonstrated that the validated assay has the ability to overcome the inherent limitations of the antibody-based tests currently employed by case-working laboratories for the detection of seminal fluid.

A seminal fluid specific monoplex assay was then developed specifically for the analysis of sexual assault samples with the goal of further enhancing the overall sensitivity for detecting trace levels of semen-specific target protein biomarkers. The use of a monoplex QQQ-MRM assay that has been optimized for sensitivity and which can detect partially degraded seminal fluid markers will enhance the ability of forensic analysts to unambiguously detect semen in two significant ways. First, this approach will enable

analysts to report the confirmatory identification of semen in post-coital samples collected as much as 8 days after intercourse. Second, this approach may allow for the confirmation of seminal fluid in samples where there may be insufficient DNA to obtain an interpretable profile (*e.g.*, in cases of vasectomy, lack of ejaculation or minimal sexual contact). In short, this will make it possible to obtain probative results from samples that might otherwise have yielded inconclusive or no results at all. This will provide the forensic and criminal justice communities in the United States and internationally with a powerful tool to aid the investigation and prosecution of sexual assault.

The functionality of the monoplex method was then enhanced, to enable the absolute quantitation of targeted high-specificity seminal fluid protein biomarkers in the panel. A major positive impact of now having a quantitative monoplex QQQ-MRM assay optimized for sensitivity is that it has enhanced the ability of forensic analysts to confidently detect seminal fluid well beyond the typical 1- to 2-day post-coital interval. By obtaining precise measurements of targeted protein levels and correlating these with the likelihood of successful DNA typing, practitioners will be able to leverage quantitative data on seminal fluid proteins in their decision making on downstream analyses for sexual assault swabs. This will enable practitioners to better identify for forensic investigators those items of evidence that are most likely to produce potentially probative results. It will also facilitate the more efficient allocation of resources by allowing analysts to focus their downstream genetic analyses efforts on those samples where protein quantitation results are predictive of successful male DNA typing. This will also have the effect of reducing the frequency with which analyst are asked to explain in a court of law the apparent

discrepancy between having an intimate sample that yields an interpretable male profile but for which serological testing was either not performed or failed to indicate the presence of seminal fluid.

Future work should evaluate a fully-automated immunoaffinity Multiple Reaction Monitoring mass spectrometry (iMRM) method for the analysis of SAK samples. The iMRM workflow uses custom antibodies to specifically enrich for targeted tryptic peptides. This would produce a highly purified final extract for analysis by LC-MSMS to reliably screen for both seminal fluid and saliva in SAK evidence. In a proposal submitted to the US Armed Forces, the use of this iMRM proteomics strategy has been proposed to set statistically supported criteria for prioritizing SAK samples for genetic analysis based on preliminary data detailed in Chapter 5. Both peptide and male-DNA quantitation thresholds would be established for predicting DNA typing success. Using a separate dataset, type I and II error rates would be compared for the overall iMRM workflow to existing Y-screen strategies for SAK sample assessment using post-coital samples. Front-end sample solubilization and fractionation procedures can also be optimized. The soak and spin methods employed in this research can be compared to new commercial products (Qiagen AllPrep) designed to fractionate DNA, RNA and protein to determine which methods produces the greatest chance of recovery for both protein and genetic material. Finally, a blind side-by-side assessment of novel serological workflows and strategies including proteomics, epigenetics and RNA-based techniques would provide the forensic community with a more informative look at the progress being made in each of these areas of research as compared to currently employed testing methodologies.

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APPENDIX I: MULTIPLEX QQQ-MRM METHOD PARAMETERS

Acquisition Method Report

Agilent Technologies

Acquisition Method Info

Method Name	35min_dMRM_All_final_rebuilt.m
Method Path	D:\MassHunter\methods\Protoemics_Project\Developmental_Validation\35min_dMRM_All_final_rebuilt.m
Method Description	10 minute blank method. hold 2 min 80% ACN

Device List

ALS
Bin Pump
TCC
MS QQQ

MS QQQ Mass Spectrometer

Ion Source	ChipCube	**Tune File**	stunes.TUNE.XML
Stop Mode	No Limit/As Pump	**Stop Time (min)**	1
Time Filter	On	**Time Filter Width (min)**	0.07

Time Segments

Index	Start Time (min)	Scan Type	Ion Mode	Div Valve	Delta EMV	Store	Cycle Time (ms)	Triggered?	MRM Repeats
1	0	DynamicMRM	Nano ESI	To MS	300	Yes	1500	No	0

Time Segment 1

Scan Segments

Cpd Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Frag (V)	CE (V)	Cell Acc (V)	Ret Time (min)	Ret Window	Polarity
AGLC [+57.0] QTFVYGG C[+57.0] R.heavy	Yes	749.843888	Unit/Enh (6490)	969.448632	Unit/Enh (6490)	130	24.1	5	16.5	10	Positive
AGLC [+57.0] QTFVYGG C[+57.0] R.heavy	Yes	749.843888	Unit/Enh (6490)	868.400953	Unit/Enh (6490)	130	24.1	5	16.5	10	Positive
AGLC [+57.0] QTFVYGG C[+57.0] R.light	No	744.839753	Unit/Enh (6490)	959.440363	Unit/Enh (6490)	130	24.1	5	16.5	10	Positive
AGLC [+57.0] QTFVYGG C[+57.0] R.light	No	744.839753	Unit/Enh (6490)	858.392684	Unit/Enh (6490)	130	24.1	5	16.5	10	Positive
ALDGING GITHAGR. heavy	Yes	464.580358	Unit/Enh (6490)	604.30631	Unit/Enh (6490)	130	8.8	5	11	10	Positive
ALDGING GITHAGR. heavy	Yes	464.580358	Unit/Enh (6490)	546.792838	Unit/Enh (6490)	130	8.8	5	11	10	Positive
ALDGING GITHAGR.l ight	No	461.244268	Unit/Enh (6490)	599.302175	Unit/Enh (6490)	130	8.8	5	11	10	Positive
ALDGING GITHAGR.l ight	No	461.244268	Unit/Enh (6490)	541.788704	Unit/Enh (6490)	130	8.8	5	11	10	Positive
DGVTGPG FTLSGGC [+57.0]C [+57.0] QGGR.hea vy	Yes	976.926866	Unit/Enh (6490)	1008.38610 8	Unit/Enh (6490)	130	31.1	5	15.7	10	Positive
DGVTGPG FTLSGGC [+57.0]C [+57.0] QGGR.hea vy	Yes	976.926866	Unit/Enh (6490)	790.844616	Unit/Enh (6490)	130	31.1	5	15.7	10	Positive
DGVTGPG FTLSGGC [+57.0]C [+57.0] QGGR.hea vy	Yes	976.926866	Unit/Enh (6490)	762.333884	Unit/Enh (6490)	130	31.1	5	15.7	10	Positive

Acquisition Method Report

Agilent Technologies

Cpd Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Frag (V)	CE (V)	Cell Acc (V)	Ret Time (min)	Ret Window	Polarity
DGVTGPGFTLSGGC[+57.0]C[+57.0]QGGR.light	No	971.922731	Unit/Enh (6490)	998.377839	Unit/Enh (6490)	130	31.1	5	15.7	10	Positive
DGVTGPGFTLSGGC[+57.0]C[+57.0]QGGR.light	No	971.922731	Unit/Enh (6490)	785.840481	Unit/Enh (6490)	130	31.1	5	15.7	10	Positive
DGVTGPGFTLSGGC[+57.0]C[+57.0]QGGR.light	No	971.922731	Unit/Enh (6490)	757.32975	Unit/Enh (6490)	130	31.1	5	15.7	10	Positive
DIFTTQDELLVYNK.heavy	Yes	853.939984	Unit/Enh (6490)	1331.69327	Unit/Enh (6490)	130	24.3	5	24.3	10	Positive
DIFTTQDELLVYNK.heavy	Yes	853.939984	Unit/Enh (6490)	1001.53937	Unit/Enh (6490)	130	30.3	5	24.3	10	Positive
DIFTTQDELLVYNK.light	No	849.932885	Unit/Enh (6490)	1323.67907	Unit/Enh (6490)	130	24.3	5	24.3	10	Positive
DIFTTQDELLVYNK.light	No	849.932885	Unit/Enh (6490)	993.525138	Unit/Enh (6490)	130	30.3	5	24.3	10	Positive
DTGILDSLGR.heavy	Yes	528.781605	Unit/Enh (6490)	670.375784	Unit/Enh (6490)	130	17.2	5	18	10	Positive
DTGILDSLGR.heavy	Yes	528.781605	Unit/Enh (6490)	557.29172	Unit/Enh (6490)	130	17.2	5	18	10	Positive
DTGILDSLGR.light	No	523.77747	Unit/Enh (6490)	660.367515	Unit/Enh (6490)	130	17.2	5	18	10	Positive
DTGILDSLGR.light	No	523.77747	Unit/Enh (6490)	547.283451	Unit/Enh (6490)	130	17.2	5	18	10	Positive
DVSQSSISFQIEK.heavy	Yes	738.37702	Unit/Enh (6490)	1046.56081	Unit/Enh (6490)	130	23.8	5	17.78	10	Positive
DVSQSSISFQIEK.heavy	Yes	738.37702	Unit/Enh (6490)	759.41268	Unit/Enh (6490)	130	20.8	5	17.78	10	Positive
DVSQSSISFQIEK.light	No	734.36992	Unit/Enh (6490)	1038.54660 2	Unit/Enh (6490)	130	23.8	5	17.78	10	Positive
DVSQSSISFQIEK.light	No	734.36992	Unit/Enh (6490)	751.398481	Unit/Enh (6490)	130	20.8	5	17.78	10	Positive
ELSELSLLSLYGIHK.heavy	Yes	570.656972	Unit/Enh (6490)	734.418491	Unit/Enh (6490)	130	12.6	5	29	10	Positive
ELSELSLLSLYGIHK.heavy	Yes	570.656972	Unit/Enh (6490)	626.381181	Unit/Enh (6490)	130	12.6	5	29	10	Positive
ELSELSLLSLYGIHK.light	No	567.985572	Unit/Enh (6490)	730.411391	Unit/Enh (6490)	130	12.6	5	29	10	Positive
ELSELSLLSLYGIHK.light	No	567.985572	Unit/Enh (6490)	622.37408	Unit/Enh (6490)	130	12.6	5	29	10	Positive
FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF.heavy	Yes	1221.91783 6	Wide / Unit (6490)	1697.80232 6	Unit/Enh (6490)	130	35.9	5	30	10	Positive
FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF.heavy	Yes	1221.91783 6	Wide / Unit (6490)	1239.58981 4	Unit/Enh (6490)	130	35.9	5	30	10	Positive
FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF.heavy	Yes	1221.91783 6	Wide / Unit (6490)	1084.51631 5	Unit/Enh (6490)	130	35.9	5	30	10	Positive
FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF.light	No	1215.23301 7	Wide / Unit (6490)	1687.77509 8	Unit/Enh (6490)	130	35.9	5	30	10	Positive
FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF.light	No	1215.23301 7	Wide / Unit (6490)	1229.56256 6	Unit/Enh (6490)	130	35.9	5	30	10	Positive

Acquisition Method Report

Agilent Technologies

Cpd Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Frag (V)	CE (V)	Cell Acc (V)	Ret Time (min)	Ret Window	Polarity
FGYGYGP YQPVPEQ PLYPQPY QPQYQQ YTF.light	No	1215.233017	Wide / Unit (6490)	1074.489087	Unit/Enh (6490)	130	35.9	5	30	10	Positive
FQELE3E TLK.heavy	Yes	616.318442	Unit/Enh (6490)	956.502617	Unit/Enh (6490)	130	17	5	12.9	10	Positive
FQELE3E TLK.heavy	Yes	616.318442	Unit/Enh (6490)	827.460024	Unit/Enh (6490)	130	17	5	12.9	10	Positive
FQELE3E TLK.heavy	Yes	616.318442	Unit/Enh (6490)	714.375965	Unit/Enh (6490)	130	17	5	12.9	10	Positive
FQELE3E TLK.light	No	612.311343	Unit/Enh (6490)	948.488418	Unit/Enh (6490)	130	17	5	12.9	10	Positive
FQELE3E TLK.light	No	612.311343	Unit/Enh (6490)	819.445825	Unit/Enh (6490)	130	17	5	12.9	10	Positive
FQELE3E TLK.light	No	612.311343	Unit/Enh (6490)	706.361761	Unit/Enh (6490)	130	17	5	12.9	10	Positive
GC[+57.0] VQDEFC [+57.0] TR.heavy	Yes	641.264939	Unit/Enh (6490)	965.402075	Unit/Enh (6490)	130	20.7	5	9.8	10	Positive
GC[+57.0] VQDEFC [+57.0] TR.heavy	Yes	641.264939	Unit/Enh (6490)	837.343498	Unit/Enh (6490)	130	17.7	5	9.8	10	Positive
GC[+57.0] VQDEFC [+57.0] TR.light	No	636.260804	Unit/Enh (6490)	955.393806	Unit/Enh (6490)	130	20.7	5	9.8	10	Positive
GC[+57.0] VQDEFC [+57.0] TR.light	No	636.260804	Unit/Enh (6490)	827.335229	Unit/Enh (6490)	130	17.7	5	9.8	10	Positive
GGYTLV3 GYPK.heavy	Yes	575.305138	Unit/Enh (6490)	872.496744	Unit/Enh (6490)	130	18.7	5	14.17	10	Positive
GGYTLV3 GYPK.heavy	Yes	575.305138	Unit/Enh (6490)	658.365001	Unit/Enh (6490)	130	15.7	5	14.17	10	Positive
GGYTLV3 GYPK.light	No	571.298038	Unit/Enh (6490)	864.482545	Unit/Enh (6490)	130	18.7	5	14.17	10	Positive
GGYTLV3 GYPK.light	No	571.298038	Unit/Enh (6490)	650.350802	Unit/Enh (6490)	130	15.7	5	14.17	10	Positive
GPYPPGP LAPPQPF GPGFVPP PPPPPYG PGR.heavy	Yes	1039.880088	Wide / Unit (6490)	1238.655588	Unit/Enh (6490)	130	29.4	5	29.2	10	Positive
GPYPPGP LAPPQPF GPGFVPP PPPPPYG PGR.heavy	Yes	1039.880088	Wide / Unit (6490)	856.459574	Unit/Enh (6490)	130	29.4	5	29.2	10	Positive
GPYPPGP LAPPQPF GPGFVPP PPPPPYG PGR.light	No	1034.539388	Wide / Unit (6490)	1228.647319	Unit/Enh (6490)	130	29.4	5	29.2	10	Positive
GPYPPGP LAPPQPF GPGFVPP PPPPPYG PGR.light	No	1034.539388	Wide / Unit (6490)	850.445765	Unit/Enh (6490)	130	29.4	5	29.2	10	Positive
GQNRPG VQTQGQA TGGAWV3 3YDR.heavy	Yes	854.076941	Wide / Unit (6490)	1137.519882	Unit/Enh (6490)	130	28.8	5	14.7	10	Positive
GQNRPG VQTQGQA TGGAWV3 3YDR.heavy	Yes	854.076941	Wide / Unit (6490)	993.46639	Unit/Enh (6490)	130	28.8	5	14.7	10	Positive
GQNRPG VQTQGQA TGGAWV3 3YDR.heavy	Yes	854.076941	Wide / Unit (6490)	962.474637	Unit/Enh (6490)	130	19.8	5	14.7	10	Positive
GQNRPG VQTQGQA TGGAWV3 3YDR.light	No	850.740851	Wide / Unit (6490)	1127.511613	Unit/Enh (6490)	130	28.8	5	14.7	10	Positive

Acquisition Method Report

Agilent Technologies

Cpd Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Frag (V)	CE (V)	Cell Acc (V)	Ret Time (min)	Ret Window	Polarity
GQNRPG VQTQGQA TGGAWVS SYDR.light	No	850.74085 1	Wide / Unit (6490)	983.45812 1	Unit/Enh (6490)	130	28.8	5	14.7	10	Positive
GQNRPG VQTQGQA TGGAWVS SYDR.light	No	850.74085 1	Wide / Unit (6490)	962.47463 7	Unit/Enh (6490)	130	19.8	5	14.7	10	Positive
GTFATLS ELHC [+57.0] DK.heavy	Yes	743.85794	Unit/Enh (6490)	1110.5339 34	Unit/Enh (6490)	130	26.9	5	15.38	10	Positive
GTFATLS ELHC [+57.0] DK.heavy	Yes	743.85794	Unit/Enh (6490)	896.40219 2	Unit/Enh (6490)	130	23.9	5	15.38	10	Positive
GTFATLS ELHC [+57.0] DK.light	No	739.85084 1	Unit/Enh (6490)	1102.5197 35	Unit/Enh (6490)	130	26.9	5	15.38	10	Positive
GTFATLS ELHC [+57.0] DK.light	No	739.85084 1	Unit/Enh (6490)	888.38799 3	Unit/Enh (6490)	130	23.9	5	15.38	10	Positive
IAEYMNH LIDIGVAG FR.heavy	Yes	643.66906 7	Unit/Enh (6490)	908.45793	Unit/Enh (6490)	130	15.3	5	28.9	10	Positive
IAEYMNH LIDIGVAG FR.heavy	Yes	643.66906 7	Unit/Enh (6490)	872.93937 3	Unit/Enh (6490)	130	15.3	5	28.9	10	Positive
IAEYMNH LIDIGVAG FR.light	No	640.33297 7	Unit/Enh (6490)	903.45379 6	Unit/Enh (6490)	130	15.3	5	28.9	10	Positive
IAEYMNH LIDIGVAG FR.light	No	640.33297 7	Unit/Enh (6490)	867.93523 9	Unit/Enh (6490)	130	15.3	5	28.9	10	Positive
IPPPPPAP YGPGIFP PPPPQP.h eavy	Yes	715.06259 5	Wide / Unit (6490)	1148.6212 21	Unit/Enh (6490)	130	17.8	5	26	10	Positive
IPPPPPAP YGPGIFP PPPPQP.h eavy	Yes	715.06259 5	Wide / Unit (6490)	735.40681	Unit/Enh (6490)	130	17.8	5	26	10	Positive
IPPPPPAP YGPGIFP PPPPQP.li ght	No	710.71893 7	Wide / Unit (6490)	1141.6040 57	Unit/Enh (6490)	130	17.8	5	26	10	Positive
IPPPPPAP YGPGIFP PPPPQP.li ght	No	710.71893 7	Wide / Unit (6490)	729.39300 1	Unit/Enh (6490)	130	17.8	5	26	10	Positive
ISPQIQLS GQTEQTQ K.heavy	Yes	897.47779 7	Unit/Enh (6490)	1127.5782 42	Unit/Enh (6490)	130	31.7	5	12.9	10	Positive
ISPQIQLS GQTEQTQ K.heavy	Yes	897.47779 7	Unit/Enh (6490)	1014.4941 78	Unit/Enh (6490)	130	31.7	5	12.9	10	Positive
ISPQIQLS GQTEQTQ K.heavy	Yes	897.47779 7	Unit/Enh (6490)	797.41975	Unit/Enh (6490)	130	25.7	5	12.9	10	Positive
ISPQIQLS GQTEQTQ K.light	No	893.47069 7	Unit/Enh (6490)	1119.5640 43	Unit/Enh (6490)	130	31.7	5	12.9	10	Positive
ISPQIQLS GQTEQTQ K.light	No	893.47069 7	Unit/Enh (6490)	1006.4799 79	Unit/Enh (6490)	130	31.7	5	12.9	10	Positive
ISPQIQLS GQTEQTQ K.light	No	893.47069 7	Unit/Enh (6490)	793.41265 1	Unit/Enh (6490)	130	25.7	5	12.9	10	Positive
IVGGWEC [+57.0] EK.heavy	Yes	543.26241 6	Unit/Enh (6490)	972.43349 2	Unit/Enh (6490)	130	20.7	5	10	10	Positive
IVGGWEC [+57.0] EK.heavy	Yes	543.26241 6	Unit/Enh (6490)	873.36507 8	Unit/Enh (6490)	130	20.7	5	10	10	Positive
IVGGWEC [+57.0] EK.light	No	539.25531 6	Unit/Enh (6490)	964.41929 3	Unit/Enh (6490)	130	20.7	5	10	10	Positive
IVGGWEC [+57.0] EK.light	No	539.25531 6	Unit/Enh (6490)	865.35087 9	Unit/Enh (6490)	130	20.7	5	10	10	Positive
LSEPAELT DAVK.hea vy	Yes	640.84482 4	Unit/Enh (6490)	951.52368 7	Unit/Enh (6490)	130	17.7	5	16	10	Positive

Acquisition Method Report

Agilent Technologies

Cpd Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Frag (V)	CE (V)	Cell Acc (V)	Ret Time (min)	Ret Window	Polarity
LSEPAELTDAVK.heavy	Yes	640.844824	Unit/Enh (6490)	854.470923	Unit/Enh (6490)	130	23.7	5	16	10	Positive
LSEPAELTDAVK.light	No	636.837725	Unit/Enh (6490)	943.509488	Unit/Enh (6490)	130	17.7	5	16	10	Positive
LSEPAELTDAVK.light	No	636.837725	Unit/Enh (6490)	846.456724	Unit/Enh (6490)	130	23.7	5	16	10	Positive
LSGLLDLALGK.heavy	Yes	554.346806	Unit/Enh (6490)	907.570243	Unit/Enh (6490)	130	15.1	5	27	10	Positive
LSGLLDLALGK.heavy	Yes	554.346806	Unit/Enh (6490)	737.464715	Unit/Enh (6490)	130	15.1	5	27	10	Positive
LSGLLDLALGK.light	No	550.339706	Unit/Enh (6490)	899.556044	Unit/Enh (6490)	130	15.1	5	27	10	Positive
LSGLLDLALGK.light	No	550.339706	Unit/Enh (6490)	729.450516	Unit/Enh (6490)	130	15.1	5	27	10	Positive
LSITGTYDLK.heavy	Yes	559.812796	Unit/Enh (6490)	918.502223	Unit/Enh (6490)	130	15.2	5	16.84	10	Positive
LSITGTYDLK.heavy	Yes	559.812796	Unit/Enh (6490)	805.418159	Unit/Enh (6490)	130	15.2	5	16.84	10	Positive
LSITGTYDLK.light	No	555.805696	Unit/Enh (6490)	910.488024	Unit/Enh (6490)	130	15.2	5	16.84	10	Positive
LSITGTYDLK.light	No	555.805696	Unit/Enh (6490)	797.404966	Unit/Enh (6490)	130	15.2	5	16.84	10	Positive
NFPSPVDAAFR.heavy	Yes	615.810696	Unit/Enh (6490)	969.502775	Unit/Enh (6490)	130	22.9	5	19.65	10	Positive
NFPSPVDAAFR.heavy	Yes	615.810696	Unit/Enh (6490)	485.255026	Unit/Enh (6490)	130	16.9	5	19.65	10	Positive
NFPSPVDAAFR.light	No	610.806562	Unit/Enh (6490)	959.494506	Unit/Enh (6490)	130	22.9	5	19.65	10	Positive
NFPSPVDAAFR.light	No	610.806562	Unit/Enh (6490)	480.250891	Unit/Enh (6490)	130	16.9	5	19.65	10	Positive
SAVTALWGK.heavy	Yes	470.770734	Unit/Enh (6490)	782.465056	Unit/Enh (6490)	130	12.5	5	16.49	10	Positive
SAVTALWGK.heavy	Yes	470.770734	Unit/Enh (6490)	683.396636	Unit/Enh (6490)	130	12.5	5	16.49	10	Positive
SAVTALWGK.light	No	466.763634	Unit/Enh (6490)	774.450851	Unit/Enh (6490)	130	12.5	5	16.49	10	Positive
SAVTALWGK.light	No	466.763634	Unit/Enh (6490)	675.382437	Unit/Enh (6490)	130	12.5	5	16.49	10	Positive
STEYGEGYAC[+57.0]DTDLR.heavy	Yes	873.861183	Unit/Enh (6490)	1080.465404	Unit/Enh (6490)	130	30.9	5	12.5	10	Positive
STEYGEGYAC[+57.0]DTDLR.heavy	Yes	873.861183	Unit/Enh (6490)	860.380612	Unit/Enh (6490)	130	30.9	5	12.5	10	Positive
STEYGEGYAC[+57.0]DTDLR.light	No	868.857048	Unit/Enh (6490)	1070.457135	Unit/Enh (6490)	130	30.9	5	12.5	10	Positive
STEYGEGYAC[+57.0]DTDLR.light	No	868.857048	Unit/Enh (6490)	850.372343	Unit/Enh (6490)	130	30.9	5	12.5	10	Positive
SVLGQLGITK.heavy	Yes	512.318048	Unit/Enh (6490)	837.528378	Unit/Enh (6490)	130	13.8	5	18.46	10	Positive
SVLGQLGITK.heavy	Yes	512.318048	Unit/Enh (6490)	724.444314	Unit/Enh (6490)	130	13.8	5	18.46	10	Positive
SVLGQLGITK.light	No	508.310949	Unit/Enh (6490)	829.514179	Unit/Enh (6490)	130	13.8	5	18.46	10	Positive
SVLGQLGITK.light	No	508.310949	Unit/Enh (6490)	716.430115	Unit/Enh (6490)	130	13.8	5	18.46	10	Positive
SYPGLTSYLVR.heavy	Yes	633.341829	Unit/Enh (6490)	918.528262	Unit/Enh (6490)	130	23.5	5	21.71	10	Positive
SYPGLTSYLVR.heavy	Yes	633.341829	Unit/Enh (6490)	508.294151	Unit/Enh (6490)	130	17.5	5	21.71	10	Positive
SYPGLTSYLVR.light	No	628.337695	Unit/Enh (6490)	908.519993	Unit/Enh (6490)	130	23.5	5	21.71	10	Positive
SYPGLTSYLVR.light	No	628.337695	Unit/Enh (6490)	503.290016	Unit/Enh (6490)	130	17.5	5	21.71	10	Positive
TLDEYWR.heavy	Yes	496.739209	Unit/Enh (6490)	778.339399	Unit/Enh (6490)	130	13.2	5	14.6	10	Positive
TLDEYWR.heavy	Yes	496.739209	Unit/Enh (6490)	663.312455	Unit/Enh (6490)	130	16.2	5	14.6	10	Positive
TLDEYWR.heavy	Yes	496.739209	Unit/Enh (6490)	534.269862	Unit/Enh (6490)	130	16.2	5	14.6	10	Positive

Acquisition Method Report

Agilent Technologies

Cpd Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Frag (V)	CE (V)	Cell Acc (V)	Ret Time (min)	Ret Window	Polarity
TLDEYWR.light	No	491.735074	Unit/Enh (6490)	768.33113	Unit/Enh (6490)	130	13.2	5	14.6	10	Positive
TLDEYWR.light	No	491.735074	Unit/Enh (6490)	653.30418 6	Unit/Enh (6490)	130	16.2	5	14.6	10	Positive
TLDEYWR.light	No	491.735074	Unit/Enh (6490)	524.26159 3	Unit/Enh (6490)	130	16.2	5	14.6	10	Positive
WYVVGLAGNAILR.heavy	Yes	721.41312	Unit/Enh (6490)	993.60790 9	Unit/Enh (6490)	130	20.2	5	28	10	Positive
WYVVGLAGNAILR.heavy	Yes	721.41312	Unit/Enh (6490)	894.53949 5	Unit/Enh (6490)	130	26.2	5	28	10	Positive
WYVVGLAGNAILR.light	No	716.408986	Unit/Enh (6490)	983.59964	Unit/Enh (6490)	130	20.2	5	28	10	Positive
WYVVGLAGNAILR.light	No	716.408986	Unit/Enh (6490)	884.53122 6	Unit/Enh (6490)	130	26.2	5	28	10	Positive
YFYNAK.heavy	Yes	407.204696	Unit/Enh (6490)	650.33878 7	Unit/Enh (6490)	130	13.5	5	8	10	Positive
YFYNAK.heavy	Yes	407.204696	Unit/Enh (6490)	503.27037 3	Unit/Enh (6490)	130	13.5	5	8	10	Positive
YFYNAK.light	No	403.197596	Unit/Enh (6490)	642.32458 8	Unit/Enh (6490)	130	13.5	5	8	10	Positive
YFYNAK.light	No	403.197596	Unit/Enh (6490)	495.25617 4	Unit/Enh (6490)	130	13.5	5	8	10	Positive

Source Parameters

Parameter	Value (+)	Value (-)
Gas Temp (°C)	350	350
Gas Flow (l/min)	4	4
Capillary (V)	1950	1700

Chromatograms

Chrom Type	Label	Offset	Y-Range
TIC	TIC	0	10000000

Instrument Curves

Actual

Autosampler

Name	ALS	**Model**	G1329A
Ordinal #	1	**Options**	THM
Stop time (min)	No Limit	**Post Time (min)**	Off
Injection Type	Standard Injection	**Injection Volume (µl)**	5
Overlap Time (min)	Disable Overlapped Injection	**Draw Position (mm)**	0
Draw Speed (µl/min)	200	**Eject Speed (µl/min)**	200
Wash Vessel	N/A		
Contact 1	Off		
Contact 2	Off		
Contact 3	Off		
Contact 4	Off		

Acquisition Method Report

Agilent Technologies

Binary Pump

Name	Bin Pump	**Model**	G1312A
Ordinal #	1	**Options**	SSV
Stop Time (min)	No Limit	**Post Time (min)**	Off
Flow (ml/min)	0	**Pressure Min (bar)**	0
Pressure Max (bar)	400	**Max Flow Gradient (ml/min)**	100
Solvent A		**Solvent B**	
Solvent Ratio A	100	**Solvent Ratio B**	0
Solvent Type A1		**Solvent Type B1**	
Solvent Type A2		**Solvent Type B2**	
Compress. A (*10-6/bar)	100	**Compress. B (*10-6/bar)**	115
Stroke A (µl)	Auto	**Stroke B (µl)**	Auto
Stroke Syncronization			
Contact 1	Off		
Contact 2	Off		
Contact 3	Off		
Contact 4	Off		

Thermostated Column Compartment

Name	TCC	**Model**	G1316A
Ordinal #	1	**Options #**	CSV
Stop time (min)	No Limit	**Post Time (min)**	Off
Left Temp. (°C)	Not Controlled	**Right Temp. (°C)**	Same as left
Left Ready (°C)	When Temp Within Set Point +/- 0.8	**Right Ready (°C)**	When Temp Within Set Point +/- 0.8
Valve Position	1		
Contact 1	Off		
Contact 2	Off		
Contact 3	Off		
Contact 4	Off		

APPENDIX II: CRITICAL VALUES FOLLOWING POST-HOC TUKEY TEST COMPARING UNSCHEDULED (METHOD 1) AND SCHEDULED (METHODS 2-6) ITERATIONS OF A SEMINAL FLUID MONOPLEX ASSAY FOR ALL TARGET PEPTIDES

ELSE df=6 P < 0.05 P > 0.05

	Method 1	Method 2	Method 3	Method 4	Method 5	Method 6
Method 1	0					
Method 2	0.113	0				
Method 3	0.116	2.54E-03	0			
Method 4						
Method 5						
Method 6						

LPSE df=8 P < 0.05 P > 0.05

	Method 1	Method 2	Method 3	Method 4	Method 5	Method 6
Method 1	0					
Method 2	0.045	0				
Method 3	0.047	1.70E-03	0			
Method 4	0.078	0.033	0.031	0		
Method 5						
Method 6						

LSEP df=10 P < 0.05 P > 0.05

	Method 1	Method 2	Method 3	Method 4	Method 5	Method 6
Method 1	0					
Method 2	0.080	0				
Method 3	0.074	6.30E-03	0			
Method 4	0.077	3.10E-03	3.20E-03	0		
Method 5	0.077	3.34E-03	2.96E-03	2.43E-04	0	
Method 6						

DVSQ df=12 P < 0.05 P > 0.05

	Method 1	Method 2	Method 3	Method 4	Method 5	Method 6
Method 1	0					
Method 2	0.027	0				
Method 3	0.025	2.51E-03	0			
Method 4	0.022	5.46E-03	2.95E-03	0		
Method 5	0.026	1.71E-03	7.92E-04	3.74E-03	0	
Method 6	0.031	3.03E-03	5.54E-03	8.50E-03	4.75E-03	0

DIFS df=12 P < 0.05 P > 0.05

	Method 1	Method 2	Method 3	Method 4	Method 5	Method 6
Method 1	0					
Method 2	0.075	0				
Method 3	0.069	5.80E-03	0			
Method 4	0.066	8.47E-03	2.67E-03	0		
Method 5	0.058	0.016	0.011	7.97E-03	0	
Method 6	0.066	9.28E-03	3.48E-03	8.11E-04	7.16E-03	0

DIFT df=8 P < 0.05 P > 0.05

	Method 1	Method 2	Method 3	Method 4	Method 5	Method 6
Method 1	0					
Method 2	0.045	0				
Method 3	0.043	1.68E-03	0			
Method 4	0.043	1.70E-03	2.14E-05	0		
Method 5						
Method 6						

APPENDIX III: QUALITATIVE MONOPLEX QQQ-MRM SEMINAL FLUID METHOD PARAMETERS

Acquisition Method Report

Agilent Technologies

Acquisition Method Info

Method Name 15min_dMRM_Final_Final.m

Method Path D:\MassHunter\Methods\Heather_Fellowship\15min_dMRM_Final_Final.m

Method Description 1 min source with trap column, HSA

Device List

- HiP Sampler
- Binary Pump
- Column Comp.
- QQQ

MS QQQ Mass Spectrometer

Ion Source	AJS ESI	**Tune File**	atunes.TUNE.XML
Stop Mode	No Limit/As Pump	**Stop Time (min)**	1
Time Filter	On	**Time Filter Width (min)**	0.03
LC>Waste Pre Row	N/A	**LC>Waste Post Row**	N/A

Time Segments

Index	Start Time (min)	Scan Type	Ion Mode	Div Valve	Delta EMV	Store	Cycle Time (ms)	Triggered?	MRM Repeats
1	0	DynamicMRM	ESI+Agilent Jet Stream	To MS	200	Yes	500	No	3

Time Segment 1

Acquisition Method Report

Agilent Technologies

Scan Segments

Cpd Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Frag (V)	CE (V)	Cell Acc (V)	Ret Time (min)	Ret Window	Polarity
DTGILDSL GR.heavy	Yes	528.78160 5	Unit/Enh (6490)	840.48131 2	Unit/Enh (6490)	380	17.2	4	5.56	6	Positive
DTGILDSL GR.heavy	Yes	528.78160 5	Unit/Enh (6490)	670.37578 4	Unit/Enh (6490)	380	17.2	4	5.56	6	Positive
DTGILDSL GR.light	No	523.77747	Unit/Enh (6490)	830.47304 3	Unit/Enh (6490)	380	17.2	4	5.56	6	Positive
DTGILDSL GR.light	No	523.77747	Unit/Enh (6490)	660.36751 5	Unit/Enh (6490)	380	17.2	4	5.56	6	Positive
GSISIQTE EQIHGK.h eavy	Yes	512.26988 8	Unit/Enh (6490)	695.87444 7	Unit/Enh (6490)	380	13.5	4	4.27	3	Positive
GSISIQTE EQIHGK.h eavy	Yes	512.26988 8	Unit/Enh (6490)	639.33241 5	Unit/Enh (6490)	380	13.5	4	4.27	3	Positive
GSISIQTE EQIHGK.h eavy	Yes	512.26988 8	Unit/Enh (6490)	464.25205 7	Unit/Enh (6490)	380	13.5	4	4.27	3	Positive
GSISIQTE EQIHGK.li ght	No	509.59848 8	Unit/Enh (6490)	691.86734 8	Unit/Enh (6490)	380	13.5	4	4.27	3	Positive
GSISIQTE EQIHGK.li ght	No	509.59848 8	Unit/Enh (6490)	635.32531 6	Unit/Enh (6490)	380	13.5	4	4.27	3	Positive
GSISIQTE EQIHGK.li ght	No	509.59848 8	Unit/Enh (6490)	461.58065 7	Unit/Enh (6490)	380	13.5	4	4.27	3	Positive
LSEPAELT DAVK.hea vy	Yes	640.84482 4	Unit/Enh (6490)	951.52368 7	Unit/Enh (6490)	380	20.7	4	6.05	3	Positive
LSEPAELT DAVK.hea vy	Yes	640.84482 4	Unit/Enh (6490)	654.39121 6	Unit/Enh (6490)	380	20.7	4	6.05	3	Positive
LSEPAELT DAVK.hea vy	Yes	640.84482 4	Unit/Enh (6490)	476.26548 1	Unit/Enh (6490)	380	20.7	4	6.05	3	Positive
LSEPAELT DAVK.light	No	636.83772 5	Unit/Enh (6490)	943.50948 8	Unit/Enh (6490)	380	20.7	4	6.05	3	Positive
LSEPAELT DAVK.light	No	636.83772 5	Unit/Enh (6490)	646.37701 7	Unit/Enh (6490)	380	20.7	4	6.05	3	Positive
LSEPAELT DAVK.light	No	636.83772 5	Unit/Enh (6490)	472.25838 2	Unit/Enh (6490)	380	20.7	4	6.05	3	Positive
QITIPSQE QEHSQK. heavy	Yes	554.28419 7	Unit/Enh (6490)	710.35133 6	Unit/Enh (6490)	380	15.1	4	3.61	3	Positive
QITIPSQE QEHSQK. heavy	Yes	554.28419 7	Unit/Enh (6490)	603.28546 5	Unit/Enh (6490)	380	15.1	4	3.61	3	Positive
QITIPSQE QEHSQK.li ght	No	551.61279 7	Unit/Enh (6490)	706.34423 7	Unit/Enh (6490)	380	15.1	4	3.61	3	Positive
QITIPSQE QEHSQK.li ght	No	551.61279 7	Unit/Enh (6490)	599.27836 6	Unit/Enh (6490)	380	15.1	4	3.61	3	Positive

Scan Parameters

Data Stg	Threshold
Centroid	0

Source Parameters

Parameter	Value (+)	Value (-)
Gas Temp (°C)	150	150
Gas Flow (l/min)	11	11
Nebulizer (psi)	30	30
SheathGasHeater	150	150
SheathGasFlow	10	10
Capillary (V)	3500	3000
VCharging	300	500

Ion Funnel Parameters

Pos High Pressure RF	150	Neg High Pressure RF	90
Pos Low Pressure RF	60	Neg Low Pressure RF	60

Chromatograms

Chrom Type	Label	Offset	Y-Range
TIC	TIC	0	10000000

Instrument Curves

Actual

Name: HiP Sampler Model: G4226A

Auxiliary	
Draw Speed	200.0 µL/min
Eject Speed	200.0 µL/min
Draw Position Offset	-3.0 mm
Wait Time After Drawing	0.0 s
Sample Flush Out Factor	5.0
Vial/Well bottom sensing	No
Injection	
Injection Mode	Injection with needle wash
Injection Volume	10.00 µL
Needle Wash	
Needle Wash Location	Rush Port
Wash Time	8.0 s
High throughput	
Automatic Delay Volume Reduction	No
Overlapped Injection	
Enable Overlapped Injection	No
Valve Switching	
Valve Movements	0
Valve Switch Time 1	
Switch Time 1 Enabled	No
Valve Switch Time 2	
Switch Time 2 Enabled	No
Valve Switch Time 3	
Switch Time 3 Enabled	No
Valve Switch Time 4	
Switch Time 4 Enabled	No
Stop Time	
Stoptime Mode	As pump/No limit
Post Time	
Posttime Mode	Off

Name: Binary Pump Model: G4220A

Flow	0.400 mL/min
Use Solvent Types	Yes
Stroke Mode	Synchronized
Low Pressure Limit	0.00 bar
High Pressure Limit	550.00 bar
Max. Flow Ramp Up	100.000 mL/min²
Max. Flow Ramp Down	100.000 mL/min²
Expected Mixer	No check
Stroke A	
Automatic Stroke Calculation A	Yes
Stop Time	
Stoptime Mode	Time set
Stoptime	15.00 min
Post Time	
Posttime Mode	Time set
Posttime	4.00 min

Solvent Composition

	Channel	Ch. 1 Solv.	Name 1	Ch2 Solv.	Name 2	Selected	Used	Percent
1	A	100.0 % Water V.03	H2O	100.0 % Water V.03	H2O	Ch. 1	Yes	95.00 %
2	B	100.0 % Acetonitrile V.03	ACN 0.1% FA	100.0 % Isopropanol V.03	IPA	Ch. 1	Yes	5.00 %

Timetable

	Time	A	B	Flow	Pressure
1	12.50 min	65.00 %	35.00 %	--- mL/min	--- bar
2	13.00 min	10.00 %	90.00 %	--- mL/min	--- bar
3	14.90 min	10.00 %	90.00 %	--- mL/min	--- bar
4	15.00 min	95.00 %	5.00 %	--- mL/min	--- bar

Acquisition Method Report

Agilent Technologies

Name: Column Comp. **Model:** G1316C

Valve Position	Port 1 ->6
Ready when front door open	Yes
Left Temperature Control	
Temperature Control Mode	Temperature Set
Temperature	45.00 °C
Enable Analysis Left Temperature	
Enable Analysis Left Temperature On	Yes
Enable Analysis Left Temperature Value	0.8 °C
Right Temperature Control	
Right temperature Control Mode	Not Controlled
Enable Analysis Right Temperature	
Enable Analysis Right Temperature On	Yes
Enable Analysis Right Temperature Value	0.8 °C
Stop Time	
Stoptime Mode	As pump/injector
Post Time	
Posttime Mode	Off