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Validation of a Deployable Proteomic Assay for the Serological Screening of Sexual Assault Samples

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Abstract

Protein mass spectrometry (MS) has emerged as a technique to supplant traditional serological tests for body fluid identification. It was hypothesized that proteomic techniques would surpass the sensitivity and specificity of traditional serological techniques. An automated workflow coupled with protein MS has been developed for the confirmatory identification of five biological fluids. A developmental validation was completed, assessing parameters such as reproducibility, sensitivity, ion suppression, and limit of detection. Implementation was determined through tandem sample processing by MS, traditional serological tests, and standard DNA profiling methods. The MS approach offered superior detection limits while also providing true confirmatory results, producing an unambiguous identification of body fluids to the point where the technology can be considered comparable to DNA profiling.

An extensive study was conducted to evaluate the effects of personal lubricants on biomarker detection in sexual assault evidence. Lubricants have the potential to inhibit protease activity, displace hydrophobic markers during solid phase extraction, and suppress ion detection during MS analysis. Three studies were performed: (1) determination of vaginal fluid biomarker detection from vaginal swabs fortified with lubricant; (2) the effect of lubricant formula on seminal fluid and saliva biomarker detection was established; and (3) the detection of biomarkers condoms. Data was interpreted by the overall peak area response (PAR) of the target biomarker, biomarker PAR in relation to internal standard, and PAR of digestion control protein.

Multi-stage workflows associated with proteomic analysis remain a major hurdle towards the adoption of the technique in caseworking laboratories. A streamlined sample-to-results workflow has been developed using peptidomic analysis, allowing for straightforward preparation versus bottom up methodologies. Low molecular weight proteins were extracted and data was acquired using an orbitrap-quadrupole HRMS. Numerous protein biomarkers have been characterized in human seminal fluid, saliva, and vaginal fluid.

In conclusion, the implementation of the protein MS approach offers an advantageous relationship between a positive identification and downstream DNA testing, including the capacity to deliver confirmatory contextual information in a criminal investigation. Furthermore, lubricant type does affect the ability to accurate identify protein biomarkers. And lastly, the research presented will demonstrate the use of peptidomic analysis for the confirmatory identification of biological fluids in SA type evidence.

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Catherine O. Brown

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CHAPTER 1: INTRODUCTION

1 Introduction

Forensic science is the application of scientific testing to criminal and civil law. The overarching goal of forensic testing is to establish an association between a piece of physical evidence and an individual, whether a victim or suspect of a crime. By exploiting the transfer of evidence, as stated by Edmond Locard [1], analysts seek to identify, compare, and associate physical evidence in an effort to provide information to a court of law to assist the trier of fact in determining the probative value and weight of forensic evidence. The probative value of physical evidence stems from the ability to determine an object's uniqueness. The capabilities of forensic testing to narrow down the range of possibilities as to what an item of evidence may be, such as a biological stain, or to whom biological evidence may belong, can help to focus an investigation.

Forensic biology is a subdiscipline within the field of forensic science that seeks to identify and individualize the source of biological stains through serological and genetic testing. Forensic serology describes the identification of biological fluids, such as blood, semen, and saliva, through the use of biochemical techniques to associate an item of evidence with a victim or suspect. Common types of crimes that utilize forensic biology testing include homicide, assault, and rape. Prior to the advent of DNA analysis, forensic biologists depended heavily on antigen and protein polymorphisms as a means of discriminatory identification. For example, the identification system of ABO blood type groups was developed during the early 20th century. The frequency and inheritance of A and B antigens present on the surface of erythrocytes were mapped and used as the first form of human identification apart from fingerprint analysis. While this discovery lead to a more advanced classification system, the use of erythrocyte antigen characterization was primarily utilized for exclusionary purposes.

1.1 Modern Genetic Typing for Forensic Identification

It was not until the late 20th century that DNA polymorphisms, in the form of variable number tandem repeats, were applied to forensic evidence successfully. This novel technique demonstrated greater individual variability than protein polymorphic markers, with DNA profiling not only capable of identifying a true perpetrator but excluding innocent suspects. With the simultaneous development of the polymerase chain reaction (PCR), the use of DNA polymorphisms quickly surpassed that of more established techniques. Rapid advancements in PCR chemistries, polymorphism fragment length, and automation have led to genetic typing being crowned the gold standard within the field of forensic biology. For example, the National DNA Index System (NDIS), launched in October 1998, contains 1,096,398 forensic profiles and over 14,492,991 offender profiles (*i.e.,* convicted offender, detainee, and legal profiles) to date [2]. Forensic uses of DNA typing expand beyond the capability of identifying potential suspects and include the identification of mass catastrophe victims, missing persons, and endangered or protected species.

The process of genetic testing follows five central procedural steps: extraction, quantitation, amplification, separation, and interpretation. It is estimated that the human genome shares 99.7% sequence homology between individuals, limiting forensic researchers to target the remaining 0.3% for discriminatory analyses. Short tandem repeat (STR) regions, a type of microsatellite, are currently targeted for forensic genetic typing as they are small in size (approximately 2 to 7 base pairs in length) and the number of repeats at a given location is highly variable among individuals. Simply, they describe a pattern of nucleotides that are repeated directly adjacent to each other, with the number of consecutive repeats varying between individuals. STR regions can be further classified as simple, non-consensus, compound, or complex repeats based on their incremental repeat patterns. Twenty STR locations are accepted into the Combined DNA Index System (CODIS), with the number increased from the original thirteen locations in the year 2017. As interest in the analysis of highly degraded evidence continued to push the lower sensitivity limits of genetic typing, the use of single nucleotide polymorphism (SNP) markers emerged to supplement STR typing. SNPs are single base pair sequence variations at a particular point in the genome that arise

due to spontaneous mutation. These mutations can be present in coding, regulatory, and intronic sequences within genes or in intergenic regions. SNP markers are attractive for forensic investigations given their short amplicon size, lack of stutter artifact formation during amplification, and their ability to predict ethnicity and phenotypic characterizations.

Common practice for DNA size fractionation traditionally involves the use of capillary electrophoresis. However, crime laboratories have begun implementing next generation sequencing (NGS) methodologies for challenging casework. NGS, also referred to as massively parallel sequencing (MPS), allows for the simultaneous sequencing of many DNA samples, instead of one sample at a time as with capillary electrophoretic separation. The benefits of this type of analysis allow for an influx of data that is not normally obtained with traditional sequencing, such as the discovery of different types of genomic features in a single sequencing run (*e.g.,* SNPs, copy number variants, structural variants). In regard to forensic genetic applications, NGS can be used for a variety of analyses such as STR identification for relationship testing, SNP identification for phenotypic characterization, and mitochondrial genome sequencing. NGS is currently used for investigative lead generation and genetic genealogy practices, seen in cases such as the Golden State Killer [3] or the identification and conviction of W.M. Talbot in Washington state for the 1987 murders of Jay Cook and Tonya Van Cuylenborg [4].

Modern genetic testing procedures have rapidly exceeded the anticipated sensitivity limits to the extent that trace DNA can be identified from an individual that had prior interaction with a person but was not involved in criminal activity. The concept of indirect transfer of genetic material describes the deposition of one's DNA onto an object or person and subsequently having their DNA relocated to a secondary object or person without physical contact. This phenomenon has been rigorously evaluated within the forensic community in an attempt to provide reason in the event of explaining alternate transfer propositions [5–8]. This limitation has prompted forensic investigators to question not *what* evidence to collect for DNA analysis, but *where* to collect on an item of evidence to obtain accurate results. For example, given the case scenario of a drug facilitated sexual assault, a victim that was unconscious during sexual activity would not have recollection of

where the offender may have removed an article of clothing or the extent of sexual acts performed. In a recent study, simulated digital sexual assault scenarios were conducted in a controlled laboratory setting and revealed that the amount of offender DNA deposited on undergarments is highly variable. Furthermore, although not an original aim of the study, the DNA of a colleague or cohabitating partner was identified in several instances [9].

The principles of DNA transfer, persistence, prevalence, and recovery are taken into consideration during data interpretation to provide insight into activity level reporting and generating descriptive probability estimates [10]. It is important to understand these variables as they relate to DNA transfer versus contamination or true instances of multiple contributors. As genetic testing methodologies have gotten more sensitive and can generate informative results from poor quality and lesser quantities of input DNA, the types of DNA profiles obtained have become exceedingly more challenging to interpret, particularly in samples with multiple low level contributors. Practitioners within the field have responded by shifting to the use of more advanced probabilistic modeling. The need for innovative interpretation strategies for low template DNA was reported two decades ago [11], and since, various types of semi-continuous and continuous probabilistic genotyping models are accepted in the forensic community [12]. In short, probabilistic genotyping has unlocked potential for deciphering challenging DNA profiles that would have otherwise been deemed uninterpretable with the use of traditional binary methods, such as combined probability of inclusion (CPI) calculations.

DNA profiling and interpretation methodologies have grown very rapidly over the past 25 years. The lack of consistency in reporting language of advanced probabilistic genotyping calculations remains a topic of debate within the greater forensic biology community. Furthermore, the proprietary source code of interpretation software, such as TrueAllele® by Cybergenetics, has received criticism within the legal community as defendants are unable to visualize how software forms decisions [13,14]. In essence, the scientific methodology behind DNA profiling has been well-demonstrated and is concrete, with limitations arising from the interpretation of generated profiles.

On the contrary, the scientific methodology for modern serological testing has not received the same attention.

1.2 Test Classifications for Traditional Body Fluid Identification

In contrast to the evolution and occasional transilience of modern genetic testing, the identification of biological stains remains rooted in historical biochemical analyses. Traditional serological procedures rely on detecting the presence or activity of macromolecules that display a level of specificity within a biological fluid. Historically, proteins are the macromolecule of interest. Proteins are composed of amino acid monomers that have been bound together through polypeptide covalent bonds. Amino acids are simple monomer subunits that contain an amine and a carboxyl functional group, providing the monomer with directionality. Amino acids are categorized according to their side chain, which extends as a substituent from the backbone structure. There are twenty unique side chains, and therefore twenty unique amino acids. Given the combination of amino acids within a protein, a nearly endless array of protein sequences can be achieved. It is the amino acid side chains, or combination of side chains, that provide a protein with specific chemical properties such as polarity and charge state. The primary structure of a protein is defined as the sequence of amino acids or unfolded polypeptide chain. The polypeptide chain begins to adopt a three-dimensional structure as amino acid side chains initiate new interactions in the form of hydrogen bonds and functional group interactions. The orientation and unique combination of amino acids at the primary level dictate the interactions formed at the secondary level. Interactions become stronger and the protein obtains its true three-dimensional shape at the tertiary level, where characteristic folds initiate a protein's function. When two or more polypeptide subunits are joined, a quaternary structure is achieved.

Protein form and function have remained a primary target for serological assays. A majority of historical serological techniques rely on detecting the presence or activity of proteins consistent with a target body fluid. Body fluids are rich in protein material, however, the challenge for forensic testing is to identify specific targets and not necessarily the most abundant targets. Achieving a desirable balance between sensitivity and specificity has proven difficult, with cross-reactivity and

false positive identifications fairly common. In general, evidence that is received in the laboratory for forensic biology testing will first be screened for the presence of biological stains. This is conducted through visualizing stains that may not be evident to the naked eye and conducting presumptive testing to narrow down the possible stain origin. For sexual assault evidence, confirmatory testing can also be performed to further discriminate the presence of biological stains. Once the origin of a stain is revealed, small cuttings or scrapings are acquired, and genetic material is extracted for analysis. Together, results from serological and genetic testing deliver associative support for a connection between an individual and a biological stain on an item of evidence. However, modern genetic testing procedures are more sensitive than their serology counterpart, and thus DNA results often take precedence in a criminal investigation.

The identification of biological fluids on forensic evidence provides important contextual information to an investigation. While genetic testing provides individualization to a biological stain, serological testing can indicate where the biological stain originated. In this section, the traditional techniques used for presumptive and confirmatory identification of biological fluids will be summarized. Screening procedures are classified according to their mechanism of action or sample preparation procedure and are characterized below into one of the following groups: alternative light source, colorimetric assays, enzymatic assays, microscopic visualization, and antibody-based assays. Although many techniques exist for each, a select grouping of procedures were chosen to exemplify each classification, with the discussion herein not designed to present an exhaustive list of historical techniques.

1.2.1 Alternate Light Source for Body Fluid Identification

A crime scene investigator or forensic analyst will rely heavily on the use of an alternative light source (ALS) for the screening of large surface areas, such as a wall or a mattress. This screening technique visually enhances biological stains that may not be visible to the naked eye and can be employed for assistance with collection, documentation, and processing of biological evidence [15]. The theory behind this investigative tool relies on emission and excitation wavelengths. Excitation wavelengths, generally in the visible range of the electromagnetic

spectrum, are absorbed by fluorophores within a stain or substrate. The fluorophore absorbs the excitation wavelength and transitions the energy to a more excited state, emitting excess energy. This excess, or emission wavelength, is detected as fluorescence. Barrier filters or colored goggles are necessary for visualization of fluorescence, as the excitation wavelength will otherwise overpower the emission wavelength. Colored goggles selectively filter light and allow the emission wavelength to pass through and be detected by the eye. ALS is utilized for gunshot residue [16], fiber [17], and fingerprint evidence [18], and within forensic biology, is a prevalent tool for localizing biological stains, such as semen, saliva, and urine.

For seminal fluid visualization, flavins and choline contribute to the fluorescence of semen as well as any bacterial growth. When irradiated, semen stains will appear bluish-white when deposited on dark materials or, in contrast, may appear as dark spots when deposited on white fabrics due to high background fluorescence. Similarly, saliva detection is indicated by a bluish-white illumination and is more difficult to visualize in comparison with other fluids given its simple composition [19]. Generally, wavelengths between 450 nm and 495 nm (blue light) are used in combination with orange filter goggles for fluorescence visualization. Benefits of ALS include the ability to scan larger items of evidence, such as a bed sheet, for trace amount of biological material. However, background interferences are common, and are attributed to substrate color and presence of whiteners or other substances applied during the manufacturing process. It is accepted practice to use ALS as a screening tool prior to further examination of forensic evidence.

1.2.2 Colorimetric Assays for Body Fluid Identification

Colorimetric assays utilize the conjugation of electrons across an indicator compound to cause a shift in the highest occupied molecular orbital-lowest unoccupied molecular orbital (HOMO-LUMO) gap to produce a wavelength of light within the visible spectrum. Blood is most frequently detected using colorimetric tests that exploit the peroxidase-like activity of the protein hemoglobin. The non-protein heme group present within hemoglobin catalyzes oxidation-reduction reactions, where colorless indicators are oxidized and cause a color change. The oxidation of a molecule describes the loss of an electron whereas the reduction of a molecule describes the gain of an

electron. Colorimetric tests for blood detection can be further separated into one-phase and two-phase procedures [19]. For one-phase procedures, a suspected stain is subjected to hydrogen peroxide and an indicator compound, such as tetramethylbenzidine (TMB), simultaneously. Although rapid to perform and fairly sensitive, one-phase procedures display a wide variety of false positives ranging from plant, animal, and bacterial sources that exhibit strong chemical oxidant/reductant properties or peroxidase-like activity [20]. In contrast, two-phase procedures subject a suspected stain first to an indicator compound followed by a separate addition of hydrogen peroxide. Positive reactions that occur prior to the addition of hydrogen peroxide are ruled negative. An example of a two-phase procedure is the phenolphthalein test, also referred to as Kastle-Meyer test [21].

Unlike TMB or phenolphthalein, oxidation-reduction reactions can produce chemiluminescence or fluorescence instead of a color change. Luminol test (3-aminophthalhydrazide) is commonly employed during crime scene investigation for the detection of minute traces of blood, even traces that have been wiped clean from surfaces. Chemiluminescence is visualized as a blue glow. This test has demonstrated unparalleled sensitivity, with a reporting limit of 5,000,000-fold blood dilution detected [22]; however, commercial bleach is a false positive [23]. Given its use on large items of evidence such as carpets, walls, and flooring, it has been demonstrated that luminol will not negatively affect polymerase chain reaction for DNA analysis [24].

1.2.3 Microscopic and Histological Analysis for Body Fluid Identification

Microscopic crystal tests were historically performed for the identification of blood but have been replaced in favor of catalytic tests and antibody-binding assays. An example of a crystal test is the Takayama test [25]. The heme group is dissociated from the hemoglobin protein unit and pyridine binds to the fifth and sixth orbital positions on the iron atom, resulting in the compound hemochromogen. Under alkaline conditions and in the presence of glucose, hemochromogen is pushed out of solution and creates rhomboid-shaped crystals (**Figure 1.1A**). Although once considered confirmatory, it was determined that crystal formation was selective for the iron

protoporphyrin ring structure within heme and not the protein itself. Furthermore, crystal growth is contingent on creating an ideal environment, which is time consuming and difficult to reproduce.

In comparison with the visualization of crystal formation, the histological staining and visualization of cells is used for semen identification and vaginal fluid detection. The detection of vaginal fluid is not common practice in operational laboratories, as the histological staining described herein is not discriminatory for vaginal epithelia. Three historical staining techniques include Periodic Acid-Schiff (PAS) reagent, Lugol's iodine, and Dane's stain. PAS is used to visualize polysaccharides present in cells, commonly glycogen and glycoproteins. This staining technique is commonly used by pathologists during routine renal biopsy evaluation and determination of alveolar sarcoma [26]; however, this stain has been retired from forensic testing of vaginal epithelia, having exhibited no differentiating power between vaginal and rectal epithelial cell types [27]. Lugol's iodine stain to detect glycogenated epithelial cells was once considered a reliable indicator of vaginal fluid. This was based on the belief that vaginal epithelial cells had a significantly higher glycogen content than other epithelial cell types [28,29]. Iodine present in the stain reacts with intra-cellular glycogen, producing a dark brown color [29] (**Figure 1.1B**). Further research revealed that (1) glycogen content varies widely during the menstrual cycle [30] and (2) epithelial cells from swabs of the glans penis and male urethral secretions contain similar glycogen levels [28,29]. As a result, Lugol's test is no longer common practice for forensic analysis as the value of this technique has diminished. Dane's stain (haematoxylin-phloxine-alcian) uses three dyes that preferentially stain mucopolysaccharides from prekeratin and keratin [31]. This technique differs from PAS and Lugol's iodine in that it can distinguish skin epithelial cells, appearing bright red upon microscopic visualization, from vaginal/buccal cells which stain orange, based on the high presence of keratin found in skin. Using a modified Dane's stain with methanol fixation, vaginal epithelia were able to be differentiated from buccal epithelia [32]. Although a promising solution to challenges encountered with PAS and Lugol's iodine, limitations arise with the analysis of mixed sample types commonly encountered in sexual assault evidence.

The only accepted confirmatory test within forensic biology is the microscopic visualization of spermatozoa in semen. Spermatozoa are unique to semen and exhibit a distinct morphology in relation to other cell types, especially epithelial cells. Specifically, the head region contains nuclear DNA and is protected by the acrosomal cap, the location of digestive enzymes that break down the zona pellucida and assist in penetration of the female ovum. The midpiece connects the head region to the tail and contains numerous mitochondria. The most defining characteristic of spermatozoa is the tail region. The flagellated cell contains a microfilament that runs the length of the tail region, creating rhythmic contractions and forward motion. The histological staining of head and tail regions, in addition to the overall morphology of the cell, attribute to the confirmatory nature of this technique.

The most commonly used histological stain is Kernechtrot-Picroindigocarmine (KPIC), also referred to as Christmas tree stain. KPIC uses a series of stains to differentially identify sperm cells from epithelial cells. Nuclear fast red dye stains the nuclear material of the head region and picric acid-indigocarmine stains the membrane and tail regions a blue-green color [33] (**Figure 1.1C**). Limitations to this technique arise due to the lack of standardization among interpretation guidelines within agencies. While there is a generally accepted scale for sperm scoring, subjectivity is introduced in the manner in which an analyst interprets the ratio of spermatozoa to epithelial cells. In addition, the major challenge faced by most examiners is not the judgement of whether a cell is a spermatozoon but rather to locate an intact cell. The severing of the head region from the tail region of the spermatozoa is not uncommon and is caused by many factors, such as analyst handling, spermicidal lubricants, and extreme temperatures. There are varying opinions in the field on whether a spermatozoon needs to be intact to prompt a positive identification. Furthermore, absence of spermatozoa in the male ejaculate can be due to hereditary, pathologic, or surgical reasons.

The product Sperm Hy-Liter™ (Independent Forensics) utilizes a fluorescently labeled, human specific, monoclonal antibody that binds to sperm head proteins and further assists with the visualization of spermatozoa. Specifically, this commercial kit contains two separate fluorescent dyes. The first, 4',6-diamidino-2-phenylindole (DAPI) stain is incorporated into nuclear material and is visualized with a DAPI compatible fluorescent filter. The second dye, green fluorescein

isothiocyanate (Alexa 488), is bound to a monoclonal antibody that targets protein antigen in the sperm nuclear membrane. With selective filters, dye components can be visualized separately (**Figures 1.1D and 1.1E**) or overlayed (**Figure 1.1F**), allowing for visualization of sperm cells in a dense vaginal epithelial sample. Due to the preferential binding of the antibody, this method is more sensitive and specific than traditional histological staining techniques [34]; however, a limitation to this method is the breakdown of sperm cells over time [35]. The reporting of a positive result is based on the occurrence of a binding event between the paratope of the antibody and the epitope of the sperm head proteins. Within the Sperm Hy-Liter™ procedure, the reducing agent dithiothreitol (DTT) is applied to the sample and functions to make the spermatozoon cell membrane more permeable to staining. As spermatozoa degrade, the specific epitope present on the cell surface may undergo a conformational change an impede antibody binding. This lack of binding poses as a limitation to basing a confirmatory technique off of the detection of cellular components alone. Furthermore, depending upon the severity of degradation, the DTT can potentially lyse cell membranes instead of increasing permeability.

In summary, this classification of serological testing contains the only accepted confirmatory technique for body fluid identification. Furthermore, in regard to semen identification, histological staining of cellular material is not applicable in instances for suspects that have undergone a vasectomy surgical procedure or those that suffer from aspermia. Histological staining has historical significance within the field of forensic biology but has largely been replaced by enzymatic and antibody based assays.

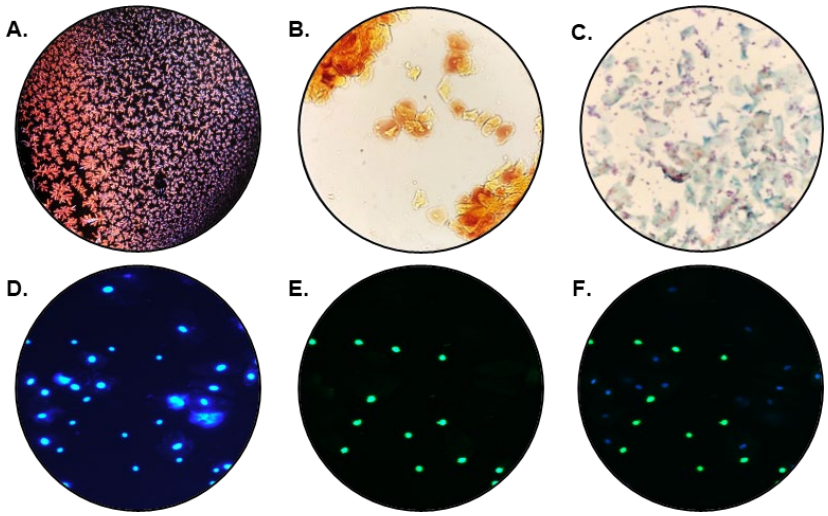


Figure 1.1. Histological stains and microscopic visualization for body fluid identification. (A) Takayama crystal formation of peripheral blood. (B) Lugol's iodine stain of a vaginal smear. (C) Kernechtrot-Picroindigocarmine stain of a vaginal swab containing spermatozoa. Spermatozoa visualized using Sperm Hy-Liter™ with fluorescent microscopy and the (D) DAPI filter displaying sperm and epithelial cells, (E) FTIC filter displaying spermatozoa, and (F) filter overlay displaying sperm and epithelia nuclei. © 2021 by Catherine Brown.

1.2.4 Enzymatic Assays for Body Fluid Identification

Enzymatic assays rely on protein form and function to remain intact in order for the test to be carried out successfully. In general, protein enzymes within a biological fluid are mixed with a cognate substrate. Through the chemical modification of the substrate, an applied dye compound will bind to the modified substrate, resulting in a visible color change. Perhaps the most widely accepted presumptive test within the enzymatic classification is the acid phosphatase test for the detection of seminal fluid, which was proposed in the early 1950s [36]. Prostatic acid phosphatase is produced in the prostate and is currently evaluated as a diagnostic marker for prostate cancer. Its physiological function is to cleave the protein semenogelin, resulting in the liquefaction of semen and creating a more conducive environment for sperm motility within the female reproductive tract. Referred to as the AP Spot Test, a seminal fluid stain is subjected to a reagent containing alpha naphthyl phosphate. The acid phosphatase protein cleaves the phosphate functional group of alpha naphthyl phosphate, resulting in alpha naphthol. A diazonium salt, such as o-dianiside (Fast Blue B), is applied and binds to alpha naphthol, producing a purple color change. This mechanism

occurs rapidly, with samples presumptively positive for seminal fluid within 60 seconds of reagent application. However, isoforms of acid phosphatase are present in vaginal secretions [37] and serum [15] (**Figure 1.2**). Although the number of false positives is relatively high, the use of AP Spot Test has historically been presumptive in nature [38] and is often performed as a quick screen for sexual assault evidence prior to sperm scoring.

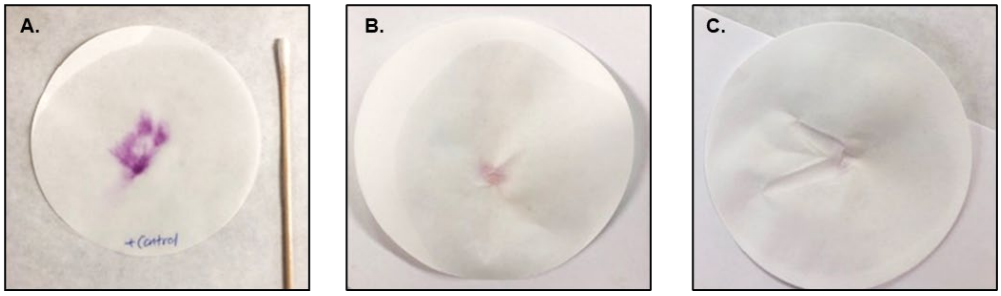


Figure 1.2. Positive experimental results of AP Spot Test on a (A) positive control swab containing semen diluted 2-fold measured at 10 seconds, (B) a vaginal swab containing semen diluted 1,000-fold measured at 45 seconds, and (C) a semen-free vaginal swab measured at 45 seconds. © 2021 by Catherine Brown.

Similarly, saliva identification relies heavily on the detection of amylase. Three classes of amylase have been characterized, with alpha amylase being of interest to forensic investigations. The isoenzyme salivary alpha amylase is produced in the parotid and submandibular glands and is responsible for the breakdown of long-chain carbohydrates. Examples of enzymatic assays for saliva detection are the starch iodine diffusion gel and commercial assays such as SALIgAE® (Abacus Diagnostics) and Phadebas® Amylase Test (Phadebas®). For starch iodine diffusion gels, agarose gel is embedded with starch molecules which are subsequently cleaved by amylase present in a sample. When stained with iodine, intact starch will turn blue, showing amylase enzyme activity by the absence of blue color (**Figure 1.3**). Similarly, SALIgAE® and Phadebas® use dye-labeled starch molecules, and when cleaved by amylase, the dye is released, and a color change is observed. The specificity of these tests is poor, as they will react with any type amylase, such as pancreatic alpha amylase or beta amylase in bacteria, producing a high false positive rate. For example, RSID™-Saliva (an antibody-based assay discussed in the next section) and SALIgAE® have been shown to cross-react with urine, breast milk, and feces [39]. A comparison of the three

enzymatic methods was performed, with a modified starch iodine test that incorporated centrifugation in absence of agarose gel. The sensitivity and specificity of the three methods was assessed in addition to a series of mixed source samples and casework-type samples. It was concluded that Phadebas® Amylase Test performed with the greatest sensitivity, detecting down to a 200-fold dilution of human saliva [40].

Enzymatic assays rely largely on color-based interpretation of results. Although it may be fairly obvious with concentrated samples, the ability to visualize a color change with trace samples is less than straightforward. Furthermore, with the testing of mixed source samples, the presence of a non-target matrix such as blood can impede the visualization of the necessary color to interpret a positive result. For example, the SALIgAE® assay is interpreted based on the change of the reagent from clear to yellow. Even with dilute blood samples containing saliva, the positive color change can appear more orange in nature.

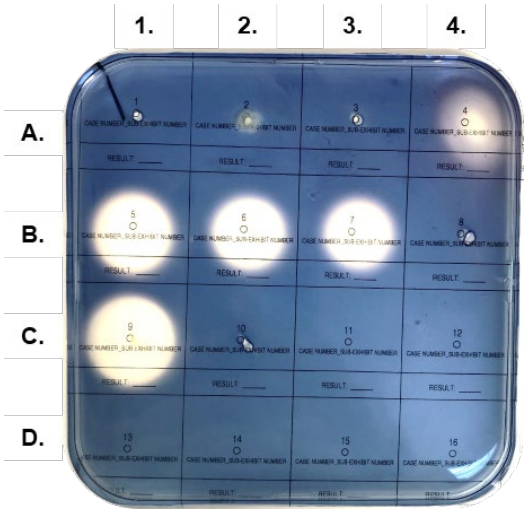


Figure 1.3. Experimental results of a starch diffusion gel depicting (C1) neat saliva, (C2) negative control, (B1) saliva diluted 10-fold, (B2) probiotic yogurt, (B3) triple hop India pale ale style beer, and (A4) human breast milk. © 2021 by Catherine Brown.

1.2.5 Antibody Assays for Body Fluid Identification

The most widely employed type of test system in operational laboratories are the antibody-based assays. There is a multitude of commercially available assays on the market, many of which achieve greater specificity in comparison with the colorimetric and enzymatic assays described

above. The theory behind these commercial assays is centered upon the binding events that occur between an antibody and its complementary antigen [15].

The production of antibodies begins with introducing a foreign antigen to a host organism, such as a rabbit, to stimulate an adaptive immune response. The foreign antigen serves as the target of the immunological response. The host organism begins producing antibody-forming cells, which can be isolated and cultured. As cells begin to divide and increase antibody production, the antibodies can be subsequently isolated for use in exogenous assays [41]. Antibodies that are capable of binding to a multivalent antigen are referred to as polyclonal antibodies. In comparison, antibodies that bind to a single epitope are defined as monoclonal antibodies. Polyclonal antibodies display stronger activity because several epitopes can be recognized for a single binding event. However, monoclonal antibodies are more specific.

Antibody-based assays, either in the form of an enzyme-linked immunosorbent assay (ELISA) or an immunochromatographic assay, employ a series of dye-labeled mono- or polyclonal antibodies. For ELISA tests, antigen is introduced to wells coated with antibody. Once bound, dye-labeled antibody conjugate is added to create a sandwich complex. Reagents are then added to cause a color change in wells containing dye-labeled antibody in the sandwich complex, which is read by a spectrophotometer. ELISA testing has largely been replaced with the use of immunochromatographic assays, also referred to as lateral flow assays. Simply, a liquid sample is introduced to the sample well of a cassette, where dye-labeled antibody present will bind target antigen in the fluid sample. As the sample migrates down the membrane, immobilized antibody in the test region of the cassette will bind, creating the sandwich complex similar to that of an ELISA. Sample well antibodies will continue migrating to the control region of the membrane and interact with immobilized antiglobulin. The control zone will exhibit a positive line because the antiglobulin will bind to the dye-labeled antibody that originated in the sample well whether antigen is present or not [15] (**Figure 1.4**). A positive identification is made by the presence of a colored line in the test zone and the control zone of the viewing window. A negative result is recorded when only a

colored line is observed at the control zone. An invalid result entails the absence of a colored line in the control zone, indicating the test should be repeated.

Commercial assays from various manufacturers, such as Independent Forensics of Illinois [42–44], Abacus Diagnostics [45,46], and Seratec [47–49], are currently on the market for the detection of peripheral blood, menstrual blood, saliva, semen, and urine. Regardless of manufacturer, a similar construction to the lateral flow assay is employed, but they vary in target antigen and antibody. For example, RSID™-Semen (Independent Forensics of Illinois) targets the protein semenogelin for seminal fluid identification, whereas ABAcard® p30 (Abacus Diagnostics) and PSA Semiquant (Seratec®) both target the protein prostate specific antigen. In general, dye-labeled monoclonal antibodies will be present in the sample well and unlabeled monoclonal antibodies will be immobilized in the test zone. Manufacturers have opted for the use of monoclonal antibodies to increase the specificity of the assay.

Lateral flow assays have proven rapid and simple to perform for screening purposes; however, they have a limited sensitivity range. False negative results can occur if too much target antigen is introduced into the sample well. This phenomenon, referred to as the high-dose hook effect, results when excess target antigen migrates along the membrane, resulting in competitive binding with bound antibody-antigen complexes at the test zone. The excess antigen binds to the immobilized antibody, preventing the formation of a sandwich complex. To avoid the high-dose hook effect, the sample should be diluted to reduce the amount of antigen applied to the membrane. Conversely, the lower sensitivity limit of lateral flow assays has been well documented. For example, a validation study of RSID™-Semen reports a sensitivity limit of 2.5 nL of semen [50], with a similar study reporting 50 nL of saliva as a lower limit of detection for RSID™-Saliva [51].

Similar to enzyme-based tests, antibody-based assays are categorized as presumptive because the binding specificity and avidity are not absolute, even with monoclonal antibodies. The reading and reporting of results create instances of analyst subjectivity when interpreting color change, particularly with low-level samples. Although these assays are marketed as confirmatory tests, they are prone to high false positive rates. For example, an internal assessment of lateral

flow assays for seminal fluid identification was previously performed. Semen-free vaginal swabs were tested using RSID™-Semen, ABAcard® p30, and PSA Semiquant. Of the 100 samples tested, RSID™-Semen returned a false positive rate for 6% and both ABAcard® p30 and PSA Semiquant demonstrated a false positive rate for 17% [52]. False positive reactions have also been reported for extracts recovered from condoms when evaluated with PSA Semiquant [53] and extracts subjected to topical lubricants with ABAcard® p30 [54]. Furthermore, when evaluated in absence of body fluid, immunochromatographic assays have been shown to display false positives in the presence of organic acids, lending insight into the mechanism behind which dyes are bound to sample well antibodies [55]. Therefore, reliance on antibody-binding as a confirmatory assay is not supported by the literature and forensic analysts should use caution in reporting of results.

In summary, traditional techniques for body fluid identification lend value to forensic investigations but suffer from numerous limitations that have suppressed the progression of the field as a whole. Regardless of test classification selected for serological screening, perhaps the greatest limitation is the lack of multiplex testing. All of the previously described classifications are single-plex assays. Furthermore, there is an obvious lack of confirmatory tests available for target fluids or lack of tests in general for fluids such as vaginal/menstrual fluid. It is for these reasons that operational laboratories have shifted to a “direct to DNA” workflow, where serological screening is omitted in favor of more sensitive genetic typing chemistries. For more than two decades, the forensic community has focused on improving the sensitivity and robustness of DNA profiling. While advances have made it possible to individualize biological traces on challenging types of evidentiary material, DNA alone does not readily indicate the body fluid source from which it was extracted. Serological testing to identify the body fluid from which a DNA profile has been generated, however, can provide vital contextual information to facilitate a successful prosecution. While this approach has a number of advantages, serological testing is especially important when the item of evidence in question (*e.g.,* a towel or bedding) does not readily lend itself to an interpretation of likely contact to the same degree as an intimate swab or underwear where the mere presence of suspect’s DNA may be sufficient.

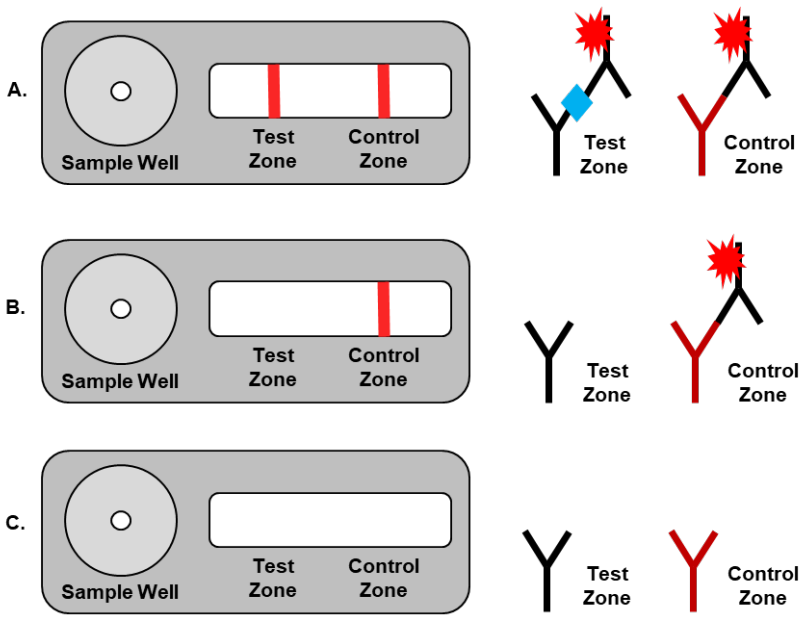


Figure 1.4. Schematic of a lateral flow assay, depicting a (A) positive identification, (B) negative identification, and (C) invalid test. Blue diamond represents target antigen. © 2021 by Catherine Brown.

1.3 Next Generation Techniques for Body Fluid Identification

Serology testing, at one point in time, was heavily regarded as a means of not only identifying a sample but individualizing a biological stain. With the advent of PCR amplification and DNA profiling, the novelty of serology screening began to fade. Practitioners, researchers, and academics have exponentially grown the capabilities of genetic typing since its first use in a criminal investigation in the mid-1980s. DNA profiling was propelled into the public eye and swiftly exceeded expectations as to the information attainable. However, this rapid change in the field of forensic biology left advancements in serology testing as an afterthought. With the ability to deconvolute profiles originating from multiple contributors, generate partial profiles from DNA quantities less than those present in a single cell nucleus, or elucidate phenotypic characteristics from SNP locations, the sensitivity limits of modern genetic typing have remained unchallenged. Given the substantial limitations characteristic of traditional serological procedures, several next generation approaches have been proposed and evaluated by researchers. DNA methylation, RNA assays,

Raman spectroscopy, and proteomic techniques seek to advance serological testing to sensitivity and specificity levels currently achievable with modern genetic typing chemistries.

1.3.1 DNA Methylation Assays for Body Fluid Identification

Epigenetics is the study of changes in gene expression that result from modifications other than those made to the DNA sequence. These changes can be categorized as those made to DNA nitrogenous bases or those made to histone proteins after the process of translation [56]. For the purposes of this section, DNA base modifications will be discussed, specifically cytosine/5'-CpG-3' methylation or CpG sites. There are approximately 30,000 CpG sites within the human genome [57]. At these locations, methyl groups are added to the 5' carbon position on cytosine rings and are adjacently followed by a guanine base pair. In vivo, this process is carried out by the enzyme methyltransferase. Modifications occur largely in promotor regions upstream of target gene sequences. In the event a promoter region becomes methylated, the region becomes less accessible by transcription factors and gene transcription is reduced or halted. Therefore, the mapping of DNA methylation regions can provide useful information, not only for human disease, but for forensic purposes. Targeting specific gene regions and mapping the presence or absence of DNA methylation has the ability to generate a methylation fingerprint for body fluid identification.

The most commonly employed method for determining DNA methylation patterns is with the use of bisulfite conversion, a chemical modification made to unmethylated cytosine residues [58]. With the addition of bisulfite, all unmethylated cytosine residues are replaced with uracil. When amplified using PCR, resulting amplicons will vary depending on the presence or absence of methylation. This change in sequence is analyzed and compared among fluids and tissues to determine methylation regions suitable for identification. Methylation sequences can be analyzed using a variety of techniques such as methylation-sensitive single-base extension assay (SNaPshot™), pyrosequencing, methylation specific PCR, and high resolution melt (HRM) analysis. Tissue-associated differences in the form of hypo- and hypermethylated regions are mapped and compared between target fluids for determination of methylation-specific patterns.

The first use of DNA methylation as a strategy for body fluid identification was reported a decade ago [59]. Restriction enzyme PCR targeting methylation regions for blood, semen, saliva, and skin identification was performed. Original research has focused on evaluating a single fluid or amplifying a single target with the use of pyrosequencing. This procedure describes a sequence by synthesis technique. Simply, a chemiluminescent signal is produced after the addition of each nucleotide during sequencing. Therefore, the signal measured is proportional to the number of nucleotide bases added [60]. Using this technique, procedures for the identification of blood, semen, saliva, and skin have been developed and validated [58,61]. The robustness of epigenetic profiling was illustrated by the analysis of samples with low input DNA, the presence of amplification inhibitors, and aged/degraded samples. More recently, with the use of microarray technology, 150 candidate markers for blood, saliva, semen, vaginal fluid, and menstrual blood identification have been described. Through more rigorous experimentation, a total of nine markers were validated for use and assay sensitivity rivaled that of contemporary STR genetic analyses [62]. In a similar study that utilized restriction enzyme PCR, eight CpG markers were identified and evaluated simultaneously with two control regions [63]. To date, a multiplex assay for blood, semen, saliva, and vaginal fluid has been developed with a single PCR procedure [64]. Upon comparison with previously developed single-plex assays, the multiplex assay produced comparable methylation trends.

Perhaps one of the most promising aspects of epigenetic analysis is the potential differentiation between peripheral and menstrual blood sources. The embryonal fyn-associated substrate (EFS) gene, which encodes for a scaffolding protein linked to immune function, was demonstrated with bisulfite pyrosequencing to have specific methylation patterns for peripheral blood when evaluated in a large population [65]. In a similar analysis of 11 CpG sites, the marker BLU2 encoded by the gene C16orf54, was identified as a discriminatory marker between peripheral and menstrual blood [66]. In regard to assay sensitivity, an original study that employed pyrosequencing illustrated promising results for blood, semen, and saliva; however, an input of 10 ng of DNA was necessary to execute the procedure [67]. The same research group continued

refining the procedure, opting for HRM analysis, citing 1 ng of DNA input to be sufficient for successful analysis [61].

Advantages to exploring DNA methylation expression patterns for serological identification include its ability to multiplex target fluids, its high sensitivity and specificity, and its compatibility with existing DNA-based methods. Nevertheless, DNA methylation patterns exhibit high rates of variability between sexes, individuals, and populations. Furthermore, forensic evidence is more often than not in some form of degraded state upon the start of analysis, with DNA loss or fragmentation being of concern for the optimization and validation of assays for body fluid identification. Additional advancements in assay sensitivity and analysis of authentic samples would be beneficial for future implementation in operational laboratories.

1.3.2 RNA Assays for Body Fluid Identification

In a similar fashion to differentially methylated regions of DNA typed for epigenetic identification, gene expression patterns have been targeted for messenger RNA (mRNA) and micro RNA (miRNA) assays for body fluid identification. Both forms of RNA assay seek to detect gene transcripts that display specificity to a cell or tissue type. RNA serves as the molecular intermediate for the translation of DNA into protein and is formed during the process of transcription. This process is catalyzed by RNA polymerase, the enzyme responsible for reading the DNA template and matching complementary nucleotides to form mRNA and other small RNAs. The transcription unit, or the stretch of DNA template that spans from the promoter region to the terminator sequence, produces a single RNA molecule. Post-transcription, the process of alternative splicing removes interfering introns and pieces together the desired exons for the generation of a unique RNA transcript fit for translation.

1.3.2.1 Messenger RNA Profiling

mRNA profiling is performed by analyzing terminally differentiated cells, where gene expression is developmentally regulated. By observing transcriptionally silent cells versus actively transcribed cells, a pattern of gene expression unique to a group of cells is established. With body fluids containing a mosaic of cell mixtures and secretions, multiple gene expression patterns and

associated mRNA markers can be exploited for the detection and identification of such fluids. The use of mRNA profiling for body fluid identification purposes was first reported in 2003, with a proof of concept study developed and candidate genes proposed for saliva identification [68]. It was demonstrated that sufficient quantities of total RNA could be isolated from cells, especially dried stains that had been solubilized, with mRNA measured through the detection of housekeeping genes such as GAPDH. This original work was further developed into a multiplex assay for the identification of blood, semen, saliva, and vaginal fluid, with each fluid characterized by two genes [69]. Gene transcripts for menstrual blood were subsequently added and an interpretation strategy was proposed to illustrate the specificity of each mRNA marker [70]].

With the promising results of this methodology, a series of collaborative studies organized by the European DNA Profiling Group (EDNAP) was initiated to further demonstrate the robustness, reliability, and sensitivity of the technique. For each study, participating laboratories could perform either an RNA extraction or an RNA/DNA co-extraction on neat stains, diluted stains, and mock casework samples of human and nonhuman origin. All of the mRNA panels described herein were simultaneously evaluated with markers for housekeeping genes. The first study focused on blood identification and employed two mRNA multiplexes: a duplex targeting genes for hemoglobin alpha and hemoglobin beta, and a pentaplex targeting genes for ALAS2, CD3G, ANK1, SPTB, and PBGD [71]. The following study tested the methodology for the identification of saliva and semen [72]. A saliva triplex included genes for histatin-3, statherin, and mucin-7. A semen pentaplex included genes for protamine 1 and 2, prostate specific antigen, semenogelin 1, and transglutaminase 4. Continuous collaborations then focused on vaginal secretions and menstrual blood [73]. Two triplex panels were selected for menstrual blood: the first containing genes for matrix metalloproteinase 7, 10, and 11, and the second triplex targeting Msh homobox 1, LEFTY2, and SFRP4. Vaginal fluid identification was based on two triplexes. The first targeting mucin-4, myozenin-1, and CYP2B7P. Interestingly, the second triplex targeted genes of *Lactobacillus* bacteria (Ljen, Lcris, and Lgas), the most abundant vaginal bacteria in the female reproductive tract. A similar panel of matrix metalloproteinases was evaluated over the course of the female uterine cycle, confirming the lack

of specificity needed to discriminate with vaginal fluid and that target abundance changes over a typical menstrual cycle [74]. The final collaborative study demonstrated the use of mRNA profiling for the detection of skin and contact traces [75]. Of the eight gene markers evaluated, two genes were consistently identified. The inconsistency of detection is likely attributed to the low quantities of genetic material deposited by shed skin cells.

Original research utilized reverse transcription-polymerase chain reaction (RT-PCR) procedures for method development [68–70]. Alternative methods using Real-Time PCR [76,77] and high resolution melt (HRM) analysis [78] were also proposed during early stages of method development. More recently, a digital gene expression method utilizing solution hybridization of NanoString® probes was explored [79]. Although RT-PCR is capable of producing informative quantitative data, assays are limited to the number of targets that can be assessed. Digital gene expression methods have the ability to count the number of individual transcripts for each sample and mimics the use of next generation sequencing for genetic analysis. This methodology was developed to assess the ability of predicting activity level of a perpetrator based on the quantity of sample present through the use of likelihood ratio statistical calculations. A custom set of genes was created: 23 gene targets for the identification of blood, menstrual blood, saliva, semen, skin, and vaginal secretions as well as an additional 10 targets for housekeeping genes. Authors note that saliva biomarkers exhibited poor specificity in relation to previously conducted work, but the remainder of targets performed as expected and exhibited high specificity and sensitivity. This method development preceded the use of massively parallel sequencing [80] and SNP characterization [81]. Reported read counts of mRNA markers for target and non-target fluids provided additional insight into the specificity of biomarkers selected in previous studies. Additional future directions for this methodology comprise the inclusion of other RNA products, such as circular RNAs [82], into multiplex panels to further investigate the sensitivity and specificity achieved with mRNA typing.

In summary, the use of mRNA profiling for forensic purposes has been well developed and evaluated. Opposition to implementing this technology in operational laboratories remain from the

fear of mRNA stability, particularly in challenging sample types. However, the quality and quantity of RNA has been demonstrated in both aged samples and samples subjected to environmental insult [83,84].

1.3.2.2 Micro RNA Profiling

Comparably, miRNA are the small non-coding RNA sequences that attenuate protein translation and have been targeted as viable biomarkers for body fluid identification, but to a lesser extent than mRNA profiling. Given their inherent size (approximately 20-25 bases in length), miRNAs emerged as an attractive marker for the identification of highly degraded body fluids. A majority of miRNA transcripts were selected based on their function within a specific tissue. However, these markers proved challenging to type using traditional primer binding strategies given their shortened length. Overall, three categories of methods are prevalent in the literature: microarray hybridization methods, quantitative reverse transcription PCR, and RNA sequencing. The combination of these techniques for miRNA profiling has led to a series of inconsistent results reported among research groups [85].

Original research into their use for forensic purposes was reported in 2009, with nine candidate miRNAs for blood, semen, saliva, vaginal secretions, and menstrual blood identified from a pool of 452 markers through the use of RT-PCR [86]. When evaluated against 21 tissue types, the panel exhibited high specificity and potential for future method development. A similar study, utilizing quantitative PCR, confirmed miRNA markers for peripheral blood and semen but described a lack of support for candidate markers for saliva, vaginal fluid, and menstrual blood [87]. Conversely, a separate research group identified and validated a proof of concept triplex of markers for both blood and saliva detection [88].

Research data presented for mRNA followed a natural evolution and build in regard to achievements and application. The same cannot be said for miRNA profiling. Original methodologies present conflicting data on the ability to characterize forensically relevant body fluids, and with large amounts of miRNA markers to evaluate and multiple methodologies performed for analysis, this area lacks a clear direction. In order to elucidate a more reliable path

forward, researchers turned to statistical modeling of data. A linear regression model for the identification of menstrual blood was generated [89]. Although trained and tested with a limited sample set, the model produced accurate identifications. But perhaps the most interesting result of this study was the reported limit of detection. The total RNA quantity for the proposed methodology was measured to be 50 pg for menstrual blood analysis, whereas comparative mRNA research performed at this time, reported an input of 5 ng of total RNA [89]. Apart from the first use of miRNA profiling [86], a full panel of biomarkers underwent rigorous experimentation prior to presenting candidate selections. From a pool of 1,700 miRNAs, a preliminary panel of 203 markers were selected for use in a microarray against blood, semen, saliva, and vaginal fluid. Once the preliminary panel was paired down, a set of eight miRNA targets were proposed as candidates: miR-484 and miR-182 for blood, miR-223 and miR-145 for saliva, miR-2392 and miR-3197 for semen, and miR-1260b and miR-654-5p for vaginal secretions [90].

More recently, statistical decision making has been continuously applied on validated panels of miRNA markers and additional interpretation strategies in normalizing data to housekeeping genes has been proposed [91–94]. Using these strategies, the greatest amount of body fluid matrices (blood, semen, saliva, vaginal fluid, menstrual blood, urine, feces, and sweat) have been successfully sequenced at quantities consistent with low-level forensic evidence [92], with authors noting interference in sequence annotations due to bacterial presence in certain fluids. A notable benefit of miRNA profiling is the ability to identify markers from DNA extracts, an attractive quality for caseworking laboratories seeking to streamline sample preparation procedures from commercially available DNA extraction kits [95].

Although research strongly supports the use of miRNA profiling for the identification of blood and semen, there is a lack of evidence encouraging its use for vaginal secretions and menstrual blood. There is conflicted data presented on the consistency of saliva miRNA marker specificity. In regard to data analysis, miRNA profiling does not lend to straightforward interpretation [93]. Furthermore, the stability of miRNA markers is affected by a myriad of factors, such as temperature, changes in pH, and radiation [85]. Disease state and the function of miRNA in

proliferation and inflammation have been well documented [96,97], illustrating that miRNA expression is not static within the human body. In conclusion, miRNA profiling requires supplementary research to provide a clear and convincing model for use as an alternative means for serological screening.

1.3.3 Raman Spectroscopy for Body Fluid Identification

Spectroscopy is a technique that measures the interaction between matter and radiation to elucidate physical structure at the molecular or atomic level. There are many different types of spectroscopy, each with a unique application and can be distinguished based on the interactions that take place. This section will focus specifically on Raman spectroscopy. This type of spectroscopy stems from inelastic Raman scattering which occurs when the matrix of interest is excited with a high-powered laser, causing the vibrational and rotational energies of the molecules to shift [98]. A photon beam is used to excite a molecule into a virtual energy state. The photon emitted from the excited molecule will be measured at a higher or lower energy state than the photon used to initiate excitement. This light scattering event results in different rotational and vibrational states of the atoms and molecules present. The emitted photon, therefore, shifts to a different frequency. The vibrational signature, or the specific change in energy, of the molecules present is recorded and used to identify the material. This technique requires minimal to no sample preparation and is nondestructive in nature; however, the target matrix must be concentrated in order to produce a strong vibrational spectrum.

In the field of forensic science, Raman spectroscopy has longstanding use for applications in drug chemistry [99,100], paint and ink analysis [101,102], and the examination of trace evidence [103,104]. For serological purposes, this next generation technique demonstrates gains in specificity when considered alongside traditional colorimetric, enzymatic, and antibody-based assays. The Raman spectra produced for forensically relevant body fluids is determined by the entire molecular composition of the specific fluid. Original research initiatives focused on using Raman spectroscopy to generate vibrational signatures of single source body fluids. It was hypothesized that the spectra produced would reflect the unique composition and complexity of

each fluid and serve as a novel tool with greater discriminatory power than historical techniques. Promising results were obtained from preliminary studies containing limited sample sizes, with experiments performed under highly controlled laboratory conditions. With the successful characterization of molecular components consistent with human semen, blood, saliva, vaginal fluid and sweat, researchers saw promise in the use of Raman spectroscopy for more challenging sample types and envisioned robust reporting with advanced statistical calculations [105,106]. When focusing specifically on human blood characterization, early studies reported the ability to make a positive identification of human blood that had been diluted 250-fold; however, the Raman signatures produced varied among individuals and even within samples supplied by a single individual [107].

More recent advancements have been illustrated through the analysis of samples that more closely resemble those collected from a crime scene. Although capable of detecting components of human blood and semen from various substrates, sensitivity was shown to decrease in relation to previous studies, with human blood detected at a 100-fold dilution. Raman spectroscopic techniques were unable to identify signatures of human blood from laundered substrates but were demonstrated to be unaffected by evidence treated with luminol [108].

Raman signatures for human blood have produced consistent spectral components of hemoglobin, heme, and tryptophan, with multiple studies demonstrating the ability to discriminate human and nonhuman blood sources [109–111]. Components used for semen identification have been reported to display vibrational signatures consistent with acid phosphatase, citric and lactic acid, urea, and zinc [112,113]. The use of non-specific signatures, such as those of lactic acid, decrease the confidence of an identification, based on endogenous levels of non-specific signatures in various fluids and tissues. However, a more recent study was successful in detection signatures indicative of prostate specific antigen. This protein is found in high concentrations in semen, with low concentrations in serum and male urine, both of which have the potential to produce a false positive result. It was successfully demonstrated that the detection of prostate specific antigen using Raman spectroscopy can eliminate a false positive detection from male urine

when simultaneously assessed with dilute semen samples [114]. Characteristic components of vaginal fluid, saliva, and sweat have been reported [113], but limited research as to the extent of substrate interference and contamination on the ability to form an accurate identification of these fluids has been conducted.

With the development of portable Raman systems, the use of this technology directly at a crime scene allows for rapid sample analysis. Traditional screening techniques for suspected blood stains, such as the use of leucomalachite green, can damage genetic material [115]. Advantages of screening with Raman spectroscopy are the technique is nondestructive and it maintains the integrity of the evidence for additional testing. The success of portable Raman spectroscopic analysis has been demonstrated for both human and nonhuman blood samples, in addition to positively characterizing a 3 month old stain [109].

Perhaps the most alluring attribute for the use of Raman spectroscopy for body fluid identification is the ability to use advanced data analysis tools to estimate error rates. The field of forensic science has been scrutinized on multiple accounts in regard to the accuracy and reliability of testing performed. As such, to determine if a methodology is valid in federal court, the procedure must not only have been tested, but potential error rates should be stated [116]. Chemometrics applies statistical theories to large and complex data sets [117]. For example, it was demonstrated that differentiation of five body fluids can be achieved with the use of interval partial least squares discriminant analysis (iPLSDA) [113]. An important aspect of this study involved the separation of datasets into those used for calibration and those used for validation. Similar practices are utilized for determining type I and type II error rates for genetic typing method validation in operational laboratories. More recently, a detection algorithm was successfully applied to the characterization of semen in the presence of substrate interferences [118]. This study sought to address one of the main limitations of Raman spectroscopy for body fluid identification. Substrates can cause interference signatures during evaluation, but given trace level sample analysis, signatures can be sufficiently more intense and mask those of the fluid components.

As such, limitations to Raman spectroscopic analysis of body fluid evidence stem from the inability to completely remove background interferences caused by substrates and sensitivity constraints caused by low-level samples. Furthermore, given the inherent complexity of biological matrices, advanced understanding of statistical theories is needed for deconvoluting components in a mixed source sample. Although the current research is supportive of this next generation technique, a majority of studies focus on human blood and semen identification, with a limited number of studies reporting on vaginal fluid [119], saliva [120], and sweat [121]. Nevertheless, this technique has been explored additionally for sex and race determination of forensic samples [122].

In summary, research into the use of Raman spectroscopy for body fluid identification has demonstrated a refined technique that is nondestructive, rapid, and confirmatory in nature. With the advent of portable instrumentation, this technique is an attractive tool for screening of biological material at a crime scene.

1.3.4 Proteomic Assays for Body Fluid Identification

The final next generation serological technique is the use of proteomic assays and will be the focus of research disseminated in later chapters. Proteomics is the large-scale study of the proteome and protein expression. It describes not just a single technique, but a family of scientific approaches that exploit protein structure and function for the purpose of further elucidating the enigma that is the human proteome. Simply, proteins are an attractive diagnostic marker because they represent intermediate phenotypes for disease and illustrate the effect of non-genetic risk factors on cellular function [123]. This concept is adopted for use in forensic biology. Forensic proteomics uses advanced analytical techniques, such as liquid chromatography and mass spectrometry, in combination with bioinformatics to analyze biological evidence. In addition to applications for historical and archeological investigations, proteomic techniques have been developed for use in the greater forensic biology workflow for the identification of body fluids, such as in cases of sexual assault and rape.

Biological evidence is, more often than not, subjected to unfavorable conditions. These conditions can include both environmental in the literal sense, such as extended UV degradation

or excessive heat, or environmental in the biological sense. In regard to the latter, natural protein function within biological systems can result in endogenous degradation of other protein material. For example, the protein prostate specific antigen in seminal fluid functions by cleaving semenogelin isoforms, resulting in the seminal fluid matrix to lose its gel-like structure. Both prostate specific antigen and semenogelin are attractive protein targets for seminal fluid identification. However, certain serological screening assays rely on protein activity, and therefore a protein's confirmational structure, for testing purposes. It would be prudent for serological identification to rely on protein presence more heavily, especially for degraded evidence. Proteomics is an attractive alternative to chemical and enzymatic assays because it can detect protein presence. Furthermore, with the inherent sensitivity of analytical techniques, proteomics has the ability to confirm protein presence based on molecular weight.

As previously discussed, proteins are highly abundant within the human body and exhibit an immense variety of functions. Therefore, proteins and their peptide fragments were a natural target for advancing serological testing strategies from traditional activity-based and ligand binding assays. One of the main challenges of proteomics is correctly determining the amino acid sequence of a protein target, as many proteins can be composed of the same amino acids but in a different order, ultimately lending to their unique properties. Liquid chromatography-mass spectrometry provides the necessary structural and molecular weight information needed for accurate identifications.

1.3.4.1 Theory of Modern Proteomic Techniques

1.3.4.1.1 Protein Digestion and Sample Preparation

Intact protein identification is challenging given the inherent size of protein molecules. For example, prostate specific antigen in seminal fluid has a mass of 28,741 Daltons. In comparison to small molecule mass spectrometry utilized for alternative forensic applications, proteins can have a molecular weight one hundred times greater than that of a single drug molecule. In addition, proteins exhibit variable charge states, sequence variants, and post translational modifications that complicate mass spectral analysis. By cleaving large protein molecules into peptide fragments,

charge state variations can be controlled, and sequence specific information can be obtained through the use of bioinformatics. Ideal peptide targets can range between 8 to 25 amino acids in length. As opposed to intact protein identification, peptides ionize well, fragment in a predicable manner, and produce good chromatography.

Protein digestion is comprised of 4 simple steps: denaturation, reduction, alkylation, and cleavage [124]. Denaturing agents can be used to compromise the quaternary, tertiary, and secondary structure of proteins, causing them to lose their native three-dimensional structure and expose amino acid side chains by breaking molecular interactions. Commonly utilized detergents, such as SDS, are not compatible with mass spectrometers. However, denaturing agents such as urea, trifluoroethanol, or guanidine thiocyanate compete for hydrogen bonding within the complex protein folds, resulting in exposure of amino acid side chains and subsequent linearization. While the protein is being denatured, bonds are simultaneously being reduced. A commonly utilized hydrophilic reducing agent is tris(2-carboxyethyl)phosphine (TCEP), selected for its solubility and stability in aqueous solutions at both acidic and alkaline pH. An SN2 nucleophilic substitution reaction occurs when TCEP is introduced to the sample. Denaturation and reduction of protein targets is necessary in order to ensure complete exposure for enzymatic digestion. Alkylation is performed in order to cap exposed and reactive cysteine thiol groups, preventing reformation of disulfide bonds or other non-specific reactions. Iodoacetamide is an irreversible alkylating agent commonly utilized during protein digestion processes. This compound causes rapid carboxymethylation of reduced cysteine residues preventing disulfide bond formation. The peptide sequence is then ready for cleavage, producing more manageable peptide fragments for analysis. Protease enzymes exhibit specificity with regard to amino acid cleavage sites. For example, the serine protease trypsin cleaves at the carboxylic junction of arginine and lysine residues, yielding predictable and specific peptide sequences.

Following digestion, denaturation agents, salts, and unwanted matrix components are removed prior to analysis via liquid chromatography-mass spectrometry. During peptide cleanup, solid phase extraction (SPE) employs a sorbent material that preferentially separates and removes

any unwanted compounds (**Figure 1.5**). A sorbent material made up of hydrocarbon chains is packed into cartridges. Simply, the sorbent is primed with an acidic organic solvent, which activates the functional groups present and removes any trapped air. The cartridge is then equilibrated using acidified water in order to maximize the sorbent's interaction with the sample matrix. Next, the digested sample material is applied to the cartridge. Slowly, the sample passes over the sorbent material which retains target peptides. Then, the cartridge is washed to remove any unbound material from the sorbent, including residual reagents from digestion processes. Lastly, peptides are eluted from the sorbent bed with acidified organic solvents. The eluant can be directly injected into the liquid chromatograph to begin analysis or lyophilized for storage or concentration.

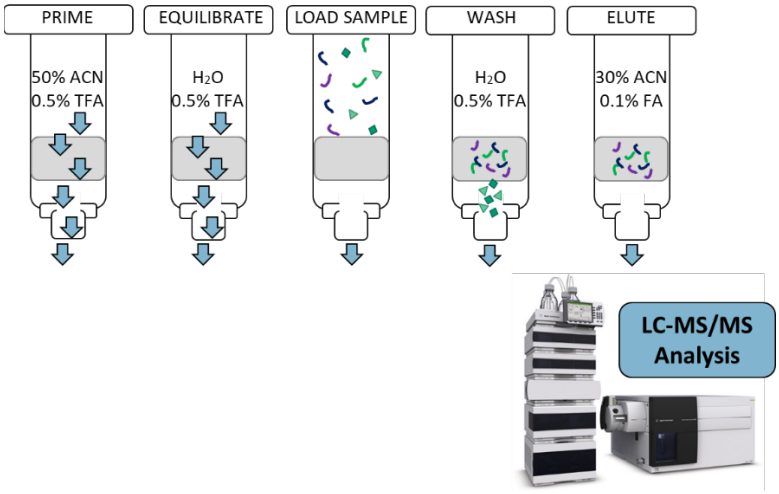


Figure 1.5. Schematic depicting a solid phase extraction procedure for post-digestion cleanup. © 2021 by Catherine Brown.

1.3.4.1.2 Chromatographic Separation and Ionization

Biological samples are subjected to separation through the use of liquid chromatography. Simply, liquid chromatography is the use of a liquid mobile phase to carry target species through a column containing a stationary phase (**Figure 1.6**). The target species will interact with the stationary phase differently than interferents or other target species. As mobile phase conditions change, the target species elute from the column based on preferential interactions. Liquid chromatography is the ideal separation method for proteins because it can analyze samples that span a wide range of analyte polarity in addition to capabilities of analyzing large molecular weight

species. In general, the mobile phase is continuously pumped through the liquid chromatograph. Pre-programmed mobile phase conditions, referred to as the mobile phase gradient, change over the course of a sample run time. For example, mobile phase starting conditions more closely resemble a high aqueous solvent, such as a 2% acetonitrile in water. Separation and elution from the analytical column occur as mobile phase conditions change, with a majority of protein material eluting at approximately 30% acetonitrile in water. Protein material that is not as strongly retained by interactions with the stationary phase of the analytical column will be carried out of the chromatograph by the mobile phase. Eluted material can either be directly detected or further fragmented in a mass spectrometer. The resulting chromatogram displays the elution sequence and amount of material present. Retention time is plotted on the x-axis and represents the time in which a specific analyte eluted from the analytical column. Intensity is represented on the y-axis. If a reference sample is available, retention time for compounds should occur within a designated amount of time.

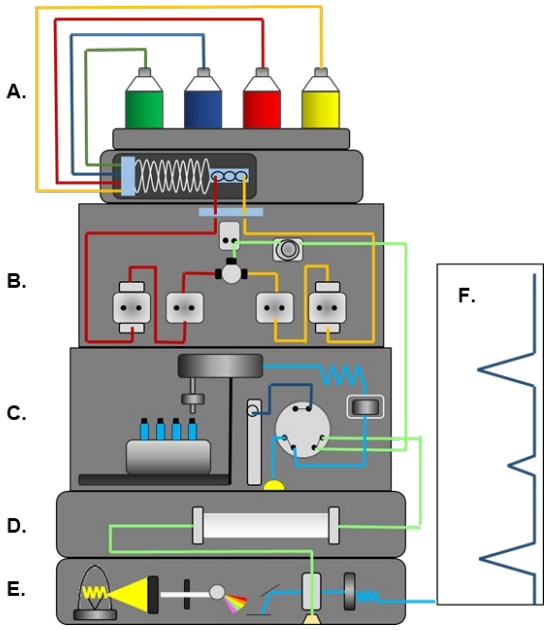


Figure 1.6. Schematic of a liquid chromatograph containing (A) mobile phase components, (B) mobile phase pumps and mixing chamber, (C) autosampler, (D) analytical column containing stationary phase, (E) detector, and (F) resulting chromatogram. © 2021 by Catherine Brown.

Multiple variables can be modified to optimize chromatographic separation. For example, mobile phase flow rate, solvent composition and pH modifiers, stationary phase composition, and column temperature are just a few factors that influence peak resolution, retention time and selectivity. Reverse phase liquid chromatography is well suited for protein separation. This mode of chromatography employs a water-based mobile phase and hydrocarbon stationary phase, such as octadecylsilyl (C18) or octylsilyl (C8) moieties. These moieties are bound to a silica support and intrude into the negative space of the analytical column to interact with analytes carried by the mobile phase. The column chemistry that ensues is fitting for the separation of compounds that are neutral, weakly acidic, and weakly basic. The retention of analytes to the stationary phase is dependent on the analyte's hydrophobicity, and by extension, an analyte's ionization state. Mobile phase is commonly supplemented with an ion pairing agent to control the retention of ionic analytes, as retention time is affected by the ionization state of an analyte.

With continuing structural elucidation using mass spectrometry, eluted analytes must first be desolvated and ionized prior to entering the mass analyzer. The process of electrospray ionization (ESI) is commonly used to transfer eluate from the chromatograph into gas phase ions suitable for mass spectrometric analysis [125] (**Figure 1.7**). A transfer line carries chromatograph eluate into the ESI source, where the eluate is sprayed from a charged capillary as a fine aerosol mist into a heated chamber. Nebulization occurs as solvent is readily evaporated and droplets are formed. The potential difference applied to the capillary allows the droplets to reduce in size. As the radius of the drop decreases, the droplet charge remains constant and repulsion forces within the droplet increase. Eventually, the repulsion stress must be released, and droplets undergo droplet jet fission to produce small, charged particles. ESI is commonly employed in proteomics research because of its ability to produce multiply-charge ions. This inherent characteristic allows additional mass-to-charge ratio measurements to be made even with a mass analyzer that have a limited mass range [126]. Instrumental parameters that can be modified to reach optimal ionization include drying gas temperature and flow rate, nebulizer pressure, and capillary voltage.

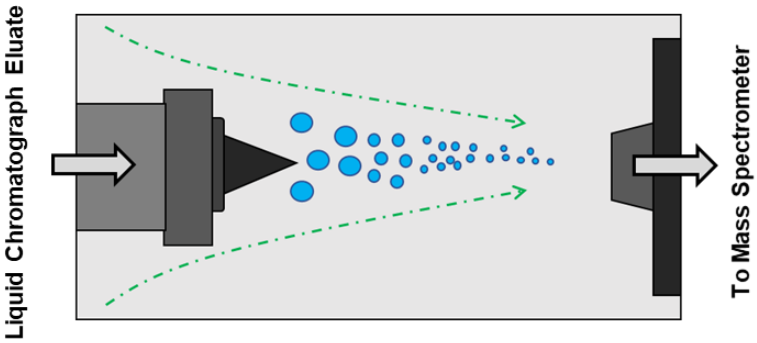


Figure 1.7. Schematic depicting electrospray ionization and the interface between the liquid chromatograph and the mass spectrometer. Green arrows represent the drying gas. Blue circles represent the charged droplets as they reduce in size due to drying and jet fission forces. © 2021 by Catherine Brown.

1.3.4.1.3 Mass Spectrometry Instrumentation and Interpretation

Within the last two decades, significant advances in mass spectrometry-based methodologies have expanded the capabilities and possibilities of scientific achievements. Parameters such as increased dynamic range and quantitative accuracy presented attractive qualities to disciplines such as clinical diagnostics and forensic science. Mass spectrometry as it applies to proteomics has assumed many forms based on the type of analyses conducted.

In a broad sense, mass analysis is the separation and filtration of ions, from which the chemical form of a species, such as its structure and ionization potential, can be elucidated. This chemical form is represented by a mass spectrum or a plot that graphs signal abundance against mass-to-charge ratio (m/z). Three qualities are evaluated when selecting a specific mass analyzer: mass resolution, mass accuracy, and mass range. Mass resolution describes the ability of a mass analyzer to separate two adjacent masses and becomes an important property when coelution of target ions may exist. Mass accuracy illustrates the mass measurement recorded to that of the true mass of the target. And lastly, mass range defines the difference between the highest and lowest measurable m/z. There are several types of mass analyzers that exhibit fundamental differences, each with a desirable application in regard to proteomics.

Quadrupole mass analyzers, which are commonly selected for targeted proteomic analysis, function by filtering ions using electrostatic potentials and selecting ions based on m/z. The quadrupole is constructed out of four poles that are oriented to create a central channel down which charged ions can travel, where they are separated and filtered by electrostatic potentials applied to the four poles. An ion will carry a stable trajectory down the central channel if it oscillates within a narrow radius. On the contrary, a collisional trajectory occurs when an ion oscillates outside of the narrow radius, resulting in collision with the poles and ejection from the central channel. Quadrupole mass analyzers are popular given their low cost and uniform performance across a wide mass range; however, quadrupole mass analyzers have relatively poor mass resolution and, in relation to other mass analyzers, demonstrate a slower speed for scanning travelling ions.

Time of flight mass analyzers exploit differences in kinetic energy to map flight paths and predict ion separation. This type of mass analyzer is attractive for discovery proteomics. Ion clusters are subjected to an accelerating voltage, where they are propelled into the flight tube. The time of flight for each ion cluster is measured from the onset of the accelerating voltage until clusters reach the detector at the end of the flight tube. The speed at which ions clusters travel through the flight tube is dependent on the m/z of the ion [127]. Although instrument calibration is more complex than a quadrupole mass analyzer, time of flight analyzers have the greatest mass range and fast ion scanning speeds.

And lastly, ion trap mass analyzers rely on applied voltages to further group ions with a specific m/z. Ions that do not favor the applied voltage do not cluster and are propelled from the ion trap. In addition, because the ion trap is filled with an inert gas, additional fragmentation of selected ions can occur [128]. Because of this property, the use of ion trap mass analyzers is popular for top down proteomics. These types of mass analyzers display excellent mass resolution and sensitivity; however, they are easily saturated and produce unusual spectra if parameters are not properly configured.

Mass analyzers can be combined in unique ways to increase ion filtration and improve qualities such as mass range and mass resolution. The goal of using multiple mass analyzers in a

single instrument is to gain additional structural information, achieve selectivity, and maximize sensitivity for quantitative analyses. Typical examples of mass analyzer combinations are triple quadrupole (*i.e.,* two quadrupole analyzers separated by a collision cell) (**Figure 1.8**) and quadrupole time of flight. The use of multiple mass analyzers lends to the process of tandem mass spectrometry.

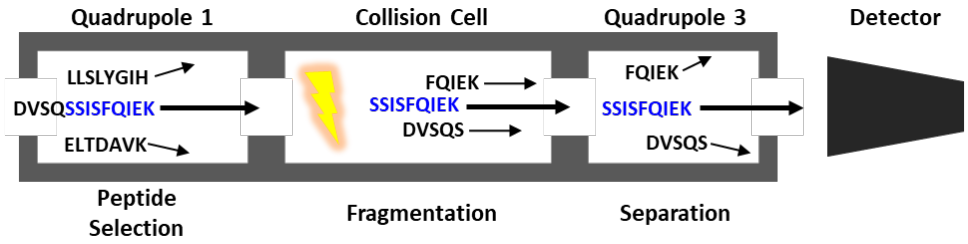


Figure 1.8. Schematic of a triple quadrupole mass spectrometer for the detection of semenogelin 2 peptide DVSQSSISFQIEK precursor and product ions. © 2021 by Catherine Brown.

1.3.4.1.4 Proteomic Methodologies

Once protein targets have been separated, it is important to consider the type of information desired from mass spectrometric analysis. Two main next generation proteomic methodologies include bottom up proteomics and top down proteomics. Bottom up proteomics describes the detection and identification of proteins that are first enzymatically digested into predictable peptide fragments. Protein material is subjected to a series of chemical modifications prior to identification. Denaturation, reduction, and proteolytic cleavage, with an enzyme such as trypsin, break complex protein domains into predictable peptide fragments suitable for mapping and targeted analysis. The retention of ionic species is achieved through the addition of ion pairing reagents, such as formic acid, into the mobile phase.

Bottom up proteomics can be further divided into discovery or shotgun proteomics and targeted analysis [129]. Discovery proteomics is utilized for the unbiased scanning of peptide ions, with specific search parameters applied after data acquisition. This proteomic application earned its title from its use in discovering protein and peptide signatures and is often performed to generate a targeted ion selection list [130]. But in order to create a targeted list, a vast amount of data must first be collected and analyzed. The two most commonly used mass analyzer combinations for data

collection are quadrupole time of flight and ion trap mass analyzers. Quadrupole time of flight (QTOF) mass spectrometry is a popular analytical platform for discovery proteomics, given its mass accuracy and wide dynamic range (**Figure 1.9**). However, in comparison with other analytical platforms, the QTOF system lacks desirable resolution. To address the limitations of QTOF mass spectrometry, the Orbitrap analyzer was introduced. Similar to QTOF, the Orbitrap has a wide mass range, but also provides high mass resolution. A benefit of bottom up discovery proteomics is the limited amount of prior knowledge necessary for data interpretation. By scanning for and measuring the masses of precursor and subsequent product ions, a roadmap of related transitions can be generated.

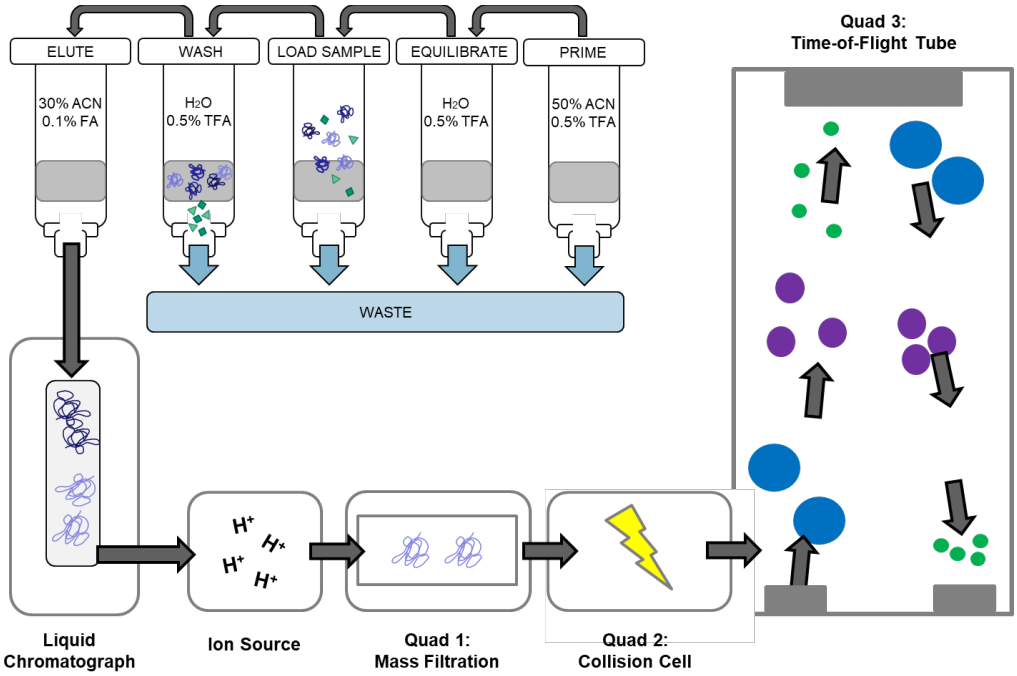


Figure 1.9. Schematic of a proteomic workflow with identification via QTOF. © 2021 by Catherine Brown.

Targeted proteomics seeks to identify an analyte or multiple analytes through the use of a selective ion list [131,132]. Although sample preparation procedures are shared between shotgun and targeted proteomics, the analytical instrumentation differs. A triple quadrupole mass analyzer is commonly selected for targeted analysis. The first quadrupole is set up to selectively scan for a given precursor mass. In the second quadrupole, or collision cell, the precursor mass is subjected

to a charged gas, resulting in further fragmentation. The third quadrupole selectively scans for a specific product ion. Single reaction monitoring (SRM) or multiple reaction monitoring (MRM) are common targeted proteomic methods for determining the absolute and relative quantification of target analytes in a given sample. Isotopically-labeled peptide standards can be simultaneously assessed [133,134]. By comparing peak area responses of endogenous peptide fragments with those of peptide standards, a relative quantitation can be calculated [135].

In comparison with bottom up proteomics, which identifies proteins that have been subjected to enzymatic cleavage, top down proteomics forgoes enzymatic cleavage [136,137]. This category of proteomics can be further classified by conducting intact protein analysis or peptidomic analysis, which describes the identification of low molecular weight proteins, naturally derived peptides, and truncated amino acid sequences of larger proteins [138]. With this type of proteomic analysis, full characterization of proteins can be achieved with increased sequence coverage because analysis is not limited to cleavage products. The eliminated laborious digestion procedures during sample preparation makes for streamlined and expedited sample analysis. Furthermore, post-translational modifications (PTMs) can remain intact and enrich data analysis.

Similar to shotgun proteomics, Orbitrap analytical platforms are a popular selection for top down proteomic analyses. The fundamental construction of the ion trap mass analyzer provides the means for intrinsic fragmentation to occur. Although tryptic peptide analysis provides immense amounts of information from complex samples, the information remains limited to cleavage sites and the length of peptide fragments produced, resulting in some information to be ignored due to lack of unambiguous identification. This becomes mitigated with top down procedures and the migration from peptide-centered to a protein-centered interpretation.

Regardless of methodology selected, peptides will fragment in a consistent manner once in a mass analyzer due to their repetitive monomer assembly. Peptides become protonated as they enter the ESI interface and are selected as precursor ions. Precursor ions are then fragmented into a variety of product ions as polypeptide backbone bonds are cleaved. Peptides are sequenced according to the bond cleavages that occur as they provide important structural information [139].

The cleavage of peptide bonds between the carboxylic acid and amine functional groups of two adjacent amino acids generates product ions that are the sum of their amino acid composition residue masses. With this type of breakage, product ions will contain at least one proton that will mobilize on either terminus group or any of the amide nitrogens within the fragment. To form multiply-charged ions, the analyte must have more than one location that can undergo ionization. Typically for peptide sequencing, basic amino acid side chains, such as those of lysine, arginine, and histidine, serve as excellent proton acceptors. By determining the number of protons present in a mass spectrum, the molecular mass, and ultimately the peptide sequence, can be determined.

1.3.4.2 Proteomics in Forensic Biology

Forensic proteomics has been well-defined for the characterization of microbial traces [140,141], species identification [142], and protein toxin detection [143,144]. However, only within the last two decades has proteomics been proposed as an alternative strategy for forensic serology applications. Research initiatives sought to address the sensitivity, specificity, and reliability of existing traditional screening techniques in addition to providing a means of identification for fluids which lack an existing technique (*i.e.,* vaginal/menstrual fluid).

Original research conducted employed discovery proteomics for the characterization of presumptive and confirmatory peptide signatures for proteins consistent with blood, semen, saliva, vaginal fluid, and menstrual blood. Protein material was cleaved into known peptide fragments, and because each protein contains a unique amino acid sequence, the mass measurements of each fragment can be read and converted into an amino acid alphabet. Perhaps the greatest attribute of proteomic analysis is the ability to selectively target characteristic biomarkers, a quality shared with RNA and methylation assay but lacking in Raman spectroscopy techniques. Body fluids are rich in protein material, a majority of which is shared among cell and tissue types. For example, immunoglobulins are expressed in blood, saliva, and vaginal fluid, given their immune function to neutralize pathogens. Although shared protein material is important, targeting fluid-specific proteins was of interest to forensic researchers. Original candidate biomarkers were selected based on their uniqueness or level of enrichment within a target fluid. Studies described candidate markers

consistent with blood identification as hemoglobin beta and alpha-spectrin; with semen identification as semenogelin-1 and semenogelin-2, prostate specific antigen, and progestogen-associated endometrial protein; and saliva identification as alpha-amylase 1, cystatin SA, and histatin-1 [145,146]. Comparable and additional biomarkers, such as submaxillary gland androgen-regulated protein 3B for saliva and hemopexin for blood, were characterized in a similar study [130]. Tissue specificity of characterized proteins were cross-referenced with existing protein databases to demonstrate the specificity of the developed methodology. In addition, the latter study evaluated reproducibility by fractionation, an important technique to ensure maximum coverage of the human proteome during discovery [130].

In order to further understand the expression patterns among individuals, candidate biomarkers were verified through population studies. With limited sample sizes utilized for biomarker characterization, it was critical to evaluate protein levels in a more diverse sample population. A population of 50 human body fluids were correctly identified by the presence of one or more candidate markers [147]. Authors make a compelling argument that, in comparison with STR markers currently targeted for genetic typing, proteins exhibit greater interindividual expression variability. Although highly specific targets may be characterized in limited samples, they may not be consistently detected or serve as the most robust biomarkers for future method development.

A majority of research aims have included the analysis of forensic-type samples, such as complex mixtures, aged stains, and stains on various substrates [145]. In regard to sexual assault and rape, evidence commonly contains fluids of mixed source. For example, a vaginal swab taken from a rape victim will inherently contain vaginal fluid, but semen and saliva may also be present. It is important to have assays sensitive and specific enough to discriminate a mixed-source sample in order to provide essential information for investigative purposes. When evaluating 2-component mixtures, proteomic techniques were successful in identifying both components, with matrix interferences observed for saliva in peripheral blood [147]. Protein material is innately separated from genetic material during solubilization of forensic evidence, with the cell pellets reserved for

genetic typing and the supernatant used for serological screening. The compatibility of proteomic techniques within a DNA-focused forensic biology workflow have been demonstrated by researchers [52,146]. One study reports the analysis of authentic crime scene samples, illustrating the successful characterization of blood in addition to producing a genetic profile with one seamless sample preparation protocol [148]. The sensitivity limits achieved with this analytical instrumentation are continuously challenged. It has been shown that laboratory-prepared laundered fabrics containing whole blood can be characterized with proteomic techniques [149]. Challenging sample type analysis will only continue to illustrate the effectiveness of proteomic analyses for body fluid identification, especially as analytical methods become more refined.

More recent studies have employed quantitative proteomic techniques to detect peptide signatures. It was originally hypothesized that semi-quantitation of biomarkers could be used to determine the amount of body fluid present in the original stain. Although biomarker concentrations were calculated, it was concluded stain amounts could not be inferred due to sufficient expression variability among individuals [150]. Similarly, external standards have been employed to monitor trypsin processivity, ionization efficiency, and matrix interferences [147]. Currently, protein mass spectrometry technology has not been implemented into operational crime laboratories for body fluid source determination application.

A main challenge of proteomic characterization is achieving a sample preparation protocol that is successful in extracting and purifying biomarker targets. Given the inherent complexity of protein material, differences in polarity and size can hinder the use of a common protocol. The conventional procedure for bottom-up targeted proteomics is time intensive to ensure adequate cleavage and recovery of protein material prior to analysis. A majority of protocols describe an overnight incubation as well as lyophilization steps; however, more recent studies explored expedited sample preparation [150,151]. In addition, as seen with other next generation techniques, the differentiation of vaginal fluid and menstrual blood remains a challenge. One area of improvement necessary for advancement of proteomic techniques is the dissemination of extensive interpretation guidelines and reporting language. Because vaginal fluid and menstrual blood

originate from the same cavity, guidelines in reporting protein biomarkers is essential to forming accurate result statements.

Benefits of proteomic techniques for body fluid identification include its ability to conduct multiplex analyses, its sensitivity range, and potential use for correlation to genetic typing success [52]. In comparison with traditional screening techniques, proteomic research has demonstrated the ability to consistently characterize five body fluids within a single assay, unlike chemical, enzymatic, and antibody-based assays which each target a single fluid. Furthermore, antibody-based assays have a limited working range, with concentrated samples producing a false negative result. Although emphasis is placed on trace level sample analysis within forensic biology, the ability to accurately characterize concentrated samples remains valuable for investigation. The inherent relationship between protein and DNA expression, characterized by the Central Dogma, illustrates the ability to utilize quantitative proteomic results as a means of predicting the quality of a genetic profile from a single stain. This relationship has been illustrated outside the realm of body fluid identification through the characterization of genetically variant peptides (GVP) and single nucleotide polymorphisms (SNP) in bone [152] and hair [153]. The strength of proteomic techniques in forensic biology are centered in its selectivity and ability to provide complimentary data to genetic testing.

1.4 Research Objectives

This dissertation research was designed to develop and rigorously validate a high-specificity forensic serology assay. The assay was designed for the simultaneous identification of five forensically-relevant body fluids: peripheral blood, seminal fluid, saliva, vaginal fluid, and menstrual blood. To achieve this, two well-established technologies, ultra-performance liquid chromatography and triple quadrupole mass spectrometry (UPLC-QQQ), were selected in combination with automated sample preparation to produce an integrated system well-suited with forensic casework needs. A previously developed prototype assay served as the model for further method development and validation. Validation procedures were conducted in accordance with the Federal Bureau of Investigation's Quality Assurance Standard for Forensic DNA Testing

Laboratories as well as guidelines published by the Scientific Working Group on DNA Analysis Methods (SWGDAM). In addition, given the analytical instrumentation employed, appropriate guidelines from the Organization of Scientific Area Committees for Forensic Science (OSAC) Chemistry: Seized Drug and Toxicology Scientific Area Committee were also considered. The final deliverable presents a fully validated analytical assay that demonstrates sensitivity and specificity gains over traditional serological screening techniques and is compatible with downstream genetic testing.

Additional assessments were formulated to better understand and mitigate the deleterious effects of personal lubricants and spermicidal products on the ability to accurately identify seminal fluid and saliva in cases of sexual assault. Supplementary measures were incorporated into the sample processing procedure to diminish competitive binding during sample purification and remove the interferents prior to proceeding with protein digestion. The multiplex assay was then used as a foundation for the development of a top down peptidomic methodology for the assessment of sexual assault evidence. With the prevalence of sexual assault evidence, an expedited sample processing workflow that is capable of generating comparable results was identified as an area that needed further attention. It was demonstrated that same-day sample preparation could be used for the identification of seminal fluid, saliva, and vaginal fluid.

The successful completion of these objectives has important implications for advancing the field of forensic serology by providing alternative means to confirming the presence of body fluids, especially in the event of sexual assault or rape.

1.5 Hypotheses

The overarching hypothesis that was tested in the course of this dissertation research is that proteomic techniques in the form of bottom up and top down methodologies will surpass the sensitivity and specificity capable of traditional serological screening techniques. The specific hypotheses that were at the core of this research therefore are:

1. The transferring of a multiplex mass spectrometry-based assay from a nanoflow to high performance chromatographic system will sufficiently increase sample throughput.

2. An automated sample processing procedure will mitigate sources of human error and contribute to increased sample throughput without a loss in peptide intensity.
3. A multiplex mass spectrometry-based assay can undergo rigorous validation and comparison to illustrate its performance and compatibility with existing forensic biology workflows.
4. Body fluid identification of samples subjected to personal lubricants can be achieved through specific sample preparation procedures and detection of protein signatures using the validated mass spectrometry-based assay.
5. An expedited proteomic analysis of body fluids consistent with sexual assault evidence can be developed using peptidomic techniques.

1.6 Dissertation Structure

Each chapter within this dissertation will contain an introduction to provide the essential background content of the research conducted and present scientific reasoning behind the experimental design. Experimental methods and a comprehensive presentation of results will be detailed. The significance of research findings will be discussed, including any caveats to the developed methodology.

Chapter 2 details the transfer of a previously developed prototype LC-MS/MS technique to a more sensitive analytical platform. In addition, the optimization and automation of the sample processing method will be discussed. Chapter 3 focuses on the full developmental validation of the LC-MS/MS technique for the identification of five forensically relevant body fluids. Chapter 4 evaluates the validated LC-MS/MS method in relation to currently utilized serological screening techniques. In addition, the compatibility of the developed LC-MS/MS methodology with downstream genetic testing protocols was assessed. Chapter 5 presents the effects of personal lubricants on the ability to accurate identify body fluids consistent with sexual assault analysis. And lastly, Chapter 6 focuses on a streamlined proteomic approach to biomarker screening for sexual assault evidence analysis.

CHAPTER 2: METHOD TRANSFER AND DEVELOPMENT OF AN AUTOMATED SAMPLE PROCESSING WORKFLOW

2 Introduction

Through previous research initiatives in the Danielson laboratory ([130,147] and unpublished research), it has been demonstrated that a targeted mass spectrometry-based multiplex assay allows for the unambiguous identification of body fluids in a forensic context. Furthermore, the sensitivity and specificity gains achieved with the developed research grade assay illustrated the significant advantages of proteomic techniques when compared with reporting limits of existing serological screening tests. However, additional areas of interest required further development to reach the readiness level expected for implementation into an operational laboratory. Areas of interest included decreasing analytical run time without sacrificing sensitivity gains, automation of preparation procedures, a direct comparison with existing serological tests, and extending the boundaries for high throughput analyses.

The research grade assay was developed on an Agilent 6430 mass spectrometer coupled to a liquid chromatograph fitted with a 1100 series nanoflow pump. Although this system was a triple quadrupole mass spectrometer capable of advanced sensitivity and specificity in comparison with other high resolution mass spectrometers, the limitation of the initial assay was the nanoflow liquid chromatograph system. While originally chosen for increased sensitivity, to ensure complete peptide biomarker separation, and reduce the likelihood of coelution, nanoflow chromatography requires inherently small flow rates to achieve greater ionization. A 30-minute analytical run time at a 400 nL/min flow rate was necessary to achieve adequate separation of biomarkers on the nanoflow system. Furthermore, a manual tryptic digestion and sample cleanup was employed, limiting the number of samples processed in a given batch. These processes incorporated multiple sample handling steps and incubation times in addition to centrifugation and lyophilization steps.

In summary, the research grade assay was limited to 12 samples per batch, which required a full day of preparation and an additional full day to acquire data.

Under this portion of the project, the previously developed research grade assay was transferred from the nanoflow chromatography system to an ultra-performance liquid chromatography tandem mass spectrometry (LC-MS/MS) platform. Specifically, an Agilent 6495 mass spectrometer coupled to a 1290 series liquid chromatograph was utilized. With the transfer of the analytical method, instrument and chromatography parameters required optimization prior to validation. During this portion of the study, synthetic peptide standards were synthesized, an internal positive control was selected and tested, and analytical operating parameters were determined.

This chapter seeks to report and establish a refined protein biomarker panel for the identification of peripheral blood, seminal fluid, saliva, and vaginal/menstrual fluid in addition to developing an automated sample processing protocol for increased sample throughput. This research was completed under three main scientific aims. The first research aim addressed the need to transfer the research grade assay to a high resolution analytical platform. The second research aim established an automated sample processing procedure and evaluated techniques for accurate protein quantification. The final research aim compared the automated processing procedure with the existing manual processing procedure for accuracy in the identification of selected biomarkers. The results outlined herein provide an optimized sample processing procedure and analytical method equipped for a full developmental validation.

2.1 Methods and Materials

2.1.1 Body Fluid Collection

All research conducted under this phase of the project was reviewed and approved by the University of Denver Institutional Review Board (IRB) for research involving human subjects. Sample collection and research was conducted in full accordance with the U.S. federal policy for the protection of human subjects. In total, 60 subjects were recruited from the graduate population at Arcadia University (Glenside, PA) and staff members employed at The Center for Forensic

Science Research & Education (Willow Grove, PA). All volunteers agreed and signed a letter of consent acknowledging that they had received, read, and understood all protocols involved in sample collection. Furthermore, all collected samples were assigned a random unique identifier to protect confidentiality.

Peripheral blood samples were collected through venipuncture under previous IRB approval. Additional blood specimens were purchased from Innovative Research, Inc. (Novi, MI).

Semen was self-collected from consenting donors. The donor deposited the semen sample into a sterile collection cup and was asked to refrigerate the sample until transport to the lab. Upon receipt, semen samples were allowed to liquify at room temperature for 30 minutes. Collection cups were vortexed and 200 µL single use aliquots were prepared and stored at -80 °C until use.

Saliva was collected from consenting volunteers who refrained from eating or drinking for 1 hour prior to collection. Salivette® collection tubes (Sarstedt, Nümbrecht, Germany) were utilized. Donors were instructed to remove the absorbent pad from the Salivette® tube and place the pad in their mouth. To stimulate saliva production, donors were instructed to gently chew on the absorbent pad. After 45 seconds, the donor placed the absorbent pad back into the Salivette® tube. The Salivette® tube was centrifuged for 10 minutes at 1,000 x g and saliva flowthrough was collected. 200 µL single use aliquots were prepared and stored at -80 °C until use.

Vaginal secretions were self-collected by consenting donors. Female participants were asked to refrain from unprotected sexual intercourse for 12 days prior to the collection of vaginal fluid. The hypoallergenic, over the counter Softdisc™ collection cup (The Flex Company, Venice, CA) was utilized. Donors inserted the collection cup into the vagina following manufacturer's instructions. It was requested the cup remain in the vagina for a minimum of 1 hour but could be left in for a period up to 12 hours. The entire Softdisc™ was placed into a sterile collection cup and transported to the laboratory. The liquid contents were placed into the collection cup and the Softdisc™ was repeatedly washed with 1 mL of ultra-pure water to remove mucous-like secretions. The liquid contents and mucous secretions were pooled and thoroughly vortexed to create a homogenous sample. 200 µL single use aliquots were prepared and stored at -80 °C until use. In

addition to vaginal secretions collected with the Softdisc™, female participants were asked to provide self-collected vaginal swabs. Consenting volunteers were provided with sterile cotton-tipped swabs. A single swab was inserted into the vaginal cavity, similar to inserting a tampon. The swab was rotated in a circular manner for 15 seconds and gently removed. Swabs were placed into sterile manila collection envelopes for transport to the laboratory. Upon receipt, swabs were dried at room temperature where the cotton tip was removed from the wooden handle and placed in a clean microcentrifuge tube. Swabs were stored at -80 °C until use.

Menstrual blood samples were self-collected by consenting donors. Female participants were asked to refrain from unprotected sexual intercourse for 12 days prior to the collection of menstrual blood. The hypoallergenic, over the counter DivaCup™ (Diva International, Inc., Ontario, Canada) was utilized. Donors inserted the DivaCup™ in accordance with manufacturer's guidelines during menses. It was requested the cup remain in the vagina for a minimum of 1 hour but could be left in for a period up to 12 hours. The entire DivaCup™ was placed into a sterile collection cup for transport to the laboratory. Upon receipt, the liquid contents were poured into the collection cup and thoroughly vortexed. 200 µL single use aliquots were prepared and stored at -80 °C until use.

2.1.2 Method Transfer and Biomarker Selection

2.1.2.1 Confirmation of Body Fluid-Specific Targets

Previously acquired data on candidate target protein and peptide sequences was reviewed in tandem with analysis of peptide standards in fluid matrix to reevaluate optimal transitions. In total, 26 peptide signatures from previously acquired data were carried over for additional analysis [130,147]. Under these previous research efforts, crude (70% purity) peptide standards were purchased, and product ions were selected via fragmentation analysis using Quadrupole Time-of-Flight (QTOF) mass spectrometry. Authentic body fluids were quantitated (Section 2.1.3.1), manually digested and purified (Section 2.1.3.3) for analysis with the target inclusion list.

2.1.2.2 Mass Spectrometer Instrument Parameters

Synthetic peptide standards prepared in Section 2.1.2.1 were combined to create a protein master mix containing peptide standards at equal concentration. Each protein master mix was

directly infused into the Agilent Jet Stream source of the 6495 mass spectrometer. Using the Agilent Source and iFunnel Optimizer software (v. B.08.00), starting values of source parameters were evaluated in a step-wise fashion. Parameters assessed included sheath gas temperature and flow rate, nozzle voltage, nebulizer pressure, drying gas temperature and flow rate, and capillary voltage. Resulting data was analyzed in MassHunter Qualitative software (v.B.04.01) for greatest peak intensities and peak shape.

Collision energy for individual peptides was determined using crude (70% purity) peptide standards prepared in Section 2.1.2.1. Skyline Proteomics Environmental software v. 20.1.0.155 (MacCoss Labs, University of Washington) was used to establish predictive *in silico* collision energy values for each peptide of interest. Values were incrementally increased and decreased around the predicted value to determine the maximum signal of each ion transition. Resulting data was analyzed using MassHunter Qualitative software.

2.1.2.3 Chromatographic Optimization

2.1.2.3.1 Preliminary Assessment

Synthetic peptide standards prepared in Section 2.1.2.1 were utilized to assess the effect of mobile phase gradient, analytical column internal diameter, and flow rate on chromatographic peak separation and signal intensity. Two analytical columns were evaluated: an Agilent Poroshell 120 EC-C18 3 mm x 100 mm column and an Agilent Poroshell 120 EC-C18 2.1 mm x 100 mm column. Flow rates assessed include 0.4 mL/min, 0.5 mL/min, and 1.0 mL/min. Due to the construction of the 2.1 mm x 100 mm column, the 1.0 mL/min flow rate could not be assessed on this column due to pressure limitations. Run times evaluated include 10, 13, and 15 minutes, each with a 3-minute post run to allow for equilibration. Two final analytical methods were selected for further analysis: a 10-minute (3.1 mm x 100 mm) method using a 1.0 mL/min flow rate and a 13-minute (2.1 mm x 100 mm) method using a 0.5 mL/min flow rate. Body fluid samples from five donors were pooled, enzymatically digested, and extracted using the AssayMAP Bravo liquid handling platform protocol (discussed in Section 2.1.3.2) and analyzed, in addition to prepared

peptide standards, on both analytical methods to ensure peak-to-peak resolution remained desirable.

2.1.2.3.2 Sensitivity Assessment

Human serum albumin (HSA) peptide (Agilent Technologies, Santa Clara, CA) was diluted using 30% ACN with 0.1% FA to the following concentrations: 100 fmol/µL, 10 fmol/µL, 1 fmol/µL, 0.5 fmol/µL, and 0.1 fmol/µL. A series of seminal fluid and vaginal fluid mixtures were created from pooled fluid from five donors. Vaginal fluid was held at a constant 100 µL while 10 µL, 1 µL, 0.1 µL, and 0.01 µL of semen were added into the vaginal fluid sample (*i.e.,* a vaginal fluid to semen ratio of 1:10 to 1:10,000). Each concentration was prepared and analyzed in triplicate between the 13-minute (2.1 mm x 100 mm) method and the 10-minute (3 mm x 100 mm) method. Samples were digested and extracted using the AssayMAP Bravo liquid handling platform (discussed in Section 2.1.3.2) and 1 µL of sample was analyzed via LC-MS/MS.

2.1.2.4 Internal Positive Control

Intact *Bos taurus* myelin basic protein was purchased at a concentration of 1 mg/mL (Millipore Sigma, Darmstadt, Germany). Upon receipt, intact myelin was diluted with 50 mM ammonium bicarbonate (ABC) to a final concentration of 10 ng/µL. Isotopically-labeled *Bos taurus* myelin basic protein peptide DTGILDSLGR was purchased (New England Peptide) at a concentration of 1 mg/mL and diluted in 30% ACN 0.1% FA for a final concentration of 0.5 mg/mL. Single use aliquots of both the intact and labeled internal positive control (IPC) were stored at -80 °C. Upon use, aliquots were thawed at room temperature for 30 minutes, vortexed, and pulse spun. Isotopically-labeled IPC stock solution was further diluted with 30% ACN and 0.1% FA in deionized water to a concentration of 1 pmol/µL.

Seminal fluid and peripheral blood were used for IPC concentration analysis as they are the most protein rich fluids analyzed within this assay. A series of four 2-fold dilutions were prepared by mixing 100 µg of body fluid matrix pooled from 5 donors with either 200 ng, 100 ng, 50 ng, or 25 ng of a 1:1 molar ratio of intact myelin basic protein and isotopically-labeled peptide standard. Samples were digested and extracted using the AssayMAP Bravo liquid handling platform

(discussed in Section 2.1.3.2) and 1 µL of sample was analyzed via LC-MS/MS. Intact IPC was added to samples prior to tryptic digestion and isotopically-labeled myelin IPC was added prior to solid phase extraction cleanup.

2.1.3 Sample Preparation Optimization

2.1.3.1 Protein Quantitation

A comparison of two protein quantitation techniques was conducted. A previously optimized protocol for the bicinchoninic acid (BCA) assay was selected (Thermo Fisher Scientific, Waltham, MA). Assay standards were prepared by diluting concentrated bovine serum albumin (BSA) in 100 mM tris-hydrochloride (tris-HCl) to the following concentrations: 1,500 µg/mL; 1,000 µg/mL; 750 µg/mL; 500 µg/mL; 250 µg/mL; 125 µg/mL; and 25 µg/mL. To a 96-well flat bottom plate, 25 µL of standard or sample was added in duplicate. Samples and standards were treated with 200 µL of working reagent containing 200 µL of Reagent A and 50 µL of Reagent B per sample. The plate was incubated at 37 °C for 30 minutes followed by 10 minutes at room temperature. Samples were read using a spectrophotometer at 652 nm wavelength. The second quantitation technique utilized a Nanodrop™ One Microvolume UV-Vis spectrophotometer (Thermo Fisher Scientific). To operate, 1 µL of sample or standard was applied to the stage. Two quantitation functions on the Nandrop™ were selected for evaluation: the 280 nm absorbance assay and the built-in BCA application.

For comparison, single use body fluid aliquots were thawed at room temperature for 30 minutes then centrifuged for 10 minutes at 10,000 x g. Supernatant was transferred to a clean microcentrifuge tube and cell pellets were discarded. Single-fluid and mixture samples were prepared. Single-fluid samples were analyzed neat and diluted 10-fold, 100-fold, and 1,000-fold in 50 mM ammonium bicarbonate. Two-fluid mixture samples were prepared by combining 50 µL of each fluid for 1:1 volume mixtures. Mixture samples were thoroughly vortexed and pulse spun prior to analysis. All samples were evaluated in triplicate. Descriptive statistics were calculated and comparisons were performed through observation of average protein concentration and standard error.

2.1.3.2 Automation Procedure Development

A previously developed manual tryptic digestion was modified and transferred to the AssayMAP Bravo liquid handling platform (Agilent Technologies). A denaturant solution of 8 M urea and 5 mM tris(2-carboxyethyl)phosphine (TCEP) was prepared in 100 mM tris-HCl. Upon procedure development, varying volumes of 40 µL, 55 µL, and 90 µL denaturant solution were added to samples for evaluation. Alkylation solution was prepared as 100 mM iodoacetamide in deionized water. 100 mM tris-HCl was utilized as diluent prior to the addition of 0.25 µg/µL sequencing grade modified trypsin (Promega Corporation, Madison, WI). With the exception of the sample load volume, the default peptide cleanup parameters on the AssayMAP Bravo software were utilized for removal of residual digestion solutions.

2.1.3.3 Workflow Testing and Optimization

A comparative assessment between the manual and automated processing procedures was conducted. For the manual tryptic digestion, lyophilized sample was reconstituted in 15 µL of 50 mM ABC, vortexed for 10 seconds, and pulse spun. Protein was denatured with 15 µL of trifluoroethanol (TFE) and reduced with 1 µL of 200 mM TCEP for 30 minutes at 55 °C. After incubation, 2 µL of 200 mM iodoacetamide (IAA) was added and samples were vortexed and pulse spun. Samples were incubated for 30 minutes in the dark at room temperature. Samples were diluted with 250 µL 50 mM ABC for a final concentration of 5mM TCEP and 10 mM IAA. Samples were treated with 10 µL of 0.25 µg/µL sequencing grade modified trypsin and incubated at 37 °C for 15 hours. Digestion was stopped with the addition of 10 µL of 10% trifluoroacetic acid (TFA). Pierce® C18 Spin Columns (Thermo Fisher Scientific) were primed with 300 µL of 50% ACN in deionized water and centrifuged at 1.4 x g for 1 minute. Cartridges were equilibrated with 300 µL of 0.1% formic acid (FA) in deionized water and centrifuged at 1.4 x g for 1 minute. Total sample volume (266 µL) was loaded into the spin column and centrifuged for 1 minute at 1.4 x g. Columns were washed with 300 µL of 0.1% FA in deionized water and centrifuged at 1.4 x g for 1 minute, for a total of 3 wash steps. Samples were eluted with 2 passes of 20 µL of 70% ACN with 0.1% FA in deionized water by centrifuging for 1 minute at 1.4 x g, for a total of 40 µL of eluate. Eluate was

lyophilized to dryness. The automated procedures were conducted as described below (Section 2.4.1). Samples were fortified with 100 ng intact IPC prior to digestion and a final concentration of 50 fmol/µL isotopically-labelled IPC prior to solid phase extraction (SPE) cleanup. Descriptive statistics were calculated and comparisons were performed through observation of peak area response and standard error.

Various consumables were evaluated to provide optimum biomarker recovery. Three sample plates were compared: a Non Binding Surface treated 96-well U-bottom plate (Corning®, Corning, NY), Lo-Bind treated plate (Eppendorf, Hamburg, Germany), and a non-treated U-bottom 96-well plate (Greiner, Monroe, NC).

2.1.4 Final Protocol for Sample Preparation

Samples were quantitated using the BCA assay. Standards and working reagent were prepared as described above (Section 2.1.3.1). If necessary, samples were diluted with 100 mM tris-HCL. Samples were fortified with 20 µL of 0.5 mg/mL intact myelin protein and lyophilized to dryness.

Lyophilized sample was reconstituted and digested using the AssayMAP Bravo liquid handling platform. Samples were denatured in 55 µL of denaturant solution (8 M urea, 5 mM TCEP in 100 mM tris-HCl) for 45 minutes at 25 °C. 6 µL of 100 mM IAA was added and samples were incubated for 30 minutes at 25 °C with a lid on the plate. After 30 minutes, 170 µL of 100 mM tris-HCl was added. Samples were treated with 10 µL of 0.25 µg/µL trypsin. The sample plate was sealed and shaken for 5 minutes prior to incubating at 37 °C for 15 hours.

The tryptic reaction was stopped with the addition of 10 µL of 25% TFA. Digested samples were manually fortified with 10 µL of 1 µg/mL isotopically-labeled IPC stock solution. The sample plate was placed back on the AssayMAP Bravo deck for SPE cleanup. C18 microextraction cartridges (Agilent Technologies) were primed with 100 µL 50% ACN 0.5% TFA at a flow rate of 300 µL/min. Cartridges were equilibrated with 50 µL of 0.5% TFA in deionized water at 10 µL/min. 200 µL of digested sample was passed over the cartridge at a flow rate of 15 µL/min. Cartridges were washed with 50 µL of 0.5% TFA in deionized water at a rate of 10 µL/min. Sample material

was eluted from the sorbent in 25 µL of 70% ACN 0.1% FA at 5 µL/min. Eluate was lyophilized to dryness and prepared for LC-MS/MS analysis.

Lyophilized samples were reconstituted in 100 µL of 2% ACN with 0.1% FA in deionized water. Acquisition was performed using 10 µL of sample per injection on an Agilent 6495 mass spectrometer coupled to a 1290 series liquid chromatograph. An Agilent Poroshell 120 EC-C18 3 mm x 100 mm analytical column was used for separation. Mobile phase A consisted of water with 0.1% FA and mobile phase B consisted of ACN with 0.1% FA. Separation initiated at 5% B followed by a linear 8-32% B gradient over 8.5 minutes, a 1-minute hold at 80% B, followed by a 3-minute re-equilibration at a 1.0 mL/min flow rate and column temperature of 50 °C. Data was analyzed using Skyline Proteomics Environmental software v. 20.1.0.155.

2.2 Results and Discussion

The objective of this phase of the research was the design and optimization of the LC-MS/MS method utilized for the remainder of the project. Specifically, human body fluid-specific biomarkers were selected; peptide standards were synthesized; an internal positive control was designated; and the Agilent 6495 LC-MS/MS operating parameters were determined. Furthermore, experimentation was conducted to determine if sample processing procedures were amenable to automation. It should be noted that all preliminary target selection included the analysis of biomarkers consistent with the identification of urine (in addition to peripheral blood, seminal fluid, saliva, and vaginal/menstrual fluids). Upon development of the sample preparation procedures outlined in Section 2.1 and in consultation with senior practitioners, the identification of urine was dropped from the analytical method to allow for consistency in sample processing and preparation. However, preliminary results for urine identification are included within portions of this chapter to demonstrate the inconsistencies of preparation chemistries at accurately identifying peptide signatures consistent with urine.

2.2.1 Method Transfer and Biomarker Selection

2.2.1.1 Confirmation of Body Fluid-Specific Targets

To maximize the sensitivity and specificity of the assay, an extensive evaluation of protein, peptide, and peptide fragment ions were identified during method development. Preliminary peptide fragments were selected from the most abundant proteins present in the target body fluids (*i.e.,* peripheral blood, seminal fluid, saliva, vaginal/menstrual fluids) through review of previously acquired data from prior research initiatives [130,147]. Proteins were reassessed using proteomic databases (*i.e.,* UniProt, NCBI gene) for species- and fluid-specificity. Selected targets were evaluated experimentally to ensure the targets were abundant and not effected by endogenous matrix interferences, and top fragmentation products were selected for inclusion in the remainder of the study. Whole proteins, peptide sequences, or ion transitions were eliminated based on signal intensity, retention time, and fragmentation observed via LC-MS/MS. High purity isotopically-labeled peptide standards were custom synthesized for the final biomarker target list and used in the remainder of the study.

Product ion transition selection was carried out using QTOF analysis of synthetic peptide standards. **Figure 2.1** provides an illustrative example of peripheral blood protein alpha 1 antitrypsin peptide LSITGTYDLK peak area response, fragmentation spectra, and product ion detection and selection. The peptide precursor m/z ratio of 555.80 was fragmented and the four most abundant product ions (*i.e.,* m/z 997.5201, 910.4880, 797.4040, 696.3563) were selected for inclusion. This process was repeated for all protein/peptide pairings prior to being transferred to the Agilent 6495 LC-MS/MS platform. The preliminary inclusion list contained 30 protein targets characterized by 136 peptides (including markers for urine identification).

The inclusion list was drastically paired down during the remainder of this portion of the study. This was performed to increase the sensitivity and specificity of the analytical method by limiting the likelihood of coelution of peptide signatures. Such a large transition list results in decreased dwell times, which in turn limits the amount of time the mass spectrometer scans for the target compound, therefore preventing informative data points from being obtained. Furthermore,

as instances of fluid cross-reactivity or interference were observed, candidate protein and peptide markers were subsequently eliminated in favor of more specific protein targets. In summary, a total of 21 proteins were selected for the final inclusion list, characterized by 45 amino acid sequences and 132 unique ion transitions. **Figures 2.2-2.6** illustrate the chromatographic separation of the final peptide inclusion list for each body fluid. A complete list of protein, peptide, and transition targets is detailed in **Appendix A**. Although representative of fluid-specific and abundant targets, the diversity in protein composition of the inclusion list presented further analytical challenges for method optimization. Peptide targets exhibited a wide range of physical properties including hydrophobicity/hydrophilicity and molecular weight.

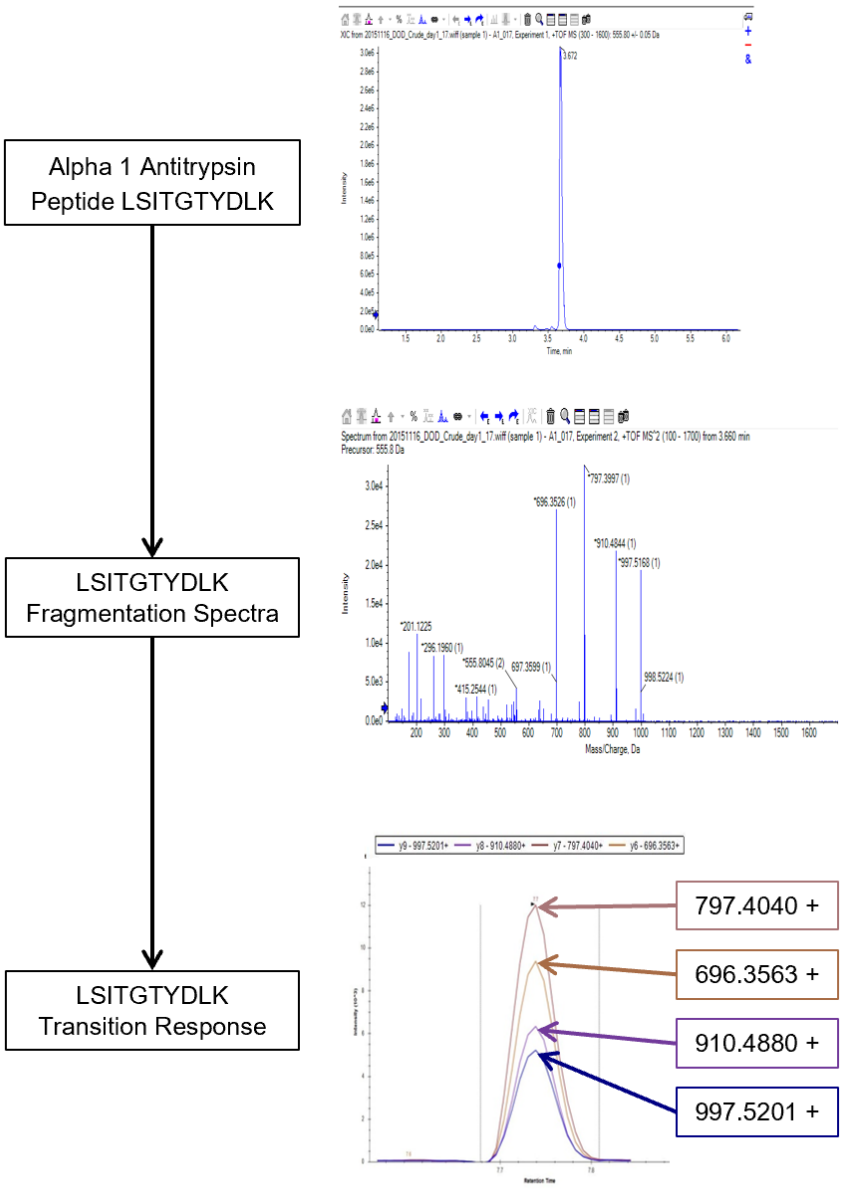


Figure 2.1. Analysis of alpha 1 antitrypsin peptide LSITGTYDLK by QTOF and LC-MS/MS. Top image represents the precursor ion response. Middle figure represents the fragmentation spectra of the precursor ion. Bottom figure represents the product ion distribution. The product ions exhibiting the greatest abundance were selected for inclusion in the targeted LC-MS/MS assay.

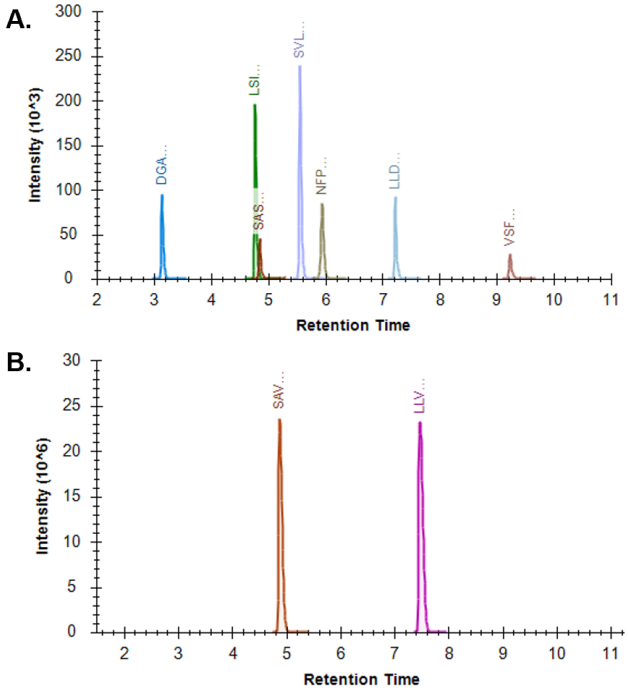


Figure 2.2. Assay results for the detection of human peripheral blood. Peaks represent selected peptides consistent with proteins (A) hemopexin, alpha 1 antitrypsin, apolipoprotein, serotransferrin, and (B) hemoglobin.

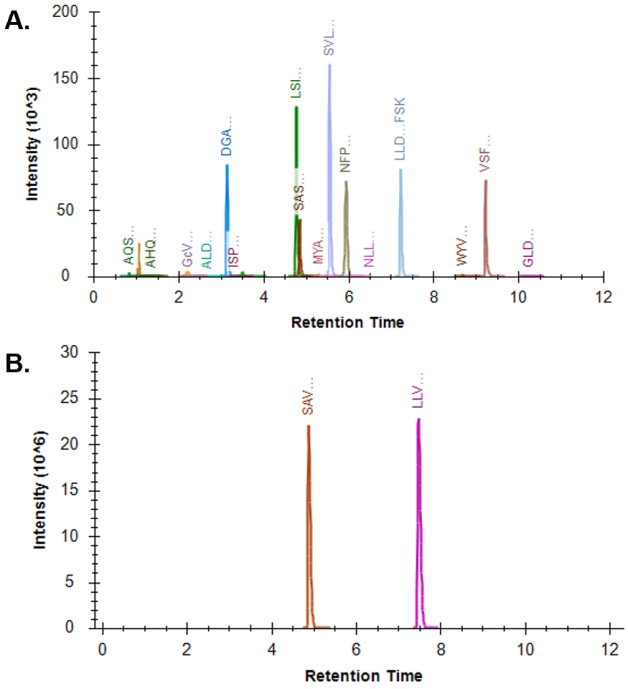


Figure 2.3. Assay results for the detection of human menstrual blood. Peaks represent selected peptides consistent with proteins (A) hemopexin, alpha 1 antitrypsin, apolipoprotein, serotransferrin, cornulin, neutrophil gelatinase, Ly6/PLAUR, suprabasin, periplakin, involucrin, small proline rich protein 3, and (B) hemoglobin.

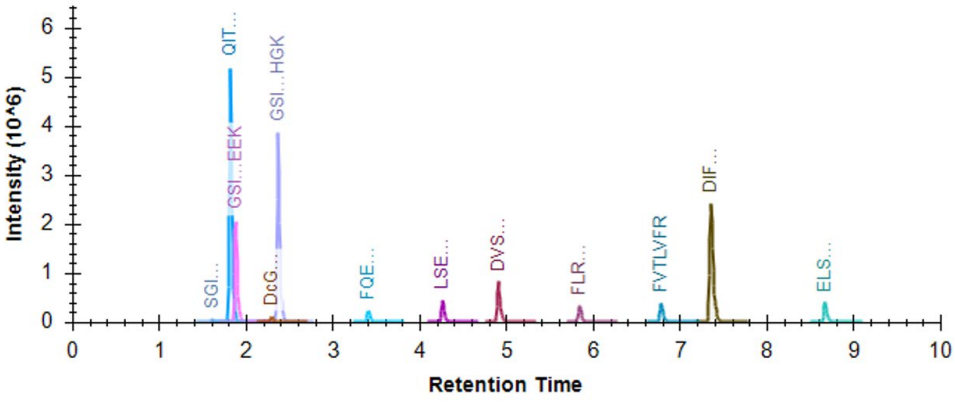


Figure 2.4. Assay results for the detection of human seminal fluid. Peaks represent selected peptides consistent with proteins semenogelin 1, semenogelin 2, prostate specific antigen, prostatic acid phosphatase, and epididymal secretory protein.

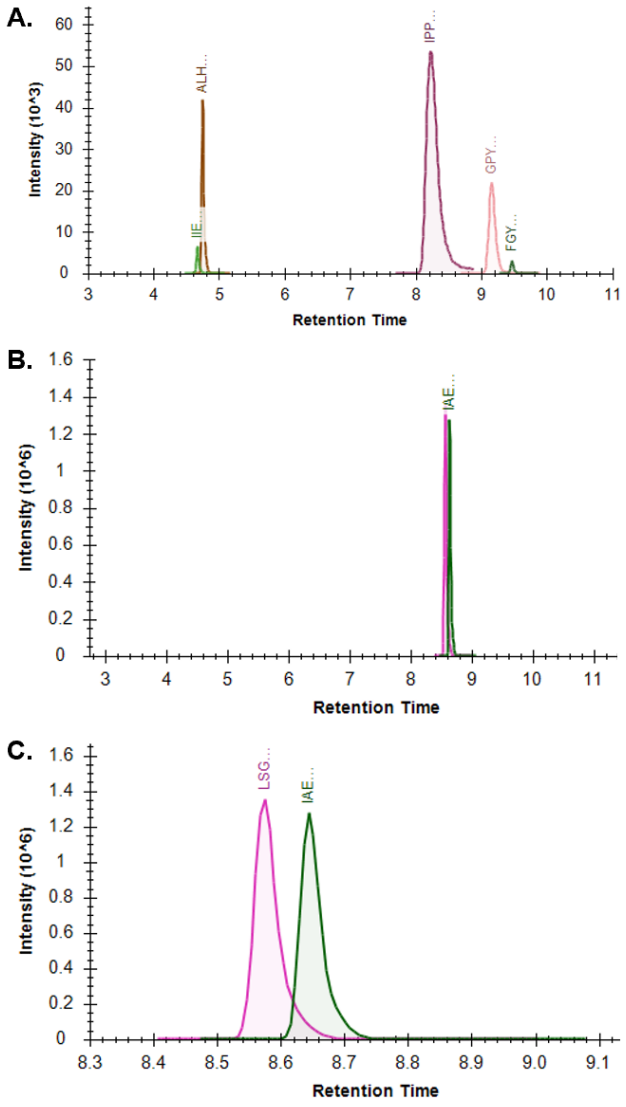


Figure 2.5. Assay results for the detection of human saliva. Peaks represent selected peptides consistent with proteins (A) submaxillary gland androgen-regulated protein 3B, cystatin SA, statherin, and (B) alpha amylase. (C) Alpha amylase peptides on a smaller retention time scale to illustrate peak resolution.

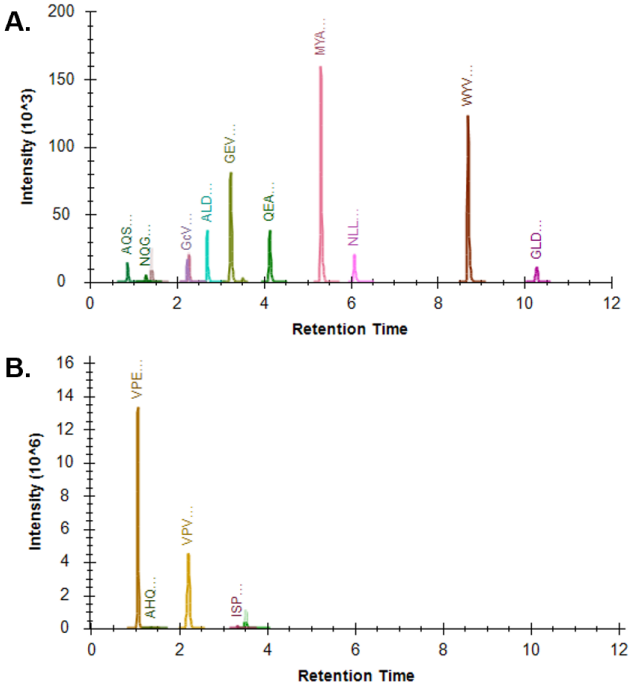


Figure 2.6. Assay results for the detection of human vaginal fluid. Peaks represent selected peptides consistent with proteins (A) neutrophil gelatinase, Ly6/PLAUR, suprabasin, periplakin, involucrin, (B) cornulin, and small proline rich protein 3.

2.2.1.2 Mass Spectrometer Instrument Parameters

Given the formation of a targeted list of target protein and peptide biomarkers, additional instrument parameters were optimized for each target to ensure optimal detection and identification. In order to optimize ionization efficiency, Agilent mass spectrometer Jet Stream source conditions were adjusted (**Figure 2.7**). The source conditions modified for maximum peak area response included nebulizing gas flow rate and temperature, sheath gas flow rate and temperature, capillary voltage, nebulizer pressure, and nozzle voltage. Conditions were optimized by injecting peptide standards and manually modifying the aforementioned parameters until a maximum peptide response signal was observed. Finalized source condition parameters are detailed in **Table 2.1**.

While source parameters were established based on response signal of the biomarker panel as a whole, the optimal collision energy was determined for each individual peptide transition.

Collision energy optimization is necessary to achieve maximum ion transmission and fragmentation within the mass spectrometer. Similar to source parameter conditions, collision energy was incrementally modified until a maximum peptide response signal was observed. Using Skyline Proteomics Environment Software, *in silico* predictions of optimal collision energy for each target peptide sequence were generated. Using synthetic peptide standards, the collision energy was modified in a stepwise manner both above and below the predicted value. A complete list of final collision energy values for target peptide sequences is detailed in **Appendix A**. For example, semenogelin 2 peptide DVSQSSISFQIEK had a predicted collision energy value of 23.8 V. The positive addition collision energies are shown in **Figure 2.8**, with 25.8 V producing the greatest peak intensity of those tested.

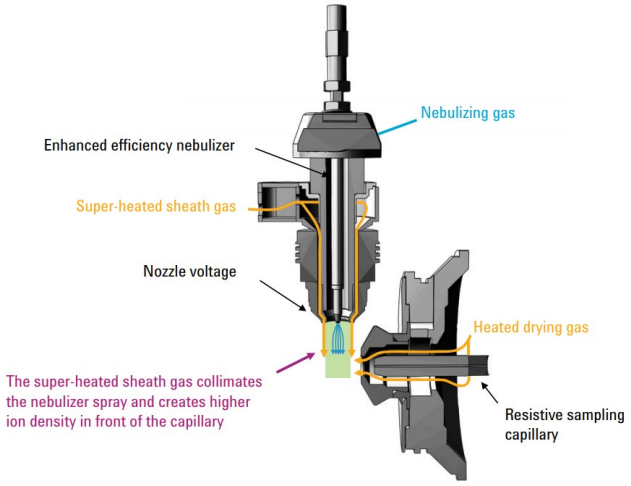


Figure 2.7. Agilent Jet Stream source schematic. Reprinted from *Agilent Jet Stream Thermal Gradient Focusing Technology* (p. 1) by A. Mordehai and J. Fjeldsted. Publication number 5990 3494. Copyright 2009 by Agilent Technologies.

Table 2.1. Optimized Agilent Jet Stream source parameters on the Agilent 6495 mass spectrometer.

Source Parameter	Source Value
Drying Gas Temperature	150 °C
Drying Gas Flow	15 liters/minute
Nebulizer Pressure	30 psi
Sheath Gas Temperature	200 °C
Sheath Gas Flow	11 liters/minute
Capillary Voltage	3500 Volts
Nozzle Voltage	300 Volts

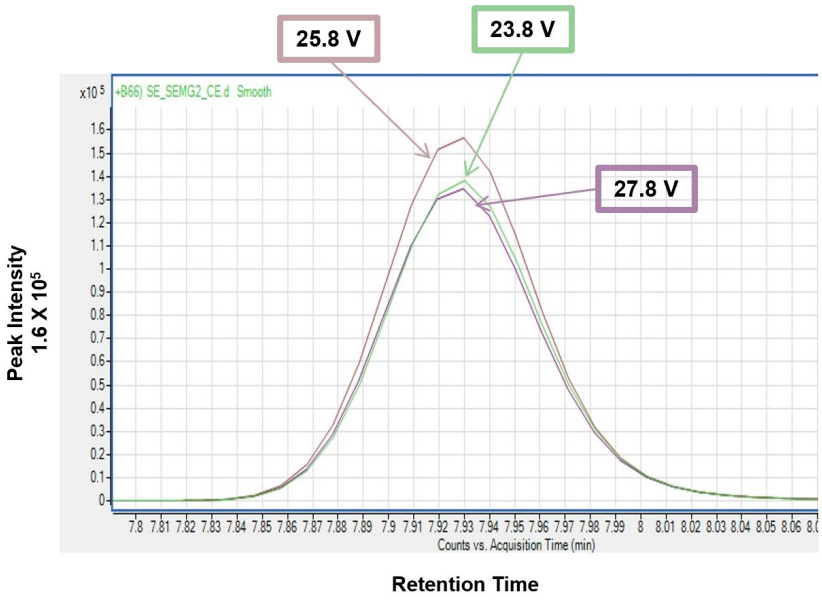


Figure 2.8. Collision energy optimization of semenogelin 2 peptide DVSQSSISFQIEK. Retention time is depicted on the x-axis and peak intensity on the y-axis. Colored peaks represent peak area intensity of the peptide at a given collision energy. The collision energy that produced the greatest peak intensity (*i.e.,* 25.8 V in tan) was selected.

2.2.1.3 Chromatographic Optimization

An area of interest that was identified for assay development in relation to the previous research grade assay was decreasing analytical run time in order to increase sample throughput. In addition to automated sample preparation (discussed in section 2.2.2.2), increasing sample throughput is an attractive quality for consideration in operational laboratories. One of the main challenges associated with a decrease in analytical run time is the loss of resolution between target

compounds due to coelution and potential ion suppression. Run time optimization and chromatographic separation were evaluated during this portion of the project using synthetic peptide standards and challenging sample types. During analysis, the LC-MS/MS mobile phase gradient, specifically for the acetonitrile organic solvent, was monitored and adjusted to prevent co-elution of peptide targets.

2.2.1.3.1 Preliminary Assessment

The starting analytical gradient was set for a 15-minute run time with a 3-minute post time at a 0.4 mL/min flow rate, for a total run time of 18 minutes. With the implementation of robotic sample preparation, the analysis of a 96-well sample plate would take approximately 30 hours. This was determined to be unsuitable for an operational environment faced with quick turn-around times, given that a single 96-well plate could not be analyzed within a single day. Therefore, two additional chromatographic run times were evaluated: a 13-minute run time and a 10-minute run time, both with a 3-minute post time (**Table 2.2**). To account for the analysis of such a large number of target compounds and the speed of analysis, two analytical columns were also assessed. The 13-minute gradient was developed using a 2.1 mm x 100 mm AdvanceBio Peptide Map column with a flow rate of 0.5 mL/min. The 10-minute gradient was developed using a 3 mm x 100 mm AdvanceBio Peptide Map column and a flow rate of 1.0 mL/min, with both columns containing a 2.7-micron pore size (**Table 2.2**). The increase in internal diameter of the analytical column, from 2.1 mm to 3 mm, allowed for increased flow rate due to the larger bore column. However, as flow rate is increased, peak-to-peak resolution will compress, generating potential challenges in maintaining assay sensitivity. A preliminary assessment of the analytical methods included the analysis of digested protein material from the target body fluids. While some compression was expected, no loss in resolution was observed with the 10-minute method for any of the body fluids. For example, **Figure 2.9** depicts chromatographic separation and resolution of a seminal fluid peptide targets for both the 13-minute (2.1 mm x 100 mm) and 10-minute (3 mm x 100 mm) methods. No consequential loss in peak resolution was observed while three minutes of instrument run time was spared per sample assayed.

Table 2.2. Chromatographic parameters for the three analytical gradients evaluated.

Parameter	15 min Run Time		13 min Run Time		10 min Run Time	
Column	2.1 x 100 mm Peptide Map		2.1 x 100 mm Peptide Map		3 x 100 mm Peptide Map	
Column Temperature	50 °C					
Mobile Phase	A: 0.1% FA in H_2O B: 0.1% FA in ACN					
Flow Rate	0.5 mL/min		0.5 mL/min		1.0 mL/min	
	Time	%B	Time	%B	Time	%B
	Initial	5	Initial	5	Initial	5
	1.1	5	0.5	8	0.5	8
	12.5	32	11.5	32	8.5	32
	13.0	80	11.7	80	8.7	80
	14.0	80	12.7	80	9.7	80
	14.2	5	12.9	5	9.9	5
	15.0	5	13.0	5	10.0	5
Stop Time	15 min		13 min		10 min	
Post Time	3 min					
Total Run Time	18.5 min		16.5 min		13.5	
Analysis Time (96 Samples)	29.6 hours		26.4 hours		21.6 hours	

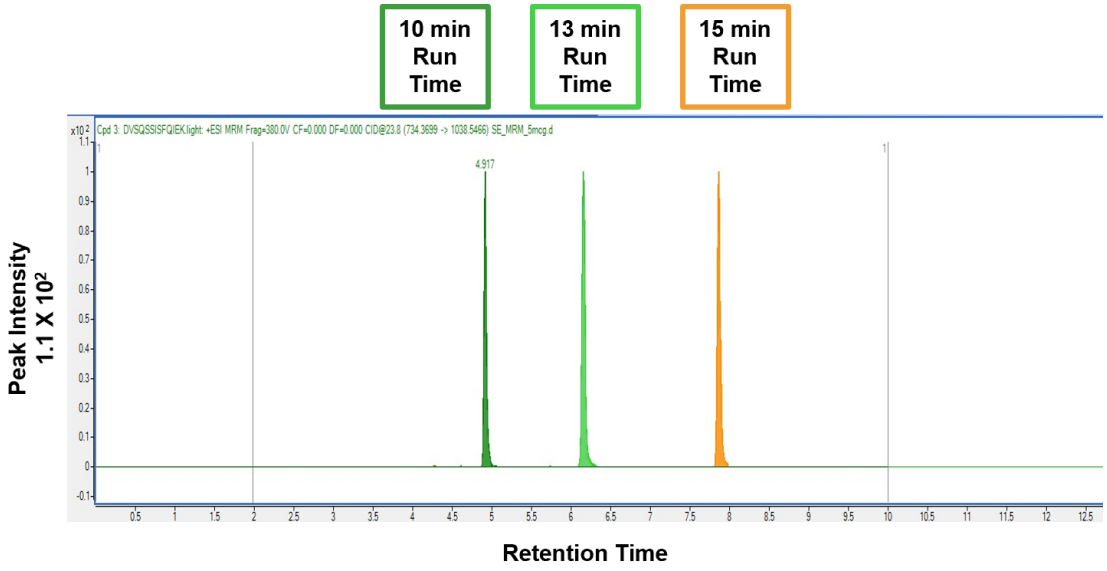


Figure 2.9. Normalized chromatographic separation of seminal fluid peptide DVSQSSISFQIEK across the three developed gradients (15-minute run time, orange; 13-minute run time, light green; 10-minute run time, dark green).

2.2.1.3.2 Sensitivity Assessment

A serial dilution of human serum albumin (HSA) was prepared and analyzed on the 13-minute and 10-minute analytical methods. A slightly greater (*i.e.,* <2%) peak area was observed in

favor of the 13-minute analytical method (**Figure 2.10**). It was determined that the 10-minute 1.0 mL/min flow rate did not negatively impact the ability of low-level sample detection and identification. Following these results, all body fluids were analyzed using the 13- and 10-minute methods to observe any consequential loss in peak-to-peak resolution due to compressed chromatographic separation. While peak compression was expected, no loss in resolution was observed with the 10-minute method for any of the fluids. For example, chromatographic separation for saliva is depicted in **Figure 2.11**. A change in elution order was observed for the two alpha-amylase peptides IAEYMNHLIDIGVAGFR and LSGLLDLALGK (green and pink peaks). The LSGLLDLALGK peptide eluted first on the 13-minute method and second on the 10-minute method. Other than compression of peak elution, no other changes in elution order were observed for the remaining body fluids.

To simulate sexual assault type samples, a series of semen dilutions in vaginal fluid were prepared. Of the four dilutions prepared, results for two are illustrated, with the 0.01 µL representing the lowest dilution point assessed. A marginal loss in peak area was observed for semenogelin 2 on the 10-minute method; however, greater peak responses for prostate specific antigen were observed on the same method (**Figure 2.12**). Based on the data obtained, the tradeoff between peptide response and sample throughput favors the faster 10-minute (3 mm x 100 mm) run time. In conclusion, the 10-minute chromatographic method was selected for the remainder of the study.

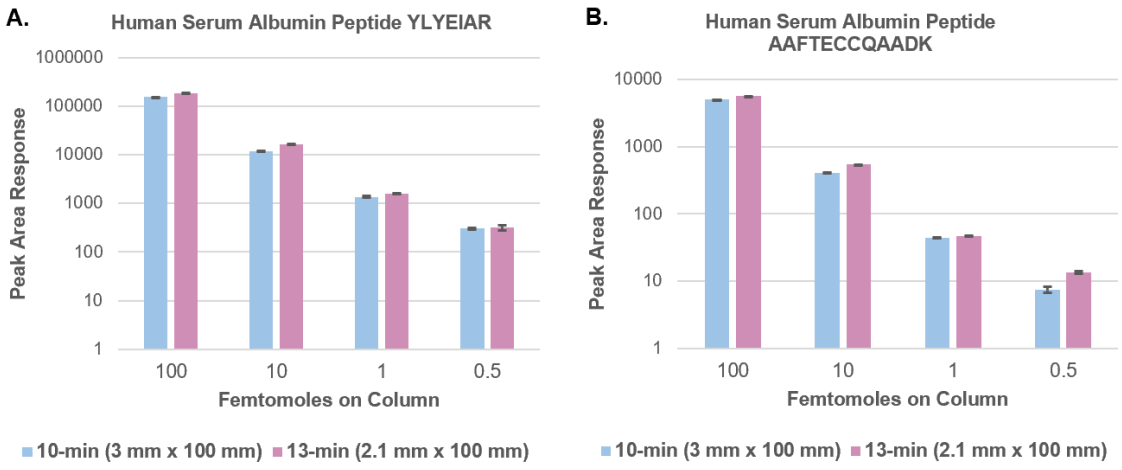


Figure 2.10. Peak area comparison between the 13-minute (purple) and 10-minute (blue) analytical methods for human serum albumin peptide (A) YLYEIAR and (B) AAFTECCQAADK. Error bars represent standard error. The amount of protein material on column (in femtomoles) is represented on the x-axis. Peak area response is represented on the y-axis.

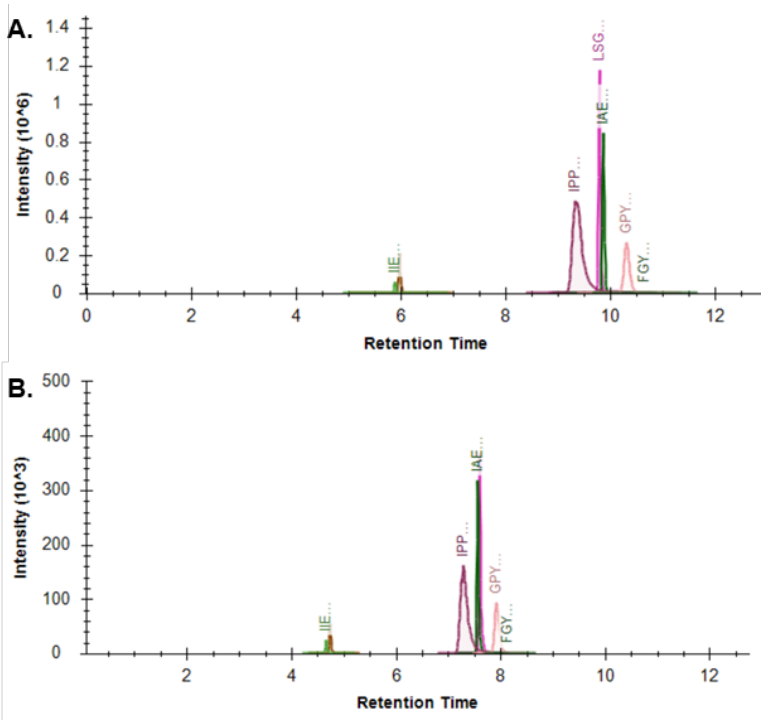


Figure 2.11. Chromatographic resolution comparison between saliva biomarker peptides on the (A) 13-minute and (B) 10-minute analytical methods.

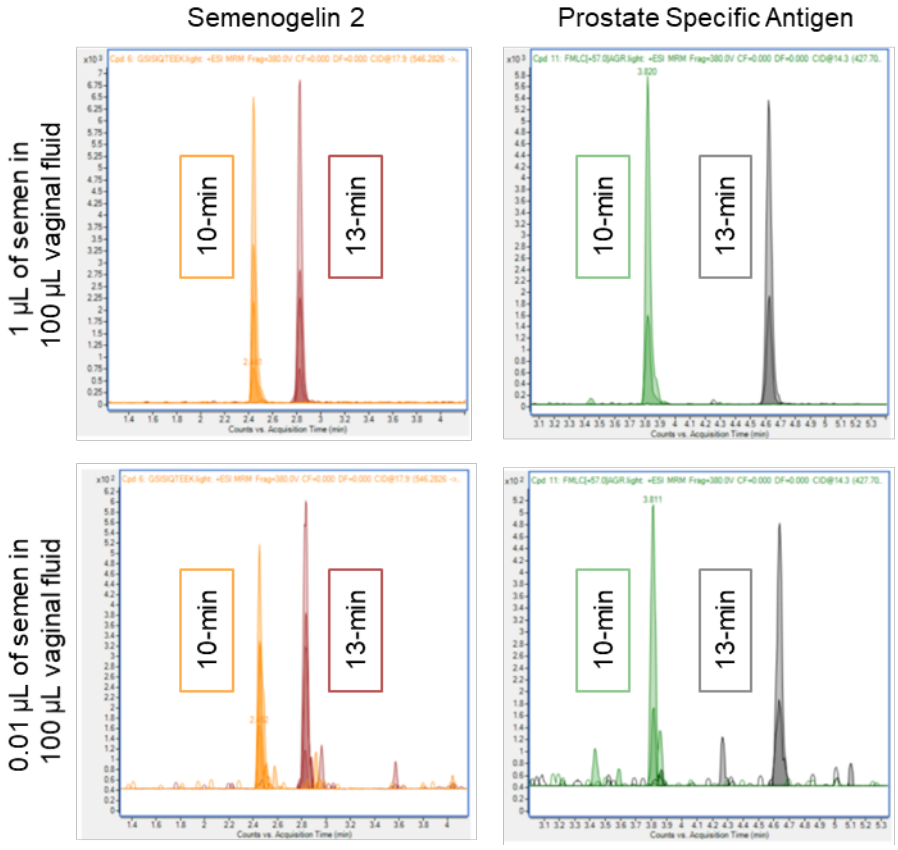


Figure 2.12. Peak area response comparison for low-level seminal fluid in a vaginal fluid matrix between the 10-minute and 13-minute analytical methods. Semenogelin 2 peptide GSISIQTEEK at the 10-minute (orange) and 13-minute (red) retention times. Prostate specific antigen peptide FMLCAGR at the 10-minute (green) and 13-minute (grey) retention times. Peak area response is represented on the y-axis.

2.2.1.4 Internal Positive Control

In order to monitor inhibition of proteolytic digestion due to the presence of sample-specific contaminants or inhibitors, an internal positive control (IPC) was designed. Two versions of the IPC were acquired: an intact protein to evaluate digestion efficiency and a heavy isotope-labeled peptide from the same protein to monitor purification and chromatographic analysis. Inhibition in protein cleavage efficiency was indicated by a reduction in the signal intensity of the control intact protein in comparison with the heavy-labeled peptide sequence of the same protein. Bovine myelin basic protein, both an intact protein version and an isotopically-labeled peptide sequence, were selected for use as the IPC (**Figure 2.13**). This protein had been used as an IPC in previous

research studies and was selected for it's conserved amino acid sequence. Using seminal fluid and peripheral blood sample matrices, a dilution series of a 1:1 molar ratio of intact myelin and labeled peptide standard were evaluated. The theory behind selecting only seminal fluid and peripheral blood for IPC evaluation rests in the inherent matrix effects and protein concentration of these two fluids.

As expected, the heavy peptide standard to digested myelin response ratios did not reach the theoretical 1:1 ratio maximum for any protein cleavage reaction in tested authentic body fluids. The ratio that was obtained, however, was constant for both the seminal fluid and peripheral blood matrices over the full range of concentrations tested. **Figure 2.14** shows the peak response for the digested myelin protein and heavy peptide standard for a 100 ng assessment in seminal fluid and peripheral blood. Both fluid matrices have matching peak heights, retention times, and a stable ratio of heavy peptide standard to digested myelin. The experiment was repeated across all body fluids using the same 1:1 molar ratio of intact myelin to labeled peptide standard, created in 100 µg of fluid matrix. Similar to results observed previous, the theoretical ratio of 1:1 was not illustrated. However, among the dilutions of each fluid matrix, the response ratios obtained had coefficients of variation (CV) values of 2% or less (**Table 2.3**). Therefore, the 100 ng quantity of intact IPC was selected for use in developmental validation studies.

The discordance in obtaining the theoretical 1:1 response ratio is best described by the relationship between enzymatic digestion of intact protein and monitoring of surrogate peptides that is characteristic of bottom up proteomic analyses [154]. The relative quantitation of surrogate peptides relies on the catalytic activity of trypsin. A 1:1 response ratio would assume a catalytic activity that displays 100% accuracy in cleaving at each arginine and lysine residue.

A. ***Bos taurus*** **Myelin Basic Protein (P02687)**

AAQKRPSQRSKYLASASTMDHARHGFLPRHR**DTGILDSLGR**FFGSDRG
APKRGSGKDGHHAARTTHYGSLPQKAQGHRPQDENPVVHFFKNIVTPR
TPPPSQGKGRGLSLSRFSWGAEGQKPGFGYGGRASDYKSAHKGLKGH
DAQGTLSKIFKLGGRDSRSGSPMARR

B. ***Homo sapiens*** **Myelin Basic Protein (P02686)**

MGNHAGKRELNAEKASTNSETNRGESEKKRNLGELSRTTSEDNEVFGE
ADANQNNGTSSQDTAVTDSKRTADPKNAWQDAHPADPGSRPHLIRLFS
RDAPGREDNTFKDRPSESDELQTIQEDSAATSESLDVMASQKRPSQRH
GSKYLATASTMDHARHGFLPRHR**DTGILDSIGR**FFGGDRGAPKRGSGKD
SHHPARTAHYGSLPQKSHGRTQDENPVVHFFKNIVTPRTPPPSQGKGR
GLSLSRFSWGAEGQRPGFGYGGRASDYKSAHKGFKGVDAQGTLSKIFK
LGGRDSRSGSPMARR

Figure 2.13. Protein sequences for myelin basic protein in (A) *Bos taurus* and (B) *Homo sapiens*. The *Bos taurus* specific peptide (DTGILDSLGR, pink) and *Homo sapiens* specific peptide (DTGILDSIGR, purple) lack 100% sequence identity, making it possible to discriminate between bovine-based IPC and endogenous human myelin basic protein.

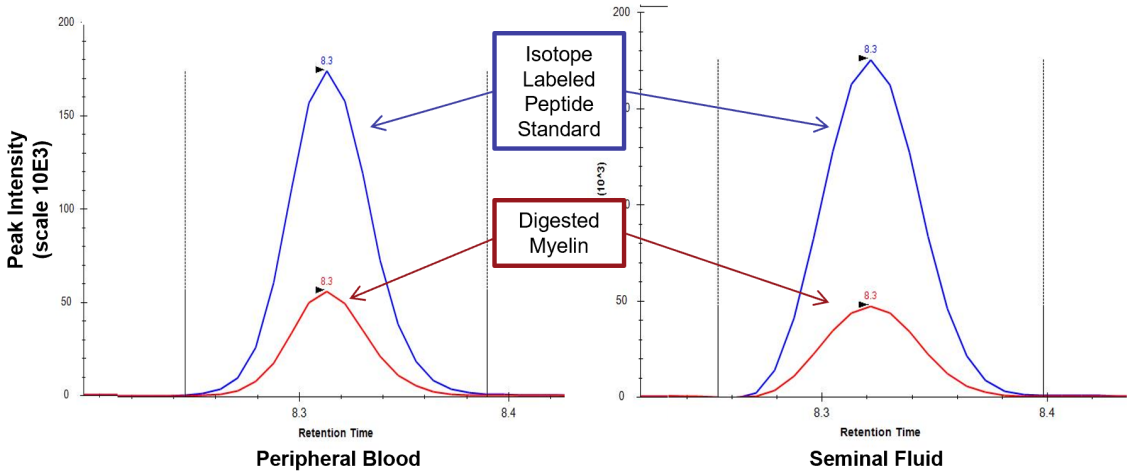


Figure 2.14. Detection of myelin bovine albumin peptide DTGILDSLGR in peripheral blood (left) and seminal fluid (right). Digested internal positive control is depicted in red (m/z 523.7775++). Heavy isotope labeled internal positive control is depicted in blue (m/z 528.7816++).

Table 2.3. Detection of IPC at 100 ng quantities. Response ratio was calculated by dividing the peak area response of the heavy peptide standard by the peak area response of the natural (intact) protein.

Replicate	DTGILDSLGR (Heavy Standard)	DTGILDSLGR (Natural Protein)				
	Peak Area	Peak Area	Response Ratio	Average	SD	%CV
Vaginal Fluid #1	7576501.5	482856.6	15.6	15.5	0.1	0.65
Vaginal Fluid #2	1753853.2	113610.3	15.4			
Saliva #1	9241114	645746.6	14.3	14.1	0.2	1.42
Saliva #2	9243275	658870.1	14.0			
Seminal Fluid #1	6891479.5	500570.6	13.7	13.5	0.2	0.57
Seminal Fluid #2	6881512.5	514243.0	13.3			
Menstrual Blood #1	2525159.7	135897.5	18.5	18.55	0.05	0.27
Menstrual Blood #2	1931371.7	103722.1	18.6			
Peripheral Blood #1	1991764.8	104990.3	18.9	18.7	0.2	1.07
Peripheral Blood #2	2369814.7	127606.9	18.5			

2.2.2 Sample Preparation Optimization

The purpose of this portion of the study was to develop an automated and expedited sample preparation protocol aside from initial swabbing or cutting of evidentiary material. This protocol was determined through the completion of three objectives. First, through evaluating two techniques for total protein quantitation. Second, development of an automated processing workflow. And lastly, comparing the automated workflow with a previously developed manual procedure.

2.2.2.1 Protein Quantitation

Accurate determination of protein quantitation is vital for biochemical experimentation, specifically for enzymatic digestion of protein material. A multitude of quantitation techniques are available [155], with two UV absorbance techniques chosen for comparison. The bicinchoninic acid assay (BCA) is a standard technique for the reliable and specific quantitation of protein content. While analytically reliable and conservative with regard to consumption of evidentiary material, newer instrumentation employing micro spectrophotometry have been promoted by some practitioners within the field. The NanoDrop™ One Microvolume UV-Vis spectrophotometer (herein referred to as NanoDrop™) was acquired and compared to a previously optimized BCA assay. It

was hypothesized that the NanoDrop™ would provide a faster alternative to obtaining protein concentration data while consuming as little as 1 µL of extract.

Three experiments were designed to compare the two quantitation procedures. Single source and mixed source body fluids were prepared for the BCA assay and NanoDrop™ method at 280 nm absorbance. The NanoDrop™ produced greater protein concentrations in comparison to the BCA reaction when assessing single source fluids (**Figure 2.15**). However, any notation of improvement between the two assays was highly inconsistent across fluids. For example, the NanoDrop™ indicated an average protein concentration for semen that was 50% greater than that obtained using the BCA assay. Moreover, this irregularity was observed across 15 samples that contained 1:1 mixtures of the target body fluids (**Figure 2.16**). The greatest discrepancy in quantitation results for both single source and mixture samples were produced by samples containing urine. Urine is primarily composed of water and non-protein compounds such as uric acid and creatinine. Both of these compounds have the ability to absorb 280 nm wavelength UV light [156].

Ultimately, the protein quantitation estimates provided by the NanoDrop™ with the 280 nm absorbance mode were determined to be inconsistent and unreliable. This was attributed to the presence of non-protein matrix components that absorb light in the 280 nm range. Measuring protein absorbance at 280 nm is routinely conducted in biological procedures given that measurements can be quickly taken and are highly reproducible when using purified sample material. It has been reported that aromatic amino acids, such as tyrosine and tryptophan, strongly absorb UV light at the 280 nm wavelength [157]. However, other aromatic ring containing structures, such as enzymatic cofactors, can also absorb UV light at the 280 nm wavelength. Furthermore, UV absorbance can be affected by protein structure, peptide sequence, and pH environment.

The NanoDrop™ had a built-in BCA application, which was run in parallel with the manual BCA assay to evaluate the inconsistent results observed with single and mixed source samples. This was conducted with only peripheral blood, seminal fluid, and saliva matrices. For this

experiment, all samples were treated with the Bradford reagent and samples were analyzed in parallel with the manual BCA assay and NanoDrop™ system. Protein concentrations were consistent between the two analyses; however, the standard spectrophotometer proved to be the faster of the two methods (**Figure 2.17**).

Due to the inconsistent results observed with the 280 nm quantitation approach, it was determined that all samples would require quantitation with the BCA assay. In addition, pipetting individual samples onto the NanoDrop™ system introduced more analyst hands-on time and sample handling than with the standard BCA analysis. The discrepancies in urine quantitation values were an underlying source of concern and attributed to the removal of urine form the target inclusion list. In conclusion, the manual BCA assay using a standard spectrophotometer was selected as the protein quantitation method for the remainder of the study.

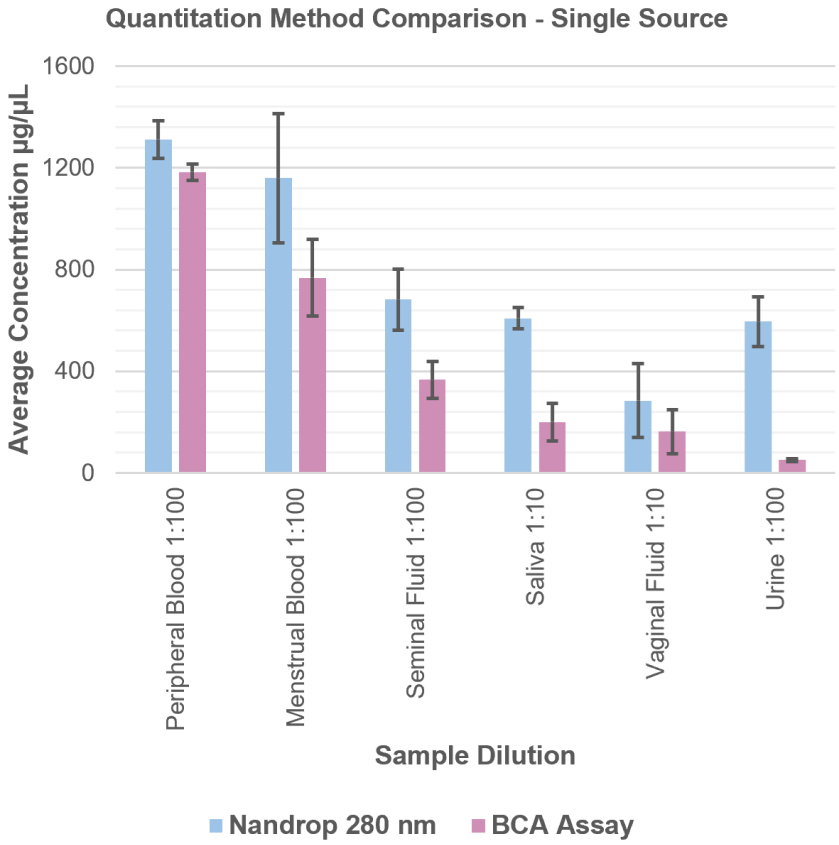


Figure 2.15. Single source body fluid comparison using the NanoDrop™ 280 nm (blue) and BCA assay (purple). Representative dilution samples are represented on the x-axis. Average protein concentration is represented on the y-axis. Error bars represent standard error.

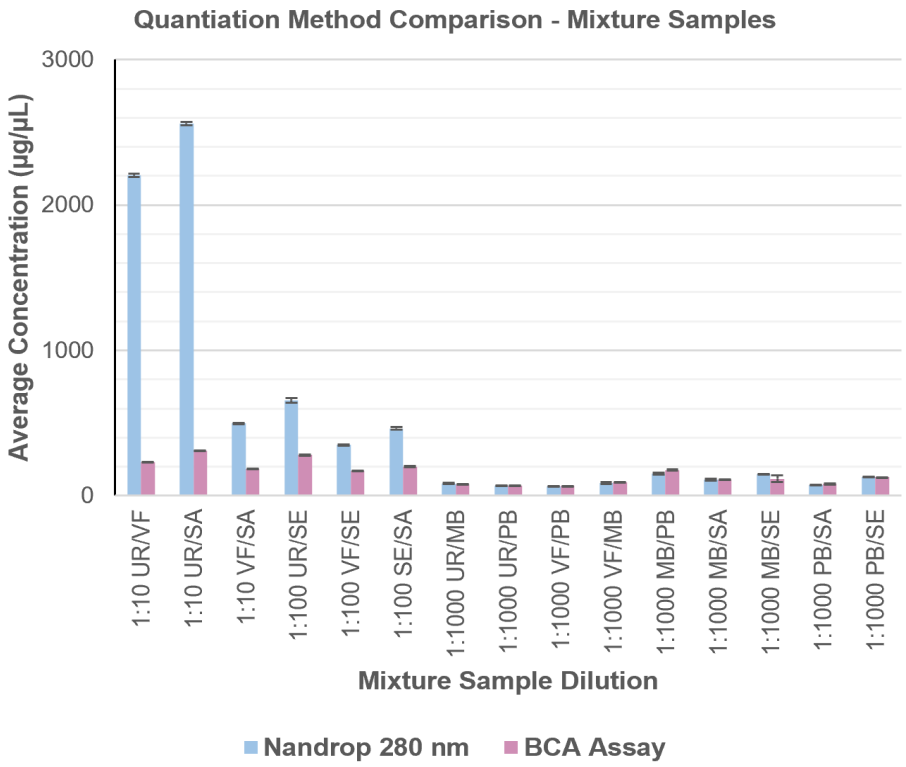


Figure 2.16. Mixture sample comparison using the NanoDrop™ 280 nm (blue) and BCA assay (purple). Mixture samples were prepared as a 1:1 ratio and are represented on the x-axis. Samples were diluted prior to analysis with quantitation methods. Average concentration is represented on the y-axis. Error bars represent standard error.

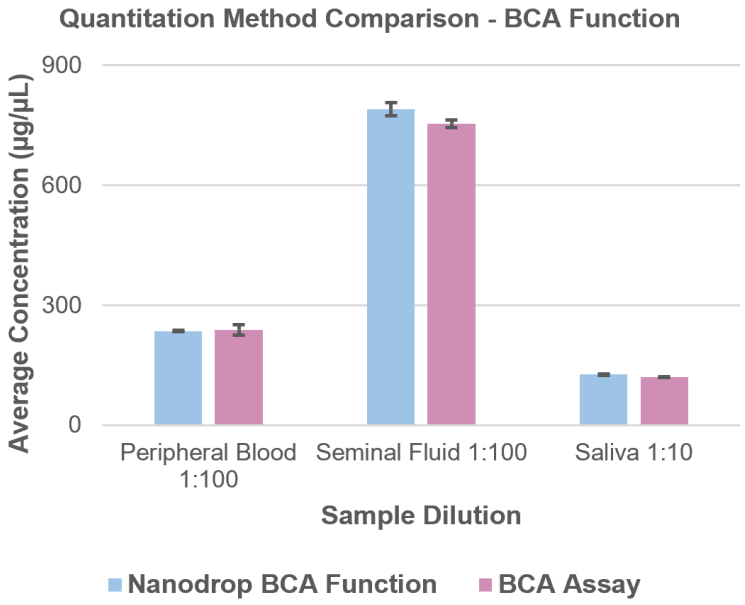


Figure 2.17. Comparison of the NanoDrop™ BCA function (blue) and manual BCA assay (purple) using peripheral blood, seminal fluid, and saliva. Average concentration is represented on the y-axis. Error bars represent standard error.

2.2.2.2 Automation Procedure Development

A previously developed tryptic digestion and SPE cleanup were reformatted to be conducted on the AssayMAP Bravo liquid handling platform. The reagent volumes and robotic processes were developed using an existing protocol [158]. Parameters such as liquid flow rates and reagent transfer volumes were evaluated to ensure optimal digestion efficiency and retention of target protein material during extraction. Specific handling parameters and deck layouts for tryptic digestion and SPE cleanup are depicted in **Figures 2.18 and 2.19**, respectively.

To demonstrate the consistency of results obtained by the automated procedure, six seminal fluid samples were prepared in duplicate and digested, desalted, and purified on the AssayMAP Bravo liquid handling platform. Representative results for the semenogelin 2 peptide GSISIQTEEK are depicted in **Figure 2.20** and **Table 2.4**. Resulting peak area responses for the quantifier ion (m/z 834.4203+) were reproducible across all 12 samples using the AssayMAP Bravo liquid handling platform, with a calculated coefficient of variation less than 15%. The same

preparation was conducted for the remaining body fluid matrices, with similar results observed as those described above (**Figure 2.21**).

Digestion variability was observed when large volumes of fluid were processed using the AssayMAP Bravo system. For example, when digesting diluted seminal fluid and saliva, lyophilized residue deposited on the side of the microplate wells was not consistently solubilized with the addition of denaturant reagent. To address this, varying volumes of denaturant (default 40 µL, 55 µL, and 90 µL) were added to wells containing lyophilized seminal fluid sample. In addition, a gentle mixing step was added to the digestion protocol. It was determined that a 15 µL increase over the default application volume produced the most consistent results (**Table 2.5**). Furthermore, the larger amount of denaturant volume (90 µL) may have inhibited trypsin activity, resulting in less consistent digestion efficiency.

In summary, the developed automation procedure for the digestion and purification of protein material was successful. Overall, the procedure was designed with respect to simplicity and speed of processing. Reagent preparation and procedure parameters were streamlined into a seamless protocol that was further evaluated in the next section.

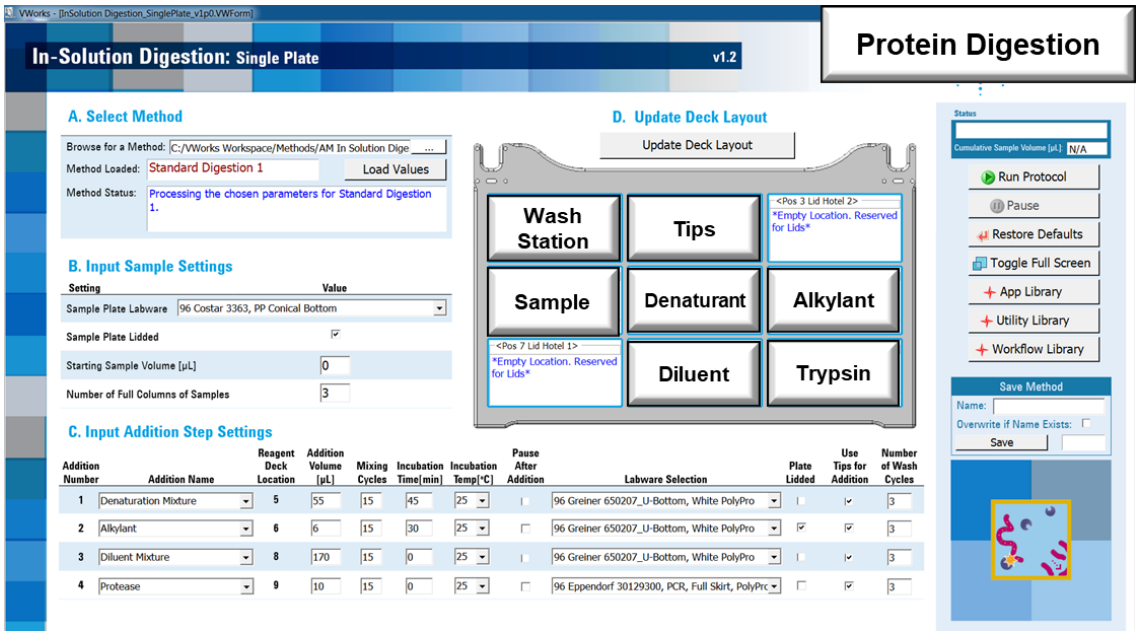


Figure 2.18. Sample process setting and deck layout for automated tryptic digestion in a 96-well plate format on the AssayMAP Bravo liquid handling platform.

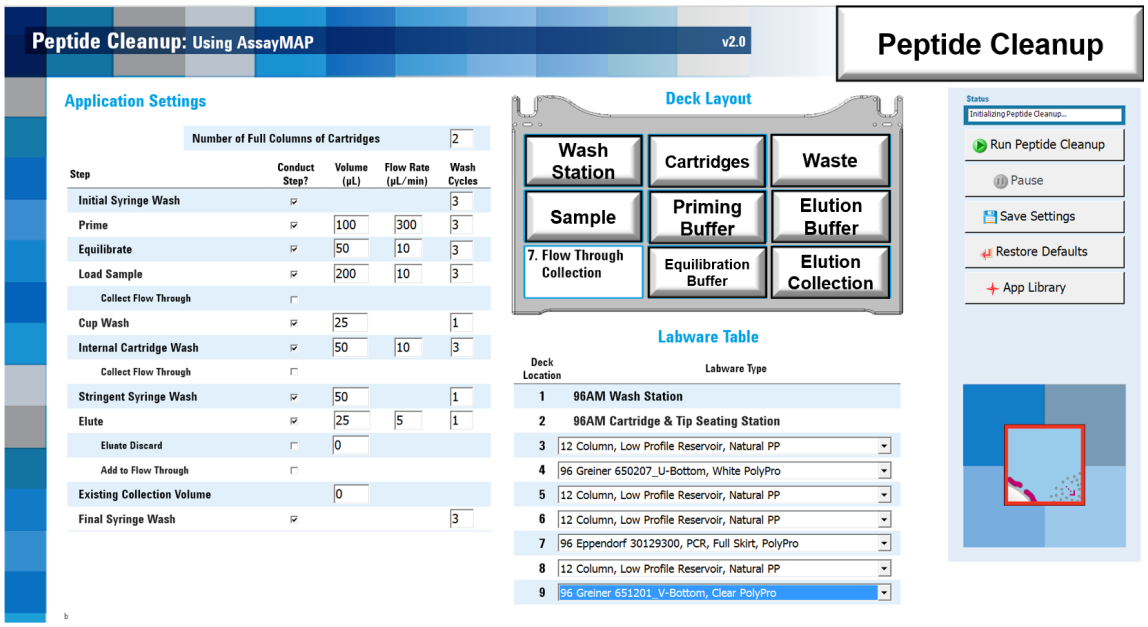


Figure 2.19. Sample process setting and deck layout for automated solid phase extraction cleanup in a 96-well plate format on the AssayMAP Bravo liquid handling platform.

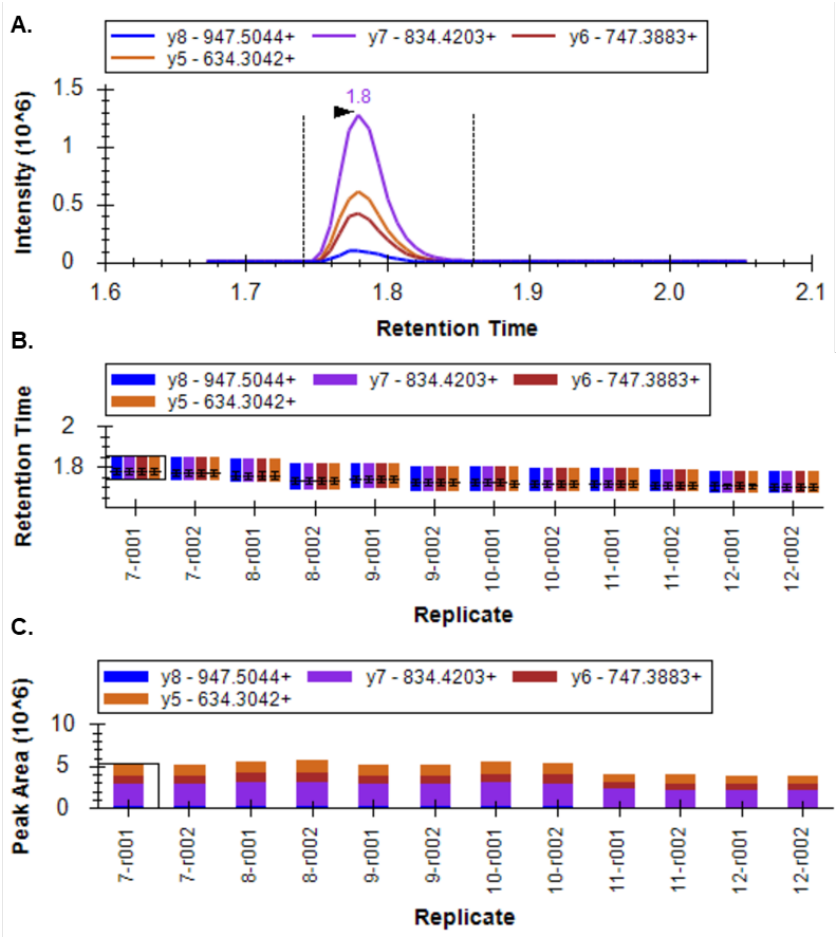


Figure 2.20. Reproducibility results for six seminal fluid samples (prepared in duplicate) processed using the automated procedure. Illustrated is protein semenogelin 2 peptide GSISIQTEEK. (A) Chromatograph of sample 7-r001, depicting retention time on the x-axis and peak area response on the y-axis. (B) Reproducibility of retention time, depicting minor drifts among the 12 replicates. (C) Reproducibility of peak area response among the 12 replicates.

Table 2.4. Peak area reproducibility for seminal fluid protein semenogelin 2 peptide GSISIQTEEK.

Sample ID	Peak Area Response
7-r001	2.80E6
7-r002	2.69E6
8-r001	2.93E6
8-r002	2.94E6
9-r001	2.69E6
9-r002	2.67E6
10-r001	2.83E6
10-r002	2.80E6
11-r001	2.17E6
11-r002	2.09E6
12-r001	2.03E6
12-r002	2.00E6
Average	2.55E6
Standard Deviation	3.66E5
%CV	14.32

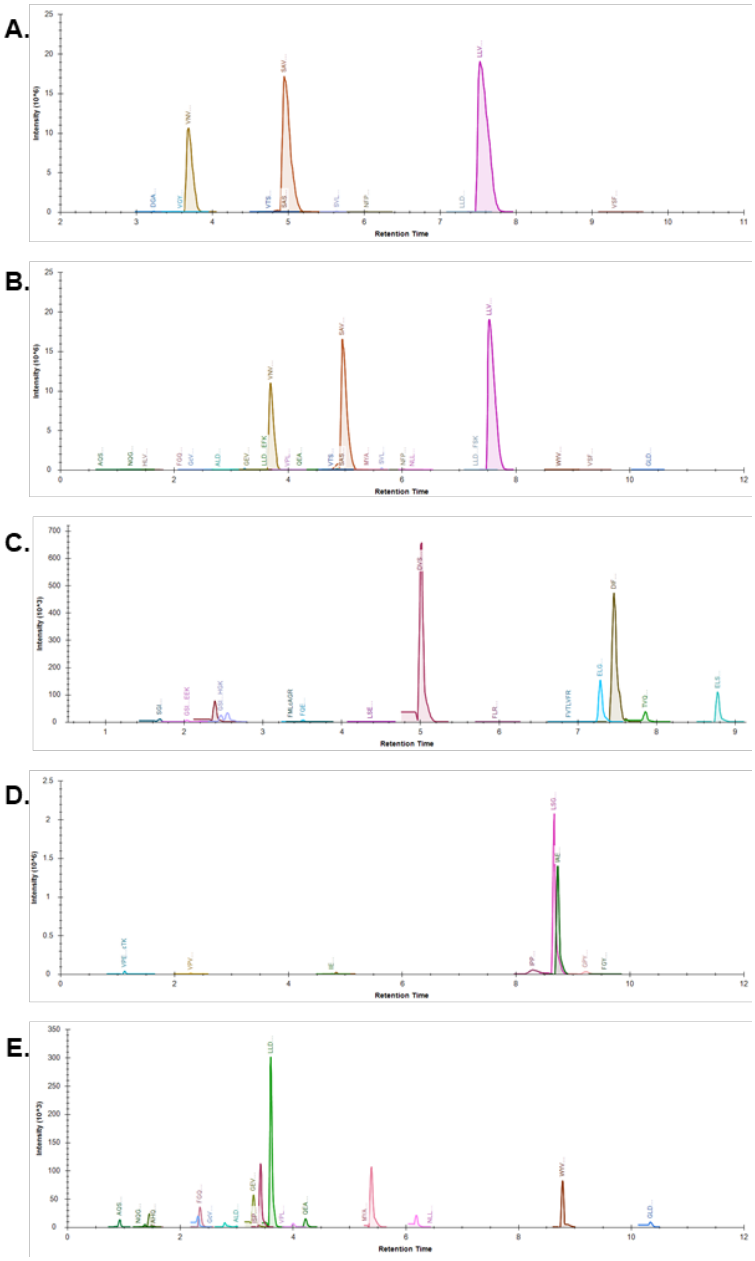


Figure 2.21. Chromatography for (A) peripheral blood, (B) menstrual blood, (C) seminal fluid, (D) saliva, and (E) vaginal fluid, illustrating successful processing with the automated procedure.

Table 2.5. Calculated CV (as a percent) for representative peptide biomarkers of seminal fluid proteins at three denaturant volumes evaluated for digestion on the AssayMAP Bravo liquid handling platform.

Protein	Peptide	Control (40 µL)	+15 Denaturant (55 µL)	+50 Denaturant (90 µL)
Prostatic Acid Phosphatase	FQELESETLK	8.38	3.57	6.13
Prostate Specific Antigen	LSEPAELTDAVK	6.38	8.34	19.40
Semenogelin 1	DIFSTQDELLVYNK	6.68	4.80	4.09
Semenogelin 2	GSISIQTEEK	7.13	3.62	4.55
Epididymal Secretory Protein	SGINCPIQK	7.19	3.65	6.03

2.2.2.3 Workflow Testing and Optimization

A comparison between the manual and automated preparation protocols was conducted to serve as the final assessment of the performance parameters for each approach. This experimentation was carried out with three single-source samples of each body fluid that had been fortified with internal standard. Digestion efficiency was determined by calculating the ratio of digested peptide in relation to the response of the internal standard. For example, two chromatograms depicting peak area responses consistent with saliva are depicted in **Figure 2.22**. As expected, the greatest peak intensity is representative of alpha-amylase peptide LSGLLDLALGK; however, peak intensity for the manual preparation was almost twice as much as that of the automated preparation. Similar results were observed for saliva protein submaxillary gland androgen-regulated protein 3B (**Figure 2.23**). This particular peptide target is more hydrophobic than a majority of the peptide sequences within the multiplex assay. Endogenous peak areas across three replicates were greater for the manual preparation in comparison with the automated platform (red bars). Internal standard (blue bars) showed greater consistency between the two preparation methods, with slightly lower peak area response for the manual preparation.

The small loss in response with the liquid handling platform that was depicted for saliva was consistent across all fluids tested (**Figure 2.24**). It was hypothesized that this loss in response could be contributed to the quality of plastic labware utilized between the two preparation methods. The manual digestion was performed using coated low-retention microcentrifuge tubes; however, the same coating was not utilized for preliminary experimentation on the AssayMAP Bravo liquid handling platform. To address this, 96-well microplates containing the same or similar low-retention coatings were acquired and evaluated. Eppendorf Lo-Bind and Corning® Non Binding Surface treated plates were evaluated, with untreated Grenier U-bottom serving as a control. Single source fluids were prepared and processed using each plate type, with the optimal plate selected based on peak intensity of peptide targets. For example, peak intensities of seminal fluid peptide DIFSTQDELLVYNK on each plate are depicted in **Figure 2.25**. The greatest peak area intensity is illustrated by the Corning® Non Binding Surface plate. Similar results were observed across peptide markers of the remaining target fluids.

Furthermore, with the manual preparation, the entire sample volume is transferred onto sorbent material during SPE cleanup and purification. With the automated platform, however, a minimum sample volume (20 µL of the 250 µL digestion product) must remain in each well to ensure that no air is injected into the micro cartridges during SPE cleanup. In summary, despite the lower peak area responses, the automated sample processing procedure was selected for use for the remainder of the study. The automated liquid handling platform deposits purified tryptic peptides directly into the 96-well autosampler plate, further streamlining the processing procedure.

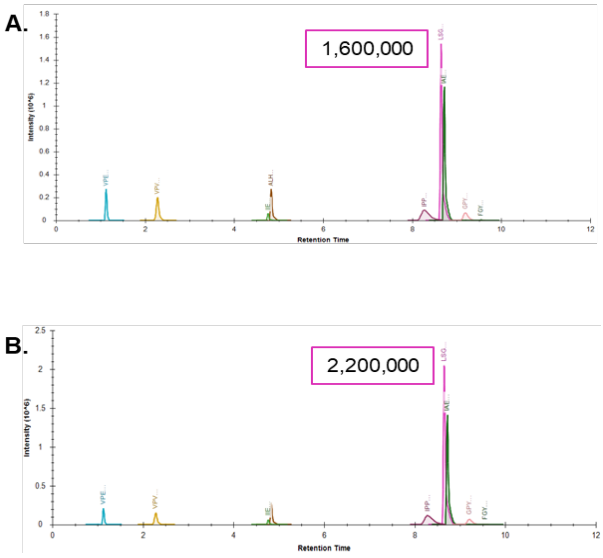


Figure 2.22. Digestion and purification of saliva via the (A) automated procedure and (B) manual procedure. Peak area response for alpha-amylase peptide LSGLLDLALGK is illustrated in pink.

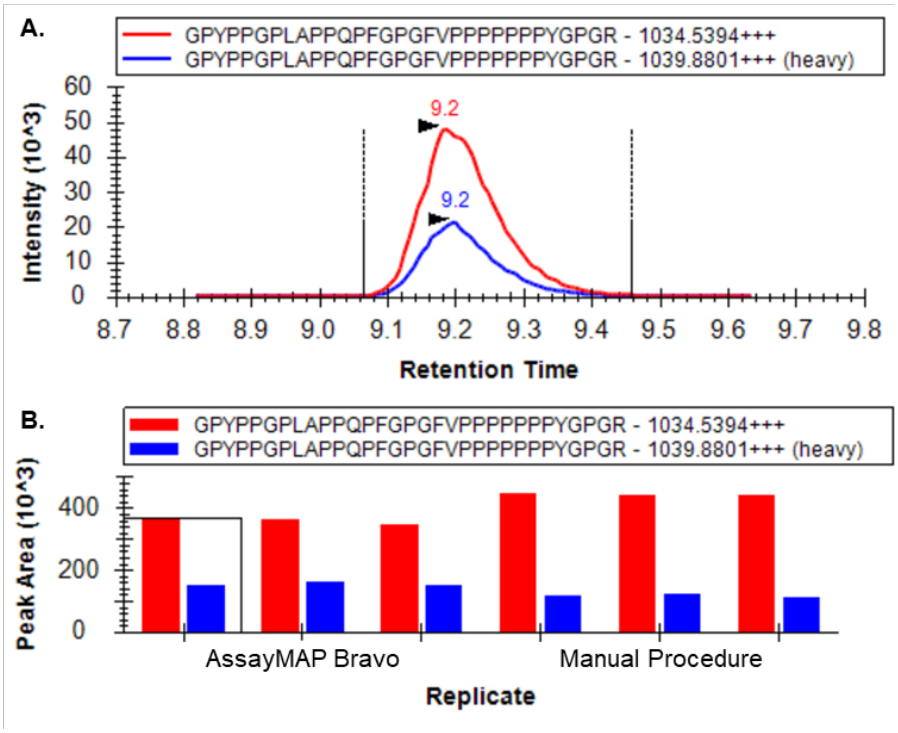


Figure 2.23. Digest and purification comparison of saliva protein submaxillary gland androgen-regulated protein 3B peptide GPYPPGPLAPPQPFGPGFVPPPPPPPYGPGR. Endogenous peptide peak area response (red) and internal standard peptide peak area response (blue) illustrated for three replicates processed on the automated platform and with the manual procedure. (A) Representative chromatography on the AssayMAP Bravo system. (B) Consistency in peak area response.

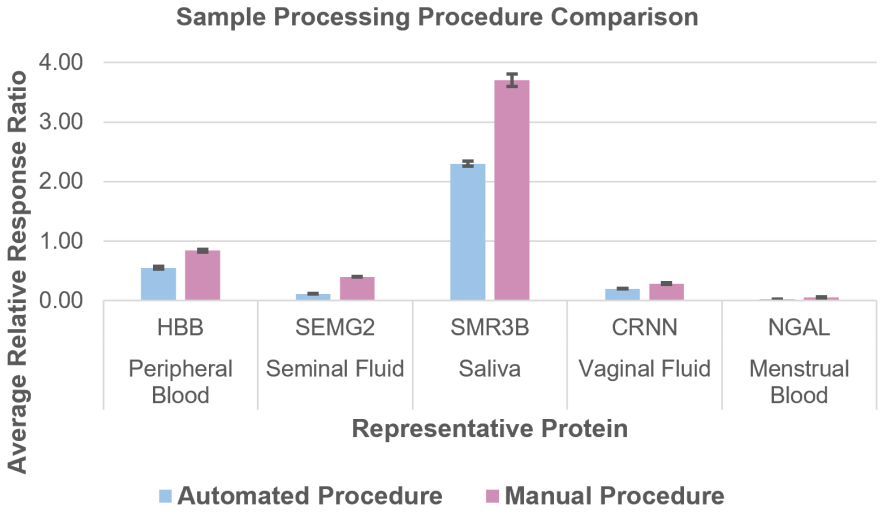


Figure 2.24. Single source body fluids processed on the AssayMAP Bravo liquid handling platform (blue) and manual procedure (purple). Representative proteins for each fluid evaluated are depicted on the x-axis (HBB, hemoglobin; SEMG2, semenogelin 2; SMR3B, submaxillary gland androgen-regulated protein 3B; CRNN, cornulin; NGAL, neutrophil gelatinase). Average relative response ratio is represented on the y-axis. Error bars represent standard error.

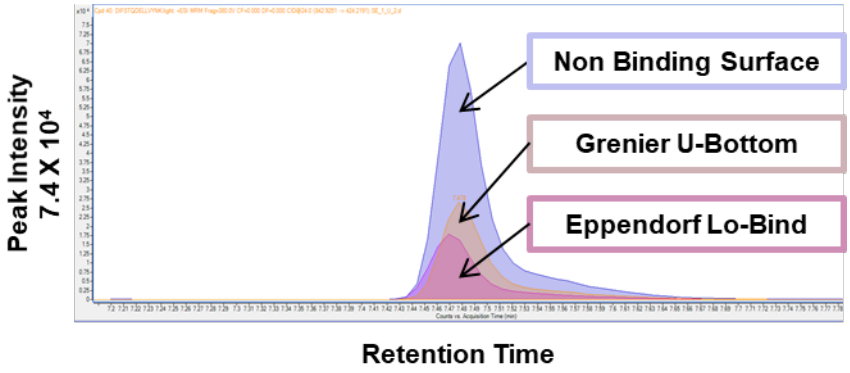


Figure 2.25. 96-well microplate comparison for use in automated digestion and purification. Three microplates were evaluated for greatest peak area intensity, depicted on the y-axis. Shown is a chromatogram for semenogelin 1 peptide DIFSTQDELLVYNK.

2.3 Concluding Remarks

In summary, the results detailed herein demonstrate the ability to automate sample processing and expedite analysis of the research grade multiplex assay without loss of sensitivity. Previously identified protein and peptide biomarker targets were subjected to further scrutiny for inclusion in the high throughput LC-MS/MS assay. With the creation of a targeted analytical method, individual parameters were sufficiently established for optimal target detection and

identification. The final biomarker target list includes both presumptive and confirmatory proteins consistent with peripheral blood, seminal fluid, saliva, and vaginal/menstrual fluid.

Implementation of an automated sample processing protocol not only sufficiently increased the number of samples amenable to preparation in a given batch but mitigated preventable sources of human error during digestion and cleanup procedures. Where small batches averaging fifteen samples were prepared for analysis using the research grade assay, the optimized protocol was constrained only to the capacity of a 96-well plate. Furthermore, by limiting the amount of sample handling time by the analyst, the automated protocol promotes efficiency of additional laboratory resources in regard to analysts' time. The benefits of automation and high throughput screening are advantageous to generating interest among practitioners for implementation of a novel technique into operational laboratories.

The following two chapters detail a full developmental validation and comparison assessment of the optimized LC-MS/MS assay and sample processing protocol described within this chapter. With a verified protein biomarker panel and preparation protocol, the analytical boundaries and limitations of the LC-MS/MS needed identifying. Therefore, a full developmental validation was conducted in accordance with standard guidelines within forensic biology and toxicology. Additionally, compatibility of the LC-MS/MS assay within the greater forensic biology workflow was demonstrated.

CHAPTER 3: DEVELOPMENTAL VALIDATION OF AN AUTOMATED MULTIPLEX ASSAY FOR THE IDENTIFICATION OF BIOLOGICAL FLUIDS

3 Introduction

The objective of this phase of the research was to validate the developed and optimized multiplex serological assay. Validation is the final phase of the proteomic workflow, allowing for efficacy and reliability of the analytical method to be established as acceptable prior to implementation into an operational environment. The experimentation performed during this phase was designed in accordance with the Quality Assurance Standards set forth by the Federal Bureau of Investigation (FBI) and recommended guidelines published by the Scientific Working Group on DNA Analysis Methods (SWGDAM) and the Scientific Working Group on Forensic Toxicology (SWGTOX). Due to the hybrid nature of experimentation and data analysis described, it was determined that both toxicological and serological guidelines were to be consulted and exercised for validation purposes. Specifically, with the use of liquid chromatography-tandem mass spectrometry (LC-MS/MS), validation guidelines were referenced via SWGTOX publications for experimental procedures such as ion suppression and enhancement, repeatability, reproducibility, and limit of detection.

This chapter reports the validation results of the previously described proteomic assay for the identification of peripheral blood, seminal fluid, saliva, and vaginal/menstrual fluid. Body fluid specific protein markers were previously selected under prior funding, where they underwent rigorous discovery and verification of the candidate markers within a greater population. The selected markers were reconfirmed as described in Chapter Two, with each protein marker and subsequent peptide targets assessed both in vitro with analytical identification and *in silico* using bioinformatics software. The successful operation of the method at each level described herein

identified working conditions and associated limitations that were acknowledged to estimate the true performance of the analytical method.

3.1 Methods and Materials

All research conducted under this phase of the project was reviewed and approved by the University of Denver Institutional Review Board (IRB) for research involving human subjects as described under section 2.1.1.

3.1.1 Repeatability and Reproducibility Sample Preparation

The research scientist prepared all body fluid aliquots for sample preparation following protocols outlined in section 2.1.1. Aliquots were stored at -80 ˚C until use. For the remainder of the study, both the research scientist and laboratory advisor performed sample processing procedures on the prepared aliquots simultaneously, including total protein quantitation, reagent preparation, plate loading for digestion and cleanup, and sample reconstitution for instrumental analysis. Protocols outlined in section 2.1.4 were followed during this portion of the study on all five target body fluids. Procedures were performed over the course of three days.

Repeatability was recorded as the variation in precision for results obtained by a single analyst. Reproducibility was recorded as the variation in accuracy obtained between analysts conducting identical protocols. Coefficient of variation (CV) of total protein quantitation in addition to the variation in internal standard were calculated for each individual and between individuals.

3.1.2 Sensitivity Sample Preparation

Target body fluid from five individuals was prepared according to parameters outlined in section 2.1.1. A serial dilution of pooled fluid was created in deionized water. For seminal fluid, saliva, and vaginal fluid, dilutions were started at 2-fold. Knowing *a priori* peripheral and menstrual blood exhibit concentrated protein material, dilutions were started at 100-fold. Dilutions were not quantitated for total protein amount. 200 µL of each dilution was lyophilized and digested followed by SPE cleanup using protocols detailed in section 2.1.4. Sample dilutions were prepared in triplicate, with 20 µL of sample injected on column. A select set of sample dilutions were prepared

and analyzed given laboratory resources. **Table 3.1** below details dilutions analyzed via LC-MS/MS per target body fluid.

Table 3.1. Dilutions analyzed for the sensitivity study of the multiplex assay validation.

Seminal Fluid	Saliva	Peripheral Blood	Menstrual Blood	Vaginal Fluid
1.02E+03	8.00E+00	1.60E+03	1.60E+03	4.00E+00
2.05E+03	1.60E+01	3.20E+03	3.20E+03	8.00E+00
4.10E+03	3.20E+01	6.40E+03	6.40E+03	1.60E+01
8.19E+03	6.40E+01	1.28E+04	1.28E+04	3.20E+01
1.64E+04	1.28E+02	2.56E+04	2.56E+04	6.40E+01
3.28E+04	2.56E+02	5.12E+04	5.12E+04	1.28E+02
6.55E+04	5.12E+02	1.02E+05	1.02E+05	2.56E+02
1.31E+05	1.02E+03	4.10E+05	4.10E+05	5.12E+02
2.62E+05	2.05E+03	8.19E+05	8.19E+05	1.02E+03
5.24E+05	4.10E+03	1.64E+06	1.64E+06	2.05E+03
	8.19E+03	3.28E+06	3.28E+06	4.10E+03
	1.64E+04	6.55E+06	6.55E+06	8.19E+03
	3.28E+04			1.64E+04
	6.55E+04			3.28E+04
				6.55E+04

3.1.3 Stability Sample Preparation

3.1.3.1 Freeze Thaw Stability

Target body fluid from five individuals was pooled and prepared in three batches containing triplicate samples according to parameters outline in section 2.1.4. The first batch of samples (Day 1) were processed and analyzed, with 2.5 µg of protein material injected on column. Remaining sample batches (Day 2 and Day 3) were stored at -80 ˚C until future use. Day 2 of Freeze Thaw Stability started by completely thawing all prepared material to room temperature and continuing with the sample processing procedure for batch two (Day 2). Batch three material was again frozen at -80 ˚C until future use. Day 3 of Freeze Thaw Stability was conducted in the same manner, allowing the final batch to thaw completely prior to sample processing. Vaginal fluid samples were analyzed for 2.5 µg and 10 µg on column, to adequately assess any loss of lower abundant target peptides.

3.1.3.2 Autosampler Stability

Target body fluid from five individuals was prepared according to parameters outlined in section 2.1.4. Samples were analyzed via LC-MS/MS over the course of 3 days, with the microplate

containing processed sample remaining in the autosampler kept at 8 ˚C for the duration of the study. 2.5 µg of material was analyzed on column. Samples were reinjected 24 hours (Day 2) and 48 hours (Day 3) after original sample preparation.

3.1.4 Mixtures Sample Preparation

Target body fluid from five individuals was pooled, filtered, and quantitated according to parameters outlined in section 2.1.1. Mixtures in a 1:1 ratio of 50 µg total protein per fluid were generated, for a total of 100 µg protein material. All possible combinations of two-fluid mixtures were created: vaginal fluid (VF) and saliva (SA); VF and seminal fluid (SE); VF and peripheral blood (PB); VF and menstrual blood (MB); MB and PB; MB and SE; SA and MB; SA and PB; SA and SE; SE and PB. Mixture samples underwent digestion and SPE procedures outlined in section 2.1.4. 10 µg of material was analyzed on column.

3.1.5 Specificity Sample Preparation

Whole blood samples from 11 different species (Rhesus monkey, horse, pig, chicken, cow, beagle, mouse, black bear, coyote, white-tailed deer, river otter) and saliva samples from 2 species (cow, Rhesus monkey) were purchased from Innovative Research™ (Novi, MI). Blood samples were treated with potassium ethylenediamine tetraacetic acid anticoagulant. In addition, oral swabs from 2 species (alpaca, tortoise) were obtained by the research scientist.

Oral swabs were resuspended in 500 µL of deionized water and allowed to solubilize for 30 minutes at room temperature with frequent vortexing. All samples (blood, saliva, oral swabs) were microcentrifuged at 12,000 x g for 10 minutes. Supernatant was filtered using Costar® Spin-X® centrifugal filters (Corning®, Corning, NY) at 10,000 x g for 2 minutes. The remaining sample preparation follows procedures outlined in section 2.1.4. 2.5 µg of material was analyzed on column.

3.1.6 Casework Type Sample Preparation

A compilation of all casework samples prepared is detailed in **Appendix C**.

3.1.6.1 Substrate Samples

Substrates tested include cotton, denim, leather, carpet, drywall, plastic beverage bottle, aluminum beverage can, and feminine hygiene menstrual pad. Substrates were cut into approximately 2 cm x 2 cm squares upon which 50 µL of single source body fluid was applied. Samples were dried at room temperature overnight. Substrates were solubilized in 1 mL deionized water for 30 minutes with frequent vortexing. Substrates were placed in a centrifugal basket and centrifuged at 10,000 x g for 10 minutes. Supernatant was transferred for analysis following procedures outlined in section 2.1.4 and cell pellets were discarded.

3.1.6.2 Environmental Contaminant Samples

Environmental contaminants tested include dirt slurry, rust slurry, 10% bleach in water solution, chewing tobacco, cigarette butt, lubricated condom with spermicide additive, and water-based personal lubricant. Dirt slurry was prepared by mixing 1 gram weather-conditioned soil with 1 mL tap water. In a similar manner, the rust slurry was prepared by mixing 500 µg rust with 500 µL of tap water. Rust was obtained by scarping the external surface of a weather-conditioned chain link fence. Neat bleach was diluted 10-fold with deionized water for a final concentration of 10% bleach. Chewing tobacco expellant was obtained from a volunteer that actively engages in using smokeless tobacco products. Water-based personal lubricant containing glycerin was purchased (CVS Pharmacy™, Woonsocket, RI).

Liquid matrix contaminants (*i.e.,* dirt slurry, rust slurry, 10% bleach solution, chewing tobacco, and water-based lubricant) were thoroughly mixed in a 1:1 v/v ratio with corresponding target body fluid. A 100 µL volume of the resulting mixture was applied to a full cotton swab and allowed to dry at room temperature overnight. Solid matrix contaminants (*i.e.,* cigarette butt, spermicide lubricated condom, menstrual pad) were cut into a 2 cm x 2 cm square prior to applying 50 µL target body fluid. Prepared samples were dried at room temperature overnight. Full swabs and 2 cm x 2 cm contaminant samples were extracted in 1 mL deionized water for 30 minutes with frequent vortexing. Substrates were placed in a centrifugal basket centrifuged at 10,000 x g for 10

minutes. Supernatant was transferred for analysis following procedures outlined in section 2.1.4 and cell pellets were discarded.

3.1.6.3 Mixtures Samples

Mixture samples were prepared using single source body fluid from consenting donors. A 50-fold two-component mixture solution was created by thoroughly vortexing 10 µL of neat body fluid serving as a minor contributor with 500 µL neat body fluid serving as the major contributor. 100 µL of mixture solution was applied to a cotton-tipped swab and allowed to dry at room temperature overnight. Full swabs were extracted in 1 mL deionized water for 30 minutes with frequent vortexing. Substrates were placed in a centrifugal basket and centrifuged at 10,000 x g for 10 minutes. Supernatant was transferred for analysis following procedures outlined in section 2.1.4 and cell pellets were reserved for genetic testing, described in Chapter 4.

Five two-component mixture samples were prepared for this portion of the project: (1) vaginal fluid in urine, (2) saliva in vaginal fluid, (3) seminal fluid in vaginal fluid, (4) saliva in menstrual blood, (5) seminal fluid in menstrual blood.

3.1.6.4 Sexual Assault Samples

3.1.6.4.1 Simulated Sexual Assault Samples

Simulated sexual assault samples were prepared by thoroughly mixing 10 µL neat semen with 1 mL neat vaginal fluid, both fluids being from a single donor. This was repeated for a total of seven samples. The semen and vaginal fluid utilized for all seven samples were donated by the same individuals to prevent inconsistency in protein expression. Samples were incubated at 37 ˚C for one day, three days, five days, seven days, nine days, and eleven days, with time zero samples immediately stored for future processing. When the designated time point was reach, samples were removed from the incubator and frozen at -80 ˚C until analysis. Samples were prepared and analyzed following procedures outlined in section 2.1.4.

3.1.6.4.2 Mock Sexual Assault Kit Samples

Vaginal, oral, and rectal swabs were obtained from a single, consenting female individual. Immediately after sampling, neat semen from a single donor was applied in order to most accurately

simulate an authentic sample collection. Semen was diluted 100-fold with deionized water prior to application. 10 µL of diluted semen was applied, for a volume equivalent of 0.1 µL semen applied to each swab. Samples were prepared in duplicate and allowed to dry at room temperature overnight. Full swabs were extracted in 1 mL deionized water for 30 minutes with frequent vortexing. Substrates were placed in a centrifugal basket and centrifuged at 10,000 x g for 10 minutes. Supernatant was transferred for analysis following procedures outlined in section 2.1.4 and cell pellets were reserved for genetic testing, described in Chapter 4. This preparation protocol was duplicated using semen provided by a vasectomized male individual, for a total of 12 mock sexual assault kit samples (6 non-vasectomized and 6 vasectomized male donors).

3.1.6.4.3 Digital Swab Samples

Three types of digital swab samples were prepared for this portion of the study: vaginal swabs, menstrual blood swabs, and saliva swabs. One consenting female donor was utilized. The volunteer thoroughly washed their hands prior to self-penetration. In the comfort of the individual's home, the volunteer was instructed to digitally penetrate the vagina both during menses and in the absence of menses using the index finger for 20 seconds. The volunteer removed their finger and allowed the deposited material to dry for approximately 5 minutes. Self-collection using a dry cotton-tipped swab was performed, with the entire area of the finger swabbed for collection. Collection was repeated in duplicate for each form of penetration, with each sample taken on a separate day. Upon receipt at the laboratory, swabs were dried at room temperature overnight to ensure consistency. Full swabs were extracted in 1 mL deionized water for 30 minutes with frequent vortexing. Substrates were placed in a centrifugal basket and centrifuged at 10,000 x g for 10 minutes. Supernatant was transferred for analysis following procedures outlined in section 2.1.4 and cell pellets were reserved for genetic testing, described in Chapter 4.

3.1.6.5 Degradation Samples

Degradation samples simulating laundered items of evidence were generated for this portion of the study. Peripheral blood and semen were the selected target body fluids. To a cotton bath towel and to denim jeans, 1 mL of single-source peripheral blood was applied to the substrate,

outlined with permanent marker, and allowed to dry at room temperature overnight. In addition, a control region to which no body fluid was applied approximately 20 cm from the stain region was marked for analysis. To a cotton-blend bed sheet and a cotton pair of women's underwear, 1 mL of single-source semen was applied to the substrate, outlined with permanent marker, and allowed to dry at room temperature overnight. In addition, a control region to which no body fluid was applied approximately 20 cm from the stain region (bed sheet) or 8 cm from the stain region (underwear) was marked for analysis. Target body fluid was applied to simulate stained items of evidence that are commonly received in caseworking laboratories.

Substrates were washed individually with 1.5 fl. oz. commercially available laundry detergent (Seventh Generation™ Free and Clear, Burlington, VT) in cold water for a 15-minute wash cycle using a household grade washing machine. Substrates were individually dried in a heated commercial dryer for approximately 40 minutes. 2 cm x 2 cm cuttings of the stain and control region were excised from the substrate. Cuttings were solubilized in 1 mL deionized water for 30 minutes with frequent vortexing. Substrates were placed in a centrifugal basket centrifuged at 10,000 x g for 10 minutes. Supernatant was transferred for analysis following procedures outlined in section 2.1.4 and cell pellets were reserved for genetic testing, described in Chapter 4.

3.1.6.6 Aged Samples

Aged swabs were prepared by spotting 50 µL of neat, single-source peripheral blood, menstrual blood, saliva, semen, or vaginal fluid on a full cotton swab. An additional set of samples were prepared using 150 µL of neat, single-source vaginal fluid. Swabs were allowed to incubate at room temperature for 35 days, with collections at time zero, Day 1, Day 3, Day 7, and Day 35. Upon collection, full swabs were frozen at -80 °C until analysis. All time points were prepared in duplicate. Samples were prepared according to procedures outlined in section 2.1.4.

3.1.6.7 Sensitivity Samples

Sensitivity samples were prepared by diluting single-source, neat body fluid in deionized water in a serial manner to cover a range of final concentrations. Dilutions prepared are detailed in **Table 3.2**. 150 µL of each dilution was spotted on a full cotton swab and allowed to dry at room

temperature. Full swabs were solubilized in 1 mL deionized water for 30 minutes with frequent vortexing. Substrates were placed in a centrifugal basket and centrifuged at 10,000 x g for 10 minutes. Supernatant was transferred for analysis following procedures outlined in section 2.1.4 and cell pellets were reserved for genetic testing, described in Chapter 4.

Table 3.2. Dilutions analyzed for the sensitivity study within casework sample analysis.

Peripheral Blood Menstrual Blood Seminal Fluid	Saliva Vaginal Fluid
Neat	Neat
1:2	1:2
1:10	1:10
1:100	1:100
1:1,000	1:500
1:2,000	1:1,000
1:5,000	1:2,000
1:10,000	1:5,000
1:20,000	1:10,000
1:40,000	

3.1.7 Limit of Detection Sample Preparation

Isotopically-labeled peptide internal standards (AQUA) were purchased (New England Peptide, Inc., Gardner, MA) for each selected peptide marker, with the exception of proteins statherin and submaxillary gland androgen-regulated protein 3B. AQUA were received as 1 nmol of material, lyophilized to dryness. Material was reconstituted in 200 µL of 30% ACN with 0.1% FA in LC-MS grade water. Standards were pooled and brought up to volume in 30% ACN with 0.1% FA for a final concentration of 100 pmol/mL. 20 pmol aliquots (200 µL) were prepared, lyophilized, and stored at -80 ºC. Upon use, 20 pmol aliquots were resuspended in 40 µL of 30% ACN with 0.1% FA for a 0.5 pmol/µL stock solution.

Blank matrix was prepared by pooling target body fluid from five individuals. 100 µg total protein underwent tryptic digestion and SPE cleanup prior to AQUA fortification. The 0.5 pmol/µL stock solution was further diluted with 30% ACN with 0.1% FA to 50 fmol/µL and 5 fmol/µL substocks, used for creation of the serial dilution. Matrix was fortified with AQUA material following

volumes and target concentrations listed in **Table 3.3**. Samples were analyzed following parameters outlined in section 2.1.4.

Table 3.3. Volumes and concentrations of internal standard used during the limit of detection experimentation.

Target Amount on Column	AQUA Fortification Amount	Volume of AQUA Substock Solution
50 fmol	2 pmol	4 µL of 0.5 pmol/µL
25 fmol	1 pmol	2 µL of 0.5 pmol/µL
10 fmol	0.4 pmol	8 µL of 50 fmol/µL
5 fmol	0.2 pmol	4 µL of 50 fmol/µL
2.5 fmol	0.1 pmol	2 µL of 50 fmol/µL
1 fmol	40 fmol	8 µL of 5 fmol/µL
0.5 fmol	20 fmol	4 µL of 5 fmol/µL

3.1.8 Ion Suppression Sample Preparation

Target body fluid from five individuals was prepared and pooled for ion suppression and enhancement analysis. 100 µg of total protein was targeted for digestion. AQUA stock solution was prepared in the same manner as detail in section 3.1.7. Two sample sets were prepared for this portion of the study. First, 20 µL of 0.5 pmol/µL AQUA stock solution was analyzed independently (*i.e.,* in the absence of blank matrix). Second, pooled body fluid was fortified with 20 µL of 0.5 pmol/µL AQUA stock for a target amount of 10 pmol, with all samples prepared in triplicate. Samples were prepared according to procedures outlined in section 2.1.4. 2.5 µg of sample was analyzed per injection.

3.1.9 Carryover Sample Preparation

Samples were prepared in triplicate for a preliminary carryover study by pooling fluid from five different individuals and digesting 100 µg of total protein. Following tryptic digestion and SPE cleanup parameters outlined in section 2.1.4, samples were reconstituted in 100 µL of 2% ACN with 0.1% FA. 10 µg, 20 µg, and 30 µg of processed sample were injected on column, with each injection followed by a blank mobile phase injection (0.1% FA in water) of the same volume. Carryover was assessed by the presence of compounds within the subsequent blank injection.

A second set of samples targeting smaller quantities of total protein were prepared in triplicate and analyzed. Following the same procedure as described above, 0.5 µg, 1 µg, 2.5 µg,

and 5 µg quantities of digested protein were evaluated per body fluid. A blank mobile phase sample (0.1% FA in water) of the same volume was injected directly after each sample.

3.1.10 Blind Sample Analysis

A series of 50 blind samples were received from the grant agency for analysis. No information was supplied with the samples received. Samples consisted of pre-halved fiber-tipped swabs, swatches of cloth, and condoms. Exhibits were taken according to a pre-defined laboratory sampling protocol. Swab samples received were cut in half (*i.e.,* approximately ¼ of a full swab), reserving the remain section for additional testing, if necessary. Cuttings measuring 2 cm x 2 cm were taken from cloth swatch samples received, reserving the remaining cloth for additional testing, if necessary. The inside and outside of condom samples were double swabbed using a sterile cotton swab wetted with 2% sodium dodecyl sulfate (SDS) followed by a dry cotton swab. Condom swabs were dried at room temperature for at least 2 hours. Wet and dry swabs were combined and processed as a single sub-exhibit. Sub-exhibits were solubilized in 500 µL of deionized water for 30 minutes with frequent vortexing. Substrates were placed in a centrifugal basket and centrifuged at 10,000 x g for 10 minutes. Supernatant was transferred for analysis following procedures outlined in section 2.1.4 and cell pellets were discarded.

3.2 Results and Discussion

3.2.1 Repeatability and Reproducibility

Reproducibility was assessed to ensure robust results could be obtained over a period of multiple analysis batches prepared by a single individual. Repeatability was evaluated to ensure reliable results could be obtained between individual analysts across a period of multiple analysis batches. For this portion of the validation, reproducibility and repeatability were assessed in tandem through the preparation, analysis, and data acquisition of samples by two analysts over the course of a three-day period. This entailed BCA quantitation, robotic digestion, SPE cleanup, and analysis via LC-MS/MS.

Body fluid samples were received and prepared for testing by a single individual within the laboratory. Pooled fluid was aliquoted into single use tubes to ensure consistency of the samples

being processed for this portion of the validation. On each day of preparation, analysts performed a BCA quantitation on a single use aliquot for each target body fluid to determine the total protein concentration prior to tryptic digestion. The necessary reagents for robotic digestion and SPE cleanup were prepared individually by the analysts. Data presented in this section is representative of the variation observed for the BCA total protein quantitation in addition to the mass spectrometry results obtained including signal response, retention time, and qualitative response ratios. Furthermore, the performance of the internal positive control between analysts and across the three-day period was evaluated for reliability and robustness.

3.2.1.1 BCA Quantitation

BCA quantitation is required prior to tryptic digestion in order to normalize the amount of total protein input into the robotic preparation process. This is to ensure that the proper ratio of digestion enzyme to protein substrate is met, in addition to monitoring the amount loaded onto the SPE cleanup cartridges and LC-MS/MS system. The average protein concentration, as well as the measured combined and individual variability of the BCA quantitation assay for each body fluid, is detailed in **Table 3.4**. Coefficient of variation (CV) values both within and between analysts was less than 10% across this portion of the validation.

Table 3.4. Calculated CV (as a percent) of average protein concentration between analysts and combined for each fluid assayed.

Fluid	Analyst 1	Analyst 2	Combined
Menstrual Blood	5.562	7.300	6.188
Peripheral Blood	4.021	6.315	5.056
Seminal Fluid	5.437	8.342	6.861
Vaginal Fluid	6.899	8.370	8.557
Saliva	4.095	7.830	7.385

3.2.1.2 Signal Response

The peak area of each peptide in the assay was assessed for all replicates as well as across the replicates for the two analysts individually. Of the 46 peptides included in the assay, 41 showed CV values less than 25%, with the majority of these targets showing less than 10%

variation. Data for this portion of the study can be found in **Appendix D**, with results for seminal fluid depicted in **Table 3.5**.

As previously stated, a small fraction of peptides did display higher degrees of peak area variability. High CV values were associated with either small hydrophilic (*i.e.,* eluting early during chromatographic separation) or large hydrophobic (*i.e.,* eluting late during chromatographic separation) peptides. For example, semenogelin 2 peptide GSISIQTEEK is one of the first eluting peptides in the assay, with a retention time of 1.9 minutes, and had a combined peak area CV of 51.764%. It is hypothesized that this specific peptide target is not captured efficiently during the SPE cleanup, resulting in more variable recovery across replicates. Likewise, it is hypothesized that more hydrophobic peptide markers will be bound, essentially irreversibly, to plastics during tryptic digestion and to the sorbent material of SPE cartridges. In addition, there may be reduced resolubilization issues after any lyophilization step. At the onset of peptide target selection, it was expected that some peptides would display more variability in recovery, solubility, and nonspecific absorption than others, simply due to the specific chemistries associated with each amino acid sequence. As a counter to this potential challenge, the ability to target multiple proteins per fluid and multiple peptides for each protein (*i.e.,* a fundamental concept underlying the design of the assay), helped to ensure that there are reliable peptide biomarkers which can be consistently detected for identification of each fluid. For example, other semenogelin 2 peptides (*e.g.,* DVSQSSISFQIEK) show consistent CV values of approximately 8%, albeit at lower overall peak areas.

Table 3.5. Calculated CV (as a percent) for peak area response of seminal fluid peptide markers between analysts and combined.

Fluid	Protein	Peptide	Analyst 1	Analyst 2	Combined
Seminal Fluid	Acid Phosphatase	FVTLVFR	87.638	41.252	95.688
		FQELESETLK	19.405	14.717	26.483
		ELSELSLLSLYGIHK	5.787	10.988	8.773
	Prostate Specific Antigen	FLRPGDDSSHDLMLLR	54.820	21.388	62.553
		LSEPAELTDAVK	34.849	17.358	45.393
	Semenogelin 2	GSISIQTEEK	38.678	19.467	51.764
		GSISIQTEEQIHGK	10.208	10.259	15.247
		DVSQSSISFQIEK	2.677	11.744	8.304
	Semenogelin 1	DIFSTQDELLVYNK	5.340	10.368	8.063
		QITIPSQEQEHSQK	6.822	7.261	7.747
	Epididymal Secretory	DCGSVDGVIK	6.243	11.758	9.125
		SGINCPIQK	9.318	9.948	9.425

3.2.1.3 Retention Time

The retention time of each peptide biomarker in the assay was assessed for all replicates as well as across the three replicates for the two analysts. Retention time averages for each analyst were used to calculate the variability. Percent CV values for this dataset were all below 0.5%. An illustrative example of results for seminal fluid is shown in **Table 3.6**. The remainder of the data are presented in **Appendix E**.

Table 3.6. Calculated CV (as a percent) for retention time of seminal fluid peptide markers between analysts and combined.

Fluid	Protein	Peptide	Analyst 1	Analyst 2	Combined
Seminal Fluid	Acid Phosphatase	FVTLVFR	0.049	0.059	0.054
		FQELESETLK	0.069	0.069	0.067
		ELSELSLLSLYGIHK	0.058	0.059	0.058
	Prostate Specific Antigen	FLRPGDDSSHDLMLLR	0.081	0.081	0.078
		LSEPAELTDAVK	0.085	0.071	0.078
	Semenogelin 2	GSISIQTEEK	0.124	0.124	0.120
		GSISIQTEEQIHGK	0.134	0.160	0.147
		DVSQSSISFQIEK	0.075	0.079	0.076
	Semenogelin 1	DIFSTQDELLVYNK	0.063	0.042	0.052
		QITIPSQEQEHSQK	0.174	0.132	0.151
	Epididymal Secretory	DCGSVDGVIK	0.138	0.157	0.148
		SGINCPIQK	0.147	0.000	0.104

3.2.1.4 Qualitative Response Ratios

The ion ratio for each peptide biomarker was assessed by dividing the peak area response of the quantifier ion (*i.e.,* more abundant transitions observed during target selection) by the peak area response of the qualifier ion(s) (*i.e.,* lower abundant transitions observed during peptide

selection). Ion ratios were assessed for all replicates as well as across the two analysts. The ion ratios for all peptides were found to be highly consistent, with a majority of calculations showing CV values less than 5%. An illustrative example of results for seminal fluid is shown in **Table 3.7**. Epididymal secretory protein peptide DCGSVDGVIK exhibited a higher CV value but was consistent between the two analysts, demonstrating a high rate of precision both within-run and between-run. The remainder of the data are presented in detail in **Appendix F**. Only neutrophil gelatinase protein in menstrual blood exhibited higher variability in the qualitative response ratio. In this case, the proteins were of very low abundance in the pooled sample being assessed.

Table 3.7. Calculated CV (as a percent) for ion ratios of seminal fluid peptide markers between analysts and combined.

Fluid	Protein	Peptide	Analyst 1	Analyst 2	Combined
Seminal Fluid	Acid Phosphatase	FVTLVFR	1.503	1.240	1.342
		FQELESETLK	4.248	3.127	3.636
		ELSELSLLSLYGIHK	4.844	2.577	4.455
	Prostate Specific Antigen	FLRPGDDSSHDLMLLR	3.828	3.360	3.677
		LSEPAELTDAVK	1.918	1.032	1.601
	Semenogelin 2	GSISIQTEEK	4.360	3.249	4.025
		GSISIQTEEQIHGK	3.749	3.127	3.505
		DVSQSSISFQIEK	6.209	3.842	5.066
	Semenogelin 1	DIFSTQDELLVYNK	3.455	4.174	3.801
		QITIPSQEQEHSQK	2.578	3.036	2.810
	Epididymal Secretory	DCGSVDGVIK	5.835	5.021	5.501
		SGINCPIQK	3.971	3.225	3.863

3.2.1.5 Internal Positive Control

The performance of the internal positive control (IPC) was assessed by comparing the response ratio of the digested myelin basic protein to that of an isotopically-labeled internal standard added to the sample being analyzed. The response ratio was found to be highly consistent across all body fluids. The variability observed both among different samples of individual body fluid and between different body fluids consistently displayed CV values less than 8% (**Table 3.8 and Figure 3.1**). Samples evaluated for this portion of the validation displayed and average peak area response ratio of 13.7±0.999.

Table 3.8. Combined calculated CV (as a percent) for the internal positive control peak area response ratio for the duration of repeatability and reproducibility experimentation.

Protein	Peptide	Analyst 1	Analyst 2	Combined
Myelin Control	DTGILDSLGR	7.158	7.095	7.546

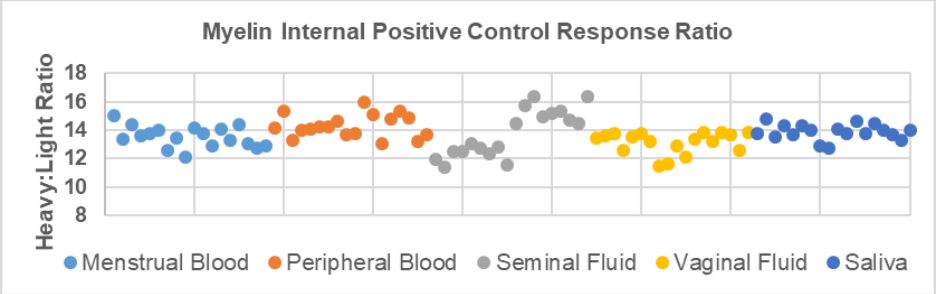


Figure 3.1. Internal positive control response ratio for the duration of repeatability and reproducibility experimentation.

3.2.2 Sensitivity

A series of dilutions were prepared for each body fluid using pooled material from multiple individuals. These samples were assayed in order to establish the minimal protein quantity in which reliable results can be obtained. Based on data acquired under previous phases of this research and the sensitivity limits observed under previously completed research (NIJ awards 2009-DN-BX-K165; 2012-DN-BX-K035) the lower end of the dilution series was considerably extended from prior studies in order to evaluate lower limits of the assay. For peripheral blood and menstrual blood, a 2-fold dilution series was made, from a 1:100 dilution to a 1:6,533,600 dilution. For seminal fluid, a 2-fold dilution series from 1:2 through 1:524,288 was made. And lastly, for saliva and vaginal fluid, a 2-fold dilution series from 1:2 to 1:65,536 was generated. Not all created dilutions were analyzed via LC-MS/MS.

Data for each body fluid is summarized in **Tables 3.9-3.13**. Results were evaluated for retention time consistency, chromatographic peak shape, peak area response, as well as calculated ion ratios. Overall, peripheral blood was confidently identified at a 1:6,533,600 dilution (**Table 3.9**) and menstrual blood at 1:6,400 dilution (**Table 3.10**). Although both peptide targets for hemoglobin were accurately detected at the lowest dilution in the peripheral blood matrix, a majority

of the remaining protein markers exhibited dropout at the 1:204,800 dilution. Similar results were observed for peripheral blood protein markers in menstrual blood; however, vaginal fluid markers in menstrual blood were shown to exhibit less sensitivity. Four protein targets failed to be detected within the menstrual blood matrix (Ly6 PLAUR, suprabasin, periplakin, and involucrin), with remaining biomarkers exhibiting dropout at the protein level after the 6,400-fold dilution. Small proline rich protein 3 was detected in each dilution assessed, however, peptide peak areas did not display negative linearity as the dilution series decreased. Peak area intensities for peptide VPEPGCTK remained consistent between the 25,600-fold dilution and the 6,533,600-fold dilution (**Figure 3.2**). Therefore, this marker was not considered when characterizing menstrual blood for biomarker sensitivity. Strong instances of peptide and protein dropout, particularly for the vaginal fluid biomarkers in this matrix, may be attributed to expression variability during the menstruation cycle.

Seminal fluid was accurately detected at the lowest dilution assessed, with three peptides identified at the 524,288-fold dilution (**Table 3.11**). All peptide targets were correctly detected at the 16,389-fold dilution, with linear peptide and protein dropout displayed as the dilution series decreased. Semenogelin 1, semenogelin 2, and acid phosphatase peptide targets met all acceptance criteria at the lowest dilution sample.

Saliva was characterized at the lowest dilution tested, with a single alpha amylase peptide detected at the 65,536-fold dilution (**Table 3.12**). All peptide markers were detected through the 256-fold dilution, and similar to seminal fluid, exhibited linear protein and peptide dropout as the dilution series decreased. With the exception of alpha amylase, remaining protein markers displayed complete dropout at the 8,192-fold dilution tested.

Vaginal fluid was identified in a two-tiered manner. Because the target biomarkers exhibit a wide range of specificity for vaginal fluid, the confidence level of accurately detecting and identifying vaginal fluid fluctuates. For this data set, vaginal fluid was confidently identified down to the 2,048-fold dilution by the presence of cornulin and neutrophil gelatinase, both of which exhibit strong specificity for vaginal fluid (**Table 3.13**). However, small proline rich protein 3 was detected

at the 32,768-fold dilution. Given the known cross-reactivity this protein exhibits, vaginal fluid detection was made with less confidence at this lower range. The use of this protein as a presumptive biomarker has been thoroughly detailed in interpretation guidelines.

Table 3.9. Observed sensitivity limit of peripheral blood. Proteins that were positively identified by all of their respective peptide markers are shown in dark green. Light green indicates where at least one peptide marker for the respective protein dropped out. White indicates complete protein dropout.

Fluid	Protein	Dilution Factor												
		1,600	3,200	6,400	12,800	25,600	51,200	102,400	204,800	409,600	819,200	1,638,400	3,276,800	6,533,600
Peripheral Blood	Hemoglobin													
	Apolipoprotein													
	Alpha-1 Antitrypsin													
	Hemopexin													
	Serotransferrin													

*T*able 3.10. Observed sensitivity limit of menstrual blood. Proteins that were positively identified by all of their respective peptide markers are shown in dark green. Light green indicates where at least one peptide marker for the respective protein dropped out. White indicates complete protein dropout.

Fluid	Protein	Dilution Factor												
		1,600	3,200	6,400	12,800	25,600	51,200	102,400	204,800	409,600	819,200	1,638,400	3,276,800	6,533,600
Menstrual Blood	Hemoglobin													
	Apolipoprotein													
	Alpha-1 Antitrypsin													
	Hemopexin													
	Serotransferrin													
	Small Proline Rich Protein													
	Cornulin													
	Neutrophil Gelatinase													
	Ly6/PLAUR													
	Suprabasin													
	Periplakin													
	Involucrin													

Table 3.11. Observed sensitivity limit of seminal fluid. Proteins that were positively identified by all of their respective peptide markers are shown in dark green. Light green indicates where at least one peptide marker for the respective protein dropped out. White indicates complete protein dropout.

Fluid	Protein	Dilution Factor									
		1,024	2,048	4,096	8,192	16,389	32,768	65,536	131,072	262,144	524,288
Seminal Fluid	Semenogelin 1										
	Semenogelin 2										
	Acid Phosphatase										
	Prostate Specific Antigen										
	Epididymal Secretory										

Table 3.12. Observed sensitivity limit of saliva. Proteins that were positively identified by all of their respective peptide markers are shown in dark green. Light green indicates where at least one peptide marker for the respective protein dropped out. White indicates complete protein dropout.

Fluid	Protein	Dilution Factor													
		8	16	32	64	128	256	512	1,024	2,048	4,096	8,192	16,389	32,768	65,536
Saliva	Alpha Amylase														
	Cystatin														
	Submaxillary Protein														
	Statherin														

Table 3.13. Observed sensitivity limit of vaginal fluid. Proteins that were positively identified by all of their respective peptide markers are shown in dark green. Light green indicates where at least one peptide marker for the respective protein dropped out. White indicates complete protein dropout.

Fluid	Protein	Dilution Factor														
		4	8	16	32	64	128	256	512	1,024	2,048	4,096	8,192	16,389	32,768	65,536
Vaginal Fluid	Small Proline Rich Protein															
	Cornulin															
	Neutrophil Gelatinase															
	Suprabasin															
	Involucrin															
	Periplakin															
	Ly6/PLAUR															

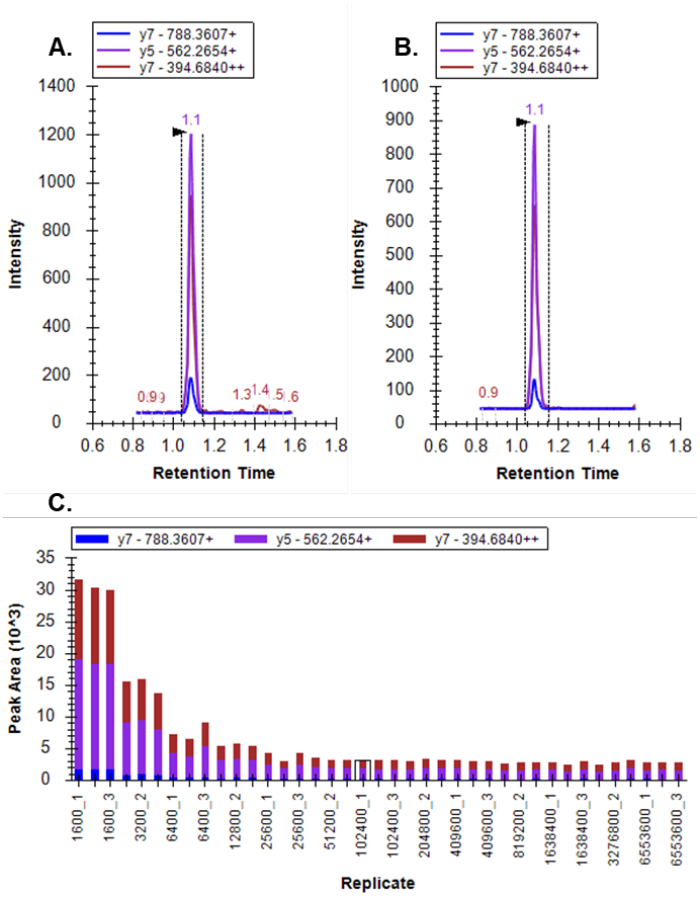


Figure 3.2. Small proline rich protein 3 peptide VPEPGCTK peak area response. (A) Peak area intensity at a 25,600-fold dilution. (B) Peak area intensity at a 6,553,600-fold dilution. (C) Peak area response across all replicates evaluated, progressing from 1,600-fold to 6,553,600-fold dilution.

3.2.3 Stability

Stability was evaluated to ensure that samples remain viable if an instrumentation error or power outage were to occur that causes samples either: (1) to remain in the autosampler overnight without being processed or (2) to be subjected to additional freeze-thaw cycles. This portion of the

validation assessed two aspects of a sample's stability. First, freeze thaw stability was evaluated using aliquots of pooled samples. Three samples were assayed immediately and served as baseline peak area response. The remaining six samples were stored at -80 ˚C. All six remaining aliquots were thawed the following day. Three of the thawed samples were analyzed on the second day and the remaining three were refrozen for subsequent thawing and analysis on day three. The second form of stability assessed, autosampler stability, was evaluated by storing processed samples in the chilled autosampler and analyzing them over a period of 3 days.

3.2.3.1 Freeze Thaw Stability

Single use aliquots of pooled body fluid were evaluated over two freeze thaw cycles to evaluate if storage would have a negative effect on the detection of a particular fluid (**Tables 3.14 and 3.15**). A significant reduction in signal intensity would indicate that samples are degrading during the freeze-thaw process and would require re-extraction, digestion, and cleanup prior to reliable analysis. Overall, only one vaginal fluid peptide was affected by repeated freeze thaw cycles (**Table 3.15**). Suprabasin peptide FGQGVHHGLSEGWK was detected in Day 1 samples but was absent after the first freeze thaw cycle (**Figure 3.3**). Because FGQGVHHGLSEGWK is a low abundant target, even minor degradation of the sample appears to have deleterious effects. In addition to the one suprabasin peptide in vaginal fluid, several markers (***e.g.,*** suprabasin, periplakin, etc.) in menstrual blood were not detected during this portion of the study. These results may be attributed to the low abundance of these markers in the menstrual blood matrix, which is consistent with results observed under subsequent aims. Furthermore, given that protein dropout was observed on Day 1 of the study, it is more likely due to low abundance in comparison with protein biomarker degradation due to instability. Finally, performance of the IPC was assessed to ensure complete sample preparation over the course of this study (**Figure 3.4**). Although a negative trend pattern is visible when plotting the IPC response ratio over time, all calculated ratios fell within acceptance criteria.

Table 3.14. Freeze thaw stability results for peripheral blood, saliva, and seminal fluid. Peptide markers that were positively identified are shown in dark green. No peptide or protein dropout was observed.

Fluid	Protein	Peptide	Day 1	Day 2	Day 3
Peripheral Blood	Alpha-1 Antitrypsin	LSITGTYDLK			
		SVLGQLGITK			
	Hemoglobin	SAVTALWGK			
		LLVVYPWTQR			
	Hemopexin	NFPSPVDAAFR			
	Apolipoprotein	LLDNWDSVTSTFSK			
		VSFLSALEEYTK			
	Serotransferrin	DGAGDVAFVK			
		SASDLTWDNLK			
Saliva	Alpha Amylase	LSGLLDLALGK			
		IAEYMNHLIDIGVAGFR			
	Statherin	FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF			
	Submaxillary Protein	GPYPPGPLAPPQPFGPGFVPPPPPPPTGPGR			
		IPPPPPAPYGPGIFPPPPPQP			
	Cystatin	IIEGGIYDADLNDER			
		ALHFVISEYNK			
Seminal Fluid	Acid Phosphatase	FVTLVFR			
		FQELESETLK			
		ELSELSLLSLYGIHK			
	Prostate Specific Antigen	FLRPGDDSSHDLMLLR			
		LSEPAELTDAVK			
	Semenogelin 2	GSISIQTEEK			
		GSISIQTEEQIHGK			
		DVSQSSISFQIEK			
	Semenogelin 1	DIFSTQDELLVYNK			
		QITIPSQEQEHSQK			
	Epididymal Secretory	DCGSVDGVIK			
		SGINCPIQK			

Table 3.15. Freeze thaw stability for vaginal fluid and menstrual blood. Peptide markers that were positively identified are shown in dark green. White indicates peptide dropout.

Fluid	Protein	Peptide	Day 1	Day 2	Day 3
Vaginal Fluid	Cornulin	LLDEDHTGTVEFK			
		ISPQIQLSGQTEQTQK			
		AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR			
	Neutrophil Gelatinase	WYVVGLAGNAILR			
		MYATIYELK			
	Ly6/PLAUR	GLDHGLLAFIQLQQCAQDR			
		GCVQDEFCTR			
	Suprabasin	ALGDINSGITHAGR			
		FGQGVHHGLSEGWK			
	Periplakin	AQSLQSAK			
		NLLDEIASR			
		NQGPQESVVR			
	Small Proline Rich Protein 3	VPEPGCTK			
		VPVPGYTK			
	Involucrin	HLVQQEGQLEQQER			
		QEAQLELPEQQVGQPK			
		GEVLLPVEHQQQK			
Menstrual Blood	Cornulin	LLDEDHTGTVEFK			
		ISPQIQLSGQTEQTQK			
		AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR			
	Neutrophil Gelatinase	WYVVGLAGNAILR			
		MYATIYELK			
	Ly6/PLAUR	GLDHGLLAFIQLQQCAQDR			
		GCVQDEFCTR			
	Suprabasin	ALGDINSGITHAGR			
		FGQGVHHGLSEGWK			
	Periplakin	AQSLQSAK			
		NLLDEIASR			
		NQGPQESVVR			
	Involucrin	HLVQQEGQLEQQER			
		QEAQLELPEQQVGQPK			
		GEVLLPVEHQQQK			
	Small Proline Rich Protein 3	VPEPGCTK			
		VPVPGYTK			
	Alpha-1 Antitrypsin	LSITGTYDLK			
		SVLGQLGITK			
	Hemoglobin	SAVTALWGK			
		LLVVYPWTQR			
	Hemopexin	NFPSPVDAAFR			
	Apolipoprotein	LLDNWDSVTSTFSK			
		VSFLSALEEYTK			
	Serotransferrin	DGAGDVAFVK			
		SASDLTWDNLK			

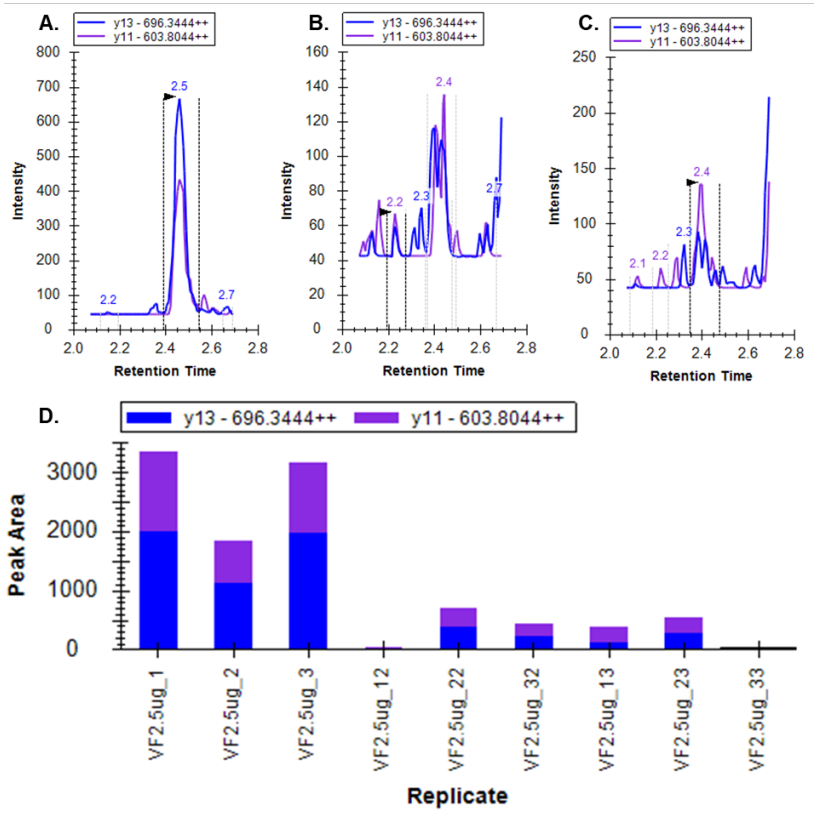


Figure 3.3. Suprabasin peptide FGQGVHHGLSEGWK peak area response in vaginal fluid. (A) Peak area intensity on Day 1. (B) Peak area intensity on Day 2. (C) Peak area response on Day 3. (D) Peak area response across all triplicates evaluated, progressing from Day 1 to Day 3.

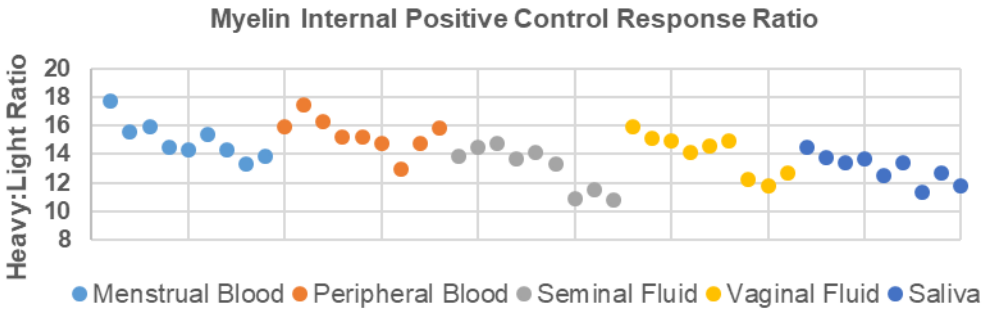


Figure 3.4. Internal positive control response ratio for the duration of freeze thaw stability experimentation.

3.2.3.2 Autosampler Stability

Three replicates of the five target body fluids were processed and evaluated over a period of 3 days. Samples were analyzed on Day 1 (*i.e.,* 0 hours) and stored on the liquid chromatograph's

chilled autosampler set at 8 ˚C. The same samples were then reinjected 24 hours (Day 2) and again at 48 hours (Day 3) after original processing to assess fluctuations in detection signal. A significant drop in signal strength or quality would indicate that any samples stored in excess of 24 hours on the autosampler would need to be re-extracted, digested, and cleaned up. Fortunately, the resulting data confirms that no such problems occurred. There was no sufficient drop in peak area response for any target peptide in the assay. On the contrary, a slight increase in peak area response was often observed. This was evident across all fluids; however, this increase is not sufficient. This was attributed to sample evaporation and concentration of target peptides that occurred after the 96-well sample plate cover was punctured for initial injection, resulting in evaporation of reconstitution solvent. Detailed results for this portion of the study are outlined in **Appendix G**. In addition, IPC ion ratios across the three days were calculated, with no significant difference between ion ratios for 0 and 24 hours (t_s=0.404, df=58, P>0.05) or for 0 and 48 hours (t_s=1.084, df=58, P>0.05). Percent CV value for the IPC are listed in **Table 3.16**. Overall, these results indicate that samples plated on the autosampler can be analyzed within two days of being processed without significant change in signal strength.

Table 3.16. Calculated CV (as a percent) for the internal positive control for the duration of the autosampler stability study.

Protein	Peptide	Day 1	+24hr	+48hr
Myelin Control	DTGILDSLGR	8.051	8.530	8.760

3.2.4 Mixtures Study

Pooled samples from five individuals were prepared in triplicate as 1:1 v/v mixtures among each of the five target fluids. The primary purpose of this mixture study was to identify potential cross-fluid interference and ion suppression that could produce a false negative or false positive result. The pairwise matrix of five body fluids includes all possible combinations of fluid interactions. Typical of toxicological assays, this study was designed to mimic interference studies performed as part of LC-MS/MS validations in operational toxicology laboratories. It is important to emphasize that the objective of this study is fundamentally different from mixture studies performed in forensic

DNA testing. In the context of DNA assay validation, the goal of a mixture study is typically to detect and resolve major and minor contributors.

In several cases, cross-fluid interferences were detected, potentially leading to difficulties in the interpretation of results. These have been identified, and using an abundance of caution, the interfering transition was either removed or replaced. For example, a major interference peak was detected with prostatic acid phosphatase peptide FVTLFR when seminal fluid was mixed with peripheral blood (**Figure 3.5**). In this case, the transition m/z 534.3398+ (purple peak) generates the interference peak. By replacing transition m/z 534.3398+ with transition m/z 247.1441+, the problem was readily eliminated in all subsequent analyses.

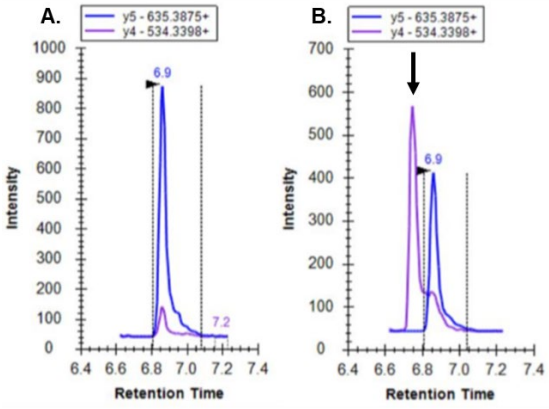


Figure 3.5. Prostatic acid phosphatase peptide FVTLFR peak area response in (A) neat seminal fluid and (B) seminal fluid and peripheral blood mixture. The m/z 534.3398+ transition (purple peak denoted with arrow) was removed and replaced.

3.2.5 Specificity Samples

The amino acid sequences of each biomarker were previously screened against protein databases to assess the possibility of obtaining positive results from non-human proteins. To remain in concordance with governing validation guidelines, additional *in vitro* experimentations was conducted to demonstrate species specificity. The data presented in this section demonstrates this empirically, through the analysis of domestic pet and livestock body fluid samples. Seven peripheral blood samples (Rhesus monkey, pig, horse, chicken, cow, mouse, and dog) were purchased and four peripheral blood samples (coyote, white-tailed deer, black bear, and river otter)

were acquired from previous projects. In addition, four saliva samples (cow, Rhesus monkey, tortoise, and alpaca) were procured.

The results of this study are outlined in **Table 3.17**. It was previously determined through investigation of database entries that the selected hemoglobin peptide sequences were not human specific. It is well known that the hemoglobin amino acid sequence is highly conserved across mammals, particularly in higher order primates (**Figure 3.6**). As hypothesized, hemoglobin was identified across most of the species assayed. In addition, there were two instances of trace vaginal fluid biomarkers, neutrophil gelatinase and Ly6 PLAUR, detected in cow and Rhesus monkey saliva (**Figure 3.7**). Trace levels of acid phosphatase and epididymal secretory protein were detected in cow and Rhesus monkey peripheral blood samples as well.

Table 3.17. Species specificity of protein biomarkers within the multiplex assay. Light green is indicative of at least one peptide marker identification for the respective protein. White indicates no protein marker was detected.

		Peripheral Blood											Saliva			
Fluid	Protein	Canine	Bovine	Equine	Porcine	Chicken	Mouse	Rhesus	Coyote	White Tailed Deer	Black Bear	River Otter	Bovine	Rhesus	Alpaca	Tortoise
Peripheral Blood	Alpha-1 Antitrypsin															
	Hemoglobin															
	Hemopexin															
	Apolipoprotein															
	Serotransferrin															
Saliva	Alpha Amylase															
	Statherin															
	Submaxillary Protein															
	Cystatin															
Vaginal Fluid/ Menstrual Blood	Cornulin															
	Neutrophil Gelatinase															
	Ly6/PLAUR															
	Suprabasin															
	Periplakin															
	Small Proline Rich Protein 3															
	Involucrin															
Seminal Fluid	Acid Phosphatase															
	Prostate Specific Antigen															
	Semenogelin 2															
	Semenogelin 1															
	Epididymal Secretory															

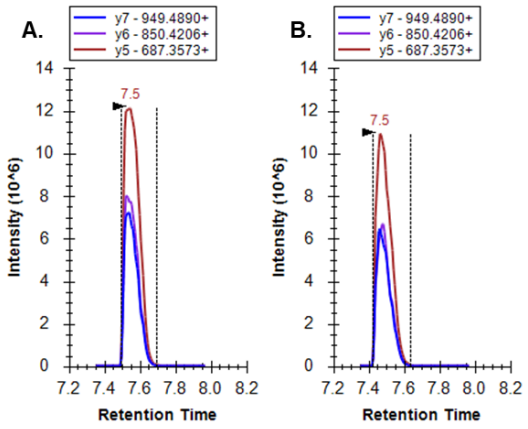


Figure 3.6. Hemoglobin peptide LLVVYPWTQR peak area response in (A) Rhesus monkey and (B) human peripheral blood.

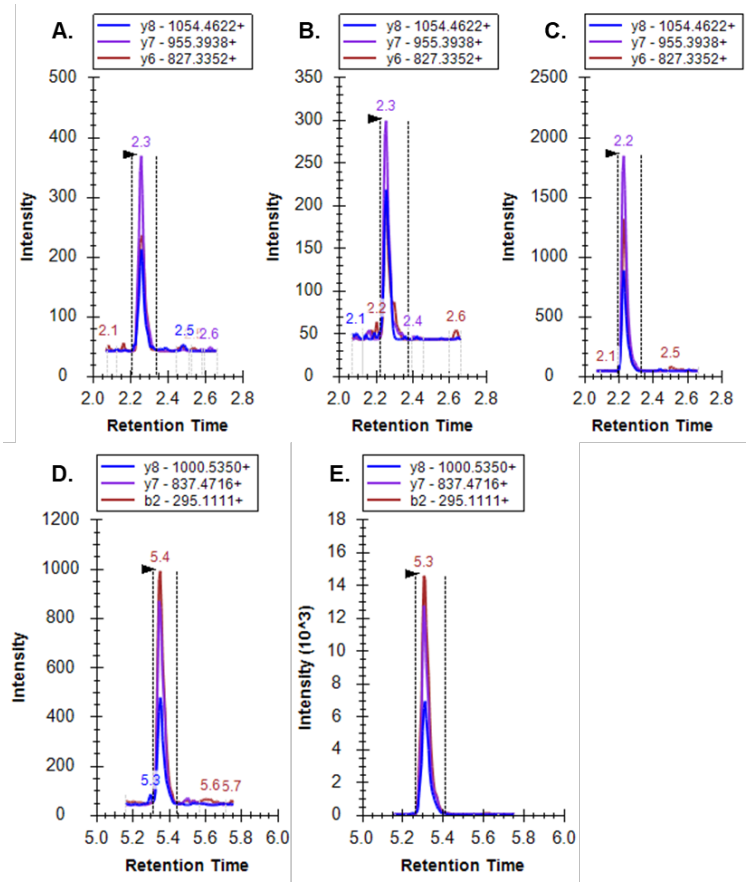


Figure 3.7. Peak area response of vaginal fluid markers in saliva samples. Ly6/PLAUR peptide GCVQDEFCTR peak area intensity in (A) bovine saliva, (B) Rhesus saliva, and (C) human vaginal fluid control samples. Neutrophil gelatinase peptide MYATIYELK peak area intensity in (D) Rhesus saliva and (E) human vaginal fluid control samples.

There were no instances of cross reactivity with the established assay for peripheral blood from chicken, coyote, or dog in addition to alpaca saliva (**Figure 3.8**). A more comprehensive assessment with the inclusion of additional target fluids from various species could present further chromatographic interferences. Overall, the results of this experimentation stress the importance of clear interpretation guidelines, particularly when examining potential trace amounts of body fluid when only one biomarker is present.

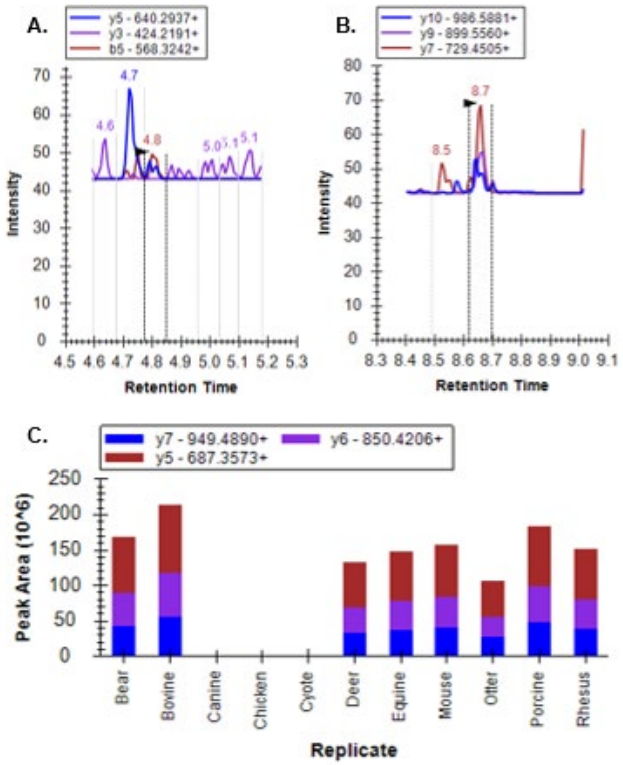


Figure 3.8. Peak area response of species specificity samples evaluated. (A) alpaca saliva sample protein cystatin peptide ALHFVISEYNK, (B) alpaca saliva sample protein amylase peptide LSGLLDLALGK, (C) hemoglobin peptide LLVVYPWTQR across all blood sample obtained.

3.2.6 Casework Type Samples

Laboratory simulated forensic casework type samples were evaluated to identify additional performance limitations, particularly with the analysis of trace level samples, and to aid in the generation of finalized Standard Operating Procedures and interpretation guidelines.

3.2.6.1 Substrate Samples

Various substrate compositions were analyzed to ensure chemical products (*i.e.,* indigo dye) or extraction efficiency off different materials (*i.e.,* cotton versus leather) would not interfere with the detection of protein biomarkers. Single source body fluids were evaluated on the following substrates: cotton, leather, denim, carpet, drywall, plastic bottle, aluminum bottle, and feminine hygiene menstrual pad. Results from this study are detailed in **Table 3.18**.

Peripheral blood was applied to cotton, denim, carpet, leather, and drywall. Upon processing, all peripheral blood peptide markers were detected and identified for each substrate type. No indication of suppression due to substrate composition was observed during analysis. Saliva was applied to plastic bottle and aluminum bottle cuttings. Analysis of the substrates showed that all saliva peptide markers were accurately identified. Similar to peripheral blood results, biomarkers were not suppressed due to substrate composition. Seminal fluid was applied to cotton and leather. All seminal fluid peptide markers were detected during analysis, indicating substrate composition did not affect the ability to accurately identify seminal fluid.

Vaginal fluid was applied to cotton, denim, and leather. Instances of vaginal fluid peptide dropout were observed during evaluation. For the cotton sample analyzed, all peptide markers with the exception of suprabasin peptide FGQGVHHGLSEGWK were detected. Similarly, peptide dropout for suprabasin peptide FGQGVHHGLSEGWK and involucrin peptide HLVQQEGQLEQQER were observed during analysis of vaginal fluid recovered from leather. The greatest instance of peptide dropout was observed with the analysis of vaginal fluid when applied to denim. Five vaginal fluid proteins, neutrophil gelatinase, Ly6/PLAUR, suprabasin, periplakin, and involucrin were incompletely detected, with dropout of at least one peptide per protein.

Similar vaginal fluid biomarker dropout was evident during menstrual blood analysis. Menstrual blood was applied to cotton, denim, and a feminine hygiene menstrual pad. Although all peripheral blood markers were positively detected, instances of vaginal fluid peptide dropout and complete protein dropout were observed. Across all substrates tested, three proteins exhibited complete dropout: suprabasin, involucrin, and Ly6/PLAUR. Furthermore, peptide dropout for

proteins cornulin and periplakin was also observed, leaving proteins neutrophil gelatinase and small proline-rich protein 3 as the only vaginal fluid markers completely identified.

Although suppression from substrate composition is a possible explanation as to the dropout of vaginal fluid peptide markers for both the analysis of vaginal fluid and menstrual blood matrices, the endogenous low-level abundance of these proteins must be taken into consideration. The dilution of protein material during extraction is a more probable explanation, causing the protein markers to be below the limit of detection. Furthermore, because the protein biomarker dropout during menstrual blood analysis was not dictated by the substrate on which the fluid was applied, the consistency across substrate type further substantiates the lower abundance of vaginal markers to protein absence during analysis. In conclusion, the composition of common substrates encountered during forensic analysis do not affect the ability for protein biomarkers to be identified using the developed workflow.

Table 3.18. Detection of body fluid markers in substrate samples. Proteins that were positively identified by all of their respective peptide markers are shown in dark green. Light green indicates where at least one peptide marker for the respective protein dropped out. Red indicates complete protein dropout. White indicates not tested.

Fluid	Protein	Peripheral Blood on Cotton	Peripheral Blood on Denim	Peripheral Blood on Carpet	Peripheral Blood on Leather	Peripheral Blood on Drywall	Saliva on Plastic Bottle	Saliva on Aluminum Can	Vaginal Fluid on Cotton	Vaginal Fluid on Denim	Vaginal Fluid on Leather	Semen on Cotton	Semen on Leather	Menstrual Blood on Cotton	Menstrual Blood on Denim	Menstrual Blood on Pad
Peripheral Blood	Alpha-1 Antitrypsin															
	Hemoglobin															
	Hemopexin															
	Apolipoprotein															
	Serotransferrin															
Saliva	Alpha Amylase															
	Statherin															
	Submaxillary Protein															
	Cystatin															
Vaginal Fluid/ Menstrual Blood	Cornulin															
	Neutrophil Gelatinase															
	Ly6/PLAUR															
	Suprabasin															
	Periplakin															
	Small Proline Rich Protein 3															
	Involucrin															
Seminal Fluid	Acid Phosphatase															
	Prostate Specific Antigen															
	Semenogelin 2															
	Semenogelin 1															
	Epididymal Secretory															

3.2.6.2 Environmental Contaminant Samples

A variety of environmental contaminants were selected and tested to evaluate potential concerns with enzymatic digestion, peptide cleanup, or to detect any instances of unanticipated

interferences during instrumental analysis. Single source body fluids were tested with the following environmental contaminants: dirt, rust, 10% bleach solution, chewing tobacco, cigarette butt, spermicide lubricated condom, and water-based personal lubricant. Results from this study are detailed in **Table 3.19**.

Peripheral blood was applied and mixed with dirt, rust, and a 10% bleach solution. All peripheral blood peptide markers met acceptance criteria and were positively identified during analysis. Similarly, saliva was applied and mixed with chewing tobacco and a cigarette butt. All saliva peptide biomarkers met acceptance criteria and were positively identified. Seminal fluid was applied and mixed with water-based personal lubricant, spermicide lubricated condom, and 10% bleach solution. With the exception of the 10% bleach solution, all seminal fluid peptide markers were positively identified. Epididymal secretory protein E1 peptide SGINCPIQK was not detected in 10% bleach solution.

Vaginal fluid was applied or mixed with water-based personal lubricant and a spermicide lubricated condom. This study was repeated with menstrual blood following equivalent sample preparation parameters. Detection of vaginal fluid peptide markers within these two matrices was determined to be specific for the environmental contaminant being assessed. Beginning with the vaginal fluid matrix, no instances of protein dropout were observed when analyzed from a spermicide lubricated condom. However, peptide and protein dropout were exhibited with the introduction of water-based personal lubricant. Complete protein dropout of suprabasin, Ly6/PLAUR, periplakin, small proline rich protein 3, and involucrin in addition to peptide dropout of protein cornulin were observed. The lack of detection when subjected to lubricant is likely due to two separate chemical interactions. First, lubricant is composed of long, lipophilic hydrocarbons that act as a competitive species during SPE cleanup. Lubricant hydrocarbons are preferentially bound to sorbent material during SPE as a result of their chemical structure. Although a simple addition of sodium dodecyl sulfate (SDS) would eliminate this interference, SDS is not compatible with mass spectrometry analysis.

Second, the specific type of personal lubricant utilized during this study contained glycerol (propane-1,2,3-triol), a compound produced from the hydrolysis of triglycerides that increases the solubility of proteins when in matrix. At concentrations greater than 10%, glycerol causes inaccurate protein quantitation [159]. Diluting the sample to decrease the interference caused by this ingredient is not suitable for analysis as the vaginal fluid peptide markers exist endogenously in low abundance, resulting in a misleading quantitation value.

Similar with menstrual blood containing samples, protein and peptide dropout was exhibited. Vaginal fluid protein biomarkers Ly6/PLAUR, suprabasin, and involucrin were not detected and peptide marker dropout was exhibited for proteins cornulin and periplakin. However, there was consistency in protein dropout observed between contaminant sample types, indicating that the natural low-level abundance of vaginal fluid markers attributes to the lack of detection. Furthermore, the protein detection for these sample types is equivalent to that reported during the substrate interference study in section 3.2.6.1.

Table 3.19. Detection of body fluid markers in samples subjected to environmental contaminants. Proteins that were positively identified by all of their respective peptide markers are shown in dark green. Light green indicates where at least one peptide marker for the respective protein dropped out. Red indicates complete protein dropout. White indicates not tested.

		Peripheral Blood			Saliva		Vaginal Fluid		Seminal Fluid			Menstrual Blood	
Fluid	Protein	Dirt	Rust	10% Bleach	Chewing Tobacco	Cigarette	Spermicide Condom	Water-based Lubricant	Water-based Lubricant	Spermicide Condom	10% Bleach	Water-based Lubricant	Spermicide Condom
Peripheral Blood	Alpha-1 Antitrypsin												
	Hemoglobin												
	Hemopexin												
	Apolipoprotein												
	Serotransferrin												
Saliva	Alpha Amylase												
	Statherin												
	Submaxillary Protein												
	Cystatin												
Vaginal Fluid/ Menstrual Blood	Cornulin												
	Neutrophil Gelatinase												
	Ly6/PLAUR												
	Suprabasin												
	Periplakin												
	Small Proline Rich Protein 3												
	Involucrin												
Seminal Fluid	Acid Phosphatase												
	Prostate Specific Antigen												
	Semenogelin 2												
	Semenogelin 1												
	Epididymal Secretory												

3.2.6.3 Mixtures

The purpose of this study was to evaluate the performance of the optimized assay when analyzing mixed samples with a major and minor component. Five two-fluid mixtures were prepared, with pairings selected based on sample types routinely tested for in operational forensic laboratories. For example, a seminal fluid minor contributor in a vaginal fluid major contributor was prepared to simulate a sexual assault type sample. Results from this study are summarized in **Table 3.20**.

With the exception of urine, for which protein biomarkers are not included within the developed assay, all major and minor body fluid contributors were correctly identified. Beginning with the vaginal fluid minor contributor in a urine major contributor, high rates of vaginal fluid protein marker dropout were observed. Fluid identification was made from the presence of small proline rich protein 3 and cornulin. The urine matrix predictably diluted the vaginal fluid markers past the limit of detection of the assay. In addition, although the average urea composition (9.5 g/L) in urine

is below the concentration incompatible with the BCA protein quantitation assay [159], the presence of urea and additional salts endogenous to urine may have negatively affected the ability to produce a reliable protein quantitation value. Therefore, if the total protein quantitation was underestimated, less then optimal protein amounts would have been utilized for testing.

The saliva minor contributor was confirmed present in a vaginal fluid major contributor based on the presence of alpha amylase and submaxillary gland androgen-regulated protein 3B. Furthermore, all vaginal fluid protein markers were accurately identified.

The third two-component mixture tested was a seminal fluid minor contributor in a vaginal fluid major contributor. Only one instance of peptide dropout was observed, with seminal fluid protein prostate specific antigen peptide FLRPGDDSSHDLMLLR being undetected. The remaining protein markers were positively identified, particularly confirmatory markers semenogelin 1 and semenogelin 2. As expected, the vaginal fluid major contributor was also positively identified by all protein biomarkers consistent with vaginal fluid.

The seminal fluid minor contributor was positively confirmed in a menstrual blood major contributor, with all protein biomarkers accurately detected and identified. However, as seen with other casework type samples, instances of peptide and protein dropout were observed for menstrual blood characterization. Although vaginal fluid proteins cornulin, neutrophil gelatinase, and small proline rich protein 3 were present, in addition to peptides consistent with periplakin and involucrin, protein dropout of Ly6/PLAUR and suprabasin was exhibited. This dropout is consistent with results obtained during Substrate and Environment Contaminant studies (sections 3.2.6.1 and 3.2.6.2, respectively), with endogenous low-level abundance negatively affecting the ability to detect these proteins in a complex matrix such as menstrual blood.

The last two-component mixture analyzed was a saliva minor contributor in a menstrual blood major contributor. The saliva contributor was identified based on the sole presence of protein alpha amylase. Because this protein has demonstrated cross-expression in other body fluids and tissues, the presence of saliva in this particular sample cannot be reported as confirmatory.

Furthermore, as observed with the seminal fluid in menstrual blood mixture previously described, there was consistent vaginal fluid protein biomarker dropout exhibited in this sample.

Table 3.20. Detection of body fluid markers in mixture samples. Proteins that were positively identified by all of their respective peptide markers are shown in dark green. Light green indicates where at least one peptide marker for the respective protein dropped out. Red indicates complete protein dropout. White indicates not tested.

Fluid	Protein	Vaginal Fluid (minor) & Urine (major)	Saliva (minor) & Vaginal Fluid (major)	Semen (minor) & Vaginal Fluid (major)	Semen (minor) & Menstrual Blood (major)	Saliva (minor) & Menstrual Blood (major)
Peripheral Blood	Alpha-1 Antitrypsin					
	Hemoglobin					
	Hemopexin					
	Apolipoprotein					
	Serotransferrin					
Saliva	Alpha Amylase					
	Statherin					
	Submaxillary Protein					
	Cystatin					
Vaginal Fluid/ Menstrual Blood	Cornulin					
	Neutrophil Gelatinase					
	Ly6/PLAUR					
	Suprabasin					
	Periplakin					
	Small Proline Rich Protein 3					
	Involucrin					
Seminal Fluid	Acid Phosphatase					
	Prostate Specific Antigen					
	Semenogelin 2					
	Semenogelin 1					
	Epididymal Secretory					

3.2.6.4 Sexual Assault Samples

3.2.6.4.1 Simulated Sexual Assault Samples

Prepared samples were allowed to incubate at 37 ˚C for up to and including eleven days after preparation. At time zero, proteins semenogelin 1, semenogelin 2, and epididymal secretory protein were positively identified (**Table 3.21**). The complete panel of seminal fluid peptide markers was not detected in full during any timepoints evaluated during this study. Only epididymal secretory protein was consistently detected over the course of the eleven-day incubation study. Furthermore, a single peptide for both semenogelin 1 and 2 were consistently identified over the course of the study (**Figure 3.9**). Peptide dropout for proteins prostatic acid phosphatase and

prostate specific antigen was observed at time zero, with complete protein dropout of prostate specific antigen observed for the remainder of the study. Prostatic acid phosphatase exhibited protein dropout after day seven.

This study demonstrates the robustness of the seminal fluid peptide markers selected for inclusion within the larger multiplex panel. For example, prostate specific antigen's inherent function is to cleave semenogelin proteins in order to liquify ejaculated semen. With the ability to detect semenogelin proteins after incubation for eleven days further exemplifies that the selected peptide markers do not fall within amino acid cleavage sites. Furthermore, the inherent hostile environment of vaginal fluid in terms of acidic pH and endogenous proteases, did not negatively affect the ability to positively characterize "invasive" seminal fluid proteins to an extent as great as originally hypothesized.

Table 3.21. Detection of body fluid markers in simulated sexual assault samples. Proteins that were positively identified by all of their respective peptide markers are shown in dark green. Light green indicates where at least one peptide marker for the respective protein dropped out. White indicates complete protein dropout.

	Day Zero	Day One	Day Three	Day Five	Day Seven	Day Nine	Day Eleven
Semenogelin 2							
Semenogelin 1							
Acid Phosphatase							
Prostate Specific Antigen							
Epididymal Secretory							

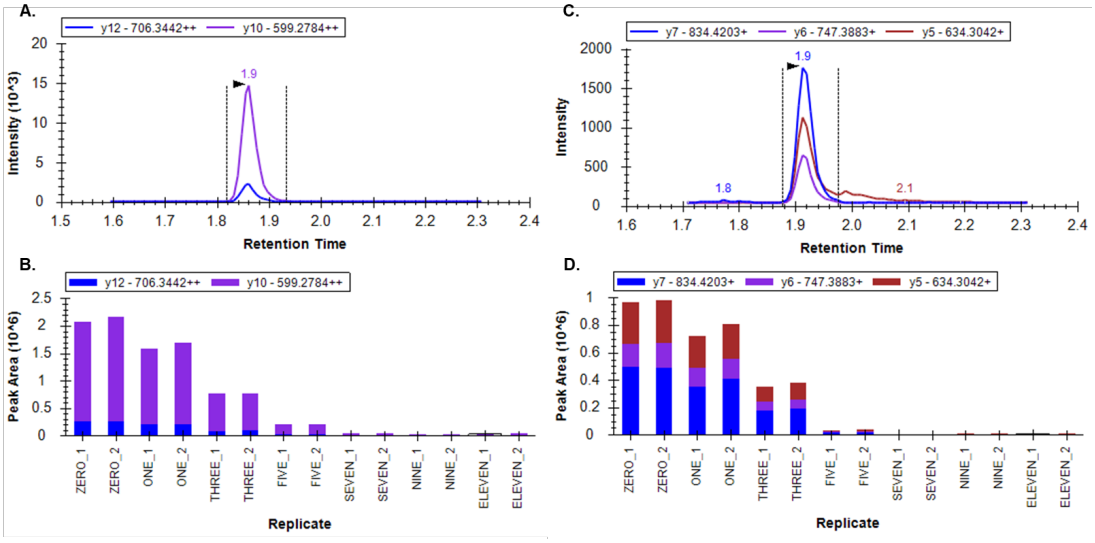


Figure 3.9. Peak area response of seminal fluid biomarkers in simulated sexual assault samples. Semenogelin 1 peptide QITIPSQEQEHSQK peak area intensity (A) at Day Eleven and (B) over the entirety of the incubation study. Semenogelin 2 peptide GSISIQTEEK peak area intensity (C) at Day Eleven and (D) over the entirety of the incubation study.

3.2.6.4.2 Mock Sexual Assault Kit Samples

Mock sexual assault kit samples were prepared in duplicate using semen provided by a vasectomized and a non-vasectomized male individual (**Table 3.22**). For sexual assault swabs prepared with semen from a non-vasectomized donor, seminal fluid was positively identified through the detection of the following protein fingerprints. Beginning with vaginal swab samples, two occurrences of seminal fluid protein dropout were recorded for a single sample (SA01_01.2). Prostate specific antigen and epididymal secretory protein were not positively identified; however, prostatic acid phosphatase, semenogelin 1, and semenogelin 2 were completely characterized. In addition, given the sample type, vaginal fluid protein markers were successfully detected and identified for both samples being assessed. Similar results were recorded for the oral swab sample types. All seminal fluid biomarkers were detected, with the exception of epididymal secretory protein, which exhibited complete dropout. Furthermore, given the sample type, all saliva biomarkers were accurately detected with no protein dropout exhibited. Rectal swab samples were shown to exhibit protein inhibition, with occurrences of seminal fluid protein dropout for both sample collections. Prostate specific antigen and epididymal secretory protein were not detected, in

addition to peptide dropout observed for prostatic acid phosphatase and semenogelin 2 (**Figure 3.10**).

Similar results were observed for samples prepared with semen from a vasectomized donor. Vaginal swab samples produced positive detection of prostatic acid phosphatase and semenogelin 1 seminal fluid proteins, in addition to vaginal fluid biomarkers. However, for both samples being analyzed, prostate specific antigen was not detected. Furthermore, semenogelin 2 exhibited peptide dropout. Second, oral swab samples were confirmed to contain seminal fluid due to the identification of semenogelin 1 and 2. The first swab analyzed (SA02_02.1) exhibited protein dropout for prostate specific antigen and epididymal secretory protein. The second oral swab (SA02_02.2) was only lacking epididymal secretory protein. Lastly, comparable with the non-vasectomized samples, the rectal swabs containing displayed occurrences of protein inhibition. For both sample collections, prostate specific antigen and epididymal secretory protein were not detected. Furthermore, peptide dropout of proteins prostatic acid phosphatase and semenogelin 2 were reported, with instances of semenogelin 1 peptide dropout for sample SA02_03.1 (**Figure 3.11**).

In summary, the LC-MS/MS assay was able to accurately identify trace amounts of seminal fluid from both non-vasectomized and vasectomized male individuals. Moreover, the protein inhibition reported from rectal swab sample analysis can be attributed to the increased presence of bacteria common for this sample type [160]. Bacteria of the human gut microbiome contain serine protease inhibitors, which may negatively affect the function of trypsin during protein digestion processes.

Table 3.22. Detection of body fluid markers in sexual assault kit samples. Proteins that were positively identified by all of their respective peptide markers are shown in dark green. Light green indicates where at least one peptide marker for the respective protein dropped out. White indicates complete protein dropout.

		Nonvasectomized Sexual Assault Kit						Vasectomized Sexual Assault Kit					
Fluid	Protein	SA01_01.1 Vaginal Swab	SA01_01.2 Vaginal Swab	SA01_02.1 Oral Swab	SA01_02.2 Oral Swab	SA01_03.1 Rectal Swab	SA01_03.2 Rectal Swab	SA02_01.1 Vaginal Swab	SA02_01.2 Vaginal Swab	SA02_02.1 Oral Swab	SA02_02.2 Oral Swab	SA02_03.1 Rectal Swab	SA02_03.2 Rectal Swab
Peripheral Blood	Alpha-1 Antitrypsin												
	Hemoglobin												
	Hemopexin												
	Apolipoprotein												
	Serotransferrin												
Saliva	Alpha Amylase												
	Stathrin												
	Submax												
	Cystatin												
Seminal Fluid	Acid Phosphatase												
	Prostate Specific Antigen												
	Semenogelin 2												
	Semenogelin 1												
	Epididymal Secretory												
Vaginal Fluid/ Menstrual Blood	Cornulin												
	Neutrophil Gelatinase												
	Ly6/PLAUR												
	Suprabasin												
	Periplakin												
	Involucrin												
	Small Proline Rich Protein 3												

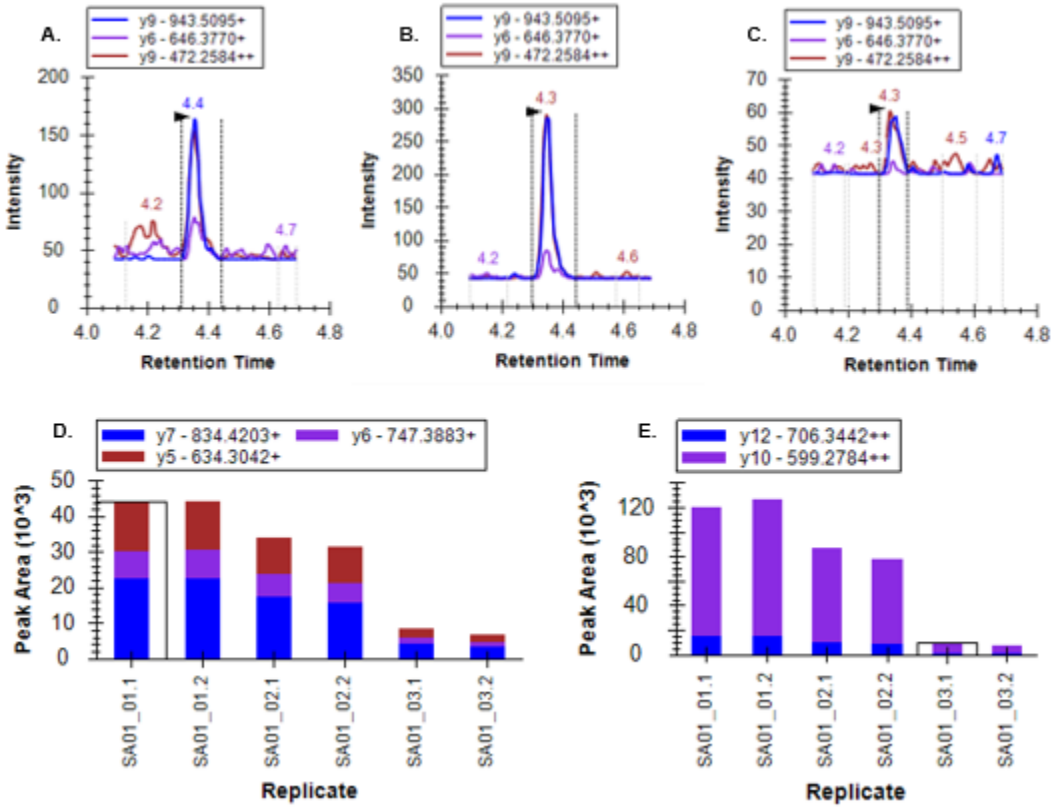


Figure 3.10. Peak area response of seminal fluid biomarkers in mock sexual assault kit samples containing semen from a non-vasectomized donor. Prostate specific antigen peptide LSEPAELTDAVK peak area intensity in (A) vaginal swab (B) oral swab (C) rectal swab. Semenogelin 2 peptide GSISIQTEEK peak area intensity (D) across vaginal, oral, and rectal swab sample replicates. Semenogelin 1 peptide QITIPSQEQEHSQK peak area intensity (E) across vaginal, oral, and rectal swab sample replicates.

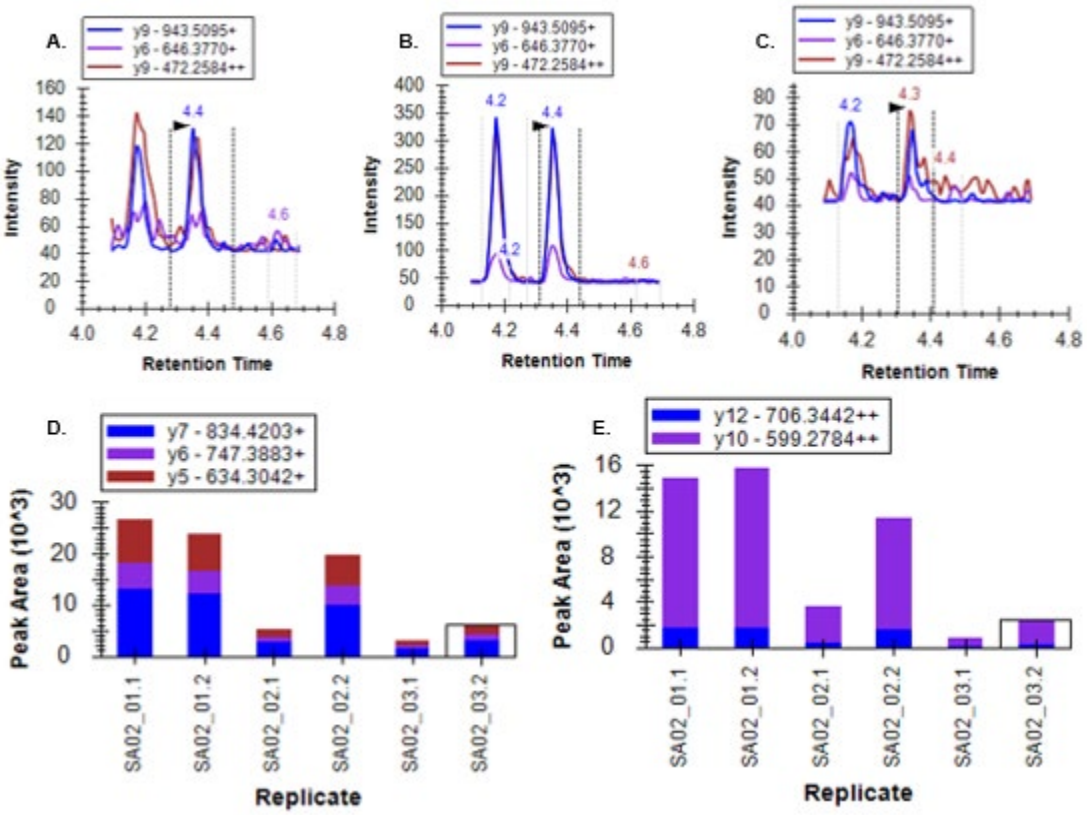


Figure 3.11. Peak area response of seminal fluid biomarkers in mock sexual assault kit samples containing semen from a vasectomized donor. Prostate specific antigen peptide LSEPAELTDAVK peak area intensity in (A) vaginal swab (B) oral swab (C) rectal swab. Semenogelin 2 peptide GSISIQTEEK peak area intensity (D) across vaginal, oral, and rectal swab sample replicates. Semenogelin 1 peptide QITIPSQEQEHSQK peak area intensity (E) across vaginal, oral, and rectal swab sample replicates.

3.2.6.4.3 Digital Swabs

Digital swab samples were collected on separate days for a total of two swab types, with each sample serving as a distinct sample collection. A summary of results is outlined in **Table 3.23**. Beginning with the oral penetration digital swabs, all saliva protein biomarkers were positively identified for both sample collections. The first sample evaluated was positive for alpha amylase and submaxillary gland androgen-regulated protein 3B, with peptide dropout observed for proteins statherin and cystatin SA. The second sample analyzed was positive for submaxillary gland androgen-regulated protein 3B and cystatin SA, with peptide dropout exhibited for alpha amylase and statherin. There were no instances of complete protein dropout observed for oral penetration digital swabs. Although inconsistencies were observed for alpha amylase detection between the

two samples received, the identification of submaxillary gland androgen-regulated protein 3B was encouraging, as this protein exhibits saliva specificity.

Vaginal penetration digital swabs were accurately identified by the presence of at least one peptide marker for all vaginal fluid markers within the assay. For the first sample analyzed, peptide dropout was observed for proteins cornulin, Ly6/PLAUR, periplakin, involucrin, and small proline rich protein 3; however, identification was made due to the presence of neutrophil gelatinase and suprabasin. Similarly, the second sample evaluated exhibited peptide dropout for proteins cornulin, periplakin, involucrin, and small proline rich protein 3. It should be noted that epididymal secretory protein, a presumptive seminal fluid protein biomarker, was detected for both vaginal penetration digital swabs.

Vaginal penetration during menses swabs were taken at both the beginning and the end of menstruation, creating two unique samples. The first digital swab was taken at the beginning of menstruation and was positively identified by both vaginal fluid and peripheral blood protein markers. Although protein dropout was observed for protein cornulin, three vaginal fluid markers were completely identified (neutrophil gelatinase, suprabasin, and involucrin). Furthermore, all peripheral blood proteins were detected, with high abundance of protein hemoglobin and alpha-1 antitrypsin reported. The second sample analyzed was collected at the end of menstruation, causing the sample to more closely resemble a vaginal swab than a menstrual swab. No instances of vaginal fluid biomarker dropout were observed. In addition, as expected, signal intensities for peripheral blood protein markers were less intense than those of the first swab evaluated.

Table 3.23. Detection of body fluid markers in digital swab samples. Proteins that were positively identified by all of their respective peptide markers are shown in dark green. Light green indicates where at least one peptide marker for the respective protein dropped out. White indicates complete protein dropout.

Fluid	Protein	Digital Swab Saliva	Digital Swab Saliva	Digital Swab Vaginal Fluid	Digital Swab Vaginal Fluid	Digital Swab Menstrual Blood	Digital Swab Menstrual Blood
Peripheral Blood	Alpha-1 Antitrypsin						
	Hemoglobin						
	Hemopexin						
	Apolipoprotein						
	Serotransferrin						
Saliva	Alpha Amylase						
	Statherin						
	Submaxillary Protein						
	Cystatin						
Seminal Fluid	Acid Phosphatase						
	Prostate Specific Antigen						
	Semenogelin 2						
	Semenogelin 1						
	Epididymal Secretory						
Vaginal Fluid/ Menstrual Blood	Cornulin						
	Neutrophil Gelatinase						
	Ly6/PLAUR						
	Suprabasin						
	Periplakin						
	Involucrin						
	Small Proline Rich Protein 3						
	Alpha-1 Antitrypsin						
	Hemoglobin						
	Hemopexin						
	Apolipoprotein						
	Serotransferrin						

3.2.6.5 Degraded Samples

Substrates were prepared to simulate authentic items of evidence that have been laundered. Upon analysis, both the stain region and the control (non-stain region) of the same substrate were processed concurrently. A complete summary of results is depicted in **Table 3.24**. Peripheral blood containing samples (cotton bath towel, denim) yielded positive identifications for all target peripheral blood biomarkers. For the towel sample, peptide dropout for apolipoprotein and serotransferrin was observed. The denim sample containing peripheral blood did not exhibit any instances of peptide or protein dropout. Both the towel and denim control regions were negative for all peripheral blood protein targets.

Seminal fluid was accurately identified on both substrates evaluated (cotton-blend bed sheet, cotton underwear). All protein markers were positively detected for the underwear stain region, with one instance of peptide dropout exhibited for prostate specific antigen. The control region for this substrate was negative for all biomarkers. The bed sheet sample had a single occurrence of protein dropout for epididymal secretory protein, with peptide dropout exhibited by prostatic acid phosphatase and prostate specific antigen. The control region for this substrate was also negative for all protein markers.

It was originally hypothesized that the use of laundry detergent may severely inhibit biomarker detection, given that detergent is commonly used to lyse cell membranes and denature protein, but have additional deleterious effects on SPE chemistries. Furthermore, detergent is generally incompatible with mass spectrometer functionality. However, laundering of the stain regions did not inhibit the ability to accurately identify target biomarkers within the validated assay. Additional studies into the effect of different detergent formulations would be of interest. A detergent marketed as “natural” was selected for this subset of samples, however, it is hypothesized that formulations containing other chemical ingredients may change the observed outcomes.

Table 3.24. Detection of body fluid markers in degraded samples. Proteins that were positively identified by all of their respective peptide markers are shown in dark green. Light green indicates where at least one peptide marker for the respective protein dropped out. White indicates complete protein dropout.

Fluid	Protein	Semen on Bed Sheet	Semen on Bed Sheet Control	Semen on Underwear	Semen on Underwear Control	Peripheral Blood on Towel	Peripheral Blood on Towel Control	Peripheral Blood on Jeans	Peripheral Blood on Jean Control
Peripheral Blood	Alpha-1 Antitrypsin								
	Hemoglobin								
	Hemopexin								
	Apolipoprotein								
	Serotransferrin								
Saliva	Alpha Amylase								
	Statherin								
	Submaxillary Protein								
	Cystatin								
Seminal Fluid	Acid Phosphatase								
	Prostate Specific Antigen								
	Semenogelin 2								
	Semenogelin 1								
	Epididymal Secretory								
Vaginal Fluid/ Menstrual Blood	Cornulin								
	Neutrophil Gelatinase								
	Ly6/PLAUR								
	Suprabasin								
	Periplakin								
	Involucrin								
	Small Proline Rich Protein 3								

3.2.6.6 Aged Samples

Aged body fluid swabs were prepared to assess the degradation of target protein markers in addition to extraction efficiency when target body fluids were not stored under ideal conditions. Protein identification results for all aged samples are outlined in **Table 3.25**. Peripheral blood biomarkers were detected at all time points collected. No instances of protein degradation were observed, with all peak intensities remaining consistent over the course of the study. Similar results regarding peripheral blood markers in menstrual blood were obtained; however, vaginal fluid marker dropout was observed. Proteins Ly6/PLAUR, suprabasin, and involucrin were not detected during this study. All other vaginal fluid markers did exhibit stability for the time points collected, with no additional protein or peptide dropout exhibited.

Seminal fluid and saliva behaved similar to peripheral blood. All target protein markers were correctly identified during each time point collected, with no instances of protein or peptide dropout over the course of the study. The amount of material present for vaginal fluid (*i.e.,* 50 µL vs. 150 µL) effected the positive identification of specific markers, as originally hypothesized. Peptide dropout was observed for protein periplakin during the study, with inconsistent peptide dropout exhibited by protein suprabasin. This suggests that the selected peptide marker AQSLQSAK for suprabasin does not exhibit the same high degree of robustness when compared with remaining vaginal fluid markers.

Table 3.25. Detection of body fluid markers in aged samples. Proteins that were positively identified by all of their respective peptide markers are shown in dark green. Light green indicates where at least one peptide marker for the respective protein dropped out. White indicates complete protein dropout.

Fluid	Protein	Day 0		Day 1		Day 3		Day 7		Day 35	
Peripheral Blood	Hemoglobin										
	Apolipoprotein										
	Alpha-1 Antitrypsin										
	Hemopexin										
	Serotransferrin										
Menstrual Blood	Hemoglobin										
	Apolipoprotein										
	Alpha-1 Antitrypsin										
	Hemopexin										
	Serotransferrin										
	Small Proline Rich Protein										
	Cornulin										
	Neutrophil Gelatinase										
	Ly6/PLAUR										
	Suprabasin										
	Periplakin										
	Involucrin										
Seminal Fluid	Semenogelin 1										
	Semenogelin 2										
	Acid Phosphatase										
	Prostate Specific Antigen										
	Epididymal Secretory										
Saliva	Alpha Amylase										
	Cystatin										
	Submaxillary Protein										
	Statherin										
Vaginal Fluid	Small Proline Rich Protein										
	Cornulin										
	Neutrophil Gelatinase										
	Suprabasin										
	Involucrin										
	Periplakin										
	Ly6/PLAUR										

3.2.6.7 Sensitivity Samples

Sensitivity samples were prepared by extracting a serial dilution of target body fluid from cotton swabs. These samples differ from those prepared under section 3.2.2 by diluting target body fluid and recovering from a substrate, in comparison with evaluating diluted target fluid directly in a test tube. A summary of results is detailed in **Tables 3.26-3.28**. Beginning with peripheral blood detection, all protein markers were successfully identified for neat, 1:2, 1:10, and 1:100 dilution samples (**Table 3.26**). Hemopexin dropout was observed at the 1:1,000 dilution, with additional protein dropout of alpha-1 antitrypsin and serotransferrin peptide dropout at the 1:2,000 dilution. From this dilution, the only protein marker consistently detected was hemoglobin, with peptide or protein dropout reported for all other markers at the 1:5,000, 1:10,000 and 1:20,000 dilutions (**Figure 3.12**). No peripheral blood biomarkers were detected at the 40,000-fold dilution.

Similar results were exhibited by menstrual blood sensitivity analysis (**Table 3.26**). All peripheral blood markers were accurately detected through the 100-fold dilution, with peptide dropout for serotransferrin and apolipoprotein at the 1:1,000 dilution. Peripheral blood marker dropout of hemopexin and alpha-1 antitrypsin was observed at the 1:2,000 dilution, with all proteins except hemoglobin not detected at the 1:5,000 dilution. Hemoglobin was detected in all menstrual blood dilution samples analyzed. As hypothesized, vaginal fluid protein markers did not exhibit the same level of sensitivity as the peripheral blood markers in menstrual blood. Protein dropout for involucrin, Ly6/PLAUR, and suprabasin was observed for all samples evaluated. Furthermore, peptide dropout for cornulin and periplakin was exhibited for all samples. These results remained consistent up to and including the 100-fold dilution sample. Additional protein dropout of periplakin and neutrophil gelatinase was reported at the 1,000-fold dilution sample. Further dilutions exhibited inconsistent protein and peptide detection. Small proline rich protein 3 was detected at the 1:10,000 and 1:20,000 dilutions (**Figure 3.12**); however, exhibited peptide dropout at the 1:5,000 and 1:40,000 dilutions. As with other casework type samples analyzed, the endogenous low abundances of target vaginal fluid protein markers attributes to the lack of detection at lower dilution samples.

Robust detection of seminal fluid biomarkers over the course of the dilution study was observed (**Table 3.26**). All protein targets were identified in the neat, 1:2, and 1:10 dilutions, with two instances of peptide dropout for proteins prostatic acid phosphatase and prostate specific antigen at the 1:100 dilution. Prostate specific antigen and epididymal secretory protein were not detected at the 1:1,000 dilution, with additional prostatic acid phosphatase dropout at the 2,000-fold dilution. Although peptide dropout was exhibited, semenogelin 1 and 2 were positively identified at the 1:5,000, 1:10,000, and 1:20,000 dilution samples. Furthermore, semenogelin 1 peptide QITIPSQEQEHSQK was accurately detected at 40,000-fold dilution extract (**Figure 3.12**).

Saliva dilution samples, as hypothesized, did not demonstrate the same degree of sensitivity as semen and peripheral blood based on the lower endogenous levels of protein quantity (**Table 3.27**). All protein markers were positively identified at the neat, 1:2, and 1:10 dilution extracts, with complete dropout of cystatin SA at 1:100. From this point, inconsistencies in biomarker detection were observed. Beginning with protein alpha amylase, peptide dropout was exhibited at the 500-fold dilution but was completely detected at the 1,000-fold dilution sample (**Figure 3.12**). Similarly, submaxillary gland androgen-regulated protein 3B demonstrated protein dropout at 1:500, with a single peptide detected at 1:1,000. The remaining samples analyzed (1:2,000; 1:5,000; 1:10,000) were negative for all target protein markers consistent with saliva.

Lastly, vaginal fluid sensitivity was evaluated (**Table 3.28**). Three instances of peptide dropout were observed at the initial 10-fold dilution analyzed: periplakin, Ly6/PLAUR, and involucrin. After this dilution, almost complete protein dropout was exhibited at 1:100, with only two peptides being accurately detected for small proline rich protein 3 and periplakin. From this point, only a single small proline rich protein 3 peptide was detected for the remainder of the samples analyzed (**Figure 3.12**). It should be restated that the dilution evaluated was initially applied to a cotton swab and subsequently extracted in 1 mL of deionized water, further diluting any protein material that was originally present. The large extraction volume was necessary to produce enough material for additional studies conducted during subsequent aim of this project, as described in Chapter 4. This decrease in recovery contributed to poor sensitivity in comparison with the

sensitivity study conducted in section 3.2.2. However, in common forensic biology laboratory protocols, swabs are extracted in sufficient liquid to ensure saturation, further concentrating present targets prior to analysis.

Table 3.26. Detection of body fluid markers in casework sensitivity samples for peripheral blood, menstrual blood, and seminal fluid. Proteins that were positively identified by all of their respective peptide markers are shown in dark green. Light green indicates where at least one peptide marker for the respective protein dropped out. White indicates complete protein dropout.

		Dilution Factor																			
Fluid	**Protein**	**Neat**		**1:2**		**1:10**		**1:100**		**1:1000**		**1:2000**		**1:5000**		**1:10,000**		**1:20,000**		**1:40,000**	
Peripheral Blood	Hemoglobin																				
	Apolipoprotein																				
	Serrotransferrin																				
	Alpha-1 Antitrypsin																				
	Hemopexin																				
Menstrual Blood	Small Proline Rich																				
	Cornulin																				
	Neutrophil Gelatinase																				
	Periplakin																				
	Involucrin																				
	Ly6/PLAUR																				
	Suprabasin																				
	Hemoglobin																				
	Serrotransferrin																				
	Apolipoprotein																				
	Hemopexin																				
	Alpha-1 Antitrypsin																				
Seminal Fluid	Semenogelin 1																				
	Semenogelin 2																				
	Acid Phosphatase																				
	Prostate Specific Antigen																				
	Epididymal Secretory																				

Table 3.27. Detection of body fluid markers in casework sensitivity samples for saliva. Proteins that were positively identified by all of their respective peptide markers are shown in dark green. Light green indicates where at least one peptide marker for the respective protein dropped out. White indicates complete protein dropout.

		Dilution Factor																	
Fluid	**Protein**	**Neat**		**1:2**		**1:10**		**1:100**		**1:500**		**1:1,000**		**1:2,000**		**1:5,000**		**1:10,000**	
Saliva	Alpha Amylase																		
	Submaxillary Protein																		
	Statherin																		
	Cystatin																		

Table 3.28. Detection of body fluid markers in casework sensitivity samples for vaginal fluid. Proteins that were positively identified by all of their respective peptide markers are shown in dark green. Light green indicates where at least one peptide marker for the respective protein dropped out. White indicates complete protein dropout.

		Dilution Factor													
Fluid	**Protein**	**1:10**		**1:100**		**1:1000**		**1:2,000**		**1:5,000**		**1:10,000**		**1:20,000**	
Vaginal Fluid	Small Proline Rich Protein														
	Periplakin														
	Cornulin														
	Neutrophil Gelatinase														
	Suprabasin														
	Ly6/PLAUR														
	Involucrin														

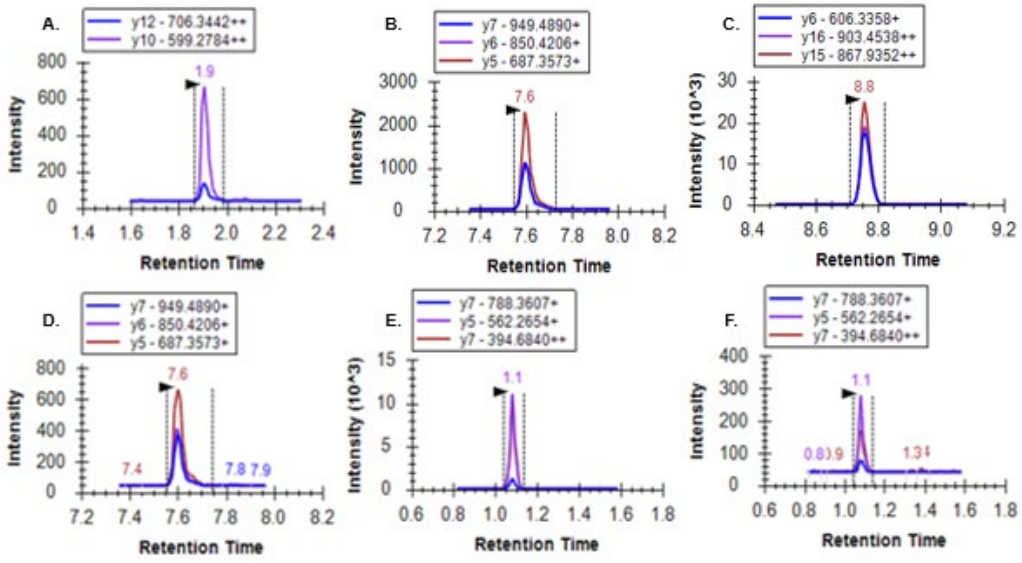


Figure 3.12. Peak area response of peptide biomarker at lowest detected sensitivity sample. (A) seminal fluid protein semenogelin 1 peptide QITIPSQEQEHSQK at 40,000-fold dilution, (B) peripheral blood protein hemoglobin peptide LLVVYPWTQR at 20,000-fold dilution, (C) saliva protein amylase peptide IAEYMNHLIDIGVAGFR at 1,000-fold dilution, (D) menstrual blood protein hemoglobin peptide LLVVYPWTQR at 40,000-fold dilution, (E) menstrual blood protein small proline rich protein 3 peptide VPEPGCTK at 20,000-fold dilution, and (F) vaginal fluid protein small proline rich proline 3 peptide VPEPGCTK at 20,000-fold dilution.

3.2.7 Limit of Detection

This study was performed to determine the detection limit for individual target peptides contained within their cognate body fluid. This aim was carried out by generating a dilution series of isotopically-labeled peptide standards, ranging from 50 femtomoles (fmol) to 500 attomoles (amol) per injection. Samples were prepared in triplicate in digested body fluid matrix. In general, the limit of detection (LOD) is described as the lowest concentration an analyte can be differentiated from analytical noise. The LOD was determined according to acceptable signal peak area response, signal to noise ratio, retention time, and qualitative response ratio between each transition.

A summary of results obtained are detailed in **Tables 3.29 and 3.30**. Overall, the LOD values for target peptide markers ranged from 1.0 to 25 femtomoles per individual peptide. Beginning with peripheral blood, the LOD for target peptide markers ranged from 2.5 fmol to 10

fmol per injection analyzed (**Table 3.29**). Hemoglobin peptides SAVTALWGK and LLVVYPWTQR were found to have a higher LOD of 10 fmol due to observed peak area fluctuations of qualifier ions. Furthermore, this higher LOD is beneficial for analysis because, as discussed later in section 3.2.9, both hemoglobin peptides were found to have high instances of carryover between sample injections.

Seminal fluid peptide marker LOD values ranged from 1.0 fmol to 25 fmol (**Table 3.29**). Similar to hemoglobin, semenogelin 1 peptide DIFSTQDELLVYNK exhibited an LOD of 25 fmol, a higher value than those observed for remaining targets, and was found to have greater instances of carryover (section 3.2.9). On the contrary, LOD values for proteins prostatic acid phosphatase and prostate specific antigen were on the lower end of the range, with peptide FVTLVFR exhibiting an LOD of 1.0 fmol.

Derived LOD values for vaginal fluid ranged from 1.0 fmol to 25 fmol, with two instances of target isotopically-labeled peptide standard dropout (**Table 3.30**). Cornulin peptide AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR and Ly6/PLAUR peptide GLDHGLLAFIQLQQCAQDR did not produce sufficient chromatographic peak shape or acceptable ion ratios at the 50 fmol fortification (**Figure 3.13**). Therefore, these peptides were not assigned a LOD and should be interpreted with caution. However, more robust protein biomarkers such as neutrophil gelatinase, exhibited acceptable LOD values at 5.0 fmol. Similar results were obtained with AQUA analysis in menstrual blood matrix, with LOD values ranging from 2.5 fmol to 25 fmol for both vaginal fluid and peripheral blood target markers (**Table 3.30**). In comparison with vaginal fluid matrix analysis, eight vaginal fluid targets produced comparable results in menstrual blood, including the dropped peptide biomarkers of cornulin and Ly6/PLAUR. However, five peptide targets were found to exhibit a greater LOD in menstrual blood than in vaginal fluid matrix. All peripheral blood target LOD values were comparable in menstrual blood, with the exception of hemopexin peptide NFPSPVDAAFR, exhibiting a greater LOD in peripheral blood than in menstrual blood matrix.

Isotopically-labeled peptide standards were not available for purchase at an acceptable purity for saliva proteins statherin and submaxillary gland androgen-regulated protein 3B. Peptide synthesis becomes exponentially more difficult as peptide length increases, with the peptide targets for these particular proteins outside of accurate synthesis range. Furthermore, peptides with a high content of certain amino acid residues (*i.e.,* proline) add additional difficulties to the synthesis process. The remaining four peptide targets (from proteins alpha amylase and cystatin SA) produced LOD values of 5 fmol or less, with LSGLLDLALGK peptide exhibiting an LOD of 2.5 fmol (**Table 3.29**).

Table 3.29. Limit of detection quantities of peripheral blood, saliva, and seminal fluid peptide biomarkers.

Fluid	Protein	Peptide	LOD (fmol)
Peripheral Blood	Alpha-1 Antitrypsin	LSITGTYDLK	5.0
		SVLGQLGITK	5.0
	Hemoglobin	SAVTALWGK	10.0
		LLVVYPWTQR	10.0
	Hemopexin	NFPSPVDAAFR	5.0
	Apolipoprotein	LLDNWDSVTSTFSK	2.5
		VSFLSALEEYTK	5.0
	Serotransferrin	DGAGDVAFVK	5.0
		SASDLTWDNLK	10.0
Saliva	Alpha Amylase	LSGLLDLALGK	2.5
		IAEYMNHLIDIGVAGFR	5.0
	Statherin	FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF	not tested
	Submaxillary Protein	GPYPPGPLAPPQPFGPGFVPPPPPPPTGPGR	not tested
		IPPPPPAPYGPGIFPPPPPQP	not tested
	Cystatin	IIEGGIYDADLNDER	5.0
		ALHFVISEYNK	5.0
Seminal Fluid	Acid Phosphatase	FVTLVFR	1.0
		FQELESETLK	5.0
		ELSELSLLSLYGIHK	10.0
	Prostate Specific Antigen	FLRPGDDSSHDLMLLR	2.5
		LSEPAELTDAVK	2.5
	Semenogelin 2	GSISIQTEEK	2.5
		GSISIQTEEQIHGK	5.0
		DVSQSSISFQIEK	10.0
	Semenogelin 1	DIFSTQDELLVYNK	25.0
		QITIPSQEQEHSQK	2.5
	Epididymal Secretory	DCGSVDGVIK	5.0
		SGINCPIQK	5.0

Table 3.30. Limit of detection quantities for vaginal fluid and menstrual blood peptide biomarkers. Not Detected (ND).

Fluid	Protein	Peptide	LOD (fmol)
Vaginal Fluid	Cornulin	LLDEDHTGTVEFK	2.5
		ISPQIQLSGQTEQTQK	25.0
		AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR	ND
	Neutrophil Gelatinase	WYVVGLAGNAILR	5.0
		MYATIYELK	5.0
	Ly6/PLAUR	GLDHGLLAFIQLQQCAQDR	ND
		GCVQDEFCTR	25.0
	Suprabasin	ALGDINSGITHAGR	1.0
		FGQGVHHGLSEGWK	25.0
	Periplakin	AQSLQSAK	2.5
		NLLDEIASR	1.0
		NQGPQESVVR	10.0
	Small Proline Rich Protein 3	VPEPGCTK	2.5
		VPVPGYTK	5.0
	Involucrin	HLVQQEGQLEQQER	10.0
		QEAQLELPEQQVGQPK	5.0
		GEVLLPVEHQQQK	2.5
Menstrual Blood	Cornulin	LLDEDHTGTVEFK	2.5
		ISPQIQLSGQTEQTQK	10.0
		AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR	ND
	Neutrophil Gelatinase	WYVVGLAGNAILR	10.0
		MYATIYELK	5.0
	Ly6/PLAUR	GLDHGLLAFIQLQQCAQDR	ND
		GCVQDEFCTR	10.0
	Suprabasin	ALGDINSGITHAGR	10.0
		FGQGVHHGLSEGWK	25.0
	Periplakin	AQSLQSAK	2.5
		NLLDEIASR	2.5
		NQGPQESVVR	10.0
	Involucrin	HLVQQEGQLEQQER	10.0
		QEAQLELPEQQVGQPK	5.0
		GEVLLPVEHQQQK	5.0
	Small Proline Rich Protein 3	VPEPGCTK	2.5
		VPVPGYTK	10.0
	Alpha-1 Antitrypsin	LSITGTYDLK	5.0
		SVLGQLGITK	5.0
	Hemoglobin	SAVTALWGK	10.0
		LLVVYPWTQR	10.0
	Hemopexin	NFPSPVDAAFR	2.5
	Apolipoprotein	LLDNWDSVTSTFSK	2.5
		VSFLSALEEYTK	5.0
	Serotransferrin	DGAGDVAFVK	5.0
		SASDLTWDNLK	25.0

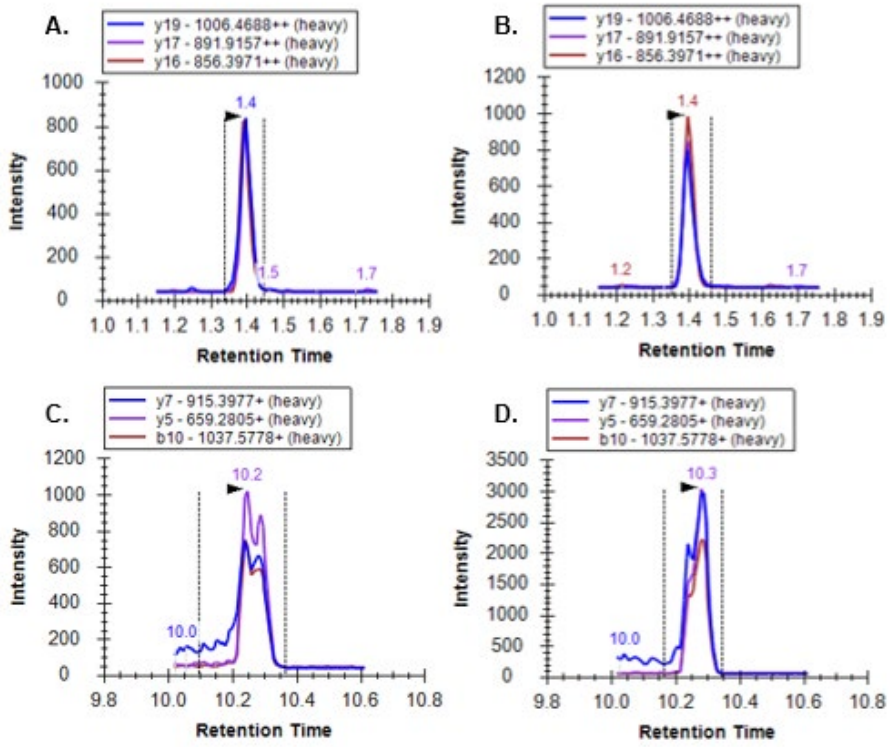


Figure 3.13. Peak area response of peptide biomarkers at 50 fmol limit of detection. (A) Menstrual blood matrix detection of cornulin peptide AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR, (B) vaginal fluid matrix detection of cornulin peptide AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR, (C) menstrual blood matrix detection of Ly6/PLAUR peptide GLDLHGLLAFIQLQQCAQDR, and (D) vaginal fluid matrix detection of Ly6/PLAUR peptide GLDLHGLLAFIQLQQCAQDR.

3.2.8 Ion Suppression

This portion of the validation was performed to assess whether coeluting compounds have a suppressing or enhancing effect on peptide response. This study was performed by preparing two samples: the first containing only isotopically-labeled peptide standards and the second containing body fluid fortified with isotopically-labeled peptide standards. As previously stated, AQUA standards were not available for saliva proteins statherin and submaxillary gland androgen-regulated protein 3B and were therefore not tested during this portion of the validation.

To assess peptide suppression or enhancement, the percent difference of peak area average between the neat standards and the AQUA fortified fluid matrix was calculated (**Tables 3.31 and 3.32**). Peptides that expressed a negative percent difference were marked as having ion

suppression, indicating the observed peak area of the peptide in the digested body fluid sample was less than that observed of the neat standard. Peptides that expressed a positive percent difference were interpreted as having ion enhancement, evident by a greater peak area response in the fortified digested body fluid than the neat standard. Substantial ion suppression was observed in 22% of peptide targets, with calculated percent difference greater than 30%. For example, the hemoglobin peptides SAVTALWGK and LLVVYPWTQR showed 55% and 94% suppression, respectively, when analyzed in neat peripheral blood (**Figure 3.14**). Similar suppression of hemoglobin peptides was also observed in menstrual blood. Substantial suppression of hemoglobin was expected, although not anticipated at such extreme rates, for both peripheral blood and menstrual blood matrices due to their endogenous complexity.

Ion enhancement was not observed as frequently as suppression, with only two instances of substantial enhancement. First, ion enhancement was observed at peptide FGQGVHHGLSEGWK from vaginal fluid protein marker suprabasin. This occurrence was observed in both vaginal fluid and menstrual blood matrix, with calculated percent difference of 79% and 67%, respectively.

Table 3.31. Calculated percent difference of peripheral blood, seminal fluid, and saliva peptide biomarkers to assess ion suppression and enhancement.

Fluid	Protein	Peptide	%Difference
Peripheral Blood	Alpha-1 Antitrypsin	LSITGTYDLK	2.03E+01
		SVLGQLGITK	-6.54E+00
	Hemoglobin	SAVTALWGK	-5.53E+01
		LLVVYPWTQR	-9.43E+01
	Hemopexin	NFPSPVDAAFR	-1.03E+01
	Apolipoprotein	LLDNWDSVTSTFSK	2.20E+01
		VSFLSALEEYTK	-2.15E+01
	Serotransferrin	DGAGDVAFVK	-3.15E-01
		SASDLTWDNLK	-1.08E+01
Seminal Fluid	Acid Phosphatase	FVTLVFR	-8.46E+00
		FQELESETLK	-8.39E+00
		ELSELSLLSLYGIHK	2.31E+00
	Prostate Specific Antigen	FLRPGDDSSHDLMLLR	-3.75E+00
		LSEPAELTDAVK	-1.44E+01
	Semenogelin 2	GSISIQTEEK	-1.10E+01
		GSISIQTEEQIHGK	-1.30E+01
		DVSQSSISFQIEK	6.93E+00
	Semenogelin 1	DIFSTQDELLVYNK	1.46E+01
		QITIPSQEQEHSQK	-2.39E+01
	Epididymal Secretory	DCGSVDGVIK	-7.27E+00
		SGINCPIQK	-1.56E+01
Saliva	Alpha Amylase	LSGLLDLALGK	-3.05E+00
		IAEYMNHLIDIGVAGFR	2.27E+01
	Statherin	FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF	Not tested
	Submaxillary Protein	GPYPPGPLAPPQPFGPGFVPPPPPPPTGPGR	Not tested
		IPPPPPAPYGPGIFPPPPPQP	Not tested
	Cystatin	IIEGGIYDADLNDER	-2.09E+00
		ALHFVISEYNK	-1.69E+01

Table 3.32. Calculated percent difference of vaginal fluid and menstrual blood peptide biomarkers to assess ion suppression and enhancement.

Fluid	Protein	Peptide	%Difference
Vaginal Fluid	Cornulin	LLDEDHTGTVEFK	-4.95E+00
		ISPQIQLSGQTEQTQK	-2.81E+00
		AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR	6.59E+00
	Neutrophil Gelatinase	WYVVGLAGNAILR	2.25E+01
		MYATIYELK	-7.88E+00
	Ly6/PLAUR	GLDHGLLAFIQLQQCAQDR	8.23E+00
		GCVQDEFCTR	-1.41E+00
	Suprabasin	ALGDINSGITHAGR	-6.21E+00
		FGQGVHHGLSEGWK	7.90E+01
	Periplakin	AQSLQSAK	-8.64E+00
		NLLDEIASR	-6.10E+00
		NQGPQESVVR	-7.83E+00
	Small Proline Rich Protein 3	VPEPGCTK	-9.73E+00
		VPVPGYTK	-4.21E+00
	Involucrin	HLVQQEGQLEQQER	-5.28E+00
		QEAQLELPEQQVGQPK	-6.87E+00
		GEVLLPVEHQQQK	-3.85E+00
Menstrual Blood	Cornulin	LLDEDHTGTVEFK	-2.17E+01
		ISPQIQLSGQTEQTQK	-2.93E+01
		AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR	-8.43E+00
	Neutrophil Gelatinase	WYVVGLAGNAILR	2.25E+01
		MYATIYELK	-1.55E+01
	Ly6/PLAUR	GLDHGLLAFIQLQQCAQDR	-1.15E+02
		GCVQDEFCTR	-2.18E+01
	Suprabasin	ALGDINSGITHAGR	-8.31E+01
		FGQGVHHGLSEGWK	6.68E+01
	Periplakin	AQSLQSAK	-1.71E+01
		NLLDEIASR	-1.26E+01
		NQGPQESVVR	-2.38E+01
	Involucrin	HLVQQEGQLEQQER	-1.85E+01
		QEAQLELPEQQVGQPK	-2.46E+01
		GEVLLPVEHQQQK	-1.02E+01
	Small Proline Rich Protein 3	VPEPGCTK	-1.77E+01
		VPVPGYTK	-3.52E+01
	Alpha-1 Antitrypsin	LSITGTYDLK	3.93E+00
		SVLGQLGITK	-1.87E+01
	Hemoglobin	SAVTALWGK	-5.11E+01
		LLVVYPWTQR	-9.26E+01
	Hemopexin	NFPSPVDAAFR	-2.40E+01
	Apolipoprotein	LLDNWDSVTSTFSK	-6.42E-01
		VSFLSALEEYTK	4.70E+00
	Serotransferrin	DGAGDVAFVK	-1.71E+01
		SASDLTWDNLK	-1.78E+01

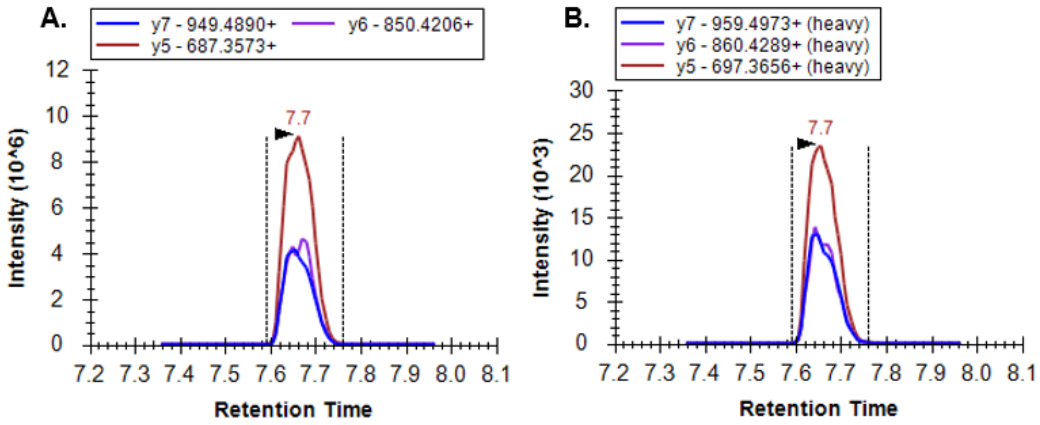


Figure 3.14. Peak area response of (A) endogenous hemoglobin peptide LLVVYPWTQR and (B) AQUA hemoglobin peptide LLVVYPWTQR.

3.2.9 Carryover

Carryover was exhaustively evaluated during validation to ensure accurate and precise detection of target biomarkers, particularly for assessment of low-level sample types that are preceded by highly concentrated samples. Carryover is defined as the unintended presence of an analyte peak in an adjacent sample analyzed after a positive sample [161].

Preliminary carryover samples included 10 µg, 20 µg, and 30 µg of prepared material analyzed on column followed by a blank injection. Carryover in the subsequent blank was observed for two protein biomarkers. First, hemoglobin in peripheral blood and menstrual blood matrices was detected in blanks after all three loading amounts evaluated (**Figure 3.15**). Peptide LLVVPWTQR produced linear peak response in blank injections, with linearity of sample injections decreasing as the loading amount increased, indicating saturation of the mass spectrometer detector. Peptide SAVTALWGK was detected in blank injections after 20 µg and 30 µg sample amounts. Second, semenogelin 1 exhibited high levels of carryover in all subsequent blank injections (**Figure 3.15**). Peptide DIFSTQDELLVYNK peak area responses produced linear results in blank injection analysis, with peak areas greater than 7,400,000 in sample injections at 10 µg loading amounts. Similar to hemoglobin peptide LLVVPQTQR, semenogelin 1 peptide DIFSTQDELLVYNK is relatively hydrophobic, eluting toward the end of the analytical run time. With reverse-phase liquid

chromatography, hydrophobic moieties are strongly retained within the stationary phase, with instances of incomplete release of hydrophobic analytes not uncommon.

Because hemoglobin and semenogelin 1 carryover was observed at substantial peak area responses, samples were reinjected using the same loading amounts previously described followed by two blank injections. This was performed to evaluate the retention of the hydrophobic peptides. As expected, both hemoglobin and semenogelin 1 were detected in the second blank injection analyzed after a 10 µg injection (**Figure 3.16**).

Preliminary carryover studies showed that 10 µg of material on column produced peak area responses that greatly exceeded what is necessary for accurate identification of target fluids. Keeping in mind common forensic biology laboratory protocol, lower injection volumes were evaluated for use in order to conserve precious sample material. For this purpose, vaginal fluid protein biomarkers were assessed as they are inherently detected in lower abundance within matrix. 10 µg injection quantities produced adequate peak area responses for all vaginal fluid peptide markers, with an average response of all peptide markers recorded as 49,800. From the data produced during this study, it was concluded that decreased loading amounts would not only limit the possibility of carryover with more abundant targets but would still yield reliable responses for target peptide markers by limiting chromatographic effects observed with column overload (*i.e.,* peak shouldering, peak tailing, etc.). Final carryover studies were performed using 0.5 µg, 1 µg, 2.5 µg, and 5 µg loading amounts followed by blank injections.

Lower sample injection amounts were found to limit instances of carryover while maintaining accurate peak area responses for fluid identification. However, hemoglobin and semenogelin 1 carryover persisted, albeit at low-level abundance (**Figure 3.17**). Although semenogelin 1 exhibited carryover at the 2.5 µg injection amount, observed peak areas were sufficiently less than those observed from hemoglobin carryover at all loading amounts evaluated. Due to these effects, hemoglobin and semenogelin 1 targets must be called at a greater intensity, regardless of ion ratio and chromatographic acceptance at lower peak areas. Therefore, a 2.5 µg loading amount was selected as the optimal amount of protein on column during analysis. Although

2.5 µg of material still produced sufficient response for less abundant targets, specifically target vaginal fluid biomarkers, two instances of protein dropout, Ly6/PLAUR and epididymal secretory protein E1, were observed at the 2.5 µg injection. A complete overview of observed carryover is outlined in **Table 3.33**. The presence of carryover was addressed in drafted interpretation guidelines, with strict detail given on the acceptance of semenogelin 1 and hemoglobin peaks above a designated value in order to prevent the false identification of carryover peaks.

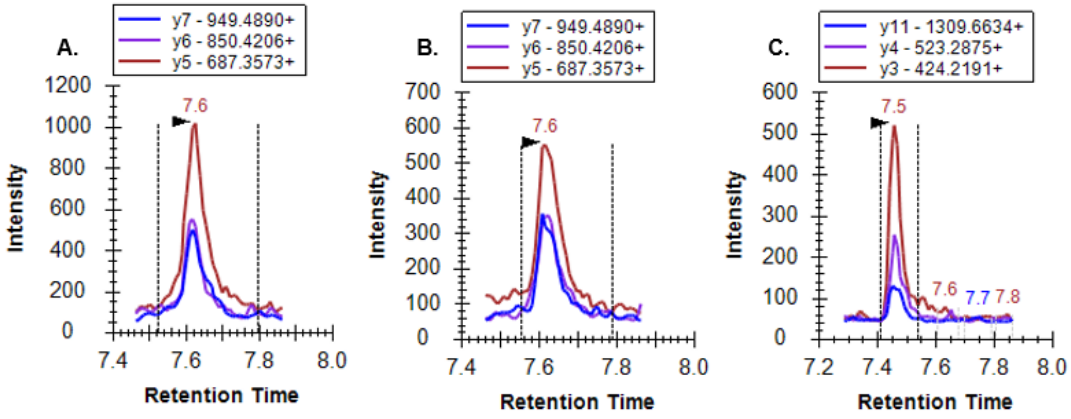


Figure 3.15. Peak area response of (A) hemoglobin peptide LLVVYPWTQR in peripheral blood, (B) hemoglobin peptide LLVVYPWTQR in menstrual blood, and (C) semenogelin 1 peptide DIFSTQDELLVYNK in seminal fluid in blank injections directly following a 10 µg sample injection.

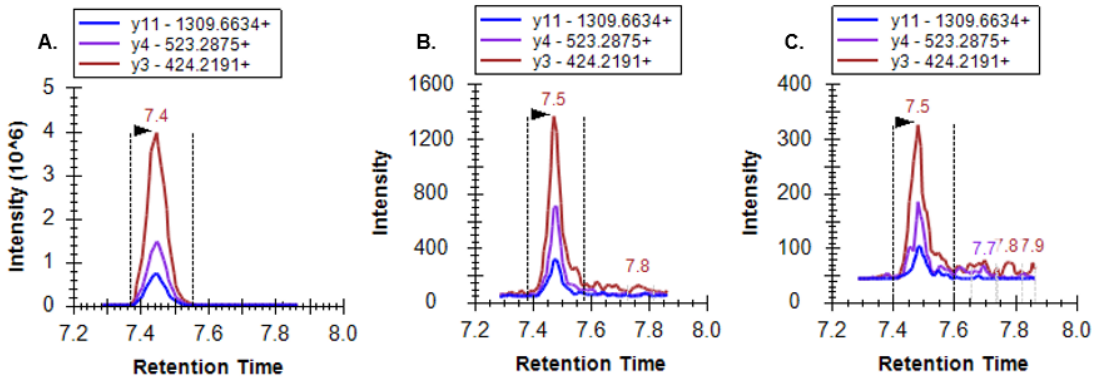


Figure 3.16. Peak area response of semenogelin 1 peptide DIFSTQDELLVYNK in (A) 10 µg sample injection, (B) first subsequent blank injection, and (C) second subsequent blank injection.

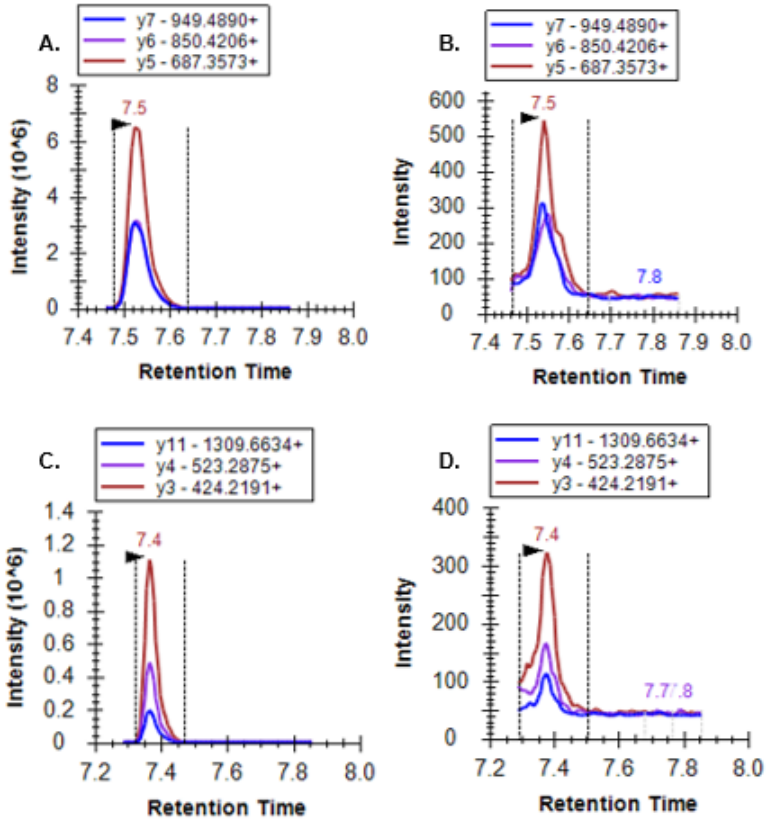


Figure 3.17. Peak area response of (A) hemoglobin peptide LLVVYPWTQR in 0.5 µg peripheral blood, (B) hemoglobin peptide LLVVYPWTQR in subsequent blank injection, (C) semenogelin 1 peptide DIFSTQDELLVYNK in 2.5 µg seminal fluid, and (D) semenogelin 1 peptide DIFSTQDELLVYNK in subsequent blank injection.

Table 3.33. Detection of body fluid markers during carryover assessment. Proteins that were positively identified by all of their respective peptide markers are shown in green. White indicates complete protein dropout.

		Sample (ug)							Blank (ug)						
Fluid	**Protein**	0.5	1	2.5	5	10	20	30	0.5	1	2.5	5	10	20	30
Peripheral Blood	Alpha-1 Antitrypsin														
	Hemoglobin														
	Hemopexin														
	Apolipoprotein														
	Serotransferrin														
Saliva	Alpha Amylase														
	Statherin														
	Submaxillary Protein														
	Cystatin														
Vaginal Fluid	Cornulin														
	Neutrophil Gelatinase														
	Ly6/PLAUR														
	Suprabasin														
	Periplakin														
	Small Proline Rich Protein 3														
	Involucrin														
Seminal Fluid	Acid Phosphatase														
	Prostate Specific Antigen														
	Semenogelin 2														
	Semenogelin 1														
	Epididymal Secretory														
Menstrual Blood	Cornulin														
	Neutrophil Gelatinase														
	Ly6/PLAUR														
	Suprabasin														
	Periplakin														
	Involucrin														
	Small Proline Rich Protein 3														
	Alpha-1 Antitrypsin														
	Hemoglobin														
	Hemopexin														
	Apolipoprotein														
	Serotransferrin														

3.2.10 Blind Sample Analysis

A series of 50 samples containing fiber-tipped swabs, swatches of cloth, and condoms were received from the grant agency for blind analysis. Utilizing the described LC-MS/MS method, body fluid identification assignments were made. In total, 41 of 50 blind samples (82%) analyzed were correctly identified when compared with true sample contents. The remaining 9 samples

(18%) were misidentified, with 3 false positive identifications and 5 false negative (*i.e.*, non-detections observed) results recorded. A comprehensive summation of experimental identifications, true identifications, and sample preparation information is outlined in **Appendix H**. For example, a cotton swab sample (Sample #15) was identified as containing biomarkers consistent with seminal fluid and vaginal fluid; however, biomarkers consistent with saliva were not detected (**Figure 3.18**). This sample was characterized as a false negative, having contained a 3-part mixture (10 µL semen and 1 µL of saliva on a vaginal swab). On the contrary, a cotton swab (Sample #22) was misidentified as containing biomarkers consistent with vaginal fluid and characterized as a false positive identification (**Figure 3.19**).

Receiving true sample contents *ex post facto*, the validated LC-MS/MS assay demonstrated robustness and reliability when challenged with non-target fluids. For example, when subjected to samples containing breast milk (Sample #49), sweat (Sample #50), or urine (Samples #23, #24, #39), no misidentifications were made. One interesting sample to note (Sample #30) contained nasal secretions. Although not a target fluid of the validated method, a peptide biomarker profile was previously acquired and utilized to make a special note regarding the experimental identification of this particular sample. Although reported as containing peripheral blood and saliva, it was specified as a note that the resulting profile may be chemically consistent with nasal secretions.

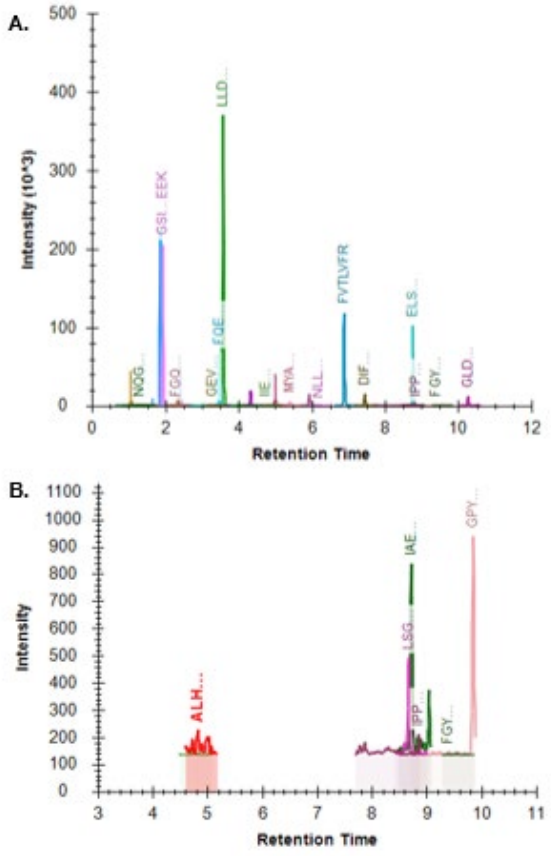


Figure 3.18. Peptide biomarker profile of Blind Sample #15. (A) Biomarkers consistent with seminal fluid and vaginal fluid and (B) lack of biomarkers consistent with saliva, prompting a false negative identification.

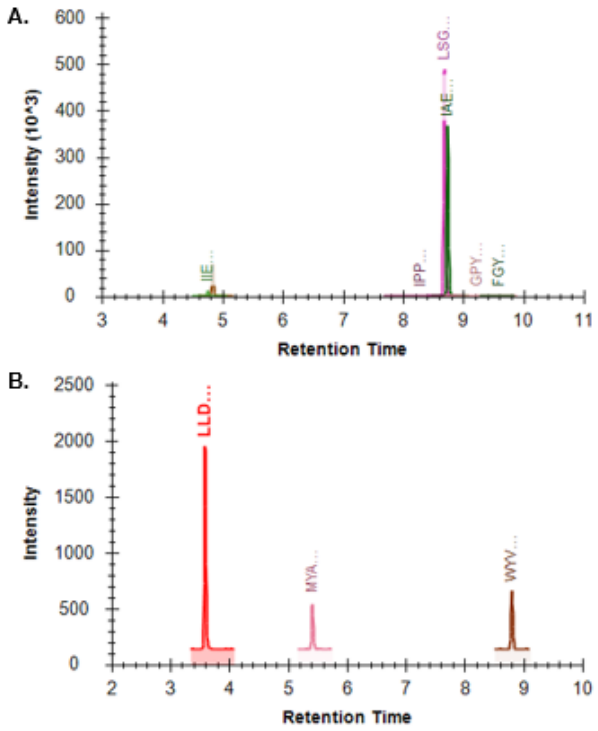


Figure 3.19. Peptide biomarker profile of Blind Sample #22. (A) Biomarkers consistent with saliva and (B) biomarkers consistent with vaginal fluid, prompting a false positive identification.

3.3 Concluding Remarks

With the completion of this phase of the research, the robustness and reliability of the multiplex assay described has been exhaustively demonstrated. In addition, the inherent limitations observed through the analysis of both routine and challenging forensic type evidence have elucidated a defined understanding of presumptive and confirmatory biomarkers utilized for identification. Overall, the culmination of results detailed within this chapter are the final product of a fully validated analytical method for the serological analysis of peripheral blood, seminal fluid, saliva, and vaginal/menstrual fluids. With achieving sufficient sensitivity coverage over several orders of magnitude, in addition to demonstrating the specificity of fluid-specific biomarkers, the use of protein mass spectrometry as an advanced serological technique offers significant advantages over currently existing catalytic and immunological assays routinely employed in operational laboratories.

The deliverable to the forensic community following the developmental validation results detailed in this chapter include not only a robust analytical multiplex assay, but comprehensive interpretation guidelines (**Appendix I**). Through meticulous evaluation of detection limits, the presence of carryover, and the suppression rates of specific biomarkers, potential avenues for misidentification or incorrect interpretation have been minimized. The purpose of the drafted interpretation guidelines is to serve as a resource for consistent and reliable identification of the body fluids assessed between forensic analysts and laboratories; however, an analyst should remain cognizant during interpretation and exercise a conservative approach, as stated within the guidelines.

The remaining two chapters of this dissertation will establish the use of the described serological assay as a vital step to the greater forensic biology workflow and further evaluate limitations caused as a result of the complexity of sexual assault evidence. By displaying the ability of the multiplex assay to compliment forensic genetic testing methodology, the implementation of such an emerging technique can further illustrate the necessity of serological results to a criminal investigation. Furthermore, a direct comparison to commercially available immunological and catalytic tests will further exemplify the unmatched sensitivity and specificity of the validated assay.

CHAPTER 4: A COMPARATIVE STUDY OF A VALIDATED PROTEOMIC ASSAY WITH EXISTING FORENSIC BIOLOGY CASEWORK MODELS

4 Introduction

The objective of this phase of research was to exemplify the use of the validated proteomic assay in an operational environment. With the limitations of the assay established in the previous chapter, experimentation conducted during this aim demonstrated the efficacy of the validated assay within a forensic biology workflow. This was evaluated in two manners; first, by comparing the validated assay to existing casework models (*i.e.,* immunochromatographic/lateral flow assays, enzymatic assays) for serological screening, and second, the impact of the validated assay on the ability to produce a genetic profile. By assessing the validated assay in tandem with existing workflow strategies, any adverse effects warranted from implementation were identified and addressed.

Current testing strategies for serological screening have historically been rooted in enzyme- and antibody-based assays that rely on protein structure confirmation to produce a positive result. However, given that forensic evidence is commonly subjected to unfavorable environmental conditions, protein structure, and therefore protein function, are likely to be compromised. In addition, given that the formation of an enzyme-substrate complex or antibody binding event lack absolute specificity, these assays are unable to confirm the presence of a target body fluid. Therefore, severely degraded samples and cross-reactivity with non-target compounds have left both the sensitivity and specificity of serological screening much to be desired, especially in comparison with modern genetic testing capabilities. The first aim of this portion of the study was to directly compare the developed proteomic assay with available serological screening tests to empirically demonstrate the sufficient gains in sensitivity and specificity.

With the lack of advancements achieved within serological screening, a shift in existing casework models has been observed within the forensic biology community. Traditionally, evidence received would undergo serological screening, with results dictating the prioritization of samples for subsequent genetic testing. However, with the lack of reliable body fluid tests, serological screening as a gatekeeper for DNA analysis has been reconsidered. More recently, laboratories have opted for a "direct to DNA" approach, with small portions of evidence quickly extracted and quantitated for genetic material. The resulting quantity and quality of DNA determines the type of genetic testing performed, with serological screening only conducted if needed. Although a practical alternative to the traditional workflow, there has been little research on the efficacy of a direct to DNA procedure, particularly with challenging sample types. By evaluating the use of the proteomic workflow within existing casework models, the use of serological screening as a gatekeeper for genetic testing has the capability of providing copious amounts of information vital to criminal investigations that would have otherwise been absent, in addition to reducing costs associated with advanced genetic analyses.

This chapter seeks to establish the viability of the validated proteomic assay within the overall forensic biology workflow. This was achieved through the completion of a side-by-side comparison of the assay with select available immunological and enzymatic tests and by demonstrating the value of proteomic results in relation to the quality of genetic profiles produced. For the purposes of direct comparison, this chapter is organized according to sample type, with serological and genetic information presented together.

4.1 Methods and Materials

All research conducted under this phase of the project was reviewed and approved by the University of Denver IRB for research involving human subjects. Sample collection and research was conducted in full accordance with the U.S. federal policy for the protection of human subjects as described in section 2.11.

4.1.1 Sample Preparation

Samples were prepared as outlined in section 3.1 and carried over to this portion of the research for operational testing. Three categories of laboratory prepared casework-type samples were reserved for serological comparison only: substrate samples (3.1.6.1), environmental contaminant samples (3.1.6.2), and aged samples (3.1.6.6). Six categories of casework-type samples were reserved for both serological comparison and genetic analysis: sensitivity samples (3.1.6.7), mixtures (3.1.6.3), simulated sexual assault samples (3.1.6.4.1), sexual assault type samples (3.1.6.4.2), digital swab samples (3.1.6.4.3), and degraded samples (3.1.6.5). Swabs or substrates were solubilized as previously described in section 3.1. Remaining supernatant was tested against existing serological models in tandem with proteomic analysis, eliminating an additional freeze/thaw cycle to ensure consistency in material evaluated. Cell pellets were preserved for genetic analysis at -20 ºC and were thawed at room temperature prior to DNA typing.

4.1.2 Traditional Serological Testing

Casework-type samples were prepared using the previously described protocol (section 2.1.4) and once solubilized, a matched liquid sample was manually processed with a corresponding antibody-based assay or enzymatic test. Specifically, antibody-based and enzyme-based tests targeting blood, seminal fluid, and saliva were selected. The immunochromatographic assays utilized for this portion of the research were as follows: RSID™ Semen, RSID™ Blood, RSID™ Saliva (Independent Forensics, Hillside, IL), ABAcard® p30 and ABAcard® HemaTrace (Abacus Diagnostics, West Hills, CA). The enzymatic test used for this portion of the research was SALIgAE® (Abacus Diagnostics) and was selected given the manufacturer does not supply an antibody-based assay for the identification of saliva. See **Table 4.1** for more specific information regarding the selected commercially available screening tests.

For sensitivity samples, the maximum volume suggested by the manufacturer was used for testing (200 µL ABAcard® assays, 100 µL RSID™ assays, 50 µL SALIgAE® assay). This set of samples was not originally quantitated for total protein amount during the validation phase of this research, as outlined in Chapter 3. For remaining sample types, matched liquid samples were

prepared as an equivalent total protein amount. The quantity observed from BCA quantitation was diluted up to maximum volume in Universal Buffer™ (Independent Forensics of Illinois). The maximum volume, and therefore an equivalent protein amount, was processed on either a lateral flow cartridge or enzyme test.

Table 4.1. Selected traditional serological screening tests for comparison with the developed proteomic assay.

Manufacturer	Test Name (Test Type)	Target Antigen/ Substrate	Dye
Abacus Diagnostics	ABAcard® HemaTrace (Antibody)	Hemoglobin	Possible Colloidal Gold, Colloidal Silver, Carbon, or Latex
	ABAcard® p30 (Antibody)	Prostate Specific Antigen	Possible Colloidal Gold, Colloidal Silver, Carbon, Latex
	SALIgAE® (Enzymatic)	Salivary Amylase	Proprietary Mechanism
Independent Forensics	RSID™ Blood (Antibody)	Glycophorin A	Colloidal Gold
	RSID™ Semen (Antibody)	Semenogelin	Colloidal Gold
	RSID™ Saliva (Antibody)	Salivary α-Amylase	Colloidal Gold

4.1.3 Forensic Genetic Testing

4.1.3.1 DNA Extraction

Given the composition of the samples assessed during this phase of the research, two DNA extraction protocols were utilized. Knowing *a priori* the makeup of each sample, all samples containing semen or seminal fluid were subjected to a previously validated manual organic differential extraction procedure. All other samples (*i.e.,* those free of semen) were subjected to a previously validated manual organic extraction protocol.

Pelleted cellular material selected for organic extraction was treated with 350 µL of master mix containing extraction buffer and proteinase K solution. Samples were incubated at 56 ºC overnight (a maximum of 18 hours) on a shaker at 850 RPM. After incubation, samples were purified and concentrated. Each sample received two iterations of purification using 300 µL of

phenol:chloroform:isoamyl alcohol reagent. An additional cleanup step was performed with the addition of 300 µL of water saturated butanol. Purified sample was passed through a Vivacon® 500 centrifugal concentrator (Vivaproducts, Inc., Littleton, MA), washed with 200 µL of TE^{-4} buffer (10mM Tris-HCl, 0.1mM EDTA, pH 8.0), and eluted in 100 µL of TE^{-4} buffer.

Pelleted cellular material selected for organic differential extraction was treated with 505 µL of master mix containing extraction buffer and proteinase K solution. Samples were incubated at 56 ºC for 40 minutes on a shaker at 850 RPM. Samples were centrifuged for 10 minutes at 14,000 x g. The supernatant (epithelial fraction) was transferred to a clean tube. Remaining cellular material (sperm fraction) was washed with 500 µL of UltraPure water and centrifuged for 10 minutes at 14,000 x g, where the wash buffer was removed. Cellular material was treated with 380 µL of master mix containing extraction buffer, 1 M dithiothreitol (DTT), and proteinase K solution. The sample was incubated at 89 ºC for 40 minutes on a shaker at 850 RPM. Epithelial and sperm fractions were purified and concentrated as described above for the organic extraction. Concentrated DNA was recovered in 100 µL of TE^{-4} buffer. Extractions were batched, with each batch being assigned a unique reagent control to monitor for any instances of contamination. Extracts were stored at -20 ºC for later use.

4.1.3.2 DNA Quantitation

Once purified, all samples were quantitated using Quantifiler™ Trio DNA Quantification Kit (Applied Biosystems™, Foster City, CA). Extracts were thawed and brought to room temperature prior to quantitation. A previously validated standard operating procedure was used. A standard curve was prepared, in addition to a negative control, for each batch of samples. Standards were prepared by diluting the DNA Standard with Quantifiler™ THP DNA Dilution Buffer to the following final concentrations: 50 ng/µL, 5.0 ng/µL, 0.5 ng/µL, 0.05 ng/µL, and 0.005 ng/µL. TE^{-4} buffer was used for the negative control. Standards, controls, and samples were treated with a master mix of kit specific Primer Mix and PCR Reaction Mix. Quantitation was performed on an Applied Biosystems™ 7500 Real-Time PCR System. Thermal cycler parameters were set to heat at 95 ºC for 2 minutes followed by 40 cycles of 9 seconds at 95 ºC and 30 seconds at 60 ºC. The

quantification batch was considered valid if the standard curve slope measured between -3.0 and -3.6 and the R^2 value was greater than or equal to 0.98.

4.1.3.3 DNA Amplification

Amplification reactions were prepared in accordance with DNA quantification results. All samples that underwent an organic extraction (*i.e.,* those free of semen) were amplified for STR analysis using GlobalFiler™ PCR Amplification Kit (Applied Biosystems™). Samples that were subjected to an organic differential extraction that produced a male quantification value were also prepared for routine STR amplification using GlobalFiler™ PCR Amplification Kit. However, remaining organic differential samples that did not produce a male quantification value were prepared for Y-STR amplification using Yfiler™ Plus PCR Amplification Kit (Applied Biosystems™).

Full scale amplification was performed, with a total of 1.0 ng of DNA targeted. Maximum extract volume (15 µL GlobalFiler™, 10 µL Yfiler™ Plus) was used for samples that did not produce a sufficient quantitation value. Manufacturer recommended protocols for GlobalFiler™ and Yfiler™ Plus were followed, with no changes made to the respective protocols. Amplification was performed on an Applied Biosystems™ 9700 Thermal Cycler. 29 amplification cycles were performed for GlobalFiler™ and 30 amplification cycles were performed for Yfiler™ Plus. Samples were batched, with each batch assigned a positive and negative control to ensure amplification was performed properly. Amplicons were stored at 4 °C until separation.

4.1.3.4 DNA Separation and Analysis

Amplicons were separated and detected using a 3500 Genetic Analyzer (Applied Biosystems™) fitted with an 8-capillary array. POP-4™polymer (Applied Biosystems™) was utilized as the separation matrix. GeneScan™ 600 LIZ™ Dye Size Standard (v2.0) and DNA Control 007 were used for both GlobalFiler™ and Yfiler™ Plus amplicons. The Yfiler™ Plus Allelic Ladder and GlobalFiler™ Allelic Ladder were employed for their respective amplicon samples.

GeneMapper® ID-X software (v.1.4) was utilized for profile interpretation. Through internal validation that independently evaluated the signal-to-noise characteristics of each dye channel, the following analytical thresholds were set for a 10 second injection with GlobalFiler™: 6-FAM™, 60

relative fluorescence units (RFUs); VIC™, 75 RFUs; NED™, 50 RFUs; TAZ™, 60 RFUs; and SID™, 60 RFUs. From a separate internal validation, the following analytical thresholds were set for a 10 second injection with Yfiler™ Plus: 6-FAM™, 50 RFUs; VIC™, 50 RFUs; NED™, 40 RFUs; TAZ™, 40 RFUs; and SID™, 40 RFUs.

4.2 Results and Discussion

For the purposes of direct comparison, results for sensitivity in the operational environment and compatibility with existing casework models have been merged for each sample type evaluated.

4.2.1 Substrate Samples

Substrate samples were designed to ensure chemical products or composition would not interfere with protein biomarker detection or recovery. Overall, the proteomic assay was successful in identifying the target body fluid of each substrate sample (**Table 3.18**), with three instances of vaginal fluid protein dropout observed for menstrual blood samples. Matched samples were compared with serological screening tests (**Table 4.2**). With the exception of two peripheral blood samples, all substrate samples were positive on traditional immunological and enzymatic based assays. Peripheral blood applied to carpet (sample ID SUB03) and to leather (sample ID SUB04) produced a negative result when evaluated with RSID™ Blood, with carpet exhibited in **Figure 4.1A**. In addition, when compared with a positive control test, a majority of peripheral blood and menstrual blood containing samples resulted in weak or very weak positive results. For example, menstrual blood on cotton resulted in a weak positive result on RSID™ Blood in comparison with ABAcard® HemaTrace (**Figure 4.1B**). However, the proteomic assay was able to positively identify blood-containing samples by all targeted peptide biomarkers. Similarly, target proteins for semen and saliva samples were positively characterized by the proteomic assay, in addition to positive identification using traditional screening tests, with examples highlighted in **Figure 4.1C** and **Figure 4.1D**, respectively. DNA analysis was not performed on this set of casework samples.

With the exception of the aforementioned negative peripheral blood samples on the single manufacturer test, the traditional assays and proteomic assay were comparable in identification of

target body fluid. However, given the inherent nature of the LC-MS/MS technology, the proteomic assay fundamentally provided precise protein identification and additional information not capable of the lateral flow assays.

Table 4.2. Summary of comparative results for substrate samples. Positive identifications are represented by a green box. Negative identifications are represented by a red box. White boxes represent no testing was performed with that specific assay. Vaginal fluid samples were removed from the table as no additional testing or comparison was conducted.

Sample	Description	RSID™ Blood	ABAcard® HemaTrace	RSID™ Semen	ABAcard® p30	RSID™ Saliva	SALIgAE®
SUB01	Peripheral Blood on Cotton	+ very weak	+ weak				
SUB02	Peripheral Blood on Denim	+	+ weak				
SUB03	Peripheral Blood on Carpet	-	+ weak				
SUB04	Peripheral Blood on Leather	-	+ weak				
SUB05	Peripheral Blood on Drywall	+ very weak	+ weak				
SUB08	Menstrual Blood on Cotton	+ weak	+				
SUB09	Menstrual Blood on Denim	+	+ weak				
SUB10	Menstrual Blood on Pad	+	+ weak				
SUB06	Semen on Cotton			+	+		
SUB07	Semen on Leather			+	+		
SUB14	Saliva on Plastic Bottle					+	+
SUB15	Saliva on Aluminum Can					+	+

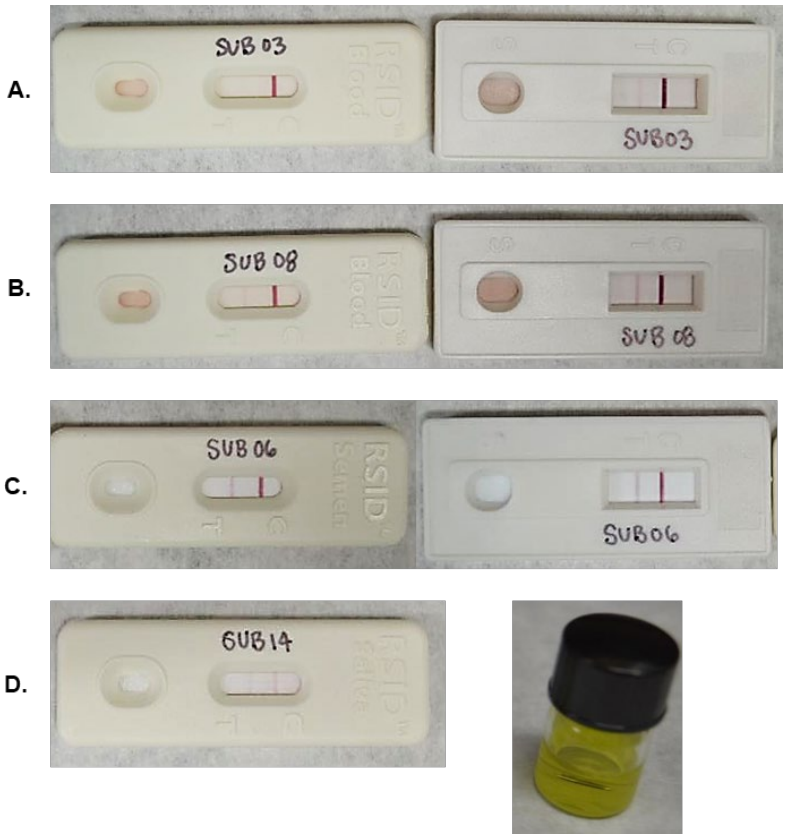


Figure 4.1. Selected serological screening test results on substrate samples. RSID™ tests are pictured on the left. ABAcard® tests and SALIgAE® are pictured on the right. Within the lateral flow test membrane window, the test line is positioned on the left and the control line on the right. Results for (A) peripheral blood on carpet, (B) menstrual blood on cotton, (C) semen on cotton, and (D) saliva on plastic bottle. On lateral flow assays, a positive result is indicated by the presence of a red line at both the test and control zones. For SALIgAE®, a positive result is indicated by a yellow colored reagent.

4.2.2 Environmental Contaminant Samples

This subset of samples was prepared to assess the effects of environmental contaminants on sample preparation chemistries of the developed proteomic workflow. When evaluated with the proteomic assay, three samples exhibited protein and peptide dropout (**Table 3.19**). Menstrual blood samples (sample ID CON04 and CON05) illustrated vaginal fluid protein and peptide dropout. The third sample, vaginal fluid and wate-based lubricant, exhibited complete protein dropout; however, this sample was unable to be directly compared with other serological screening tests as there are none commercially available. As observed with substrate samples, the traditional and proteomic assays produced comparable results in regard to target fluid identification. A summary

of serological screening results is outlined in **Table 4.3**. Overall, samples subjected to chemical insult did not inhibit the ability to produce a positive result with traditional serological assays. A very weak positive was observed for peripheral blood and 10% bleach (**Figure 4.2A**), which contradicts the positive protein identifications observed during proteomic analysis. It was originally hypothesized that the presence of personal lubricant would decrease advantageous protein binding events and result in an increase in negative reporting. However, immunological assays accurately characterized semen and menstrual blood samples containing personal lubricant (**Figure 4.2B and 4.2C**). There was no inhibition of salivary amylase, with both saliva case samples characterized as exhibited by saliva extracted from a cigarette filter (**Figure 4.2D**). DNA analysis was not performed on this set of casework samples. In summary, comparable results between the proteomic method and traditional methodologies were observed; however, the proteomic method provides a greater depth of coverage in specific biomarker identifications.

Table 4.3. Summary of comparative results for environmental contaminant samples. Positive identifications are represented by a green box. White boxes represent no testing was performed with that specific assay. Vaginal fluid samples were removed from the table as no additional testing or comparison was conducted.

Sample	Description	RSID™ Blood	ABAcard® HemaTrace	RSID™ Semen	ABAcard® p30	RSID™ Saliva	SALIgAE®
CON01	Peripheral Blood & Dirt	+	+				
CON02	Peripheral Blood & Rust	+	+				
CON03	Peripheral Blood & 10% Bleach	+ very weak	+				
CON04	Menstrual Blood & Lube	+	+				
CON05	Menstrual Blood & Spermicide Condom	+	+				
CON08	Semen & Lube			+	+		
CON09	Semen & Spermicide Condom			+	+		
CON10	Semen & 10% Bleach			+	+		
CON11	Saliva & Tobacco					+	+
CON12	Saliva & Cigarette					+	+

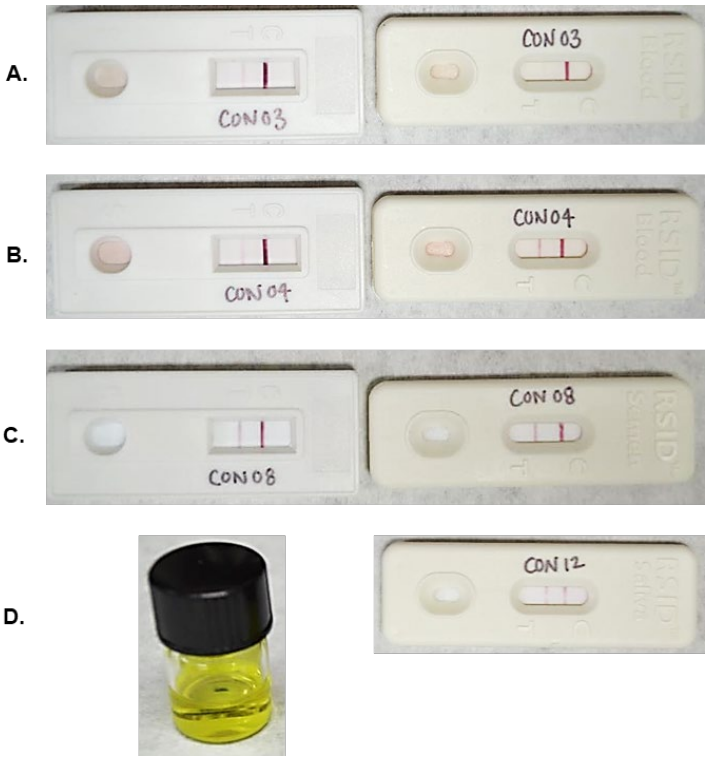


Figure 4.2. Selected serological screening test results for environmental contaminant samples. ABAcard® test and SALIgAE® are pictured on the left. RSID™ test is pictured on the right. Within the membrane window, the test line is positioned on the left and the control line on the right. Results for (A) peripheral blood and 10% bleach, (B) menstrual blood and personal lubricant, (C) semen and personal lubricant, and (D) saliva on a cigarette. On lateral flow assays, a positive result is indicated by the presence of a red line at both the test and control zones. For SALIgAE®, a positive result is indicated by a yellow colored reagent.

4.2.3 Mixture Samples

For this portion of the study, mixture samples evaluated are separate from those used during validation to determine transition interference. Casework-type mixture samples were designed to assess the capability of each testing strategy to detect a minor contributor when in the matrix of a major contributor, simulating commonly received sample types in a forensic biology laboratory. Of the five mixture samples assessed, the minor contributor was characterized when evaluated with the proteomic assay. When directly comparing a matched sample using traditional techniques, two instances of negative detection were observed (**Table 4.4**). With the detection of a minor saliva contributor, the enzymatic test SALIgAE® was unable to characterize the presence of the target fluid in either a vaginal fluid or menstrual blood matrix; however, RSID™ Saliva

exhibited a positive result for both MIX01 and MIX04 samples (**Figure 4.3**). It should be noted that the inherent color of the MIX04 extract caused the SALIgAE® buffer to become discolored, potentially masking the visualization of a positive result. There were no reported discrepancies for the detection of seminal fluid with either antibody-based assay (**Figure 4.4**). Due to the lack of commercially available serological tests for vaginal fluid, a matched sample for MIX05, vaginal fluid in a urine major contributor, was not evaluated using antibody-based or enzymatic tests.

Allele calls produced by mixtures samples for short tandem repeat (STR) and Y chromosome STR (Y-STR) analyses are outlined in **Tables 4.5 and 4.6**, respectively, with alleles unique to the major contributor removed for ease of comparison. The allele calls presented are unique to the minor contributor or shared with the major contributor. For sample MIX01, a minor saliva contributor with a major vaginal fluid contributor, 2 out of 4 salivary protein biomarkers were detected with the proteomic assay; however, the DNA profile generated was consistent with the vaginal fluid major contributor. The DNA profile produced was single source, and even with allele sharing between the two contributors, the profile did not indicate instances of peak imbalance or YINDEL/DYS391 detection (**Table 4.5**). For samples MIX02 and MIX03, a minor semen contributor in a major vaginal fluid and menstrual blood contributor, respectively, were extracted using an organic differential protocol. The sperm fractions were analyzed for Y-STR's and produced full haplotypes (**Table 4.6**). Furthermore, all 5 target seminal fluid biomarkers were detected with the proteomic assay for these samples. For sample MIX04, a minor saliva contributor in a major menstrual blood contributor, no unique alleles consistent with the minor contributor were detected. And lastly, MIX05, a minor vaginal fluid contributor in a major urine contributor, produced a complete profile for the minor contributor, with 2 out of 7 vaginal fluid markers detected with the proteomic assay.

Table 4.4. Summary of comparative results for mixture samples. Positive identifications are represented by a green box. Negative results are represented by a red box. White boxes indicate no testing with that specific assay.

Sample	MIX01	MIX04	MIX02	MIX03	MIX05
Description	Saliva in Vaginal Fluid	Saliva in Menstrual Blood	Semen in Vaginal Fluid	Semen in Menstrual Blood	Vaginal Fluid in Urine
Target Fluid	Saliva	Saliva	Semen	Semen	Vaginal Fluid
Mass Spec Protein ID	2 out of 4	1 out of 4	5 out of 5	5 out of 5	2 out of 7
Minor Contributor Unique # Alleles			Y-STR 27 of 27	Y-STR 27 of 27	22 of 22
RSID™ Saliva	+	+			
SALIgAE®					
RSID™ Semen			+	+	
ABAcard® p30			+	+	

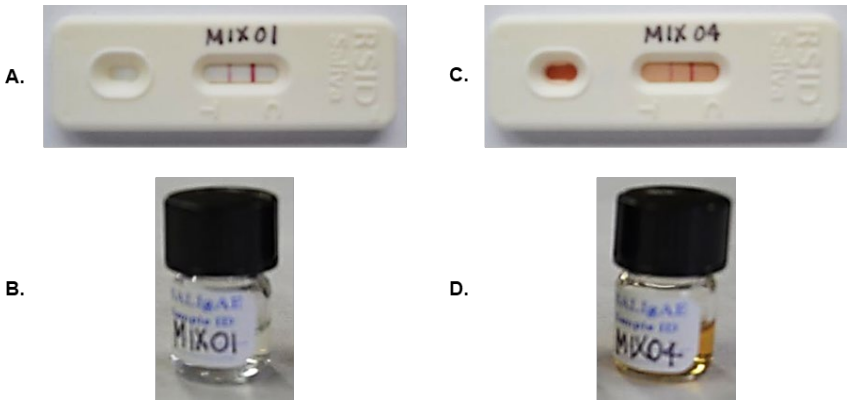


Figure 4.3. Serological screening test results for mixture samples containing saliva as a minor contributor. Within the membrane window, the test line is positioned on the left and the control line on the right. Results for saliva in a vaginal fluid major contributor using (A) RSID™ Saliva and (B) SALIgAE®. Results for saliva in a menstrual blood major contributor using (C) RSID™ Saliva and (D) SALIgAE®. On lateral flow assays, a positive result is indicated by the presence of a red line at both the test and control zones. For SALIgAE®, a positive result is indicated by a yellow colored reagent.

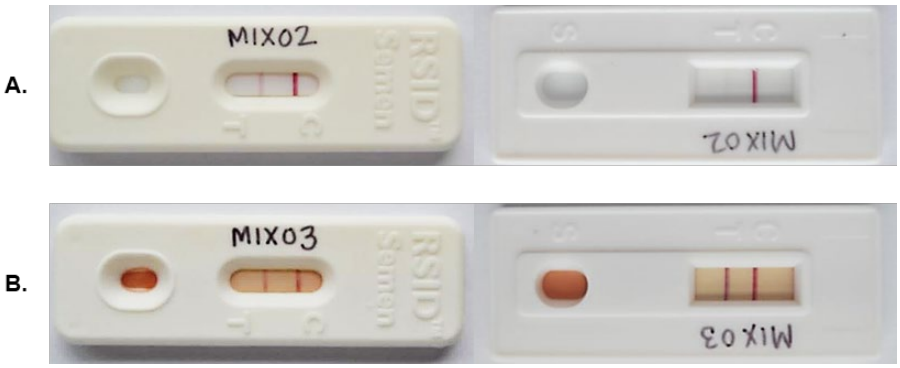


Figure 4.4. Serological screening test results for mixture samples containing semen as a minor contributor. RSID™ Semen test is pictured on the left. ABAcard® p30 test is pictured on the right. Within the membrane window, the test line is positioned on the left and the control line on the right. Results for (A) semen in a vaginal fluid major contributor and (B) semen in a menstrual blood major contributor. A positive result is indicated by the presence of a red line at both the test and control zones.

Table 4.5. STR genetic results for mixture samples. Unique allele ratio for the minor contributor was calculated using reference profiles. For clarity, unique major contributor alleles have been removed from case samples. Alleles depicted represent shared and/or unique minor contributor alleles.

Mixtures		Semen, Saliva, Urine Reference	Vaginal Fluid Reference	Menstrual Blood Reference	MIX01 Saliva in Vaginal Fluid	MIX02 Semen in Vaginal Fluid	MIX03 Semen in Menstrual Blood	MIX04 Saliva in Menstrual Blood	MIX05 Vaginal Fluid in Urine
BLUE	D3S1358	15,17	16,17	16, 18	17	17	-	-	16
	VWA	17,18	15,18	16	18	18	-	-	15
	D163539	11,12	9,12	13, 14	12	12	-	-	9
	CSF1PO	10,11	11,14	12, 13	11	11	-	-	14
	TPOX	8,11	8,11	9, 12	8,11	8,11	-	-	-
GREEN	YINDEL	2	-	-	-	-	2	-	-
	Amelogenin	XY	X	X	X	X	X	X	X
	D831179	10,13	12,13	13, 14	13	13	13	13	12
	D21S11	31.2,32.2	27,30	27, 30	-	-	-	-	27,30
	D18S51	11,15	10,14	12, 13	-	-	-	-	10,14
	DYS391	12	-	-	-	-	-	-	-
YELLOW	D2S441	11,14	11,12	10, 11	11	11	11	11	12
	D19S433	13,14	14,15	13	14	**13**,14	13	13	15
	THO1	6,7	9,9.3	6, 9.3	-	-	6	6	9,9.3
	FGA	20,24	20	20, 24	20	20	20,24	20,24	-
RED	D22S1045	15,16	11,15	15, 16	15	15	15,16	15,16	11
	D53818	11,12	11,12	12, 13	11,12	11,12	12	12	-
	D13S317	10,12	11,12	10, 11	12	12	10	10	11
	D73280	10,12	10,12	10, 11	10,12	10,12	10	10	-
	SE33	21,28.2	17,26.2	28.2*, 29.2	-	-	-	-	17,26.2
PURPLE	D10S1248	14,15	14,15	13, 15	14,15	14,15	15	15	-
	D1S1656	15	14,17	15, 16.3	-	-	15	15	14,17
	D12S391	15,16	18,20	15, 22	-	-	15	15	18,20
	D2S1338	17,24	17,19	19, 24	17	17	24	24	19
Minor Contributor Unique Allele Ratio		-	-	-	0/24	1/24	1/27	0/27	22/22

Table 4.6. Y-STR genetic results for mixture samples with semen as a minor contributor. Y-STR analysis was performed based on quantification values.

Mixtures		Reference	MIX02	MIX03
BLUE	DYS576	17	17	17
	DYS389I	14	14	14
	DYS635	20	20	20
	DYS389II	30	30	30
	DYS627	23	23	23
GREEN	DYS460	10	10	10
	DYS458	17	17	17
	DYS19	14	14	14
	YGAT	13	13	13
	DYS448	19	19	19
	DYS391	12	12	12
YELLOW	DYS456	17	17	17
	DYS390	23	23	23
	DYS438	12	12	12
	DYS392	13	13	13
	DYS518	40	40	40
RED	DYS570	17	17	17
	DYS437	15	15	15
	DYS395	11,14	11,14	11,14
	DYS449	28	28	28
PURPLE	DYS393	13	13	13
	DYS439	13	13	13
	DYS481	22	22	22
	DYF387S1	35,36	35,36	35,36
	DYS533	12	12	12
Profile Percentage		-	100%	100%

4.2.4 Sexual Assault Samples

4.2.4.1 Simulated Sexual Assault Samples

Simulated sexual assault samples were designed to imitate the environment of the vaginal cavity and its effects on the breakdown of semen. The ability to identify constituents of seminal fluid post-coitus, particularly after 48 hours, is advantageous to forensic practitioners. The poor sensitivity of currently available immunological screening assays has been reported in the literature for the detection of seminal fluid in an extended post-coital interval [50,162]. A sufficient difference in sensitivity between manufacturers was observed for this subset of samples (**Table 4.7**). Consistent with proteomic identification, RSID™ Semen positively characterized seminal fluid for all time points evaluated. However, ABAcard® p30 failed to detect seminal fluid via prostate specific antigen after time point zero (**Figure 4.5**).

In total, samples from all time points assessed produced at least 20 of 24 (83%) unique male alleles, with four samples generating 100% unique male alleles (**Table 4.8**). Time points at days three, five, seven, and nine report complete detection of the 24 unique alleles from the male contributor, with day zero returning the smallest number of unique alleles, with a minimum of 20 alleles detected from the male contributor.

Although simulating the environment of the vaginal cavity, breakdown products are not being removed in the process. Loss of sample due to natural removal of target material through vaginal drainage as well as breakdown of biomarkers by endogenous protease enzymes within the vaginal vault make the detection of seminal fluid and saliva in sexual assault samples more challenging. Protein denaturation via naturally occurring protease activity is one limitation affecting post-coital detection intervals for immunochromatographic assays. It is important to understand whether proteolytic digestion of seminal fluid and saliva targets in the vaginal vault occurs within a target peptide sequence. From the results of this study, the endogenous breakdown of semenogelin does not affect the positive characterization when using RSID Semen™; however, it can be hypothesized that the lack of p30 detection using ABAcard® p30 test can be attributed to unfavorable protein degradation that compromises the targeted protein epitope confirmation. In comparison with proteomic assay results, it can be concluded that the selected amino acid sequences of target seminal fluid biomarkers are not negatively affected by endogenous breakdown and cleavage of protein material. Furthermore, a decrease in allele characterization would also be expected with vaginal drainage.

Table 4.7. Summary of comparative results for simulated sexual assault samples. Positive identifications are represented by a green box. Negative results are represented by a white box.

Sample	Day Zero	Day One	Day Three	Day Five	Day Seven	Day Nine	Day Eleven
Mass Spec Protein ID	5 out of 5	4 out of 5	4 out of 5	4 out of 5	4 out of 5	3 out of 5	3 out of 5
No. Unique Male Alleles	20 of 24	23 of 24	24 of 24	24 of 24	24 of 24	24 of 24	22 of 24
RSID™ Semen	+	+	+	+	+	+	+
ABAcard® p30	+						

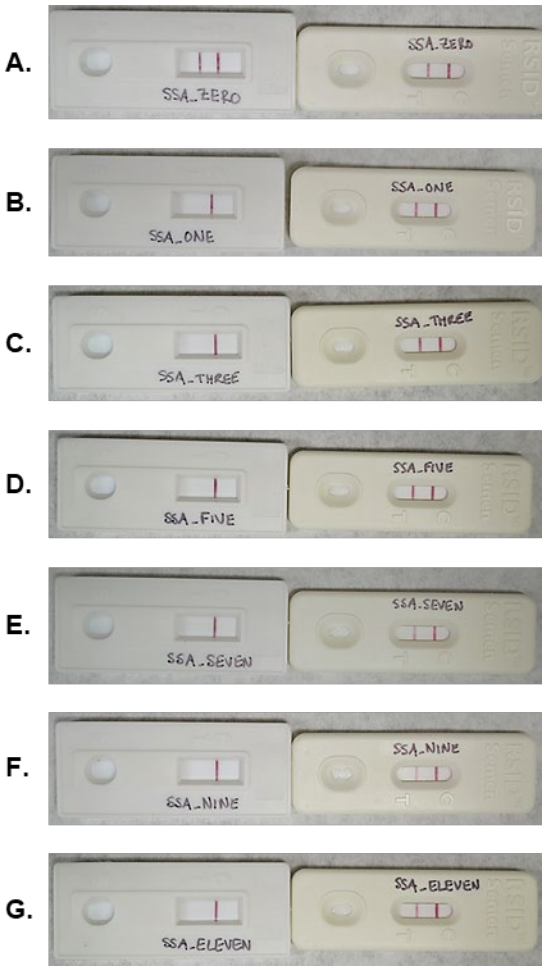


Figure 4.5. Immunochromatographic results for simulated sexual assault samples. ABAcard® p30 test is pictured on the left. RSID™ Semen test is pictured on the right. Within the membrane window, the test line is positioned on the left and the control line on the right. Results for (A) timepoint zero, (B) day one, (C) day three, (D) day five, (E) day seven, (F) day nine, and (G) day eleven. A positive result is indicated by the presence of a red line at both the test and control zones.

Table 4.8. STR genetic results for simulated sexual assault samples. The male reference sample depicts unique male alleles in bold.

		Male Reference	Female Reference	Day Zero	Day One	Day Three	Day Five	Day Seven	Day Nine	Day Eleven
BLUE	D3S1358	**15**,17	16,17	15,17	15,17	15,17	15,17	15,17	15,17	15,17
	VWA	**17**,18	15,18	17,18	17,18	17,18	17,18	17,18	17,18	17,18
	D163539	**11**,12	9,12	11,12	12	11,12	11,12	11,12	11,12	11,12
	CSF1PO	**10**,11	11,14	10,11	10,11	10,11	10,11	10,11	10,11	10,11
	TPOX	8,11	8,11	8,11	8,11	8,11	8,11	8,11	8,11	8,11
GREEN	YINDEL	**2**	-	2	2	2	2	2	2	2
	Amelogenin	XY	X	X	XY	XY	XY	XY	XY	XY
	D831179	**10**,13	12,13	10,13	10,13	10,13	10,13	10,13	10,13	10,13
	D21S11	**31.2,32.2**	27,30	31.2,32.2	31.2,32.2	31.2,32.3	31.2,32.2	31.2,32.3	31.2,32.2	31.2,32.2
	D18S51	**11,15**	10,14	11,15	11,15	11,15	11,15	11,15	11,15	11,15
	DYS391	**12**	-	-	12	12	12	12	12	12
YELLOW	D2S441	11,**14**	11,12	11	11,14	11,14	11,14	11,14	11,14	11,14
	D19S433	**13**,14	14,15	13,14	13,14	13,14	13,14	13,14	13,14	13,14
	THO1	**6,7**	9,9.3	7	6,7	6,7	6,7	6,7,	6,7	6,7
	FGA	20,**24**	20	20	20,24	20,24	20,24	20,24	20,24	20
RED	D22S1045	15,**16**	11,15	15,16	15,16	15,16	15,16	15,16	15,16	15,16
	D53818	11,12	11,12	11,12	11,12	11,12	11,12	11,12	11,12	11,12
	D13S317	**10**,12	11,12	10,12	10,12	10,12	10,12	10,12	10,12	10,12
	D7S830	10,12	10,12	10,12	10,12	10,12	10,12	10,12	10,12	10,12
	SE33	**21,28.2**	17,26.2	21,28.2	21,28.2	21,28.2	21,28.2	21,28.2	21,28.2	21,28.2
PURPLE	D10S1248	14,15	14,15	14,15	14,15	14,15	14,15	14,15	14,15	14,15
	D1S1656	**15**	14,17	15	15	15	15	15	15	-
	D12S391	**15,16**	18,20	15,16	15,16	15,16	15,16	15,16	15,16	15,16
	D2S1338	17,**24**	17,19	17,24	17,24	17,24	17,24	17,24	17,24	17,24
Unique Male Allele Ratio		-	-	20/24	23/24	24/24	24/24	24/24	24/24	22/24

4.2.4.2 Mock Sexual Assault Kit Samples

Both sets of laboratory-prepared sexual assault samples, one set containing semen from a non-vasectomized donor and the second set containing semen from a vasectomized donor, were reserved for analysis during this portion of the study (**Table 4.9**). When screening for seminal fluid using the proteomic assay, at least 3 of 5 target seminal fluid protein markers were detected across all swabs. Immunological lateral flow assays produced comparable results among the two sample sets evaluated. A positive result was observed for all vaginal swabs on RSID™ Semen and ABAcard® p30, with oral swabs producing a positive result on ABAcard® p30 only (**Figure 4.6**). However, all rectal swab samples exhibited a negative result when evaluated with a lateral flow assay.

For genetic analysis, all samples were subjected to an organic differential extraction protocol. Samples containing semen from a non-vasectomized individual produced Total Human DNA quantitation values and were analyzed for routine STR typing; however, samples containing semen from a vasectomized individual were analyzed for Y-STR markers. As expected, no genetic

profiles were generated for samples containing semen from a vasectomized individual (**Table 4.9, Appendix J**). Nonetheless, mass spectrometry results show at least 3 seminal fluid biomarkers detected in each sample type of this group. Complete and partial DNA profiles were observed for samples containing semen from a non-vasectomized individual (**Table 4.10**). The variation in profile generated can be attributed to each sample having been prepared individually (*i.e.,* not prep replicates), in order to best simulate sample-to-sample variability. For example, case sample SA01_02.1, an oral swab, generated 9 out of 24 unique male alleles; however, the second oral swab produced a full STR profile.

Table 4.9. Summary of comparative results for mock sexual assault samples containing semen from a non-vasectomized and a vasectomized donor. Positive identifications are represented by a green box. Negative results are represented by a white box.

	Description	Mass Spec Protein ID	RSID™ Semen	ABAcard® p30	No. Unique Male Alleles
Non-vasectomized Male Donor	Vaginal Swab	5 out of 5	+	+	24 of 24
	Vaginal Swab	3 out of 5	+	+	4 of 24
	Oral Swab	4 out of 5		+	9 of 24
	Oral Swab	4 out of 5		+	24 of 24
	Rectal Swab	3 out of 5			22 of 24
	Rectal Swab	3 out of 5			24 of 24
Vasectomized Male Donor	Vaginal Swab	4 out of 5	+	+	
	Vaginal Swab	3 out of 5	+	+	
	Oral Swab	3 out of 5		+	
	Oral Swab	4 out of 5		+	
	Rectal Swab	3 out of 5			
	Rectal Swab	3 out of 5			

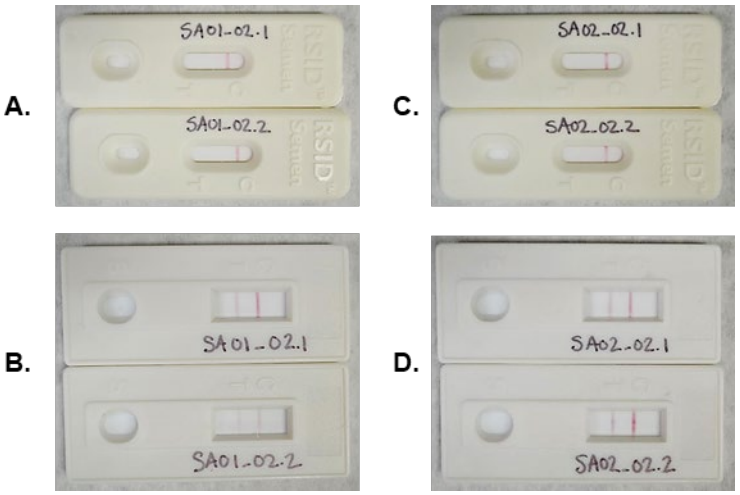


Figure 4.6. Immunochromatographic results for mock sexual assault samples. Within the membrane window, the test line is positioned on the left and the control line on the right. Results for (A) oral swab replicates using RSID™ Semen, (B) oral swab replicates using ABAcard® p30, (C) oral swab replicates containing semen from a vasectomized donor using RSID™ Semen, and (D) oral swab replicates containing semen from a vasectomized donor using ABAcard® p30. A positive result is indicated by the presence of a red line at both the test and control zones.

Table 4.10. STR genetic results for simulated sexual assault samples. The male reference sample depicts unique male alleles in bold.

		Male Reference	Female Reference	SA01_01.1	SA01_01.2	SA01_02.1	SA01_02.2	SA01_03.1	SA01_03.2
				Vaginal Swab	Vaginal Swab	Oral Swab	Oral Swab	Rectal Swab	Rectal Swab
BLUE	D3S1358	**15**,17	16,17	15,17	17	17	15,17	17	15,17
	VWA	**17**,18	15,18	17,18	-	-	17,18	17,18	17,18
	D163539	**11**,12	9,12	11,12	11	11	11,12	11,12	11,12
	CSF1PO	**10**,11	11,14	10,11	-	-	10,11	10,11	10,11
	TPOX	8,11	8,11	8,11	-	8	8	8,11	8,11
GREEN	YINDEL	**2**	-	2	-	2	2	2	2
	Amelogenin	XY	X	XY	X	XY	XY	XY	XY
	D831179	**10**,13	12,13	10,13	10,13	13	10,13	10,13	10,13
	D21S11	**31.2,32.2**	27,30	31.2,32.2	-	-	31.2,32.2	31.2,32.2	31.2,32.2
	D18S51	**11,15**	10,14	11,15	-	11	11,15	11,15	11,15
	DYS391	**12**	-	12	-	12	12	-	12
YELLOW	D2S441	11,**14**	11,12	11,14	11	11	11,14	11,14	11,14
	D19S433	**13**,14	14,15	13,14	-	13,14	13,14	13,14	13,14
	THO1	**6,7**	9,9.3	6,7	-	6	6,7	6,7	6,7
	FGA	20,**24**	20	20,24	-	20	20,24	20,24	20,24
RED	D22S1045	15,**16**	11,15	15,16	-	-	15,16	15,16	15,16
	D53818	11,12	11,12	11,12	11	12	11,12	11,12	11,12
	D13S317	**10**,12	11,12	10,12	-	-	10,12	10,12	10,12
	D7S830	10,12	10,12	10,12	-	12	10,12	10,12	10,12
	SE33	**21,28.2**	17,26.2	21,28.2	-	-	21,28.2	21,28.2	21,28.2
PURPLE	D10S1248	14,15	14,15	14,15	14,15	14	14,15	14,15	14,15
	D1S1656	**15**	14,17	15	15	15	15	15	15
	D12S391	**15,16**	18,20	15,16	15	16	15,16	15,16	15,16
	D2S1338	17,**24**	17,19	17,24	17	17,24	17,24	17,24	17,24
Unique Male Allele Ratio		-	-	24/24	4/24	9/24	24/24	22/24	24/24

4.2.4.3 Digital Swab Samples

Self-collected digital swab samples were obtained to mimic casework type samples as closely as possible. However, it should be noted that because the samples were self-collected, the likelihood of a mixed genetic profile was reduced as the body fluid and digit were of the same individual. Overall, serological tests produced comparable results with the proteomic assay and full genetic profiles were obtained from all samples (**Table 4.11**). Both oral cavity replicates produced a positive result from RSID™ Saliva and SALIgAE® tests (**Figure 4.7A**). When assessed for the presence of blood, menstrual blood was positively characterized by RSID™ Blood, with a negative result produced using ABAcard® HemaTrace for a single replicate (**Figure 4.7B**). A complete DNA profile consistent with the female contributor was produced by all digital swab samples (**Table 4.12**).

Although this subset of samples provided insight into the advantages of the proteomic assay over traditional techniques, especially for vaginal fluid samples, authentic digital swab samples would need to be evaluated for a veritable comparison. Nevertheless, the proteomic assay produced consistent identifications between replicates that were complimentary to genetic testing results.

Table 4.11. Summary of comparative results for digital swab samples. Positive identifications are represented by a green box. Negative results are represented by a red box. White boxes indicate no testing was performed.

Sample	DS01.1 Digital Swab Saliva	DS01.2 Digital Swab Saliva	DS02.1 Digital Swab Vaginal Fluid	DS02.2 Digital Swab Vaginal Fluid	DS03.1 Digital Swab Menstrual Blood	DS03.2 Digital Swab Menstrual Blood
Mass Spec Protein ID	4 out of 4	4 out of 4	8 out of 12	9 out of 12	11 out of 12	10 out of 12
DNA % Profile	100	100	100	100	100	100
RSID™ Saliva	+	+				
SALIgAE®	+	+				
ABAcard® HemaTrace					+	
RSID™ Blood					+	+

Figure 4.7. Selected serological screening test results for digital swab samples. ABAcard® test and SALIgAE® are pictured on the left. RSID™ test is pictured on the right. Within the membrane window, the test line is positioned on the left and the control line on the right. Results for (A) digital swab saliva and (B) digital swab menstrual blood. On lateral flow assays, a positive result is indicated by the presence of a red line at both the test and control zones. For SALIgAE®, a positive result is indicated by a yellow colored reagent.

Table 4.12. STR genetic results for digital swab samples.

		Female Reference	DS01.1	DS01.2	DS02.1	DS02.2	DS03.1	DS03.2
			Oral Penetration	Oral Penetration	Vaginal Penetration	Vaginal Penetration	Vaginal Penetration (Menstrual)	Vaginal Penetration (Menstrual)
BLUE	D3S1358	16,17	16,17	16,17	16,17	16,17	16,17	16,17
	VWA	15,18	15,18	15,18	15,18	15,18	15,18	15,18
	D163539	9,12	9,12	9,12	9,12	9,12	9,12	9,12
	CSF1PO	11,14	11,14	11,14	11,14	11,14	11,14	11,14
	TPOX	8,11	8,11	8,11	8,11	8,11	8,11	8,11
GREEN	YINDEL	-	-	-	-	-	-	-
	Amelogenin	X	X	X	X	X	X	X
	D831179	12,13	12,13	12,13	12,13	12,13	12,13	12,13
	D21S11	27,30	27,30	27,30	27,30	27,30	27,30	27,30
	D18S51	10,14	10,14	10,14	10,14	10,14	10,14	10,14
	DYS391	-	-	-	-	-	-	-
YELLOW	D2S441	11,12	11,12	11,12	11,12	11,12	11,12	11,12
	D19S433	14,15	14,15	14,15	14,15	14,15	14,15	14,15
	THO1	9,9.3	9,9.3	9,9.3	9,9.3	9,9.3	9,9.3	9,9.3
	FGA	20	20	20	20	20	20	20
RED	D22S1045	11,15	11,15	11,15	11,15	11,15	11,15	11,15
	D53818	11,12	11,12	11,12	11,12	11,12	11,12	11,12
	D13S317	11,12	11,12	11,12	11,12	11,12	11,12	11,12
	D7S830	10,12	10,12	10,12	10,12	10,12	10,12	10,12
	SE33	17,26.2	17,26.2	17,26.2	17,26.2	17,26.2	17,26.2	17,26.2
PURPLE	D10S1248	14,15	14,15	14,15	14,15	14,15	14,15	14,15
	D1S1656	14,17	14,17	14,17	14,17	14,17	14,17	14,17
	D12S391	18,20	18,20	18,20	18,20	18,20	18,20	18,20
	D2S1338	17,19	17,19	17,19	17,19	17,19	17,19	17,19

4.2.5 Degradation Samples

Laundered substrates, composed of a set of substrates containing semen and a set of clothing containing peripheral blood, were carried over to this section of research. As noted in section 3.1.6.5, all samples and control regions were prepared, laundered, dried, and analyzed. Overall, the expected results were observed for the proteomic assay and genetic analyses (**Table 4.13**). All samples produced a full genetic profile (**Table 4.14**). For the set of samples containing semen, the proteomic assay was capable of detecting protein signatures on both the bed sheet and underwear, with only a single protein biomarker (epididymal secretory protein) not detected on the bed sheet (**Table 4.13**). Conversely, immunological lateral flow assays failed to detect trace levels of seminal fluid (**Figure 4.8A** and **Figure 4.8B**). Both control regions exhibited negative results for both the proteomic assay and immunochromatographic assays. The second set of degraded samples, a cloth bath towel and a pair of denim jeans, targeted peripheral blood identification. Both samples produced comparable results. All protein biomarkers were detected with the proteomic assay and weak positive results were observed with lateral flow assays (**Table 4.13, Figure 4.8C, Figure 4.8D**). Interestingly, the extracted control samples from both the towel and the jeans produced a positive and weak positive result on RSID™ Blood, respectively. Using the LC-MS/MS method as a confirmation, these immunological assay results were not able to be verified, as this approach produced no identification of peripheral blood biomarkers. Furthermore, no genetic profile was produced from these control samples, even with manual analysis of electropherograms identifying no true alleles below the established analytical threshold (**Appendix J**).

Table 4.13. Summary of comparative results for degraded samples. Positive identifications are represented by a green box. Negative results are represented by a white box.

	Description	Mass Spec Protein ID	RSID™	ABAcard®	Male DNA % Profile
Semen	Bed Sheet	4 out of 5			100
	Bed Sheet Control				
	Underwear	5 out of 5	+	+	100
	Underwear Control				
Peripheral Blood	Towel	5 out of 5	+ weak	+ weak	100
	Towel Control		+		
	Jeans	5 out of 5	+ weak	+ weak	100
	Jeans Control		+ weak		

Table 4.14. STR genetic results for degraded samples.

		Semen on Bed Sheet	Semen on Underwear	Peripheral Blood on Towel	Peripheral Blood on Jeans
BLUE	D3S1358	15,17	15,17	15,17	15,17
	VWA	17,18	17,18	16,17	16,17
	D163539	11,12	11,12	11,13	11,13
	CSF1PO	10,11	10,11	9,11	9,11
	TPOX	8,11	8,11	8,9	8,9
GREEN	YINDEL	2	2	2	2
	Amelogenin	XY	XY	XY	XY
	D831179	10,13	10,13	13	13
	D21S11	31.2,32.2	31.2,32.2	28,30	28,30
	D18S51	11,15	11,15	17	17
	DYS391	12	12	11	11
YELLOW	D2S441	11,14	11,14	11,14	11,14
	D19S433	13,14	13,14	14,15.2	14,15.2
	THO1	6,7	6,7	9	9
	FGA	20,24	20,24	18,22	18,22
RED	D22S1045	15,16	15,16	11,17	11,17
	D53818	11,12	11,12	12,13	12,13
	D13S317	10,12	10,12	11,12	11,12
	D73280	10,12	10,12	11	11
	SE33	21,28.2	21,28.2	18,30.2	18,30.2
PURPLE	D10S1248	14,15	14,15	12,14	12,14
	D1S1656	15	15	15,17	15,17
	D12S391	15,16	15,16	20,22	20,22
	D2S1338	17,24	17,24	19,20	19,20

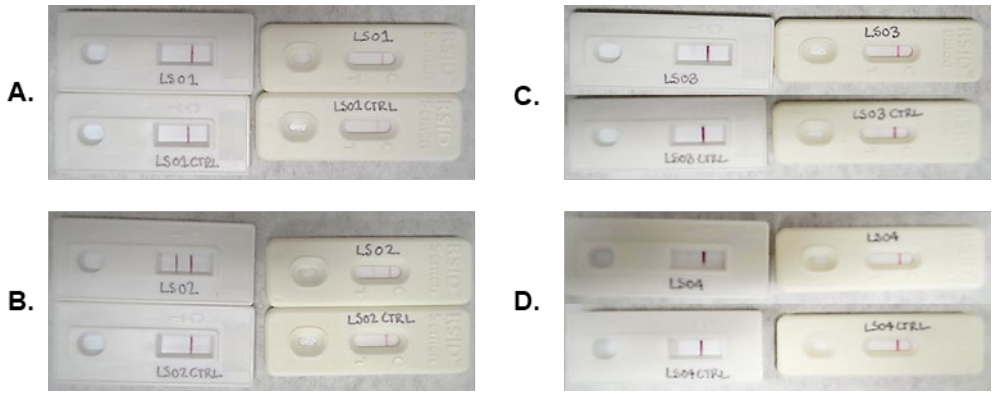


Figure 4.8. Immunochromatographic results for degraded samples. ABAcard® tests are pictured on the left. RSID™ tests are pictured on the right. Question samples are oriented on the top of each frame. Control samples are oriented on the bottom of each frame. Within the membrane window, the test line is positioned on the left and the control line on the right. Results for (A) semen extracted from a bed sheet, (B) semen extracted from underwear, (C) blood extracted from a towel, and (D) blood extracted from denim jeans. A positive result is indicated by the presence of a red line at both the test and control zones.

4.2.6 Aged Samples

Swabs fortified with neat body fluid were kept at room temperature for time points zero, three, five, seven, and thirty-five days. Comparative results are outlined in **Table 4.15** for this portion of the study. No instances of negative detection were recorded for evaluation using traditional screening techniques or the proteomic assay (**Figure 4.9**); however, weak and very weak positives were observed for ABAcard® HemaTrace® for peripheral and menstrual blood samples. No change in detection was observed over the 35-day period. DNA analysis was not performed on this set of casework samples.

The traditional serological tests and the proteomic assay produced comparable results. However, the weak and very weak positive results exhibited for peripheral blood, menstrual blood, and saliva detection with traditional assays pose a cause for concern. With complete and almost complete identification of protein material for each target fluid, the LC-MS/MS assay produced greater confirmation in fluid identification based on the specificity of the peptide biomarkers detected.

Table 4.15. Summary of comparative results for aged samples. Positive identifications are represented in green.

Sample		Day Zero	Day One	Day Three	Day Seven	Day Thirty-Five
Peripheral Blood	Mass Spec Protein ID	5 out of 5	5 out of 5	5 out of 5	5 out of 5	5 out of 5
	RSID™ Blood	+	+	+	+	+
	ABAcard® HemaTrace	+ weak	+ very weak	+ weak	+ very weak	+ very weak
Menstrual Blood	Mass Spec Protein ID	9 out of 12	9 out of 12	9 out of 12	9 out of 12	9 out of 12
	RSID™ Blood	+	+	+	+	+
	ABAcard® HemaTrace	+ weak	+ weak	+ weak	+ weak	+ weak
Seminal Fluid	Mass Spec Protein ID	5 out of 5	5 out of 5	5 out of 5	5 out of 5	5 out of 5
	RSID™ Semen	+	+	+	+	+
	ABAcard® p30	+	+	+	+	+
Saliva	Mass Spec Protein ID	4 out of 4	4 out of 4	4 out of 4	4 out of 4	4 out of 4
	RSID™ Saliva	+ weak	+ weak	+ weak	+ weak	+ weak
	SALIgAE®	+	+	+	+	+
Vaginal Fluid	Mass Spec Protein ID	7 out of 7	7 out of 7	7 out of 7	7 out of 7	7 out of 7

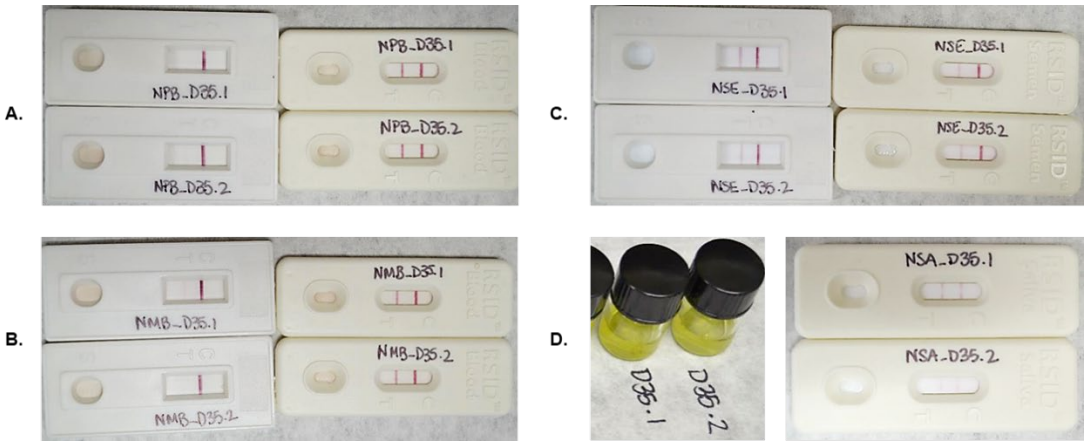


Figure 4.9. Serological screening test results for aged samples at Day 35. ABAcard® test and SALIgAE® are pictured on the left. RSID™ test is pictured on the right. Within the membrane window, the test line is positioned on the left and the control line on the right. Results for (A) peripheral blood, (B) menstrual blood, (C) semen, and (D) saliva. On lateral flow assays, a positive result is indicated by the presence of a red line at both the test and control zones. For SALIgAE®, a positive result is indicated by a yellow colored reagent.

4.2.7 Sensitivity Samples

This particular cohort of sensitivity samples were designed differently than those used for analytical sensitivity determination of the LC-MS/MS assay. Diluted body fluid was spotted on cotton swabs prior to analysis, resulting in further dilution of target material during sample solubilization, representing an inherent limitation to the preparation workflow. A summary of proteomic, serological, and genetic testing results for each target fluid are outlined in **Tables 4.16-4.20**. The lowest diluted sample to still produce a positive result when screened with serological tests are picture in **Figure 4.10**. Reviewing proteomic results, peripheral blood was characterized at the 20,000-fold dilution with the detection of a single hemoglobin peptide (**Table 4.16**). With processing a matched sample on commercially available lateral flow assays, the RSID™ Blood and ABAcard® HemaTrace tests produced a positive result up to and including the 100-fold dilution. Conversely, STR analysis results were more consistent with the rate of peptide identification using the proteomic assay (**Table 4.17**). A complete STR profile was recovered through the 100-fold dilution, mirroring the complete characterization of peripheral blood peptide markers to the same dilution. The capability of STR profiling drastically decreased at a 5,000-fold and 10,000-fold

dilutions, with samples producing a 7% and 5% STR profile, respectively. Remaining samples did not generate an STR profile; however, as previously stated, hemoglobin was still detected at the 20,000-fold dilution with the proteomic assay. Overall, the proteomic assay was demonstrated to be 200 times more sensitivity than immunological assays for the detection of peripheral blood. In addition, the proteomic assay exceeded the sensitivity of routine STR typing chemistries.

Menstrual blood was characterized down to the 40,000-fold dilution sample on the proteomic method, with the detection of a single hemoglobin peptide, similar to peripheral blood described above (**Table 4.18**). But only 4 of 7 vaginal fluid markers were accurately characterized with the proteomic assay. Cornulin, a more specific vaginal fluid marker, was detected at the 100-fold dilution, with small proline rich protein 3 sporadically identified for the remainder of the dilution series (**Table 3.27**). Interestingly, two instances of high dose hook effect were observed with immunochromatographic testing, with the neat and 2-fold dilution samples exhibiting a negative result on the antibody-based assays (**Table 4.18**). In regard to identifying the blood component of menstrual blood, the proteomic assay outperformed the antibody-based tests by a factor of 400. Furthermore, with the absence of a vaginal fluid screening test, the proteomic assay inherently provided additional pertinent information down to the 100-fold dilution. A complete STR profile was obtained through the 100-fold dilution, with informative partial profiles produced at the 1,000- and 2,000-fold dilutions (**Table 4.22**). Sufficient allele and locus dropout were recorded for the remaining samples.

Seminal fluid was accurately identified at the lowest dilution on the proteomic assay, with detection of semenogelin peptide QITIPSQEQEHSQK (**Table 4.20**). However, in comparison, the lateral flow assays exhibited poor sensitivity. RSID™ Semen failed to produce a positive result after the 100-fold dilution (**Figure 4.10C**). Furthermore, ABAcard® p30 demonstrated decreased sensitivity limits, reproducibly exhibiting a positive result only to the 10-fold dilution evaluated. In comparison, the proteomic assay demonstrated comparable results with STR and Y-STR genetic typing (**Tables 4.23 and 4.24**). With all protein biomarkers positively identified through the 100-fold dilution and a complete STR profile generated, seminal fluid identification using the validated assay

strongly mirrors the abilities of routine genetic testing. As the number of peptides detected inherently decreases, so did the number of alleles characterized. In regard to genetic testing, samples beginning with the 10,000-fold dilution failed to produce a male quantitation value and were therefore amplified for Y-STR typing (**Table 4.24**). Few Y-STR alleles were characterized at the lower end of the dilution series, with the 20,000-fold and 40,000-fold samples producing only a 7% and a 4% Y-STR profile, respectively. Overall, the proteomic assay demonstrated a sensitivity level 400 times more sensitivity than immunological tests but exhibited a similar sensitivity range of that of STR and Y-STR chemistries.

Diluted saliva was characterized reproducibly at the 1,000-fold dilution, with the detection of a single amylase peptide (**Table 4.19**). One antibody-based test and one enzymatic test were acquired for traditional serological testing for this portion of the study. Both RSID™ Saliva and SALIgAE® tests performed equally, with positive results observed up to and including the 10-fold dilution samples (**Figure 4.10D**). As previously described, the proteomic assay kept pace with the ability to generate an STR profile; however, the capabilities of genetic typing and reaching a larger sensitivity range outweigh those of the proteomic assay (**Table 4.25**). Identification of peptide markers began to decrease at the 100-fold dilution, where a full STR profile was still generated. Furthermore, out to the 2,000-fold dilution, a 70% complete STR profile was reported whereas the proteomic assay failed to detect saliva for the remainder of samples evaluated. To conclude, the proteomic assay demonstrated a sensitivity range 100 times more robust than that of the traditional screening assays. Conversely, routine genetic testing remained more sensitive than the validated assay.

Vaginal fluid was characterized by all target protein markers at the 10-fold dilution, with 2 of 7 markers detected at the 100-fold dilution and only a single protein (small proline rich protein 3) detected for the remaining samples (**Table 4.20**). In comparison, a full STR profile was produced down to the 500-fold dilution, with partial profiles observed for the remaining samples (**Table 4.26**). For the 100-fold dilution sample, no quantitation value was recorded and no DNA profile was produced. This result is inconsistent with the remaining data, and is therefore, being viewed as an

outlier. However, no matched comparison could be generated given the lack of a commercially available assay for vaginal fluid screening.

Overall, the validated proteomic assay greatly outperformed existing serological screening tests. It should be noted that samples were not solubilized in kit-specific buffer provided by the commercial entities for immunochromatographic testing. Although solubilization in buffer is recommended by the manufacturers, concentrated samples did not seem to inhibit the functionality of the assays (with the exception of the high dose hook effect for menstrual blood). Research has been conducted on the effect of pH and the ability to produce false positive and false negative results on the lateral flow assays. It was determined that extreme acidic or alkaline pH is not the sole contributor to false positive or false negative results. One study found that it is the presence of additional organic acids that causes disruption in the antibody-dye labeling mechanism [55].

Table 4.16. Summary of comparative results for peripheral blood sensitivity samples. Positive identifications are represented by a green box. Negative results are represented by a white box.

	Dilution Factor									
	Neat	**2**	**10**	**100**	**1,000**	**2,000**	**5,000**	**10,000**	**20,000**	**40,000**
Mass Spec Protein ID	5 out of 5	5 out of 5	5 out of 5	5 out of 5	4 out of 5	3 out of 5	3 out of 5	2 out of 5	1 out of 5	
Mass Spec % Peptide ID	100	100	100	100	89	56	44	33	22	
DNA % Profile	100	100	100	100	90.2	46.3	7.3	4.9		
RSID™ Blood	+	+	+	weak positive						
ABAcard® HemaTrace	+	+	+	weak positive						

Table 4.17. Summary of comparative results for menstrual blood sensitivity samples. Positive identifications are represented by a green box. Negative results are represented by a white box.

	Dilution Factor									
	Neat	**2**	**10**	**100**	**1,000**	**2,000**	**5,000**	**10,000**	**20,000**	**40,000**
Mass Spec Protein ID	9 out of 12	9 out of 12	9 out of 12	9 out of 12	7 out of 12	3 out of 12	2 out of 12	2 out of 12	2 out of 12	2 out of 12
Mass Spec % Peptide ID	58	58	58	58	38	15	12	15	15	12
DNA % Profile	100	100	100	100	75.6	82.9	17.1	36.6	9.7	4.9
RSID™ Blood			+							
ABAcard® HemaTrace	+	+	+	weak positive						

Table 4.18. Summary of comparative results for seminal fluid sensitivity samples. Positive identifications are represented by a green box. Negative results are represented by a white box.

	Dilution Factor									
	Neat	2	10	100	1,000	2,000	5,000	10,000	20,000	40,000
Mass Spec Protein ID	5 out of 5	5 out of 5	5 out of 5	5 out of 5	3 out of 5	2 out of 5	2 out of 5	2 out of 5	2 out of 5	1 out of 5
Mass Spec % Peptide ID	100	100	100	83	50	42	17	17	17	17
DNA % Profile	100	100	100	100	83.7	76.7	27.9	Y-STR 25.9	Y-STR 7.4	Y-STR 3.7
RSID™ Semen	+	+	+	+						
ABAcard® p30	+	+	+							

Table 4.19. Summary of comparative results for saliva sensitivity samples. Positive identifications are represented by a green box. Negative results are represented by a white box.

	Dilution Factor								
	Neat	2	10	100	500	1,000	2,000	5,000	10,000
Mass Spec Protein ID	4 out of 4	4 out of 4	4 out of 4	3 out of 4	1 out of 4	3 out of 4			
Mass Spec % Peptide ID	100	100	100	57	14	57			
DNA % Profile	100	100	100	100	95.3	86	51.1		13.9
RSID™ Saliva	+	+	+						
SALIgAE®	+	+	+						

Table 4.20. Summary of comparative results for vaginal fluid sensitivity samples. Positive identifications are represented by a green box. Negative results are represented by a white box.

	Dilution Factor						
	10	100	500	1,000	2,000	5,000	10,000
Mass Spec Protein ID	7 out of 7	2 out of 7	1 out of 7	1 out of 7	1 out of 7	1 out of 7	1 out of 7
Mass Spec % Peptide ID	71	12	6	6	6	6	6
DNA % Profile	100		100	87.8	90.2	29.2	7.3

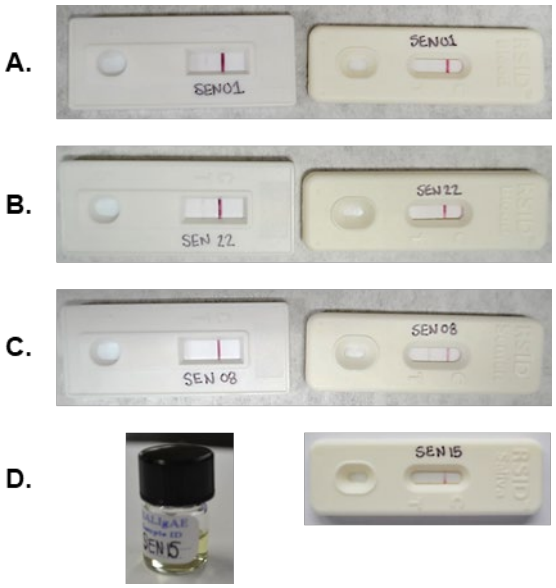


Figure 4.10. Serological screening test results for sensitivity samples. ABAcard® test and SALIgAE® are pictured on the left. RSID™ test is pictured on the right. Within the membrane window, the test line is positioned on the left and the control line on the right. Results for (A) 100-fold dilution of peripheral blood, (B) 100-fold dilution of menstrual blood, (C) 100-fold dilution of semen, and (D) 10-fold dilution of saliva. On lateral flow assays, a positive result is indicated by the presence of a red line at both the test and control zones. For SALIgAE®, a positive result is indicated by a yellow colored reagent.

Table 4.21. STR genetic results for peripheral blood sensitivity samples. Complete locus identification represented by a green box. Partial locus identification represented by a grey box. Locus dropout represented by a white box.

Peripheral Blood		Neat	2	10	100	1,000	2,000	5,000	10,000	20,000	40,000
BLUE	D3S1358	15,17	15,17	15,17	15,17	17					
	VWA	18,21	18,21	18,21	18,21	21					
	D163539	9,11	9,11	9,11	9,11	9,11					
	CSF1PO	12,13	12,13	12,13	12,13	12,13	13				
	TPOX	8,9	8,9	8,9	8,9	8,9	9				
GREEN	YINDEL	2	2	2	2	2					
	Amelogenin	XY	XY	XY	XY	XY	X				
	D831179	14,15	14,15	14,15	14,15	14,15	15				
	D21S11	28	28	28	28	28	28	28	28		
	D18S51	16,17	16,17	16,17	16,17	16,17	16				
	DYS391	11	11	11	11	11	11				
YELLOW	D2S441	11,12	11,12	11,12	11,12	11,12	11,12				
	D19S433	11,12	11,12	11,12	11,12	11	12		11		
	THO1	7	7	7	7	7	7				
	FGA	25,27	25,27	25,27	25,27	25,27	25				
RED	D22S1045	11,15	11,15	11,15	11,15	11,15	11				
	D53818	12	12	12	12	12		12			
	D13S317	12,13	12,13	12,13	12,13	12	12,13	13			
	D73280	10,12	10,12	10,12	10,12	10,12	10				
	SE33	21,26.2	21,26.2	21,26.2	21,26.2	21,26.2					
PURPLE	D10S1248	12,13	12,13	12,13	12,13	12,13	13				
	D1S1656	12,16.3	12,16.3	12,16.3	12,16.3	12,16.3					
	D12S391	17,20	17,20	17,20	17,20	17,20	20				
	D2S1338	23,26	23,26	23,26	23,26	23,26	23,26				
Profile Percentage		100	100	100	100	90.2	46.3	7.3	4.9	0	0

Table 4.22. STR genetic results for menstrual blood sensitivity samples. Complete locus identification represented by a green box. Partial locus identification represented by a grey box. Locus dropout represented by a white box. Menstrual blood for this subset of samples was provided by two donors (Reference #1 and Reference #2).

Menstrual Blood		Reference #1	Neat	2	10	Reference #2	100	1,000	2,000	5,000	10,000	20,000	40,000
BLUE	D3S1358	16, 18	16,18	16,18	16,18	16,17	16,17	16,17	17				
	VWA	16	16	16	16	15,18	15,18	15,18	15		18		
	D163539	13, 14	13,14	13,14	13,14	9,12	9,12	9,12			9		
	CSF1PO	12, 13	12,13	12,13	12,13	11,14	11,14		11,14				
	TPOX	9, 12	9,12	9,12	9,12	8,11	8,11	8,11	8,11		11		8
GREEN	YINDEL	-	-	-	-	-	-	-	-	-	-	-	-
	Amelogenin	X	X	X	X	X	X	X	X	X		X	
	D831179	13, 14	13,14	13,14	13,14	12,13	12,13	12,13	12,13	12,13	12,13		
	D21S11	27, 30	27,30	27,30	27,30	27,30	27,30	27,30	27,30	27,30			30
	D18S51	12, 13	12,13	12,13	12,13	10,14	10,14	10,14	10,14				
	DYS391	-	-	-	-	-	-	-	-	-	-	-	-
YELLOW	D2S441	10, 11	10,11	10,11	10,11	11,12	11,12	11,12	11,12		11,12	11	
	D19S433	13	13	13	13	14,15	14,15	14,15	14,15		15		
	THO1	6, 9.3	6,9.3	6,9.3	6,9.3	9,9.3	9,9.3	9,9.3	9,9.3	9.3	9.3		
	FGA	20, 24	20,24	20,24	20,24	20	20	20	20		20	20	
RED	D22S1045	15, 16	15,16	15,16	15,16	11,15	11,15	11,15					
	D53818	12, 13	12,13	12,13	12,13	11,12	11,12	11,12	11,12				
	D13S317	10, 11	10,11	10,11	10,11	11,12	11,12		11,12		11,12	12	
	D73280	10, 11	10,11	10,11	10,11	10,12	10,12		10,12		10		
	SE33	29.2	29.2	29.2	29.2	17,26.2	17,26.2		17,26.2	17	17		
PURPLE	D10S1248	13, 15	13,15	13,15	13,15	14,15	14,15		14				
	D1S1656	15, 16.3	15,16.3	15,16.3	15,16.3	14,17	14,17	14,17	14,17				
	D12S391	15, 22	15,22	15,22	15,22	18,20	18,20	18,20	18,20				
	D2S1338	19, 24	19,24	19,24	19,24	17,19	17,19	17,19	17,19	19	17	17	
Profile Percentage		-	100	100	100	-	100	75.6	82.9	17.1	36.6	9.7	4.9

Table 4.23. STR genetic results for seminal fluid sensitivity samples. Complete locus identification represented by a green box. Partial locus identification represented by a grey box. Locus dropout represented by a white box.

Semen		Reference	Neat	2	10	100	1,000	2,000	5,000
BLUE	D3S1358	15,17	15,17	15,17	15,17	15,17	15,17	15	15
	VWA	17,18	17,18	17,18	17,18	17,18	17,18	17,18	
	D163539	11,12	11,12	11,12	11,12	11,12	11,12	12	
	CSF1PO	10,11	10,11	10,11	10,11	10,11	10,11	10,11	
	TPOX	8,11	8,11	8,11	8,11	8,11	8,11	11	
GREEN	YINDEL	2	2	2	2	2		2	2
	Amelogenin	XY	XY	XY	XY	XY	X	Y	
	D831179	10,13	10,13	10,13	10,13	10,13	10	10,13	10
	D21S11	31.2,32.2	31.2,32.2	31.2,32.2	31.2,32.2	31.2,32.2	31.2,32.2	32.2	31.2
	D18S51	11,15	11,15	11,15	11,15	11,15	11,15	11,15	15
	DYS391	12	12	12	12	12	12	12	
YELLOW	D2S441	11,14	11,14	11,14	11,14	11,14	11	11	11,14
	D19S433	13,14	13,14	13,14	13,14	13,14	13	13	
	THO1	6,7	6,7	6,7	6,7	6,7	7	6,7	
	FGA	20,24	20,24	20,24	20,24	20,24	20,24	20,24	24
RED	D22S1045	15,16	15,16	15,16	15,16	15,16	15,16	15,16	
	D53818	11,12	11,12	11,12	11,12	11,12	11,12	12	
	D13S317	10,12	10,12	10,12	10,12	10,12	10,12	10,12	12
	D73280	10,12	10,12	10,12	10,12	10,12	10,12	10,12	12
	SE33	21,28.2	21,28.2	21,28.2	21,28.2	21,28.2	21	21,28.2	
PURPLE	D10S1248	14,15	14,15	14,15	14,15	14,15	14,15	15	
	D1S1656	15	15	15	15	15	15	15	15
	D12S391	15,16	15,16	15,16	15,16	15,16	15,16	15,16	
	D2S1338	17,24	17,24	17,24	17,24	17,24	17		17
Profile Percentage		-	100	100	100	100	83.7	76.7	27.9

Table 4.24. Y-STR genetic results for seminal fluid sensitivity samples. Complete locus identification represented by a green box. Partial locus identification represented by a grey box. Locus dropout represented by a white box.

Semen		Reference	10,000	20,000	40,000
BLUE	DYS576	17			
	DYS389I	14		14	
	DYS635	20			
	DYS389II	30			
	DYS627	23			
GREEN	DYS460	10	10		
	DYS458	17			
	DYS19	14	14		
	YGAT	13	13		
	DYS448	19	19		
	DYS391	12			
YELLOW	DYS456	17			
	DYS390	23		23	
	DYS438	12			
	DYS392	13	13		
	DYS518	40			
RED	DYS570	17			
	DYS437	15	15		
	DYS395	11,14			
	DYS449	28			
PURPLE	DYS393	13			
	DYS439	13			
	DYS481	22			
	DYF387S1	35,36	35		35
	DYS533	12			
Profile Percentage		-	25.9%	7.4%	3.7%

Table 4.25. STR genetic results for saliva sensitivity samples. Complete locus identification represented by a green box. Partial locus identification represented by a grey box. Locus dropout represented by a white box.

	Saliva	Reference	Neat	2	10	100	500	1,000	2,000	5,000	10,000
BLUE	D3S1358	15,17	15,17	15,17	15,17	15,17	15,17	15	15		
	VWA	17,18	17,18	17,18	17,18	17,18	17,18	17,18			
	D163539	11,12	11,12	11,12	11,12	11,12	11,12	12			
	CSF1PO	10,11	10,11	10,11	10,11	10,11	10,11	10,11			
	TPOX	8,11	8,11	8,11	8,11	8,11	8,11		8		8
GREEN	YINDEL	2	2	2	2	2	2	2			
	Amelogenin	XY	XY	XY	XY	XY	XY	X	X		
	D831179	10,13	10,13	10,13	10,13	10,13	10,13	10,13	13		
	D21S11	31.2,32.2	31.2,32.2	31.2,32.2	31.2,32.2	31.2,32.2	31.2,32.2	31.2,32.2	31.2,32.2		
	D18S51	11,15	11,15	11,15	11,15	11,15	11,15	11,15	11		
	DYS391	12	12	12	12	12	12	12	12		
YELLOW	D2S441	11,14	11,14	11,14	11,14	11,14	11,14	11,14	11,14		11
	D19S433	13,14	13,14	13,14	13,14	13,14	13,14	13,14	13,14		
	THO1	6,7	6,7	6,7	6,7	6,7	6,7	6	6,7		
	FGA	20,24	20,24	20,24	20,24	20,24	20,24	20,24	20,24		
RED	D22S1045	15,16	15,16	15,16	15,16	15,16	15,16	15,16			
	D53818	11,12	11,12	11,12	11,12	11,12	11,12	11,12	11,12		11
	D13S317	10,12	10,12	10,12	10,12	10,12	10,12	10,12	12		10
	D73280	10,12	10,12	10,12	10,12	10,12	10	10,12			10
	SE33	21,28.2	21,28.2	21,28.2	21,28.2	21,28.2	21,28.2	21,28.2	28.2		
PURPLE	D10S1248	14,15	14,15	14,15	14,15	14,15	14	14			15
	D1S1656	15	15	15	15	15	15	15	15		
	D12S391	15,16	15,16	15,16	15,16	15,16	15,16	15,16	16		
	D2S1338	17,24	17,24	17,24	17,24	17,24	17,24	17,24	17		
Profile Percentage		-	100	100	100	100	95.3	86	51.1	0	13.9

Table 4.26. STR genetic results for vaginal fluid sensitivity samples. Complete locus identification represented by a green box. Partial locus identification represented by a grey box. Locus dropout represented by a white box.

Vaginal Fluid		Reference	10	100	500	1,000	2,000	5,000	10,000
BLUE	D3S1358	16,17	16,17		16,17	16,17	16,17	17	
	VWA	15,18	15,18		15,18	15,18	15,18	15	
	D163539	9,12	9,12		9,12	9,12	9,12		
	CSF1PO	11,14	11,14		11,14	11,14	11		
	TPOX	8,11	8,11		8,11	8,11	8,11	11	
GREEN	YINDEL	-	-	-	-	-	-	-	-
	Amelogenin	X	X		X	X	X	X	
	D831179	12,13	12,13		12,13	12,13	12,13	13	13
	D21S11	27,30	27,30		27,30	27,30		30	
	D18S51	10,14	10,14		10,14	10,14	10,14		
	DYS391	-	-	-	-	-	-	-	-
YELLOW	D2S441	11,12	11,12		11,12	11,12	11,12	11,12	
	D19S433	14,15	14,15		14,15	14,15	14,15	14,15	
	THO1	9,9.3	9,9.3		9,9.3	9,9.3	9	9.3	9
	FGA	20	20		20	20	20	20	20
RED	D22S1045	11,15	11,15		11,15	11	11,15	11	
	D53818	11,12	11,12		11,12	11,12	11,12		
	D13S317	11,12	11,12		11,12	12	11,12		
	D73280	10,12	10,12		10,12	10	12		
	SE33	17,26.2	17,26.2		17,26.2	17	17,26.2		
PURPLE	D10S1248	14,15	14,15		14,15	14,15	14,15		
	D1S1656	14,17	14,17		14,17	17	14,17		
	D12S391	18,20	18,20		18,20	18,20	18,20		
	D2S1338	17,19	17,19		17,19	17,19	19		
Profile Percentage		-	100	0	100	87.8	90.2	29.2	7.3

4.3 Concluding Remarks

In conclusion, the data demonstrated that the validated proteomic assay exhibits sufficient gains in sensitivity over currently utilized antibody- and enzyme-based screening techniques. Although various types of samples were deployed for this portion of the assessment, perhaps the greatest discrepancies between the traditional and proteomic analyses were observed with the sensitivity samples. The proteomic assay greatly outperformed traditional tests and provided detailed information on the proteins/peptides identified. This information can present other avenues for interpretation and subsequent testing to a forensic analyst. Still, one particular area of advancement worth highlighting is with the analysis of seminal fluid from a vasectomized individual.

When considering the scenario of a suspected rape, the ability to accurately characterize semenogelin off a vaginal swab that will test negative for sperm cells has the potential to provide critical information to an investigation. This data further supports the necessity for confirmatory seminal fluid identification, given that not all male perpetrators may contain sperm cells that could be visualized using routine microscopic techniques.

Furthermore, it was established that the proteomic assay can be seamlessly integrated into the larger forensic biology workflow, having no discernible effect on the ability to produce a genetic profile. With the idea of reverting back to a traditional workflow in place of direct to DNA, it can be argued that protein characterization may correlate to the likelihood of producing an informative genetic profile, particularly with sexual assault samples. For more than two decades, the forensic community has focused on improving the sensitivity and robustness of DNA profiling. While advances have made it possible to individualize biological traces on challenging types of evidentiary material, DNA alone does not readily indicate the body fluid source from which it was extracted. Serological testing to identify the body fluid from which a DNA profile has been generated, however, can provide vital contextual information to facilitate a successful prosecution. With sensitivity and specificity gains established by the developed proteomic method, the combination of proteomic screening with advanced genetic profiling presents a model workflow for adoption in forensic biology laboratories.

The remaining two chapters of this dissertation will focus on the proteomic analysis of sexual assault samples. As seen previous and revisited within this chapter, the presence of personal lubricants can have deleterious effects on the ability to positively identify vaginal fluid. The impact of this particular contaminant will be further explored in the next chapter, in addition to a greater assessment on its impact for the identification of seminal fluid and saliva biomarkers. In addition, renewed insight into intact protein identification for expedited serological analysis will be discussed in the final chapter.

CHAPTER 5: ASSESSMENT OF THE POTENTIAL INHIBITORY EFFECTS OF PERSONAL LUBRICANTS ON BODY FLUID IDENTIFICATION

5 Introduction

The Federal Bureau of Investigation's Uniform Crime Reporting Program solicits information from law enforcement agencies to provide reliable and uniform crime statistics for the United States [163]. Currently, more than 18,000 agencies across the United States voluntarily submit data to this program on an annual basis. For the year 2019, the most current report available, there were 64,048 reported rapes and a rate of offense estimated at 42 offenses per 100,000 people. Of the 61,531 offenses where the sex of the offender was reported, 95.6% of offenses had a male offender (equivalent to 58,853 reported offenses). Within a single year, a 6.4% increase was observed in reported rape offenses, with 59,945 offenses reported in 2018. Over a six-year period, an increase of 37.92% of rape offenses is reported. Although the number of submitting agencies has increased by 15% since the year 2013, the prevalence of rape and sexual assault has continued to grow in the United States. This data was compiled using the 2013 revised definition of rape, with a single report referring to an instance of rape, attempted rape, or assault with intent to rape [163]. In the United States, approximately 1 in 6 women will be a victim of attempted or completed acts of sexual assault, with females under age 24 three times more likely to be a victim of sexual violence than the general population [164,165].

For more than two decades, the forensic community has focused on improving the sensitivity and robustness of DNA profiling. While advances have made it possible to individualize biological traces on challenging types of evidentiary material, DNA alone does not readily indicate the body fluid source from which it was extracted. Serological testing to identify the body fluid from which a DNA profile has been generated, however, can provide vital contextual information to

facilitate a successful prosecution. In the event of a sexual assault or rape, a Suspect Sexual Assault Evidence Collection Kit is taken by a trained nurse and submitted for forensic testing. Kits generally include vaginal, oral, and rectal swabs, in addition to articles of clothing and supplemental swabs submitted on a case-by-case basis (*e.g.,* breast, labia, inner thigh swabs). In addition, objects such as condoms or items used for vaginal penetration can be submitted for testing. Given the persistent sexual assault kit backlogs and that the sensitivity of post-coital DNA testing now exceeds that of contemporary serological tests, many labs have adopted a "direct to DNA" workflow. While this approach has a number of advantages, serological testing is especially important when the item of evidence in question (*e.g.,* a towel or bedding) does not readily lend itself to an interpretation of likely sexual contact to the same degree as an intimate swab or underwear, where the mere presence of suspect's DNA may be sufficient for criminal proceedings.

In the previous chapters, it was demonstrated that protein mass spectrometry techniques exhibit increased sensitivity and specificity to conduct accurate screening of evidentiary items for the presence of biological fluids. This approach has proven the LC-MS/MS analytical method to be robust and reliable in the detection of protein signatures, particularly for sexual assault type samples. With the developed workflow, it was demonstrated that protein components were able to be identified with a level of sensitivity comparable to that of STR/Y-STR profiling. The Uniform Crime Report states that in the year 2019, 17% of rape offenses were also linked with instances of sodomy and 6.5% with instances of sexual assault with a foreign object [163]. In the context of sexual assault sample screening, the identification of seminal fluid in addition to saliva and vaginal fluid play a significant role in sample processing and generation of critical sample information. Of the laboratory-generated case samples utilized during validation and implementation assessments, a particular set of samples, those treated with personal lubricant as part of the environmental contaminant subset, were identified for additional analyses. In particular, peak area responses of vaginal fluid biomarkers were inhibited or complete protein dropout was observed with samples subjected to a personal lubricant.

The objective of this phase of research was to assess the potential inhibitory effects of personal lubricants on the sensitivity of mass spectrometry-based body fluid identification. A two-prong approach was designed to elucidate potential deleterious effects. First, target biological fluids were subjected to multiple lubricant types at varying volumes to illustrate the changes in protein biomarker detection. Second, body fluids recovered from lubricated condoms were evaluated. In combination, the results of this research provide an inclusive assessment of sample contaminants and alternate methodologies for processing challenging sample types received as forensic evidence. This research was completed under three scientific aims using the validated LC-MS/MS assay previously described. The first aim evaluated the effects of personal lubricant on the detection of vaginal fluid protein biomarkers. Similarly, the second aim assessed the effects of personal lubricant on seminal fluid and saliva identification, both individually and as mixed source samples. And lastly, the final aim evaluated condoms containing lubricant and spermicide elements.

5.1 Methods and Materials

5.1.1 Sample Collection and Materials

All research conducted under this phase of the project was reviewed and approved by the University of Denver IRB for research involving human subjects. Sample collection and research was conducted in full accordance with the U.S. federal policy for the protection of human subjects as described in section 2.11. In total, 10 subjects were recruited from the graduate population at Arcadia University (Glenside, PA) and staff members employed at The Center for Forensic Science Research & Education (Willow Grove, PA).

Five classes of personal lubricant were selected for this study: water-based lubricant with glycerin (Astroglide®), water-based lubricant without glycerin (Sliquid® H_2O), hybrid lubricant (Sliquid® Silk), silicone lubricant (Swiss Navy®), and natural oil-based lubricant (Coconut Oil). Lubricant details are described in **Table 5.1**.

Table 5.1. Personal lubricants utilized for this study. Notes on lubricant ingredients, formula, and pH compiled from patent material and/or product descriptions.

Lubricant Class	Lubricant Make and Model	Abbreviation	Notes
Water-Based without Glycerin	Sliquid® H_2O Natural Intimate Lubricant	Sliquid® H_2O	•Glycerin and paraben free formula •Water soluble •Unflavored •Unscented •pH balanced (pH 4.0-4.4)
Water-Based with Glycerin	Astroglide® Liquid Water-Based Personal Lubricant	Astroglide®	Contains Purified Water, Glycerin, Propylene Glycol, Polyquaternium 15
Hybrid	Sliquid® Silk Intimate Hybrid Lubricant	Sliquid® Silk	•Water and silicon blend •Glycerin and paraben free formula •Unflavored •Unscented •pH balanced (4.0-4.4 pH)
Silicone	Swiss Navy® Silicone Lubricant	Swiss Navy®	Contains Cyclopentasiloxane, Dimethicone, Tocopheryl Acetate (Vitamin E).
Natural Oil	Majestic Pure™ Cosmeceuticals Fractionated Coconut Oil (Cocos Nucifera)	Coconut Oil	•Fractionation process removed long chain fatty acids •High concentration of capric acid and caprylic acid •Hexane, paraben, and sulfate free formula

5.1.2 Lubricants and Vaginal Fluid Biomarker Detection

5.1.2.1 Experimental Sample Assembly

Two experimental design plans were constructed for the processing and preparation of vaginal swab samples subjected to personal lubricants. The original experimental design consisted of full vaginal swabs that were cut in half, with one half serving as a control and the second half fortified with a set volume of personal lubricant (**Figure 5.1**). Lubricant volumes of 1 µL, 5 µL, or 15 µL were added to half swabs. Control half swabs were not fortified with lubricant. Swab halves were dried at room temperature overnight. Half swabs were solubilized in 500 µL of deionized water for 30 minutes with frequent agitation. Swabs were placed in a centrifugal basket and centrifuged at 10,000 x g for 10 minutes. Supernatant was transferred to a clean microcentrifuge tube and cell pellets were discarded. Supernatant was reserved for analysis via LC-MS/MS.

For the second experimental design, vaginal swabs were solubilized and extract was fortified with personal lubricant (**Figure 5.2**). Swab tips were solubilized in 500 µL of deionized

water for 30 minutes at room temperature with frequent agitation. Swabs were placed in a centrifugal basket and spun for 10 minutes at 10,000 x g to remove excess liquid. Supernatant was transferred and pooled for further preparation. Processed swabs and cell pellets were discarded. Pooled vaginal extract was fortified with increasing volumes of personal lubricant. For each lubricant type evaluated, 700 µL of pooled vaginal extract was fortified with either 7 µL, 35 µL, or 105 µL of personal lubricant. These volumes simulate targeting 1 µL, 5 µL, and 15 µL of personal lubricant per sample replicate. Control samples consisted of pooled vaginal extract and were not subject to any personal lubricant manipulation. Mixtures were thoroughly vortexed and 100 µL aliquots were transferred to clean microcentrifuge tubes for triplicate analysis.

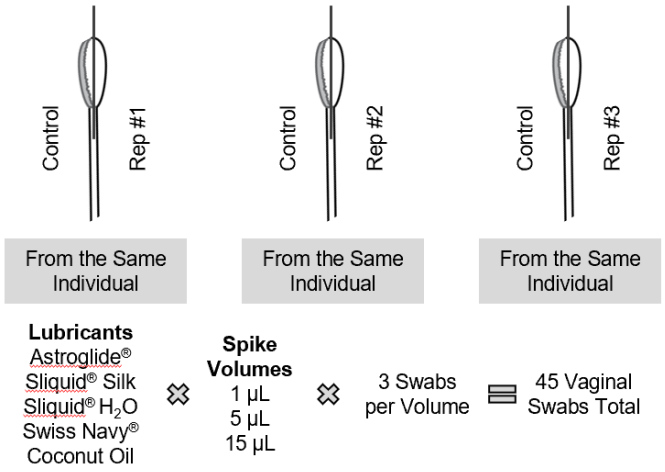


Figure 5.1. Original sample preparation strategy using vaginal swabs fortified with lubricant types. One vaginal swab was cut in half, with each paired half representing a control and an experimental sample.

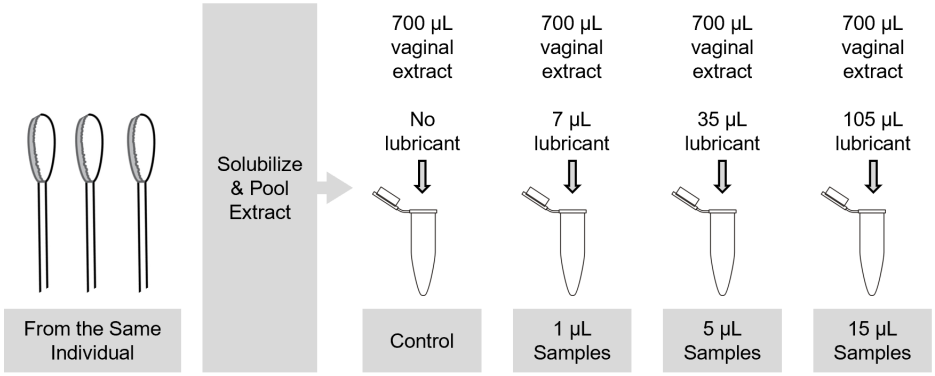


Figure 5.2. Modified sample preparation strategy developed to mitigate expression inconsistencies in vaginal fluid proteins. Vaginal swabs were solubilized and pooled prior to mixing with lubricant types.

5.1.2.2 Pre-Digestion Cleanup

Prepared sample aliquots were diluted with 400 µL of 0.5% trifluoroacetic acid (TFA) in deionized water. A positive pressure manifold (Biotage® PRESSURE+ 48) was fitted with Waters HLB 1cc extraction cartridges (Waters Corporation, Milford, MA). Cartridges were primed with 1 mL of 50% acetonitrile (ACN) 0.5% TFA in deionized water. Cartridges were equilibrated with 1 mL of 0.5% TFA in deionized water. The full 500 µL of diluted sample was applied to and passed through the extraction cartridge, which was then washed with 1 mL of 0.5% TFA in deionized water. Material was eluted from the cartridge in 200 µL of 70% ACN 0.1% formic acid (FA) in deionized water.

5.1.2.3 Sample Processing and Analysis

Extracted samples were quantitated using the BCA assay (section 2.1.3.1) and 100 µg total protein was lyophilized to dryness. Samples were subjected to the previously described 8 M urea tryptic digestion and SPE purification on the AssayMAP Bravo liquid handling platform (section 2.1.4). Eluate was fortified with 20 µL of 0.5 mg/mL intact myelin protein prior to tryptic digestion and 2.5 pmol of isotopically-labeled internal standard prior to SPE cleanup. Eluate was lyophilized to dryness and reconstituted in 100 µL of 2% ACN 0.1% FA in deionized water for LC-MS/MS analysis. Prepared samples were injected onto the 6495 triple quadrupole mass spectrometer

coupled to a 1290 series liquid chromatograph (Agilent Technologies). Data was acquired using the validated analytical method developed under Chapter 2, with data analysis performed in Skyline Software.

5.1.3. Lubricants and the Detection of Semen and Saliva Biomarkers

Vaginal swabs were collected as previously described. Pooled vaginal extract was fortified with various amounts of lubricant and either semen, saliva, or a mixture of semen and saliva. Personal lubricant volumes of 1 µL, 5 µL, and 15 µL were also utilized for this portion of the assessment. In addition, target volumes of 1 µL of semen and 10 µL of saliva were utilized for single fluid experimental samples. For example, 400 µL of pooled extract was fortified with either 4 µL, 20 µL, or 60 µL of personal lubricant in addition to 4 µL of semen or 40 µL of saliva (**Figures 5.3 and 5.4**). Mixture samples were prepared in a similar manner. Pooled vaginal extract was fortified with personal lubricant as previously described in addition to 4 µL of semen and 40 µL of saliva (**Figure 5.5**). Control samples were fortified with semen and/or saliva but absent of personal lubricant. Samples were thoroughly vortexed and 100 µL of sample was aliquoted for further processing. Samples underwent pre-digestion cleanup, tryptic digestion, and SPE purification as described in section 5.1.2.2.

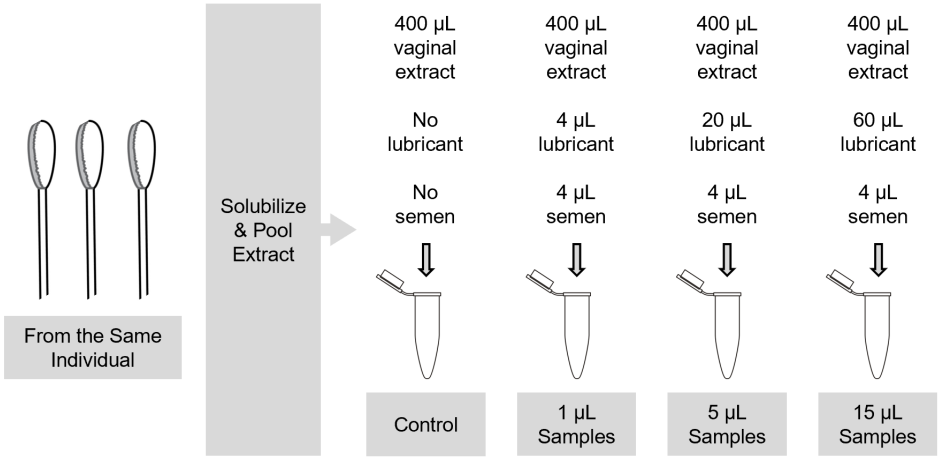


Figure 5.3. Sample preparation strategy for control and experimental samples containing semen.

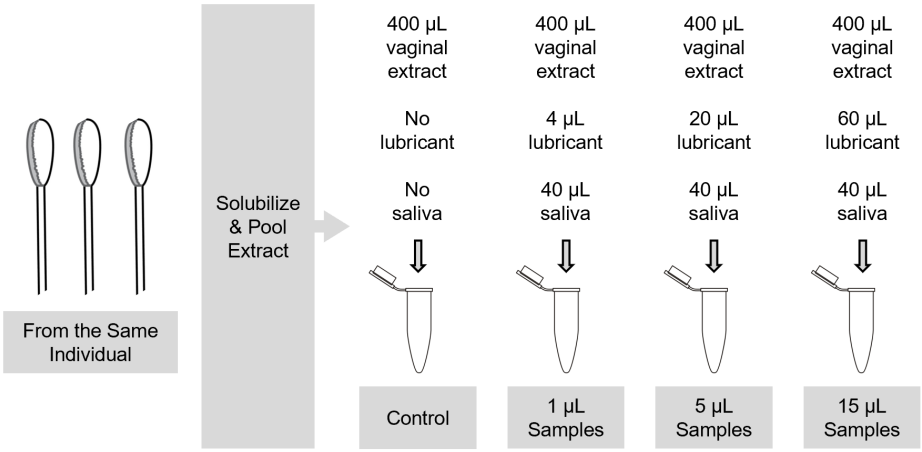


Figure 5.4. Sample preparation strategy for control and experimental samples containing saliva.

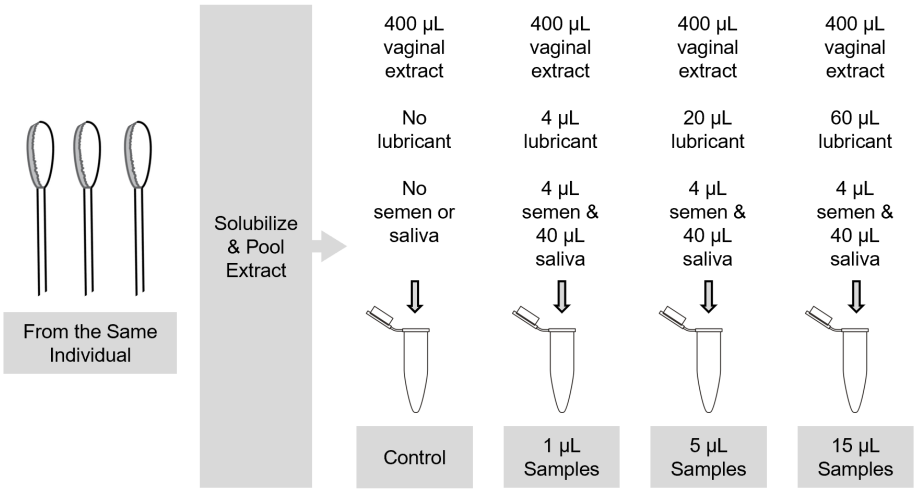


Figure 5.5. Sample preparation strategy for control and experimental samples containing both semen and saliva.

5.1.4 Vaginal Fluid Biomarker Detection from Condoms

Four condom types were selected for evaluation: Trojan™ ENZ™ unlubricated condoms, SKYN® Original condoms, SKYN® Extra Lube condoms, and Durex® Performax® condoms. These condoms were selected because they were marketed to cover a range of lubricant amounts. A summary of condom types and product information is outlined in **Table 5.2**.

Condoms were removed from the packaging, unraveled, and placed on a clean piece of butcher paper. Simulating authentic casework, only the external surface (*i.e.,* the surface that

contacts the vaginal cavity when inserted) was swabbed for this study. Vaginal swabs were obtained from female volunteers and dried at room temperature. A single swab was moistened with two drops of deionized water and used to swab the entire external surface of one condom. This was repeated in triplicate, with one vaginal swab per condom. Swabs were allowed to dry at room temperature for 30 minutes. Swabs were solubilized in 500 µL deionized water for 30 minutes with frequent agitation. Swabs were placed in a centrifugal basket and centrifuged for 10 minutes at 10,000 x g. Supernatant was transferred and pooled in a clean microcentrifuge tube for further preparation. Cell pellets and processed swabs were discarded. Supernatant was subjected to pre-digestion cleanup, tryptic digestion, and SPE purification as previously described. Eluate was lyophilized to dryness and reconstituted in 100 µL of 2% ACN 0.1% FA. 10 µL of sample was injected on column for LC-MS/MS analysis.

Table 5.2. Condom brands utilized for this study. Notes on condom lubricant (if present) and material compiled from patent information and/or product descriptions.

Condom Class	Condom Make and Model	Abbreviation	Notes
Unlubricated	Trojan™ ENZ™ Non-Lubricated Condoms	Trojan™ ENZ™	•Made from latex
Lubricated	SKYN® Original	SKYN® Original	•Made from SKYNFEEL™ non-latex material
Extra Lubricated	SKYN® Extra Lube	SKYN® Extra Lube	•Made from SKYNFEEL™ non-latex material •Marketed to contain 40% more lubricant than SKYN® Original
Ribbed	Durex® Performax® Intense Lubricated Condoms	Durex® Performax®	•Ribbed, dotted, and lubricated •Made from latex •Contains Benzocaine 5%

5.2 Results and Discussion

5.2.1 Lubricants and Vaginal Fluid Biomarker Detection

5.2.1.1 Digest Efficiency

The total protein concentration of control and experimental samples was determined using the BCA assay. Quantitation was used to normalize the amount of total protein input into the enzymatic digestion procedure. Should the presence of personal lubricants negatively impact the

ability to accurately determine the total protein quantity in a sample, the protein input for tryptic digestion would be incorrect. This would result in insufficient digestion or missed cleavages. No effect on digest efficiency was observed across and lubricant type or lubricant volume, resulting in no sufficient variation in protein concentration (**Figure 5.6**). A median protein concentration was measured at 266.42 µg/mL and 272.18 µg/mL for control and experimental samples, respectively.

Trypsin performance was monitored by assessing peak area ratios of intact internal positive control to digested internal positive control (IPC). Acceptance criteria outlined during validation studies (Chapter 3) were employed during this assessment to ascertain IPC performance. IPC ratios that fell within ratio bounds of 15.8 (upper bound) and 11.5 (lower bound) were considered acceptable and unaffected by experimental procedures. Digest performance was impacted by several personal lubricant types prior to implementing a supplemental pre-digestion cleanup (**Figure 5.7**). Greatest protease inhibition prior to supplemental cleanup was observed for Sliquid® H_2O, Coconut Oil, and Swiss Navy® lubricants. For example, the average IPC ratio for 1 µL coconut oil samples was 19.15, a ratio that falls outside the acceptable range. With the addition of the pre-digestion cleanup, this ratio fell to an acceptable 12.42. Interestingly, Sliquid® Silk demonstrated an inverse dose-response relationship, with 1 µL volumes of personal lubricant above the established range, 5 µL volumes within the established range, and 15 µL volumes below the established range. Protease activity was not negatively affected by Astroglide®. Acceptable IPC ratios for all lubricants assessed were restored with the addition of the pre-digestion cleanup to the sample processing protocol.

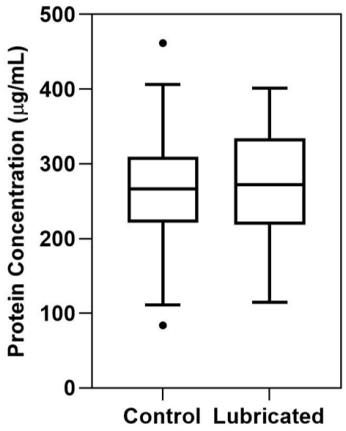


Figure 5.6. Quantitative results comparing total protein concentration of control and lubricated vaginal swab samples. Box = 25th and 75th percentiles; bars = Tukey fences. Outliers were calculated as any values outside ± 1.5 Inter Quartile Range (IQR).

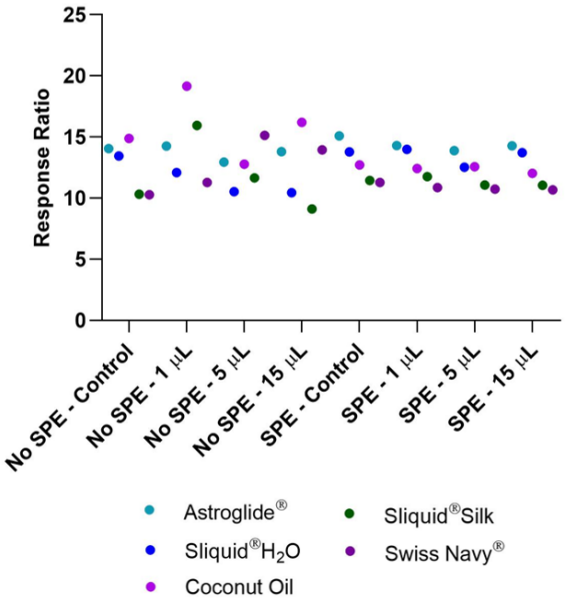


Figure 5.7. Internal positive control response ratio for control and lubricated samples which have or have not undergone an additional SPE purification during sample preparation. Response ratios were averaged across three replicates.

5.2.1.2 Biomarker Detection

The effect of personal lubricant on biomarker detection was assessed by calculating the percent difference of endogenous and internal standard peak area responses for all samples

evaluated. Similar to the results observed for digestion efficiency, biomarker detection was negatively affected by the presence of personal lubricant, particularly prior to the addition of the pre-digestion cleanup. Overall, impact of personal lubricant was sufficiently reduced at all lubricant volumes with pre-digestion cleanup (**Figure 5.8**).

The greatest deleterious effects were observed for Sliquid® H_2O, Coconut Oil, and Sliquid® Silk lubricants. Sliquid® H_2O, the water-based lubricant without glycerin, exhibited a dose-response relationship for both endogenous and internal standard biomarkers. When fortified with lubricant, a median percent difference for endogenous vaginal fluid biomarkers of 0.31%, −11.28%, and −37.54% were observed at the 1 µL, 5 µL, and 15 µL lubricant volumes, respectively. The greater percent difference at the 15 µL volume was mitigated with a pre-digestion cleanup, resulting in a percent difference of −14.28% (**Figure 5.8B**). A similar dose-response relationship was exhibited by samples treated with Sliquid® Silk (**Figure 5.8D**). Samples treated with Coconut Oil exhibited negative impact on biomarker detection regardless of lubricant volume. A percent difference of −25.04%, −22.15%, and −25.03% were observed for 1 µL, 5 µL, and 15 µL volumes, respectively. However, a wide range in response ratios was demonstrated. The lower whisker extends to −80.94% and the median lies at the top of the inter quartile range (IQR). Internal standard responses exhibited a negative percent difference, but not to the same extent as the endogenous biomarkers (**Figure 5.8C**).

To further illustrate the negative effects of certain lubricants on specific endogenous biomarkers, **Figure 5.9** depicts the response of neutrophil gelatinase peptide WYVVGLAGNAILR when subjected to each lubricant evaluated. This biomarker was selected given the specificity of neutrophil gelatinase in vaginal fluid and the hydrophobic nature of the WYVVGLAGNAILR peptide (retention time of 8.7 minutes). The response of this specific peptide was unaffected by Astroglide® and Sliquid® Silk; however, sufficient decrease in response was exhibited with Sliquid® H_2O, Coconut Oil, and Swiss Navy®. With the addition of a pre-digestion cleanup, inconsistencies were not entirely eliminated, but minimized in most cases. For example, **Figure 5.10** illustrates the

increase in peak area response of endogenous neutrophil gelatinase peptide WYVVGLAGNAILR with the addition of the pre-digestion cleanup.

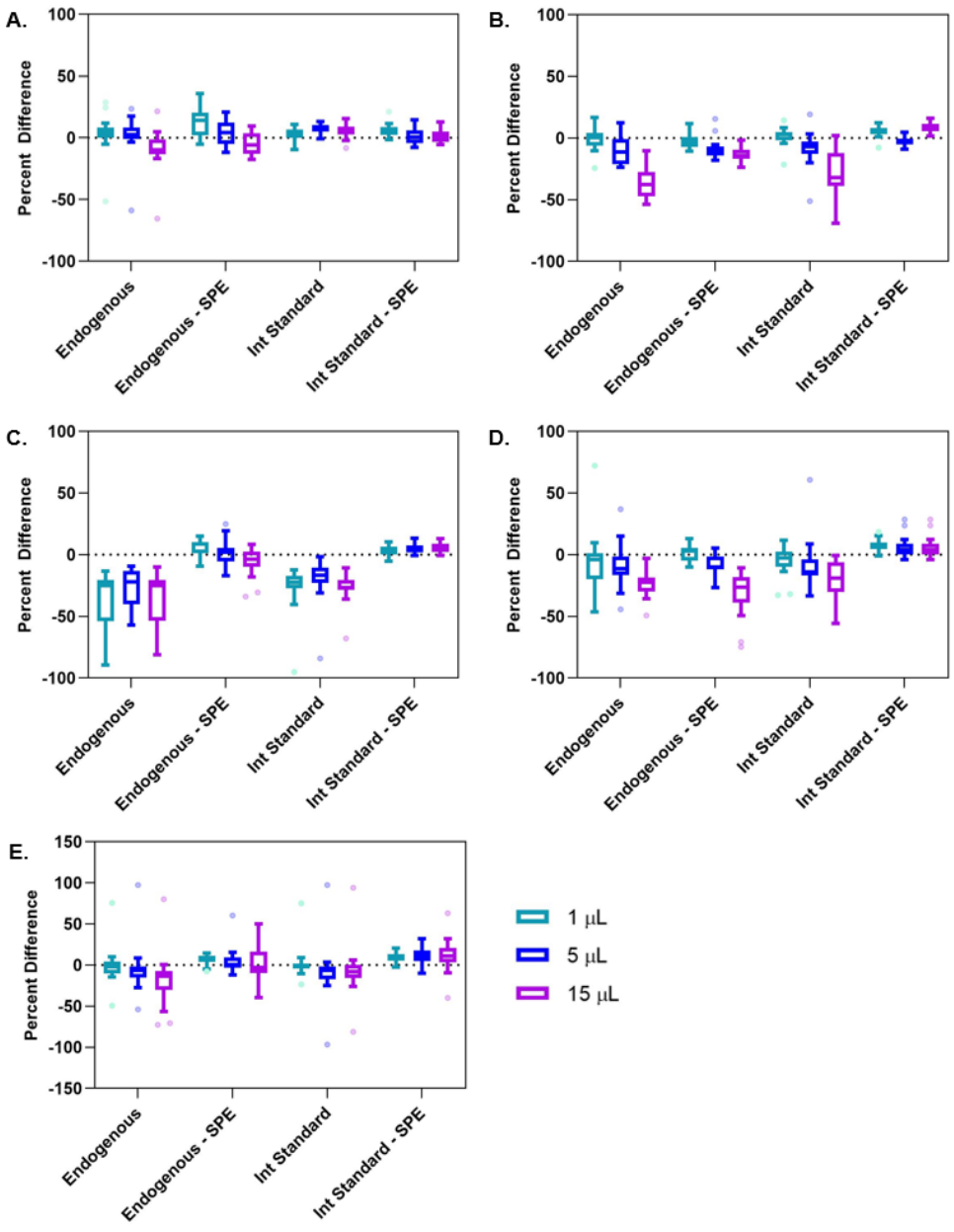


Figure 5.8. Effect of (A) Astroglide®, (B) Sliquid® H_2O, (C) Coconut Oil, (D) Sliquid® Silk, and (E) Swiss Navy® lubricants measured as percent difference on peak area response of endogenous vaginal fluid proteins and isotopically-labeled internal standards both with and without pre-digestion SPE cleanup. Box = 25th and 75th percentiles; bars = Tukey fences. Outliers were calculated as any values outside ± 1.5 IQR.

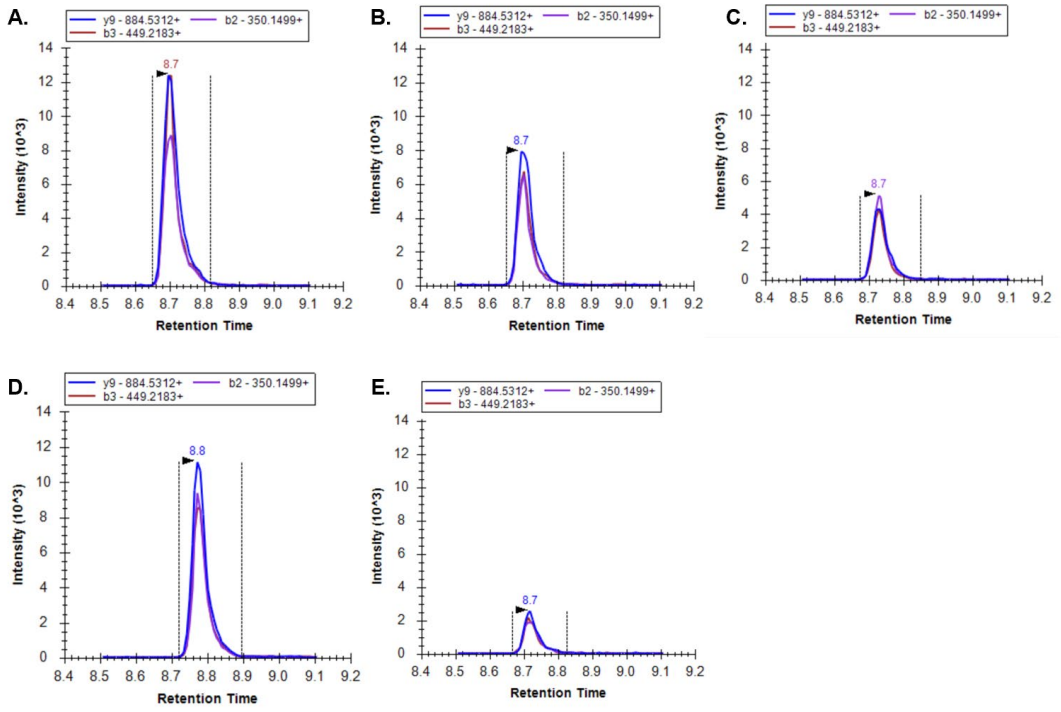


Figure 5.9. Chromatographic peak response for endogenous neutrophil gelatinase peptide WYVVGLAGNAILR when subjected to 15 µL of (A) Astroglide®, (B) Sliquid® H_2O, (C) Coconut Oil, (D) Sliquid® Silk, and (E) Swiss Navy®.

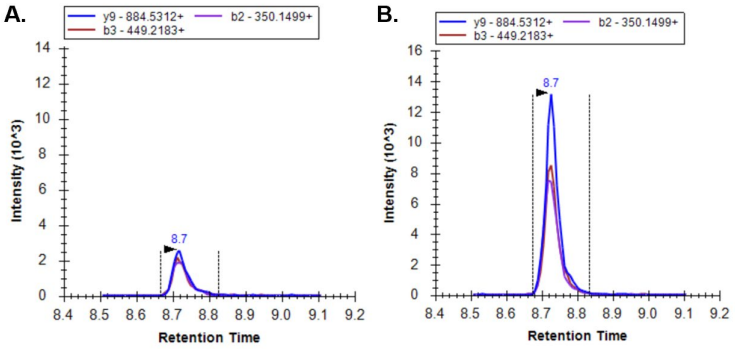


Figure 5.10. Chromatographic peak response for endogenous neutrophile gelatinase peptide WYVVGLAGNAILR (A) without pre-digestion cleanup and (B) with pre-digestion cleanup, when subjected to 15 µL of Swiss Navy®.

5.2.2 Lubricants and the Detection of Semen and Saliva Biomarkers

The overall effect of personal lubricant was further assessed with mock sexual assault samples. Generally, when sexual assault evidence is received in a forensic laboratory, it will be screened for the presence of seminal fluid and/or saliva. Therefore, in addition to the effect of

personal lubricant on the detection of vaginal fluid, it was vital to establish any deleterious effects that may inhibit the identification of seminal fluid and/or saliva. To simulate sexual assault samples, pooled vaginal extract was fortified with seminal fluid, saliva, and a mixture of both target fluids in addition to increasing amounts of personal lubricant. All lubricant classes and selected volumes were evaluated in triplicate.

In summary, all seminal fluid and saliva protein biomarkers were detected at all lubricant volumes and for all lubricant classes evaluated, in both the prepared single source and mixture samples. For prepared samples containing saliva, marginal suppression or enhancement effects were observed for samples fortified with Astroglide®, Sliquid® H_2O, and Coconut Oil. In comparison with the detection of vaginal fluid markers, sufficient losses in peptide response were observed for samples fortified with Sliquid® Silk, even with the pre-digest cleanup (**Figure 5.11A**). A median saliva biomarker signal decrease of −37.0%, −54.7%, and −93.3% at the 1 µL, 5 µL, and 15 µL volumes was observed with the Sliquid® Silk lubricant. Furthermore, at the 15 µL volume, sufficient enhancement effects were observed for Swiss Navy®, with a median signal increase of 120.5%. These observed effects were consistent with recorded peak area responses of the internal standards of single source saliva samples (**Figure 5.11B**). The greatest suppression effects were illustrated by endogenous seminal fluid protein biomarkers, with consistent suppression noted for Astroglide®, Sliquid® H_2O, Coconut Oil, and Sliquid® Silk. A median signal decrease of −23.3% and −85.7% were observed at the 5 µL and 15 µL Sliquid® Silk volumes, respectively. These two samples exhibit a large IQR. However, small instances of enhancement were recorded for the 15 µL Sliquid® Silk and 1 µL Swiss Navy® lubricants, with signal increases of 4.9% and 4.8%, respectively (**Figure 5.11C**). Conversely, minimal suppression and enhancement effects were observed for seminal fluid internal standard responses (**Figure 5.11D**).

Interestingly, the severe instances of peak response suppression for Sliquid® Silk were not as evident in samples containing a mixture of seminal fluid and saliva (**Figure 5.12A and 5.12C**) Although an average peptide response loss of −49.7% for saliva and −26.4% for seminal fluid were observed at the 15 µL volume of Sliquid® Silk, peak response enhancement for Coconut Oil was

illustrated (**Figure 5.12A and 5.12C**). Internal standard response demonstrated limited suppression for both target fluids, with enhancement observed for Coconut Oil and Sliquid® Silk lubricants (**Figure 5.12B and 5.12D**). For example, decrease in peak area response for semenogelin 2 and alpha amylase is depicted in **Figure 5.13**. Endogenous peak area response (red peaks) demonstrates a linear decrease while the internal standard response (blue peaks) remains relatively unchanged across lubricant volumes.

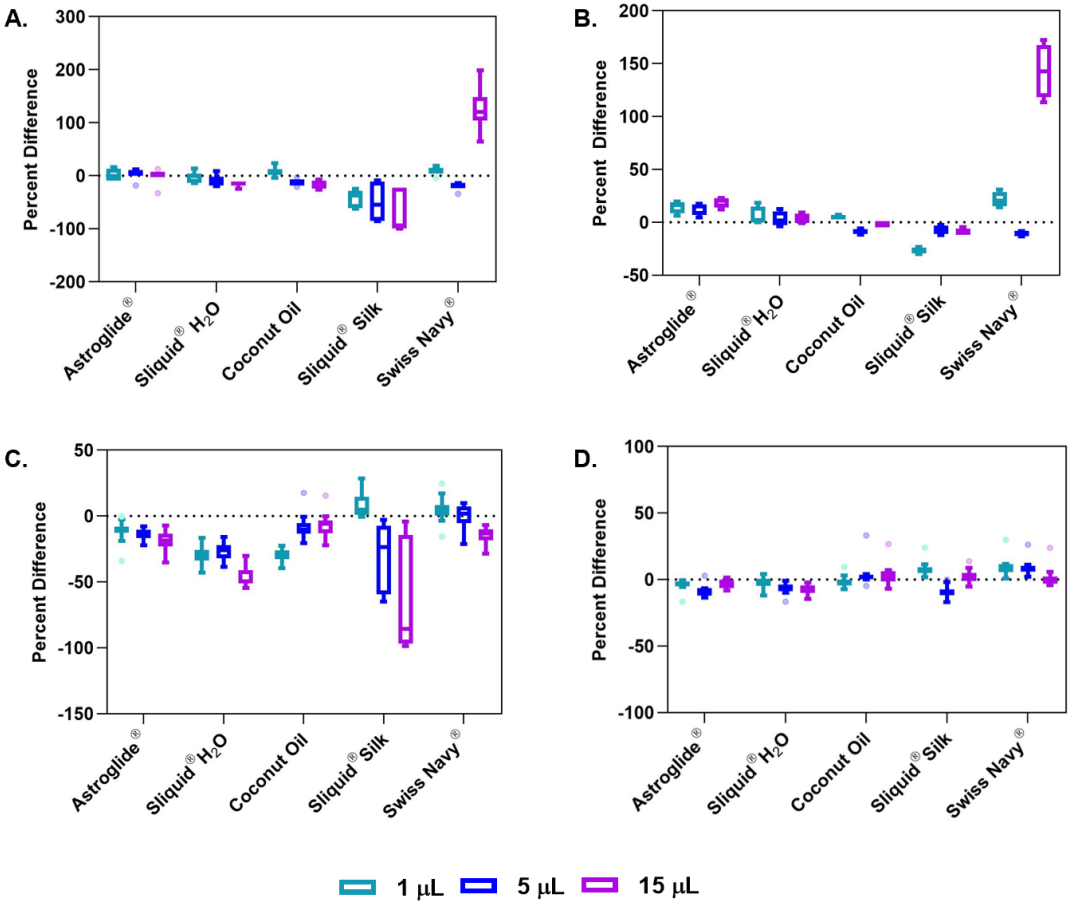


Figure 5.11. Effects of lubricant on (A) endogenous saliva biomarkers, (B) internal standard saliva biomarkers, (C) endogenous seminal fluid biomarkers, and (D) internal standard seminal fluid biomarkers. Plotted is the percent difference of average signal intensity for control versus experimental samples for all target biomarkers. Box = 25th and 75th percentiles; bars = Tukey fences. Outliers were calculated as any values outside ± 1.5 IQR.

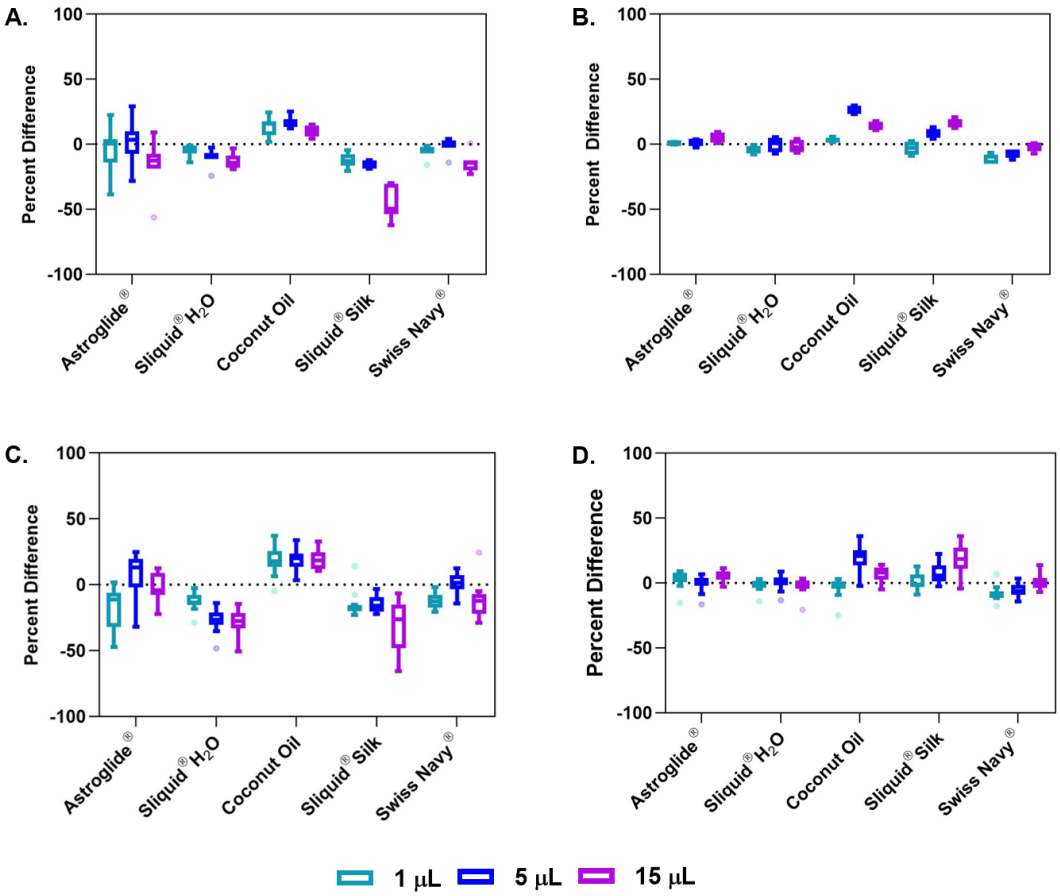


Figure 5.12. Effects of lubricant on (A) endogenous saliva biomarkers, (B) internal standard saliva biomarkers, (C) endogenous seminal fluid biomarkers, and (D) internal standard seminal fluid biomarkers in mixture samples. Plotted is the percent difference of average signal intensity for control versus experimental samples for all target biomarkers. Box = 25th and 75th percentiles; bars = Tukey fences. Outliers were calculated as any values outside ± 1.5 IQR.

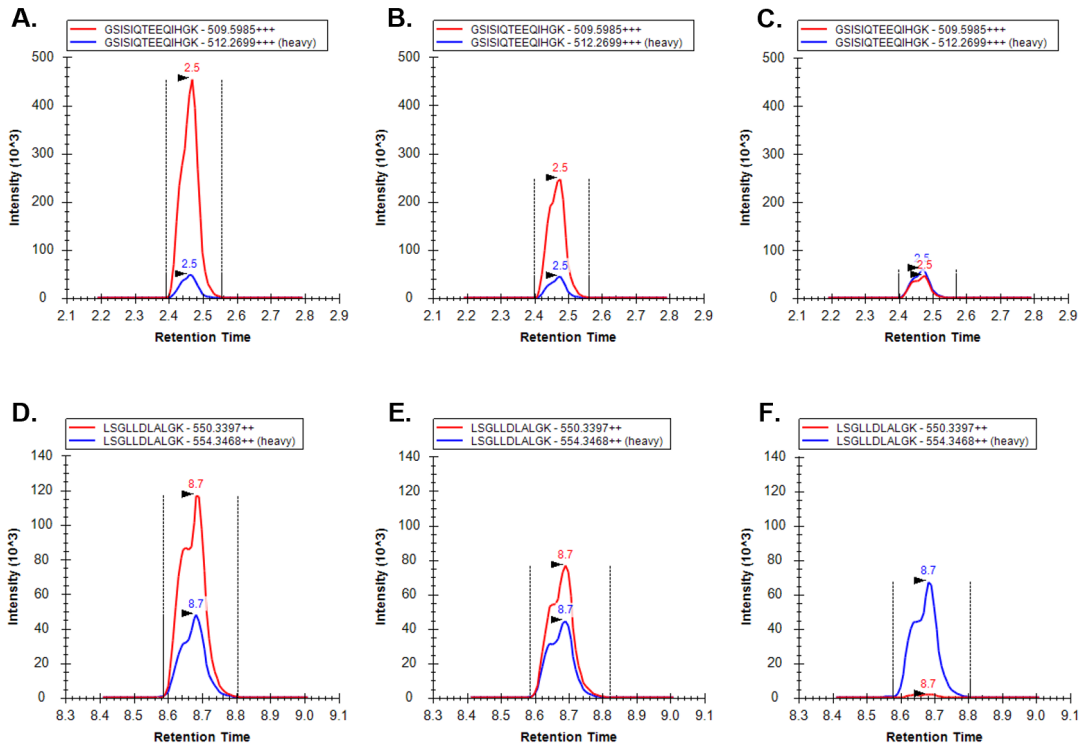


Figure 5.13. Semenogelin 2 peptide GSISIQTEEQIHGK peak area response in mixture samples containing (A) 1 µL, (B) 5 µL, and (C) 15 µL volumes of Sliquid® Silk lubricant. Alpha amylase peptide LSGLLDLALGK peak area response in mixture samples containing (D) 1 µL, (E) 5 µL, and (F) 15 µL volumes of Sliquid® Silk lubricant. Red peaks represent endogenous peptide peak area response. Blue peaks represent internal standard peak area response.

5.2.3 Vaginal Fluid Biomarker Detection from Condoms

Pre-lubricated condoms typically contain a proprietary lubricant which may include the addition of spermicidal additives, such as the non-ionic detergent nonoxol-9. It was hypothesized that such detergents and additives may decrease binding capacity of solid phase extraction cartridges. The sorbent material of the validated microextraction cartridges were hydrophobic C18 hydrocarbons bonded to silica beads. The presence of detergents can introduce competitive binding, resulting in a decrease in capture and recovery of hydrophobic markers during analysis. Furthermore, detergents have the potential to act as ion suppressants when introduced into a mass spectrometer.

To further assess this hypothesis, lubricated condoms both with and without spermicidal additives were evaluated. The external surface of each condom was swabbed with a vaginal swab to capture the proprietary lubricant and/or spermicide additive at concentrations commonly encountered in forensic casework. Unlubricated condoms (Trojan™ ENZ™) served as the condom control, in addition to pooled, untreated vaginal extract serving as the negative control. In addition to the unlubricated condom type, SKYN® Original, SKYN® Extra Lube, and Durex® Performax® condoms were selected for this portion of the study. After initial observations made during sample handling, the Durex® Performax® condoms contained the greatest amount of surface lubricant and, although marketed as unlubricated, the Trojan™ ENZ™ condoms did contain a minimal amount of lubricant on the external surface.

Overall, target peptide response of vaginal fluid markers was greater across all condom types in comparison to negative control samples (**Figure 5.14**). It should be noted that inconsistency in sample data was observed, as depicted by the percent difference range of endogenous targets and large IQR of internal standard response. For endogenous biomarkers, the calculated percent differences of peak area response were evenly distributed between the first and third quartiles. However, outliers greater than 1.5 IQR were observed for Durex® Performax®, SKYN® Original, and SKYN® Extra Lube condom samples. A median 50% gain in peptide response was exhibited by vaginal fluid markers recovered from swabs subjected to the surfaces of SKYN® Extra Lube and Trojan™ ENZ™ condoms (**Figure 5.14A**). Overall, peak response of isotopically-labeled internal standard was enhanced in comparison with control samples (**Figure 5.14B**). The internal standard response demonstrated greater agreement among sample replicates than endogenous biomarkers responses, evident by the absence of outliers and a more consistent IQR. Chromatographic endogenous and internal standard peak area responses across condom replicate samples are depicted in **Figure 5.15**.

An active ingredient in Durex® Performax® condoms that may attribute to the larger IQR and presence of outlier data points is benzocaine. Benzocaine is a common ingredient in over-the-counter topical pain ointments, such as those used for oral and otic pain. The mechanism of action

of benzocaine that results in pain relief has also been leveraged for use in contraceptives. Benzocaine acts by inhibiting voltage-dependent sodium channels, preventing an action potential from propagating down a neuron membrane. When applied to condoms, benzocaine can prevent premature ejaculation by decreasing sensitivity of male sex organs. Given the compound's structure, it is hydrophobic in nature and readily soluble in alcohol-based solvents. Therefore, benzocaine has great affinity for the C18 sorbent material of solid phase extraction cartridges and for the acetonitrile-based elution solvent.

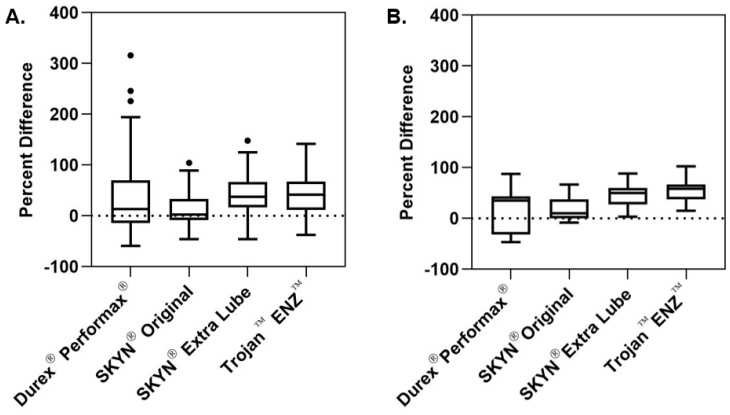


Figure 5.14. Effects of condom lubricant on (A) endogenous vaginal fluid biomarkers and (B) internal standard vaginal fluid biomarkers. Plotted is the percent difference of average signal intensity for control versus experimental samples for all target biomarkers. Box = 25th and 75th percentiles; bars = Tukey fences. Outliers were calculated as any values outside ± 1.5 IQR.

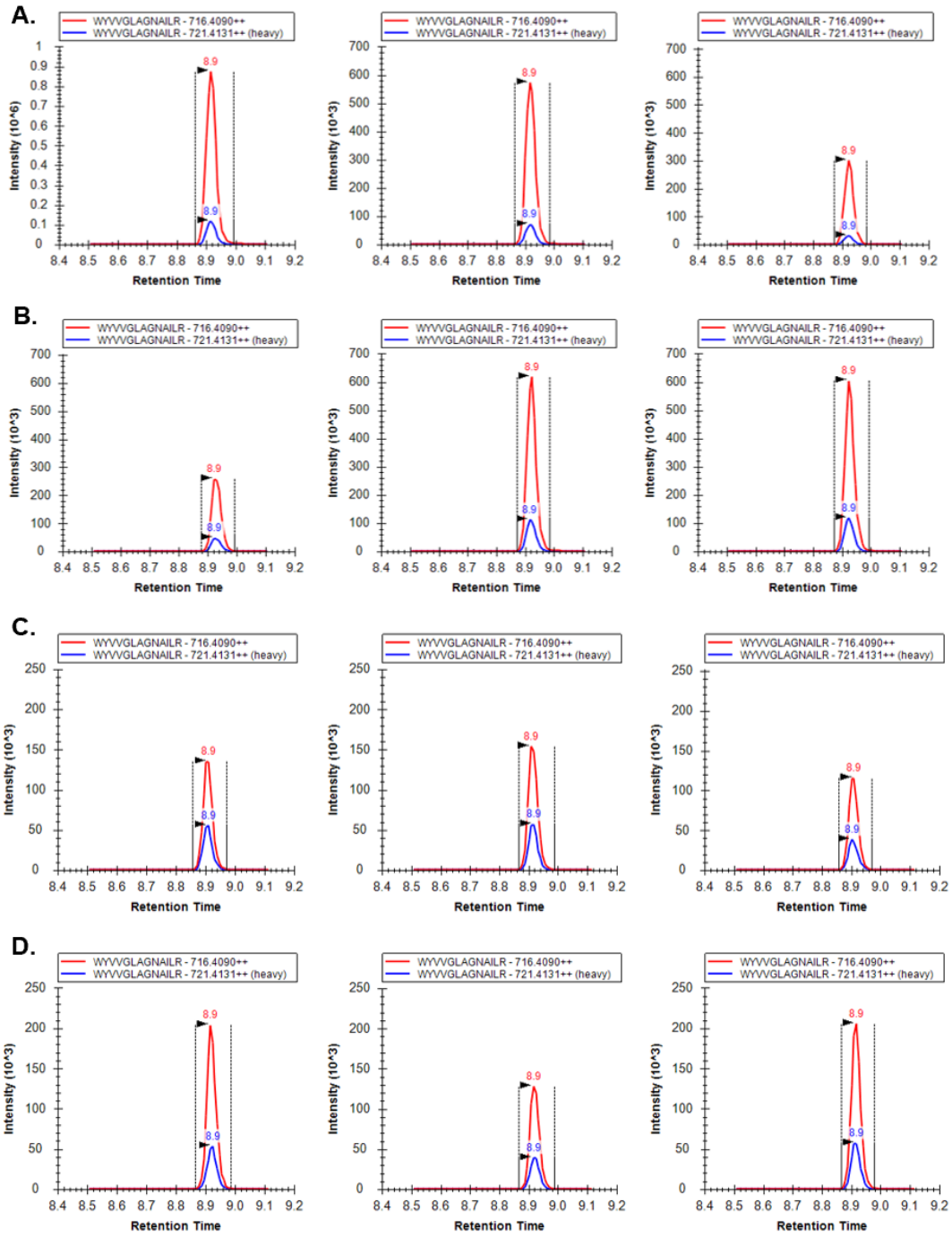


Figure 5.15. Neutrophil gelatinase peptide WYVVGLAGNAILR peak area response for (A) Durex® Performax®, (B) SKYN® Original, (C) SKYN® Extra Lube, and (D) Trojan™ ENZ™ condom triplicate samples. Red peaks represent endogenous peptide peak area response. Blue peaks represent internal standard peak area response.

5.3 Concluding Remarks

With the completion of this phase of research, it has been demonstrated that the deleterious effects of personal lubricants, specifically oil and silicone-based formulas, can be mitigated through the addition of a second solid phase extraction cleanup. When implemented prior to digestion, this cleanup eliminated interferents that would otherwise negatively affect trypsin processivity and mass spectrometry detection.

Extraction cartridge sorbent material was selected based on chemical interactions between the sorbent functional groups with functional groups of target protein biomarkers. These functional groups are considerably different from other structural elements and functional groups of interference compounds, such as surfactants. The functional group interaction between the target analyte and its surrounding environment was crucial in modifying the sample preparation protocol for this portion of the study. Given the presence of interfering compounds present in personal lubricants, such as surfactants and medium chain fatty acids, additional steps were needed to ensure maximum recovery of target biomarkers.

Reverse phase solid phase extraction was used for additional sample cleanup. Reverse phase employs a polar mobile phase and a nonpolar sorbent stationary phase. Given that serological samples are generally maintained in water or saline solution, reverse phase extraction was a clear solution to mitigating the effects of hydrophobic compounds common in lubricant formulas. A C18 sorbent material was selected for solid phase extraction cleanup and optimized as part of the developed protocol under the validation of the LC-MS/MS assay. C18 sorbents are nonpolar in nature and therefore attract and retain nonpolar analytes in solution, such as nonpolar peptide side chains or nonpolar interferent compounds.

Astroglide® contains the compound (1-(3-chloroallyl)-3,5,7-triaza-1-azoniaadamantane chloride), referred to as polyquaternium-15. Although water soluble, this compound has surfactant properties. Surfactants, even at low concentrations, have the ability to severely suppress ionization during mass spectrometry analysis. Swiss Navy®, the silicone lubricant selected for this study, contains the compound dimethicone that has surfactant properties as well. Dimethicone is a

hydrophobic compound, as is Vitamin E acetate (α-tocopheryl acetate) and cyclopentasiloxane. These three compounds would be retained during reverse phase solid phase extraction. In order to selectively remove retained proteins from interferents, a relatively polar elution solvent was necessary. In comparison with other organic solvents such as ethyl acetate or acetone, acetonitrile is a polar solvent. Therefore, protein material would have greater affinity for the elution solvent than severely hydrophobic interferents, such as dimethicone, which would stay retained to the sorbent material. In summary, it was demonstrated that the analysis of sexual assault samples subjected to personal lubricants or condom forms of contraception was achievable with modified sample preparation protocols.

The remaining chapter of this dissertation will further delve into advancing proteomic techniques as they apply to the analysis of sexual assault evidence. Thus far, the protein markers targeted using the validated LC-MS/MS assay have been manipulated by the protease trypsin, producing distinct peptide fragments. This process has the potential to eliminate high quality peptide targets that may fall within or outside of a tryptic peptide. Although it has been demonstrated that this bottom up proteomic process can be amenable to high throughput analyses, the multi-day workflow is a substantial drawback to the adoption of this workflow by forensic practitioners. Therefore, an enzyme-free proteomic identification method of seminal fluid, saliva, and vaginal fluid has been developed and assessed.

CHAPTER 6: DEVELOPMENT OF A PEPTIDOMIC METHOD FOR BODY FLUID IDENTIFICATION

6 Introduction

It has been well-established that protein mass spectrometry methodologies demonstrate the desired sensitivity and specificity for the confirmatory identification of biological fluids, particularly in comparison with traditional serological screening techniques. The research presented thus far utilized a bottom up proteomic approach, where protein material was subjected to a multi-step workflow of denaturation, enzymatic digestion, cleanup, and analysis. Although this bottom up workflow reliably identified biological stains and was amenable to automation, the multi-day workflow presents a substantial impediment to the implementation of this next generation technique in operational forensic laboratories. In contrast to traditional bottom up approaches, top down techniques measure intact proteins, naturally derived peptides, or breakdown products of larger proteins. Driven primarily by the demands of the biopharmaceutical and biomedical industries, it is possible to reliably measure small to medium sized peptides in complex biological matrices [166]. Procedural methods are straightforward and require minimal sample preparation to remove abundant endogenous proteins, such as albumin. Typically, only a one-step solid phase extraction is required prior to detection by high resolution mass spectrometry. Data analysis employs advanced bioinformatics software capable of deconvoluting and matching fragmentation spectra of large peptides. These technical advances allow for a streamlined same day workflow, an attractive alternative to the more laborious bottom up procedures currently required for proteomic body fluid identification.

A multiplex assay using high-specificity protein biomarker panels for the identification of five forensically-relevant body fluids (*i.e.,* peripheral blood, saliva, seminal fluid, vaginal/menstrual fluids) was developed and validated, as described in Chapters 2 and 3. This approach has proven

to be broadly robust and reliable, particularly for the processing of sexual assault-type samples. With this workflow, the protein components, and by association the body fluid source, of stains can be detected with a level of sensitivity comparable to that of DNA profiling. The assay has been rigorously validated in accordance with the Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology [167] and the FBI's Quality Assurance Standards (QAS) [168] to establish the reliability and limitations of the methodology. Studies on assay performance with casework-type samples have shown that the method is fully compatible with downstream genetic testing and that assay sensitivity greatly exceeds that of traditional lateral flow assays, particularly with trace level samples. Additional studies, discussed in Chapter 5, that targeted the potential impact of substances commonly encountered in sexual assault-type evidence (*e.g.,* personal lubricants and spermicidal compounds) provided additional insight on how best to maximize the successful detection of body fluids in casework.

The objective of this phase of research was to develop a peptidomic assay for the expedited identification of vaginal fluid, saliva, and seminal fluid in sexual assault evidence by high resolution mass spectrometry. This research was completed under four scientific aims. First, various sample processing protocols were evaluated and compared to ensure optimal biomarker recovery from solubilized stains. The second aim sought to adapt and transfer an existing prototype assay to a high-flow operational platform. The third aim employed a population study for the determination of candidate protein and peptide biomarkers. And lastly, a performance assessment was conducted using a targeted acquisition method created with the selected biomarkers for each biological fluid. The final deliverable would contain an expedited proteomic methodology, with the length enzymatic digestion step eliminated, where surrogate peptides could serve as a means of body fluid source determination. This peptidomic approach streamlines body fluid identification by coupling direct protein analysis of whole-body fluids in lieu of workflows that employ time-consuming enzymatic digestion prior to sample analysis. Additionally, it enabled the swift but

accurate identification and verification of body fluid-specific biomarkers with a same-day sample processing workflow for sexual assault analysis.

6.1 Methods and Materials

All research conducted under this phase of the project was reviewed and approved by the University of Denver IRB for research involving human subjects. Sample collection and research was conducted in full accordance with the U.S. federal policy for the protection of human subjects as described in section 2.11. In total, 10 subjects were recruited from the graduate population at Arcadia University and staff members employed at The Center for Forensic Science Research & Education.

Semen was self-collected from consenting donors as described in section 2.1.1. Collection cups were vortexed and 200 µL single use aliquots were prepared and stored at -80 °C until use. Saliva was collected from consenting volunteers who refrained from eating or drinking for 1 hour prior to collection. Salivette® collection tubes (Sarstedt, Nümbrecht, Germany) were utilized as described in section 2.1.1. 200 µL single use aliquots were prepared and stored at -80 °C until use. Single use body fluid aliquots (semen or saliva) were thawed at room temperature for 30 minutes then centrifuged for 10 minutes at 10,000 x g. Supernatant was transferred to a clean microcentrifuge tube and cell pellets were discarded. For this study, vaginal secretions were collected using only the swabbing method as described in section 2.1.1. Upon receipt, swabs were dried at room temperature where the cotton tip was removed from the wooden handle and placed in a clean microcentrifuge tube. Swabs were stored at -80 °C until use. Full cotton swabs were solubilized in 600 µL of deionized water for 30 minutes with frequent agitation. Swabs were placed into centrifugal baskets and centrifuged at 10,000 x g for 10 minutes. Supernatant was pooled and transferred to a clean microcentrifuge tube.

Given laboratory and IRB restrictions in place out of caution for the SARS-CoV-2 virus, additional biological matrices were purchased from both Lee Biosolutions, Inc. (Maryland Heights, MO) and Innovative Research, Inc. (Novi, MI) for the population assessment. Upon ordering, it was requested that each sample was obtained from a unique donor, with no repetitive donors. Biological

material was shipped on dry ice and immediately stored at -80 °C until use. Saliva and semen samples were thawed at room temperature for 30 minutes, vortexed, and pulse spun. 200 µL was removed for analysis and treated as described above. Supernatant was transferred to a clean microcentrifuge tube and cell pellets were discarded. Full vaginal swabs were cut and treated as described above.

Body fluid material was quantitated using the bicinchoninic acid (BCA) assay as described in section 2.1.4. Protein material was extracted using a method described below.

6.1.1 Sample Processing Development and Comparison

For this portion of the research, single source body fluid samples from at least two individuals were prepared in triplicate for analysis as described above. Final preparation protocol comparison (Agilent Bond Elut Plexa plate *versus* Agilent RP-S Cartridges) was performed with single source body fluid from two individuals prepared in quadruplicate.

6.1.1.1 Protein Precipitation

A total protein amount of 200 µg was diluted with equal volume of cold acetonitrile (maximum volume of 100 µL) and agitated. Samples were centrifuged at 10,000 x g for 15 minutes at 4 °C. Supernatant was transferred to a clean microcentrifuge tube and lyophilized to dryness. Precipitated protein (pelleted material) was discarded. The procedure was repeated with 150 µg, 50 µg, 10 µg, and 1 µg protein amounts. Protein quantities smaller than 50 µL volume equivalent were brought up to a final volume of 50 µL with 100 mM Tris-HCl prior to acetonitrile addition.

6.1.1.2 Centrifugal Filtration

Two centrifugal filters were evaluated: Spin-X^{R} UF 500 (Corning®, Corning, NY) and Amicon® Ultracel® (Millipore Sigma, Darmstadt, Germany), both of which had a molecular weight cutoff of 10 kDa. A total protein amount of 200 µg was diluted with 30% acetonitrile (ACN) with 0.1% formic acid (FA) to a final volume of 500 µL. Centrifugal filters were centrifuged at 10,000 x g for 10 minutes. Eluate was lyophilized to dryness prior to analysis.

6.1.1.3 Automated Solid Phase Extraction

Two microextraction cartridges compatible with the AssayMAP Bravo liquid handling platform were selected for evaluation: C18 sorbent and RP-S sorbent, both purchased from Agilent Technologies (Santa Clara, CA). A total protein amount of 100 µg was diluted with 0.5% trifluoroacetic acid (TFA) in deionized water for a final volume of 250 µL. Microextraction cartridges were primed with 100 µL 50% ACN 0.5% TFA at a flow rate of 300 µL/min. Cartridges were equilibrated with 50 µL of 0.5% TFA in deionized water at 10 µL/min. 220 µL of diluted sample was passed over the cartridge at a flow rate of 15 µL/min. Cartridges were washed with 50 µL of 0.5% TFA in deionized water at a rate of 10 µL/min. Sample material was eluted from the sorbent in 25 µL of 30% ACN 0.1% FA at 5 µL/min. Eluate was lyophilized to dryness prior to analysis.

6.1.1.4 Manifold Solid Phase Extraction

A positive pressure manifold was fitted with either a Bond Elut Plexa or a Bond Elut Plexa PCX plate (Agilent Technologies). Plates were received in a 96-well cartridge format. Cartridges were primed with 500 µL of 50% ACN with 0.5% TFA in deionized water and washed with 500 µL of 0.5% TFA in deionized water. A total protein amount of 100 µg was diluted with 0.5% TFA in deionized water for a final volume of 500 µL and loaded onto the cartridge. Sorbent material was washed with 500 µL of 0.5% TFA in deionized water. Sample material was eluted with 100 µL of 30% ACN with 0.1% FA in deionized water. This step was repeated for a total of 200 µL of eluate, which was lyophilized to dryness prior to analysis.

6.1.2 Development of Data Acquisition Method and Analytical Parameter Optimization

Mixed-source samples containing various protein quantities of semen and saliva were prepared. Neat biological fluid was quantitated using the BCA assay previously described. Two sets of samples were generated. First, a saliva major contributor with a semen minor contributor, with total protein quantities ranging from 80 µg to 99 µg for the major contributor. All mixture samples contained a total of 100 µg total protein. Second, a semen major contributor with a saliva minor contributor series was created, prepared in an equivalent manner.

An inclusion list containing desirable peptide biomarkers was generated from data acquired during the population study, discussed in the section below. The inclusion list was prepared in Skyline Proteomics Environmental software v. 20.2.0.343 (MacCoss Labs, University of Washington) and imported into Xcaliber™ control software on the Q Exactive™ Quadrupole-Orbitrap™ mass spectrometer (Thermo Scientific, Waltham, MA). Analytical parameters on the Q Exactive™ Quadrupole-Orbitrap™ mass spectrometer were optimized using single source biological fluids. Recommended instrument settings outlined by the University of Washington Proteomics Resource (UWPR) were used as a starting point for parameter optimization.

6.1.3 Population Study and Performance Assessment

Twenty semen samples were purchased from Innovative Research and thirty semen samples were purchased from Lee Biosolutions, for a total of 50 semen donors. An additional 5 semen samples from vasectomized donors were purchased from Lee Biosolutions. Twenty-five saliva samples were purchased from Innovative Research and Lee Biosolutions for a total of 50 saliva donors. Twenty-five vaginal swabs were purchased from Lee Biosolutions and seven vaginal swabs were collected under IRB, for a total of 32 vaginal swab donors. Regardless of donor source, received vaginal swabs were stored at -80 ºC upon receipt. Population samples were purchased due to SARS-CoV-2 restrictions put in place for collection of biological samples under the approved IRB. Performance assessment samples were divided into six categories: sensitivity, aged, substrates, contaminants, mixtures, and simulated sexual assault kit. All samples were prepared in triplicate as described in **Table 6.1**. Substrates were washed with Tide Natural detergent in cold water and dried prior to use to ensure consistency between samples.

Population samples were quantitated using the BCA assay and extracted using the Bond Elut Plexa plate protocol as described above. Performance assessment samples were extracted using Waters Oasis HLB cartridges (Milford, MA), the manufacturer equivalent of the Bond Elut Plexa chemistry. This switch was induced by time constraints and backordered consumables due to SARS-CoV-2 testing. 100 µg total protein was used for analysis. Extraction procedures between the two manifold cartridges were equivalent, with the exception of increasing the organic elution

solvent to 35% ACN with 0.1% FA with the Waters Oasis HLB cartridges, per manufacturer recommendations. Eluate was lyophilized to dryness for LC-MS/MS analysis.

Table 6.1. Performance assessment sample preparation parameters. Room temperature (RT). Samples were prepared in triplicate, with the exception of laundered sheets (substrates).

Category	Sample	Description
Negative Control	Blank Cotton Swab	No body fluid applied
	Blank Vaginal Swab	No semen or saliva applied
	Blank Oral Swab	No semen or vaginal fluid applied
Sexual Assault Kit	Vaginal Swab	Vaginal swab + 2.5 uL Semen
	Oral Swab	Oral swab + 2.5 uL Semen
	Rectal Swab	Rectal swab + 2.5 uL Semen
	Vaginal Swab	Vaginal swab + 10 uL Saliva
	Breast Swab	Cotton swab + 2 drops water, rubbed on skin for 10 seconds + 10 uL Saliva
	Rectal Swab	Rectal swab + 10 uL Saliva
Mixtures	Mixture 1	Half swab + 10 uL Saliva + 2.5 uL Semen
	Mixture 2	Half swab + 50 uL Saliva + 2.5 uL Semen
	Mixture 3	Half swab + 10 uL Saliva + 25 uL Semen
	Mixture 4	Half swab + 10 uL Saliva + 10 uL Semen
Substrates	Cotton Underwear	Worn by female for 8 hours + 2.5 uL Semen applied to crotch area
	Denim	100% Cotton denim jeans + 2.5 uL Semen, dried RT
	Denim	100% Cotton denim jeans + 10 uL Saliva, dried RT
	Polyester Sheet	100% polyester bed sheet + 2.5 uL Semen, dried RT
	Polyester Sheet	100% polyester bed sheet + 10 uL Saliva, dried RT
	Poly Blend Sheet	60% poly 40% cotton sheet + 2.5 uL Semen, dried RT
	Poly Blend Sheet	60% poly 40% cotton sheet + 10 uL Saliva, dried RT
	Laundered Polyester Sheet	1 mL semen, dried RT, laundered with Tide Natural
	Laundered Poly Blend Sheet	1 mL semen, dried RT, laundered with Tide Natural
	Bed Sheet Controls	Cutting of laundered bedsheet 2 ft. from semen stain
Contaminants	Lubricant	Vaginal swab, swab half + 2.5 uL Semen + 5 uL water-based lubricant
	Lubricant	Vaginal swab, swab half + 2.5 uL Semen + 5 uL silicon-based lubricant
	Lubricant	Vaginal swab, swab half + 2.5 uL Semen + 5 uL natural-based lubricant
	10% Bleach	10% bleach prepped in water, cotton swab + 25 uL 10% bleach + 2.5 uL Semen
	10% Bleach	10% bleach prepped in water, cotton swab + 25 uL 10% bleach + 10 uL Saliva
	Dish Soap	1 drop Dawn blue in 1 mL water, cotton swab + 25 uL soap solution + 2.5 uL Semen
	Dish Soap	1 drop Dawn blue in 1 mL water, cotton swab + 25 uL soap solution + 10 uL Saliva
	Menstrual Blood	Vaginal swab procured during menses, swab half
	Menstrual Blood	Vaginal swab procured during menses, swab half + 2.5 uL Semen
	Menstrual Blood	Vaginal swab procured du menses, swab half + 10 uL Saliva
Aged Swabs	Vaginal Swab	Collection +2 days, swab half
		Collection +3 days, swab half
		Collection +7 days, swab half
		Collection +30 days, swab half
	Semen + Vaginal Swab	Vaginal Swab Collection, swab half + 2.5 uL Semen +2 days
		Vaginal Swab Collection, swab half + 2.5 uL Semen +3 days
		Vaginal Swab Collection, swab half + 2.5 uL Semen +7 days
		Vaginal Swab Collection, swab half + 2.5 uL Semen +30 days
	Saliva + Vaginal Swab	Vaginal Swab Collection, swab half + 10 uL Saliva +2 days
		Vaginal Swab Collection, swab half + 10 uL Saliva +3 days
		Vaginal Swab Collection, swab half + 10 uL Saliva +7 days
		Vaginal Swab Collection, swab half + 10 uL Saliva +30 days
	Semen	Half swab + 2.5 uL Semen + 2 days
		Half swab + 2.5 uL Semen + 3 days
		Half swab + 2.5 uL Semen + 7 days
		Half swab + 2.5 uL Semen + 30 days
	Saliva	Half swab + 10 uL saliva + 2 days
		Half swab + 10 uL saliva + 3 days
		Half swab + 10 uL saliva + 7 days
		Half swab + 10 uL saliva + 30 days
Sensitivity Swabs	Seminal Fluid	1/2 swab + 150 uL 1:100 Semen
		1/2 swab + 150 uL 1:1,000 Semen
		1/2 swab + 150 uL 1:2,000 Semen
		1/2 swab + 150 uL 1:5,000 Semen
		1/2 swab + 150 uL 1:10,000 Semen
	Saliva	1/2 swab + 150 uL 1:2 Saliva
		1/2 swab + 150 uL 1:10 Saliva
		1/2 swab + 150 uL 1:50 Saliva
		1/2 swab + 150 uL 1:100 Saliva
		1/2 swab + 150 uL 1:500 Semen
	Vaginal Fluid	1/2 swab + 150 uL 1:2 Vaginal Extract (full swab + 500 uL water)
		1/2 swab + 150 uL 1:10 Vaginal Extract (full swab + 500 uL water)
		1/2 swab + 150 uL 1:50 Vaginal Extract (full swab + 500 uL water)
		1/2 swab + 150 uL 1:100 Vaginal Extract (full swab + 500 uL water)
		1/2 swab + 150 uL 1:500 Vaginal Extract (full swab + 500 uL water)

6.1.4 Protein Identification by LC-MS/MS

Lyophilized samples were reconstituted in 50 µL of 2% ACN with 0.1% FA in deionized water. Acquisition was performed using 5 µL of sample per injection on a Q Exactive™ Quadrupole-Orbitrap™ mass spectrometer (Thermo Scientific, Waltham, MA). A Waters® ACQUITY UPLC BEH C18 analytical column (1 x 100 mm, 1.7 µm, 130Å) was used for separation (Waters, Milford, MA). Mobile phase A consisted of water with 0.1% FA and mobile phase B consisted of ACN with 0.1% FA. Separation initiated at 5% B followed by a linear 5-35% B gradient over 17 minutes, a 3-minute hold at 90% B, followed by a 5-minute re-equilibration at a 0.40 mL/min flow rate and column temperature of 50 °C.

Acquired data was searched using Protein Metrics Byonic™ (v. 3.8) software (Protein Metrics Inc., Cupertino, CA). A no enzyme search method was created with the following criteria: for sample digestion parameters, the cleavage site was left blank, the cleavage side was set to C-terminal, the digestion specificity as nonspecific (slowest), and the number of missed cleavages was set to 2; for instrument parameters, the precursor mass tolerance was input as 5 ppm, fragmentation type was set to QTOF/HCD, fragment mass tolerance input as 10 ppm, and recalibration selected as none; for modification parameter, the total common max was input as 2 and the total rare max was input as 1, with pyro-Glu, oxidation, and deamidation set as fixed modifications. No parameters were input for glycans, S-S Xlink bonded pairs, or inclusion. Under the advanced option, the maximum precursor mass was set to 4,000, with the maximum number of precursors per MS2 input as 1, and smoothing width set to 0.01 m/z. The whole human proteome (UniProt ID UP000005640) was utilized for data searches during preliminary assessments. A focused database containing the amino acid sequences of selected protein biomarkers was generated for performance assessment data acquisition. Data files searched against either the whole human proteome or focused database were further analyzed using Protein Metrics Byologic® (v. 3.9-32) software (Protein Metrics, Inc.). Searched Byonic™ files were imported into Byologic® for additional peptide sequence elucidation.

6.2 Results and Discussion

6.2.1 Sample Processing Development and Comparison

When analyzing complex matrices such as seminal fluid, saliva, and vaginal fluid, pretreatment steps are necessary to isolate peptide material from unwanted salts, intact proteins, or cellular debris, as well as environmental contaminants. Traditional sample processing techniques for cleanup employ reverse phase solid phase extraction. A previously developed Waters Oasis® HLB cartridge extraction protocol using a positive pressure manifold [169] was replaced with an improved preparation protocol. Several preparation protocols were developed for comparison, including two automated reverse phase extraction approaches on the AssayMAP Bravo liquid handling platform, two manifold solid phase extraction plate-based approaches, centrifugal filtration methods, and a precipitation method. These various approaches were evaluated using a set of single source body fluid matrix samples. Data was acquired using the Thermo Q Exactive™ Quadrupole-Orbitrap™ mass spectrometer (herein referred to as the Q Exactive). In general, high confident identifications were observed for multiple salivary markers including statherin (STAT), submaxillary gland androgen-regulated protein 3B (SMR3B), histatin-1 (HIS1) and -3 (HIS3), cystatin-SN (CST1), and multiple proline-rich protein isoforms (PRP_1, PRP_2, PRP_3, PRP_4, and PRPC). Several seminal fluid biomarkers including semenogelin 1 (SEMG1), semenogelin 2 (SEMG2), prostate specific antigen (KLK3), and prostatic acid phosphatase (PPAP) were detected. Multiple vaginal fluid proteins including cornulin (CRNN), suprabasin (SBSN), and involucrin (IVL) were identified as well.

Developing a single uniform preparation protocol compatible with all matrices of interest proved challenging. Of the preparation protocols initially developed, both centrifugal filtration as well as the protein precipitation protocols were removed from further consideration. Centrifugal filtration methods utilized molecular weight cutoff (MWCO) filters ranging from 10,000 to 50,000 Daltons (Da). This methodology was consistently unable to identify the majority of seminal fluid markers (**Figure 6.1**), with dropout of proteins KLK3 and PPAP observed. Regarding the protein precipitation protocol, when lower quantities (*i.e.,* below 10 µg of total protein) were prepared, no

precipitation occurred, leading to large amounts of 10 kDa or greater proteins binding to the analytical column.

Two automated approaches using the AssayMAP Bravo liquid handling platform were developed (C18 and RP-S microextraction cartridges) as well as two manifold-based approaches (Agilent Bond Elut Plexa and Bond Elut Plexa PCX plate-based cartridges). Here, the C18 microextraction cartridges consistently clogged when saliva was applied to the sorbent material, to the extent that clogged cartridges did not produce eluate for analysis. This occurred regardless of protein quantities applied (50 to 150 µg) and is contributed to the viscosity of the biological matrix, even once cellular material was removed. In contrast, the RP-S cartridges did not exhibit the same issue, regardless of biological fluid evaluated (**Figures 6.2-6.4**). Regarding the manifold-based procedures, the Bond Elut Plexa consistently provided greater coverage of protein biomarkers, albeit at lower intensities than the Plexa PCX sorbent, as exhibited in the saliva matrix in **Figure 6.5**. Furthermore, the Bond Elut Plexa displayed greater coverage of peptide signatures (**Figure 6.6**). Moving forward, the automated RP-S microextraction cartridge and Bond Elut Plexa manifold protocols were selected for further assessment using multiple biological replicates.

Sample processing protocols were compared using consistent protein quantities to assess biomarker identification rates. Four replicate samples from two individuals for a total of eight samples per preparation protocol (RP-S and Bond Elut Plexa) were prepared and analyzed on the Q Exactive. In summary, the Bond Elut Plexa plate was selected as the optimal sample processing protocol for the remainder of the study. Biomarker identification in vaginal fluid was generally comparable between the two processing protocols (**Figure 6.7**). Cornulin and suprabasin were consistently identified whereas involucrin was absent from two of four replicates from the Donor #1 Bond Elut Plexa preparation and one of four replicates of Donor #1 RP-S cartridge preparation. All seminal fluid biomarkers were identified across all samples and preparation protocols (**Figure 6.8**). Slightly elevated peak intensities were observed for SEMG1 with the Bond Elut Plexa preparation whereas SEMG2, KLK3, and PPAP intensities were slightly elevated with the RP-S cartridge protocol. Similar to vaginal fluid and seminal fluid, most salivary biomarkers were detected across

all individuals and sample preparation protocols but with varying signal intensities (**Figure 6.9**). However, statherin and histatin-1, which are critical for saliva identification, were absent from a subset of the replicates prepared from Donor #2 using the RP-S cartridges (**Figure 6.10**). Since the detection of these proteins is critical for a confirmatory serological identification of saliva, the Bond Elut Plexa plate was selected for the remainder of research development.

The unbalanced distribution of detected proteins is attributed to the various sorbent material chemistries of the manifold plate protocols. The Bond Elut Plexa PCX plate utilizes strong cation ion exchange chemistries and was designed for the concentration and retention of alkaline analytes. In contrast, the Bond Elut Plexa employs a standard nonpolar sorbent, allowing the analyst to control retention based on the type and pH of mobile phase passed over the sorbent column.

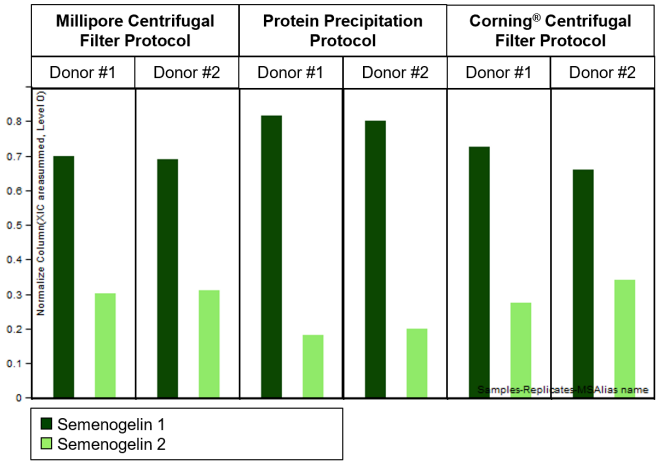


Figure 6.1. Area comparison between Millipore centrifugal filters, protein precipitation, and Corning® centrifugal filters for the detection of seminal fluid from two donors. Proteins KLK3 and PPAP were not detected using these preparation protocols.

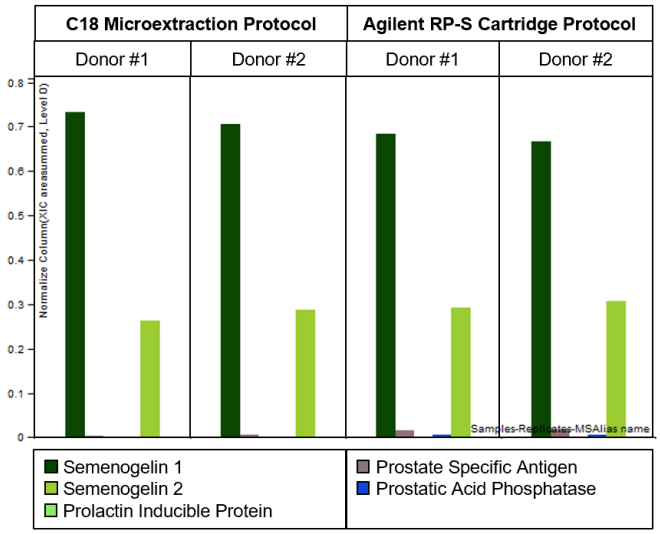


Figure 6.2. Area comparison between C18 and RP-S microextraction cartridges using the AssayMAP liquid handling platform for the detection of seminal fluid from two donors.

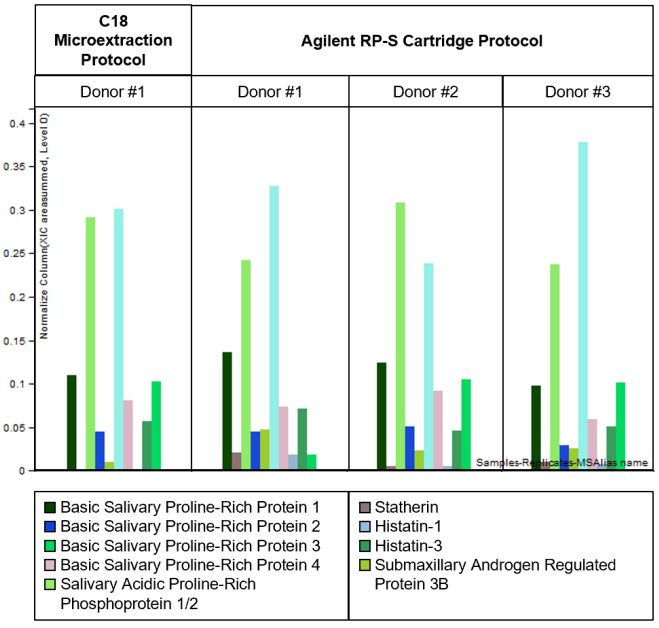


Figure 6.3. Area comparison between C18 and RP-S microextraction cartridges using the AssayMAP liquid handling platform for the detection of saliva from three donors. Two samples prepared with C18 cartridges did not produce eluate for analysis and is reflected by the single sample set reported.

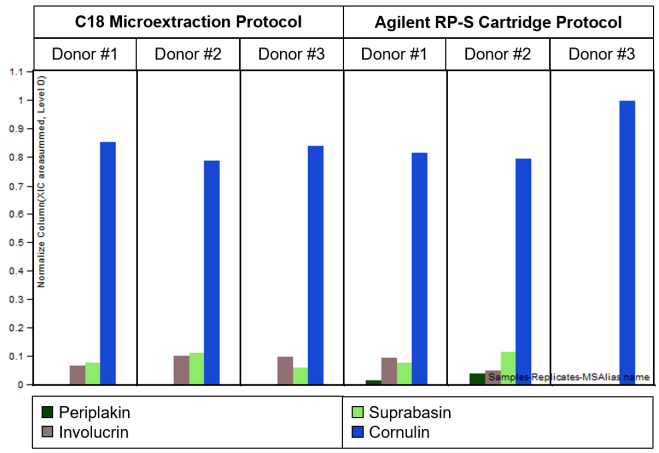


Figure 6.4. Area comparison between C18 and RP-S microextraction cartridges using the AssayMAP liquid handling platform for the detection of vaginal fluid from three donors.

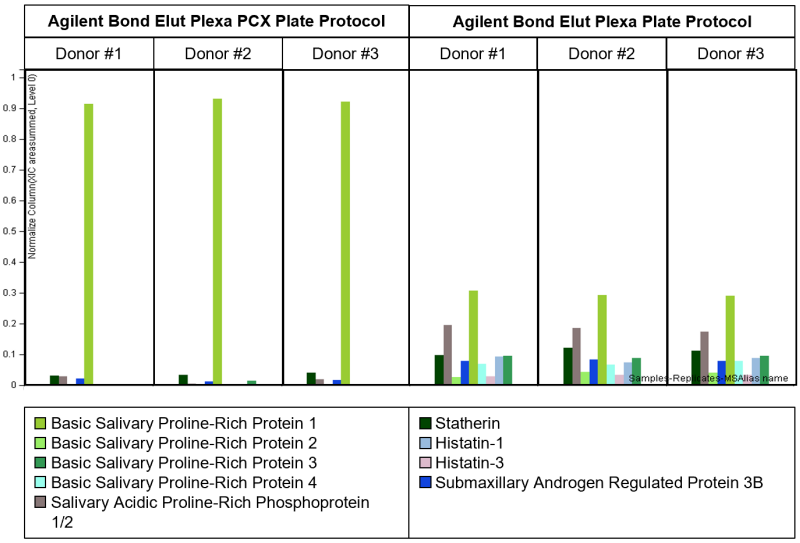


Figure 6.5. Area comparison between Bond Elut Plexa PCX and Bond Elut Plexa plates on a positive pressure manifold for the detection of saliva from three donors.

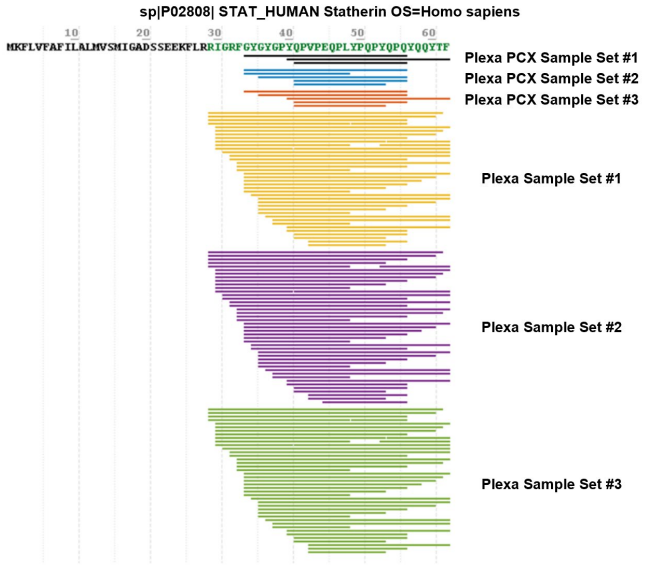


Figure 6.6. Peptide coverage of salivary protein statherin for Bond Elut Plexa PCX (black, blue, and red bars) and Bond Elut Plexa (yellow, purple, and green bars) using triplicate samples.

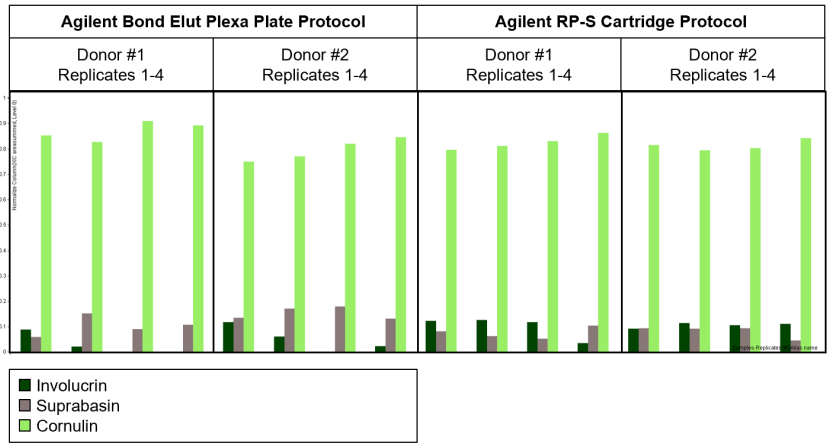


Figure 6.7. Area comparison between Bond Elut Plexa plate and RP-S microextraction cartridge protocol for the detection of vaginal fluid from two donors prepared in quadruplicate.

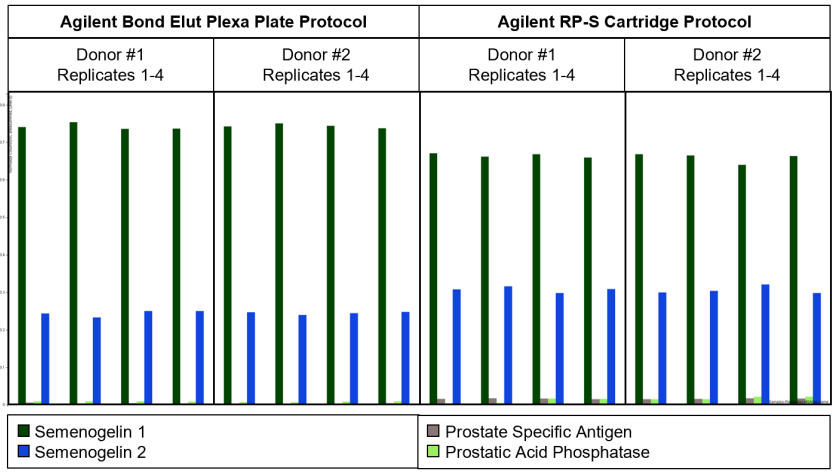


Figure 6.8. Area comparison between Bond Elut Plexa plate and RP-S microextraction cartridge protocol for the detection of seminal fluid from two donors prepared in quadruplicate.

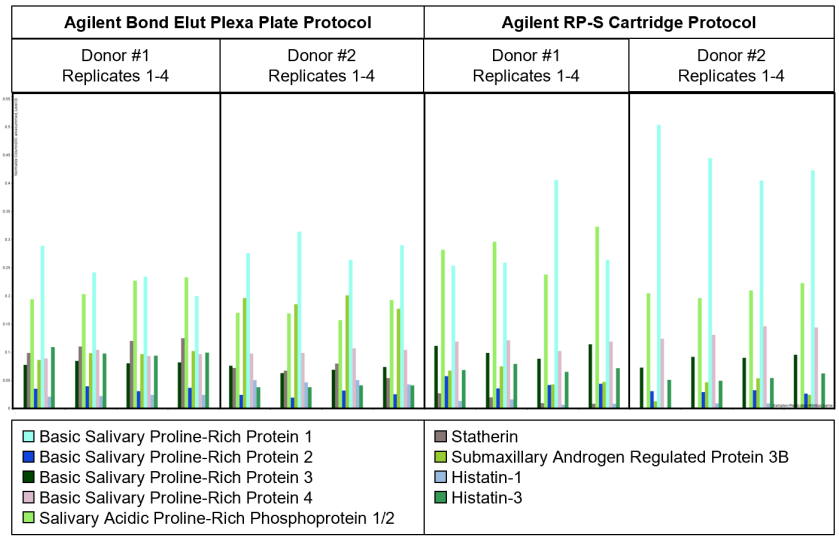


Figure 6.9. Area comparison between Bond Elut Plexa plate and RP-S microextraction cartridge protocol for the detection of saliva from two donors prepared in quadruplicate.

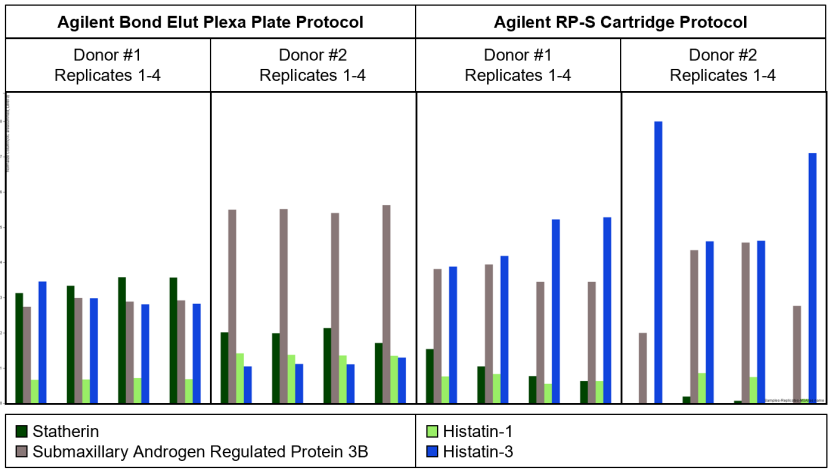


Figure 6.10. Area comparison between Bond Elut Plexa plate and RP-S microextraction cartridge protocol for the detection of saliva with focus on highly specific salivary biomarkers. Elevated statherin (dark green) and histatin-1 (light green) bars were observed for Bond Elut Plexa plate protocol.

6.2.2 Development of Data Acquisition Method and Analytical Parameter Optimization

A previously designed high-flow (0.40 mL/minute flow rate) analytical method that employed a 2.1 mm internal diameter analytical column [169] was transferred to a 1 mm internal diameter microbore analytical column. The original method was developed for a quadrupole time-of-flight (QTOF) mass spectrometer and was transferred to the Q Exactive. A Waters® BEH C18 analytical column (300Å, 1.7 µm, 1 mm X 150 mm) was utilized to develop the initial run conditions; however, in order to increase throughput, a Waters® BEH C18 1 x 100 mm analytical column was employed to create the final 25-minute analytical run (**Table 6.2**). In total, 35 minutes of run time per sample injection was saved through optimization of the analytical method. Representative chromatography for each target biological fluid can be seen in **Figure 6.11**. Analytical columns are manufactured in a multitude of diameters, lengths, and stationary phase materials. Column selection has the greatest effect on efficiency and speed of analysis, as seen by the shortening of run time during optimization. Although the stationary phase material remained the same, by decreasing the internal diameter, the flow rate was subsequently lowered. These reductions allowed for increased chromatographic efficiency and improved resolution.

Table 6.2. Separation gradient of 25-minute analytical method on the Q Exactive analytical platform.

Time (min)	Flow (mL/min)	%B
0.00	0.040	5.0
17.40	0.040	35.0
17.50	0.040	90.0
20.00	0.040	90.0
20.10	0.040	5.0
20.25	0.150	5.0
24.25	0.150	5.0
24.5	0.040	5.0
25	Stop	5.0
Column Temperature		60 °C

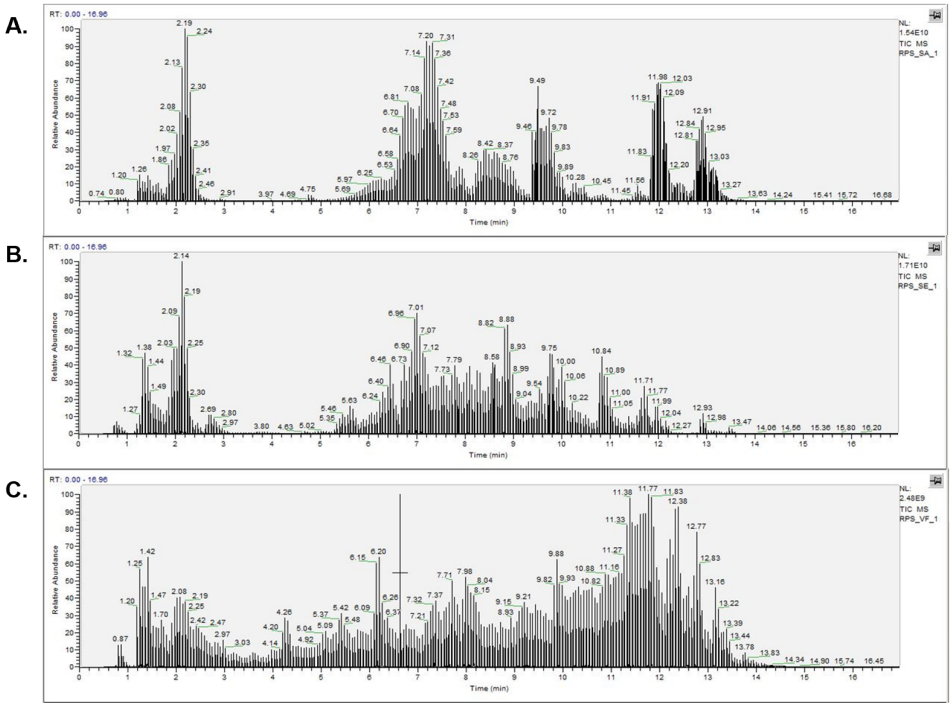


Figure 6.11. Representative chromatography for (A) saliva, (B) seminal fluid, and (C) vaginal fluid biological matrices on the Q Exactive analytical platform.

The Q Exactive was selected for this research given its enhanced sensitivity limits when compared with the original QTOF platform available. Given the inherent construction of the orbitrap mass analyzer, the selection and transmission of precursor to product ions was of interest. Optimizing acquisition parameters for the Q Exactive was conducted to ensure optimal detection of fragmentation products was achieved (**Table 6.3**). Low-abundance targets, such as salivary

protein statherin, were found to have increased signal regardless of matrix complexity. In order to ensure the highest ionization efficiency, source conditions including sheath gas flow rate, nozzle voltage, and gas temperatures were evaluated for optimal performance. The final source and tune parameters for the analytical method are outlined in **Table 6.4**. Overall, a majority of parameters were unchanged from those recommended by UWPR, with the exception of the Normalized Collision Energy ((N)CE) and Minimum Automatic Gain Control (AGC) Target.

To preferentially select desired peptide biomarkers further, a targeted acquisition method was developed for use during the performance assessment. The peptide match parameter under dd settings of the Q Exactive was set as 'on' and a peptide inclusion list was generated in Skyline software. Skyline is a vendor-neutral software that assisted with organizing selected peptide biomarkers, their m/z ratio, and associated retention times into a file type that could be imported into the Thermo Xcaliber™ software. The inclusion list was input into the targeted acquisition; however, the Q Exactive MS/MS parameters were still able to detect peptides not within the inclusion list, but preference was set for the inclusion list peptides. Although the Q Exactive prioritized the inclusion list biomarkers, it was of interest to gather additional information outside of the inclusion list for future method validation and additional studies regarding biomarker specificity. A series of mixed-source samples were prepared to further evaluate the use of a targeted acquisition method (**Figure 6.12**). Although the targeted and data dependent acquisition methods behaved similarly, the detected of additional peptide markers from minor contributor biological fluids was observed. For example, when 1 µg of saliva was mixed with 99 µg of semen, one additional peptide was identified with the targeted acquisition method. The targeted approach routinely identified more peptide biomarkers for a saliva minor contributor in comparison with the data dependent acquisition method. Therefore, the peptide biomarkers of the inclusion list were verified with reference material and the targeted approach was utilized for the performance assessment portion of this research. Verification of peptide mass, charge, retention time, and MS2 spectrum quality were all taken into consideration prior to generating the final inclusion list.

Historically, the relationship between chromatography and depth of coverage achieved via mass spectrometry was an inverse relationship, with a trade-off for speed of separation in relation to depth of proteome coverage considered. However, with advancements in analytical instrumentation, separation science, and software applications, the gap between sacrificing proteome coverage for speed of analysis has been minimized. The proteome is complex in nature and introduces inherent analytical challenges, particularly with untargeted characterization. Enhancing the chromatographic separation of protein constituents therefore increases the efficiency of detection using mass spectrometry techniques, allowing for greater depth of proteome coverage.

Table 6.3. MS and MS/MS performance parameters selected on the Q Exactive analytical platform.

MS Settings	
Resolution	70,000
AGC Target	3.00E+06
Maximum IT	50 ms
Scan Range	350 to 2000
dd-MS^2 / dd-SIM Settings	
Microscans	1
Resolution	17,500
AGC Target	1.00E+05
Maximum IT	100 ms
Loop Count	10
Isolation Window	2.2 m/z
(N)CE	27
dd Settings	
Minimum AGC Target	1.00E+03
Peptide Match	Preferred
Exclude Isotope	On
Dynamic Exclusion	6 s

Table 6.4. Source parameters optimized on the Q Exactive analytical platform.

Scan Range	400 to 2,000 m/z
Polarity	Positive
Resolution	70,000
Sheath Gas Flow Rate	35
Auxiliary Gas Flow Rate	10
Auxiliary Gas Temperature	200 °C
Sweep Gas Flow Rate	1
Spray Voltage	3.5 kV
Capillary Temperature	250 °C

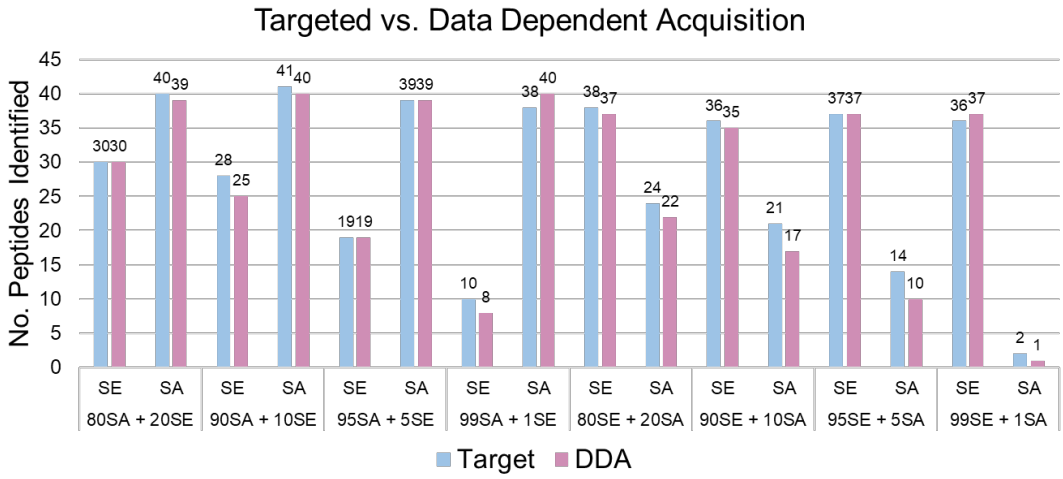


Figure 6.12. Comparison between a targeted acquisition (blue bars) and a data dependent acquisition (purple bars) method using a series of mixed-source samples. Samples were prepared by total protein amount and are represented on the x-axis. Methods were compared according to the number of selected peptides identified.

6.2.3 Biomarker Selection and Population Study

In order to accurately assess the developed analytical method and preliminary biomarkers, a population assessment was performed. This experiment was designed to conclude if a candidate biomarker was routinely identified within a larger sample population, further supporting its use as a specific biomarker for body fluid identification. For this portion of the study, an evaluation was conducted at the protein level. Parameters taken into consideration for overall assessment were percent coverage of total amino acid sequence, number of unique peptides, signal intensity, and best score. Percent coverage describes the portion of the protein sequence identified by the software. Using the complete amino acid sequence, percent coverage represents the number of

amino acids detected in relation to the full sequence. The number of unique peptides represents the number of peptide spectrum matches and does not include duplicate identifications made by the software. Signal intensity simply describes the total intensity of the MS/MS peaks produced. And lastly, best score represents the quality of the peptide spectrum match and serves as an indicator of correctness.

In summary, purchased semen samples from healthy male donors displayed an average donor age of 31.6 years, with an age range of 18 to 54 years. A majority (80%) of semen samples were collected from Caucasian donors, with 3 donors of African American descent, 3 donors of Hispanic descent, 2 donors of Asian descent, and 2 donors from Asian/Caucasian descent. Semen purchased from vasectomized male donors displayed an average donor age of 51.6 years, with an age range of 39 to 61 years. All vasectomized samples were donated by individuals of Caucasian descent. Overall, this approach consistently identified SEMG1, SEMG2, KLK3, and PPAP across the population. Summary search results for non-vasectomized and vasectomized sample sets can be found in **Tables 6.5 and 6.6**, respectively, with complete detailed search results outlined in **Appendix K**. Only a single instance of protein dropout was observed within the sample population (sample ID 31978-07), with KLK3 not detected in this particular sample. Otherwise, all non-vasectomized and vasectomized samples were positive for the four target protein biomarkers stated previous. There was little difference between the two sample populations. A slightly greater average percent coverage was exhibited by the non-vasectomized population for biomarkers KLK3 and PPAP, displaying a 15.18% and 15.48% increase, respectively. There was no change in percent coverage observed for SEMG2 and a minute 2.48% increase in coverage for vasectomized samples for SEMG1. A similar trend was illustrated for the average number of unique peptides identified by the bioinformatics software. Overall, the developed sample preparation protocol was able to characterize semen-specific protein biomarkers in both non-vasectomized and vasectomized samples. These results were used to identify surrogate peptides that are consistently identified across all individuals, as described in the next section.

Purchased saliva samples from healthy donors displayed an average donor age of 40.38 years, an age range of 19 to 70 years, with 48% female and 52% male donors represented. Racial decent was not provided for this sample set. Similar to the semen samples, consistency in protein coverage was observed with multiple protein biomarkers being confidently identified in the saliva sample population. Summary search results can be found in **Table 6.7** with complete detailed search results outlined in **Appendix L**. Several proteins were identified across all samples (*e.g.,* STAT, SMR3B, and HIS1). Other targets, such as HIS3 and CST1 were identified in 64% and 68% of samples, respectively. While more protein loss was observed with the saliva population in comparison with the semen population, it is likely that this is a function of the data dependent acquisition approach utilized. A targeted method would likely detect even the proteins which were not identified in each sample. This is due to certain salivary biomarkers being of lower abundance in relation to the limited number of precursor ions which the instrument can measure in parallel.

Unexpected results were observed for the protein alpha amylase-1. Although it was detected in 90% of the sample population, this protein was characterized with low percent coverage and by a single unique peptide. It was anticipated that the source parameters selected were not adequate at producing complete fragmentation of this particular protein. However, given the poor specificity of this protein for the saliva biological matrix, parameters were not changed in order to continuously identify more characteristic salivary biomarkers. In summary, the developed sample preparation protocol was able to characterize saliva-specific protein biomarkers. As with the semen targets, these results will be used to identify signature peptides that are both sensitive and specific for the creation of a targeted analytical method.

A total of 32 individuals were retained for the vaginal fluid population assessment. In summary, purchased vaginal swab samples (25 samples) from healthy donors displayed an average donor age of 39.16 years, with an age range of 23 to 61 years. All vaginal swab samples were collected from Caucasian donors. No identifying information was recorded for IRB collected samples (7 samples). Summary search results can be found in **Table 6.8**, with complete detailed search results outlined in **Appendix M**. Overall, no candidate protein biomarkers were consistently

detected in all samples of this population; however, cornulin displayed the highest occurrence rate with accurate detection in 31 of 32 samples evaluated. Furthermore, this protein demonstrated an average coverage rate of 52%. Secondary candidate biomarkers identified during sample preparation protocol development, suprabasin and involucrin, exhibited favorable population occurrence of 78% and 81%, respectively. The protein periplakin, a biomarker included within the previously described bottom up proteomic method (Chapters 2 and 3), displayed poor detection and protein coverage, prompting reevaluation of biomarker inclusion. With previous experimentation conducted on vaginal fluid, the lower sample occurrence, percent coverage, and number of unique peptides was anticipated to be less desirable than the results of seminal fluid and saliva evaluation. Nevertheless, the proteins detected are consistent with those of previous studies, indicating the reproducibility of biomarker recovery and identification. Of the 32 samples evaluated within this population assessment, the four candidate biomarkers were not identified within one sample (sample ID C1). As with the semen and saliva targets, these results will be used to identify surrogate peptides that are both sensitive and specific for the creation of a targeted analytical method.

A piece of data worthy of consideration is the detection of protein biomarker glycodelin in a single sample of the vaginal swab population (sample ID 5488). Although detected in only a single sample, this identification is promising for future development of top down proteomic methodologies. This particular protein is expressed in multiple body fluids, such as vaginal and seminal fluid, but displays variation in N-linked glycosylation patterns that can be exploited for fluid specificity [170]. With a challenging body fluid such as vaginal fluid, targeting additional biological properties, such as glycan chains, may be beneficial to identifying specific protein biomarkers, and therefore, increasing the specificity of the overall proteomic assay.

Table 6.5. Descriptive statistics of non-vasectomized semen population.

		% Coverage	# Unique Peptides	Intensity	Best Score	% Samples Detected
SEMG1	Minimum	71.43	234	7.41E+08	1016.10	100%
	Maximum	95.02	465	9.33E+09	1345.90	
	Median	87.01	367	3.66E+09	1157.50	
	Average	86.83	360	3.91E+09	1165.05	
SEMG2	Minimum	65.81	153	5.57E+08	1112.30	100%
	Maximum	85.60	331	4.93E+09	1491.40	
	Median	78.52	264	2.52E+09	1359.00	
	Average	77.87	261	2.57E+09	1348.34	
KLK3	Minimum	0.00	0	0.00E+00	0.00	98% (49/50)
	Maximum	44.83	33	2.25E+08	705.00	
	Median	34.87	11	2.12E+07	469.40	
	Average	32.27	12	4.43E+07	443.41	
PPAP	Minimum	3.10	1	1.43E+05	206.50	100%
	Maximum	53.63	64	4.32E+08	698.40	
	Median	25.39	11	3.08E+07	439.00	
	Average	26.98	16	5.70E+07	452.17	

Table 6.6. Descriptive statistics of vasectomized semen population.

		% Coverage	# Unique Peptides	Intensity	Best Score	% Samples Detected
SEMG1	Minimum	87.88	323	2.42E+09	1114.90	100%
	Maximum	90.69	438	4.48E+09	1180.30	
	Median	89.83	356	3.21E+09	1146.50	
	Average	89.31	372	3.33E+09	1147.86	
SEMG2	Minimum	74.57	210	1.63E+09	1350.80	100%
	Maximum	82.65	308	2.72E+09	1391.20	
	Median	76.46	299	2.65E+09	1375.90	
	Average	77.87	269	2.30E+09	1372.38	
KLK3	Minimum	12.26	2	7.74E+05	137.50	100%
	Maximum	23.75	8	1.27E+07	483.60	
	Median	16.86	5	5.24E+06	460.40	
	Average	17.09	5	6.60E+06	400.76	
PPAP	Minimum	3.63	1	3.76E+05	174.00	100%
	Maximum	20.98	9	1.08E+07	428.90	
	Median	11.66	3	3.17E+06	307.60	
	Average	11.50	4	4.34E+06	301.86	

Table 6.7. Descriptive statistics of saliva population.

		% Coverage	# Unique Peptides	Intensity	Best Score	% Samples Detected
PRP_1	Minimum	0.00	0	0.00E+00	0.00	98% (49/50)
	Maximum	95.92	213	2.01E+09	1115.80	
	Median	88.45	67	2.44E+08	750.95	
	Average	65.31	83	4.61E+08	730.27	
PRP_2	Minimum	0.00	0	0.00E+00	0.00	96% (48/50)
	Maximum	95.67	140	5.78E+08	1068.00	
	Median	47.84	23	4.41E+07	591.00	
	Average	46.64	33	1.05E+08	587.88	
PRP_3	Minimum	6.84	1	2.79E+05	125.50	100%
	Maximum	81.55	96	7.75E+08	1051.10	
	Median	67.80	44	1.79E+08	783.30	
	Average	61.67	45	2.28E+08	749.83	
PRP_4	Minimum	0.00	0	0.00E+00	0.00	96% (48/50)
	Maximum	85.42	94	8.56E+08	1068.20	
	Median	45.97	30	7.76E+07	569.85	
	Average	44.74	34	1.38E+08	589.48	
PRPC	Minimum	8.43	4	5.33E+06	274.80	100%
	Maximum	90.36	161	1.65E+09	1105.80	
	Median	90.36	91	5.33E+08	734.35	
	Average	80.51	90	5.90E+08	743.71	
STAT	Minimum	43.55	27	1.44E+07	246.80	100%
	Maximum	69.35	176	5.48E+08	623.00	
	Median	60.49	96	1.44E+08	478.90	
	Average	59.93	94	1.62E+08	468.67	
SMR3B	Minimum	65.82	29	2.08E+07	429.80	100%
	Maximum	72.15	140	1.18E+09	635.20	
	Median	72.15	79	4.06E+08	538.40	
	Average	71.32	81	4.58E+08	539.35	
HIS1	Minimum	24.56	4	9.17E+05	273.20	100%
	Maximum	66.67	117	3.03E+08	605.20	
	Median	47.37	44	4.27E+07	495.10	
	Average	51.65	49	7.59E+07	481.03	
HIS3	Minimum	0.00	0	0.00E+00	0.00	64% (32/50)
	Maximum	62.74	31	1.30E+08	683.30	
	Median	43.14	4	1.82E+06	376.00	
	Average	33.49	5	9.75E+06	302.25	
CST1	Minimum	0.00	0	0.00E+00	0.00	68% (34/50)
	Maximum	30.50	13	1.26E+07	520.10	
	Median	11.35	2	9.56E+05	330.20	
	Average	10.70	3	1.72E+06	258.02	
AMY1A	Minimum	0.00	0	0.00E+00	0.00	90% (45/50)
	Maximum	21.33	16	3.64E+06	555.00	
	Median	2.15	1	4.29E+05	351.65	
	Average	3.18	1	5.96E+05	315.77	

Table 6.8. Descriptive statistics of vaginal swab population.

		% Coverage	# Unique Peptides	Intensity	Best Score	% Samples Detected
CRNN	Minimum	0.00	0	0.00E+00	0.00	96.8% (31/32)
	Maximum	75.76	95	3.85E+08	1105.70	
	Median	57.58	56	9.72E+07	944.85	
	Average	52.02	55	1.14E+08	792.23	
SBSN	Minimum	0.00	0.00	0.00E+00	0.00	78.1% (25/32)
	Maximum	57.29	35.00	3.85E+07	683.60	
	Median	12.03	5.00	2.96E+06	224.85	
	Average	13.98	7.66	6.38E+06	267.29	
IVL	Minimum	0.00	0.00	0.00E+00	0.00	81.2% (26/32)
	Maximum	45.47	45.00	6.61E+07	675.40	
	Median	8.72	3.50	2.55E+06	274.95	
	Average	13.29	8.00	7.03E+06	267.00	
PPL	Minimum	0.00	0.00	0.00E+00	0.00	46.8% (15/32)
	Maximum	5.01	5.01	6.38E+06	482.20	
	Median	0.00	0.00	0.00E+00	0.00	
	Average	0.77	0.77	5.50E+05	113.89	

6.2.4 Target Peptide Biomarker Selection

Biomarkers were selected for inclusion in body fluid-specific panels under this portion of the study and are reported on the peptide level. Here, optimal peptide biomarkers are specific to the target body fluid and demonstrate consistency of expression across the sample population evaluated under the previous section. Additionally, relative abundance of peptide and protein biomarkers within the target matrix was used to facilitate inclusion within the target library.

To recapitulate, SEMG1, SEMG2, KLK3, and PPAP were consistently identified across the semen population samples. Salivary proteins SMR3B, STAT, and HIS1 were identified in all individuals with basic salivary proline-rich protein isoforms 1, 2, 3, and 4 (PRP_1, PRP_2, PRP_3, and PRP_4), salivary acidic proline-rich protein 1/2 (PRPC), and HIS3 being identified in most samples. And lastly, vaginal fluid was characterized by cornulin, suprabasin, involucrin, and periplakin. With these protein biomarkers selected, specific peptide fragments were identified for inclusion and reference material was synthesized for verification purposes. Purified reference material was purchased and employed to confirm the target biomarkers to ensure no erroneous identifications were made with the bioinformatics software searches. In addition, reference material was utilized to confirm the peptide retention times as well as to develop the targeted method, as discussed later in this section.

The following criteria were set for peptide filtering: peptide fragments must be within 8 to 25 amino acid residues in length, preference for peptides detected in at least 80% of the sample population, high MS1 signal intensity, high quality MS2 spectra, and those that contain minimal potential modification sites (*i.e.,* asparagine, methionine, and cysteine amino acid residues). However, these criteria were not concrete, as certain markers may exhibit high quality spectra with a lower population occurrence. Therefore, peptide length and sample occurrence were taken into consideration, but were not the sole criteria for including or eliminating a peptide marker. Signal intensity, spectrum quality, and limiting modification sites were given additional weight in decision making.

Using the criteria stated above, a preliminary list of targets included 77 semen peptides, 92 saliva peptides, and 30 vaginal fluid peptides, for which reference material was purchased and evaluated. The salivary protein HIS3 was removed from the peptide list due to low quality spectra and sample occurrence. All other listed protein biomarkers were represented in the peptide list. Furthermore, prolactin inducible protein (PIP) found in seminal fluid was re-introduced. Although not originally described during the population assessment, this protein exhibited higher quality spectra with a range of sample occurrence that prompted additional interest in this particular biomarker. In total, 38 seminal fluid peptides, 44 saliva peptides, and 12 vaginal fluid peptides were selected for the final inclusion library (**Tables 6.9-6.11**).

Table 6.9. Peptide markers for seminal fluid. Prostate specific antigen (KLK3), prolactin inducible protein (PIP), prostatic acid phosphatase (PPAP), semenogelin 1 (SEMG1), and semenogelin 2 (SEMG2). RT = retention time, AA = amino acid.

Protein	Sequence	Mass (m/z)	Charge (z)	RT	Start AA	End AA	Peptide Length
KLK3	F.LRPGDDSSHDLML.L	485.9014	3	8.80	111	123	12
	R.FLRPGDDSSHDLML.L	534.9242	3	10.60	110	123	13
	L.LRLSEPAELTD.A	622.3301	2	8.70	124	134	10
	L.LRLSEPAELTDA.V	657.8486	2	9.20	124	135	11
	W.GSIEPEEFLTPK.K	673.8456	2	10.50	158	169	11
PIP	Y.TIEILKVE.-	472.7868	2	6.60	139	146	7
	F.YTIEILKVE.-	554.3184	2	11.70	138	146	8
PPAP	F.AELVGPVIPQDW.S	662.3508	2	13.85	357	368	11
	F.GIWSKVYDPLY.C	670.8479	2	12.65	204	214	10
	F.GQLTQLGMEQHYEL.G	823.8958	2	10.55	68	81	13
	F.GQLTQLGMEQHYELGEY.I	998.4595	2	10.70	68	84	16
SEMG1	Y.DLNALHKTTKSQRH.L	412.9774	4	1.50	86	99	13
	Q.HGSHGGLDIVIIE.Q	449.5736	2	11.30	428	440	12
	Y.GENGVQKDVSQRS.I	468.5673	3	1.60	361	373	12
	V.VEVREEHS.S	492.7409	2	2.85	224	231	7
	T.NREQDLLSHEQKGRHQ.H	494.5018	4	2.55	412	427	15
	I.TIPSQEQEHSQKA.N	494.9110	3	1.30	331	343	12
	Q.TEKLVAGKSQ.I	530.8035	2	1.70	378	387	9
	Q.NVVEVREEH.S	555.7805	2	3.05	222	230	8
	N.TEERLWVHG.L	563.7856	2	6.35	162	170	8
	R.EQDLLSHEQKGRHQ.H	568.9519	3	2.50	414	427	13
	Q.NVVEVREEHS.S	599.2966	2	3.05	222	231	9
	Y.SQTEKLVAGKSQ.I	638.3488	2	2.40	376	387	11
	Q.NVVEVREEHSSK.V	706.8601	2	2.05	222	233	11
	Q.STNREQDLLSHEQKGRHQ.H	721.6932	3	2.65	410	427	17
SEMG2	V.DINDHDWTR.K	391.1757	3	4.75	72	80	11
	Q.NVVDVREEHS.S	395.1949	3	3.30	222	231	9
	V.DINDHDWTRK.S	433.8741	3	3.85	72	81	9
	Q.NVVDVREE.H	480.2433	2	4.30	222	229	7
	Y.NEDRNPIST.-	523.2491	2	3.50	574	582	8
	Y.DLNALHKATKSKQH.L	530.9610	3	2.05	86	99	13
	Q.NVVDVREEHSSKLQ.T	547.2848	3	4.30	222	235	13
	K.DVSQSSISFQIEKLVEGKSQ.I	553.0391	4	13.35	488	507	19
	S.SISFQIEKLVEGKSQ.I	564.9771	3	11.40	493	507	14
	Q.IEKLVEGKSQ.I	565.8244	2	3.15	498	507	9
	Y.HVDINDHDWTRK.S	768.3711	2	4.60	70	81	11
	K.DVSQSSISFQIEKLVEGKSQIQ.T	817.4306	3	13.80	488	509	21
	Y.VLQTEELVVNKQQRETK.N	1021.5657	2	6.05	195	211	16

Table 6.10. Peptide markers for saliva. Histatin-1 (HIS1), basic salivary proline-rich protein 1 (PRP_1), basic salivary proline-rich protein 2 (PRP_2), basic salivary proline-rich protein 4 (PRP_4), salivary acidic proline-rich protein 1/2 (PRPC), submaxillary androgen-regulated protein 3B (SMR3B), and statherin (STAT). RT = retention time, AA = amino acid.

Protein	Sequence	Mass (m/z)	Charge (z)	RT	Start AA	End AA	Peptide Length
HIS1	H.SHREFPF.Y	460.2247	2	8.25	39	45	6
	H.REFPFYGDY.G	597.2682	2	10.58	41	49	8
	K.HHSHREFPF.Y	597.2836	2	6.35	37	45	8
	S.HREFPFYGDY.G	665.7962	2	9.85	40	49	9
	F.YGDYGSNYLYDN.-	722.2886	2	7.33	46	57	11
PRP_1	Q.GGNKPQGPPPPPGKPQ.G	518.2792	3	3.09	43	58	15
	Q.GPPQQGGNRPQ.G	568.2828	2	0.99	242	252	10
	A.GNPQGPSPQGGNKPQ.G	731.8553	2	1.42	34	48	14
PRP_2	Q.GPPSPPGKPQ.G	481.2587	2	2.85	49	58	9
	Q.GGNKPQGPPSPPGKPQ.G	514.9390	3	3.24	43	58	15
	A.GNPQGAPPQGGNKPQ.G	723.8578	2	1.52	34	48	14
PRP_4	Q.RPPPPPGKPQ.G	535.8089	2	1.39	49	58	9
	Q.GPPPPPQGGRPP.R	577.3093	2	3.77	289	300	11
	Q.SHRPPPPPGKPE.R	648.3469	2	1.26	194	205	11
PRPC	Q.GPPQGQSPQ.-	448.2170	2	1.23	158	166	8
	Q.GPPPPPPGKPQ.G	534.7955	2	2.76	137	147	10
	Q.QGPPPPQGKPQ.G	565.8013	2	1.50	82	92	10
	Q.GPPQQGGHPPPPQGRPQ.G	577.9606	3	2.07	93	109	16
	Q.GPPQQGGHPRPP.R	612.8153	2	2.13	110	121	11
SMR3B	P.RGPYPPGPL.A	477.2648	2	7.98	27	35	8
	P.GIFPPPPPQP.-	523.7877	2	10.65	70	79	9
	G.RIPPPPPAPY.G	552.8137	2	7.69	58	67	9
	G.FVPPPPPPPY.G	554.2973	2	9.58	45	57	12
	P.GRIPPPPPAPY.G	581.3244	2	7.55	57	67	10
	P.GFVPPPPPPPY.G	582.8080	2	10.66	45	57	12
	Y.GPGIFPPPPPQP.-	600.8242	2	10.74	68	79	11
	P.YPPGPLAPPQPF.G	640.8373	2	12.45	30	41	11
	L.APPQPFGPGFVPPPPPPPY.G	652.6733	3	13.54	36	54	18
	Y.GPGRIPPPPPAPY.G	658.3615	2	8.13	55	67	12
	F.GPGFVPPPPPPPY.G	659.8458	2	11.06	42	54	12
	R.GPYPPGPLAPPQPF.G	717.8744	2	12.78	28	41	13
	F.GPGFVPPPPPPPYGPG.R	765.3930	2	10.82	44	57	13
	P.RGPYPPGPLAPPQPF.G	795.9250	2	11.89	27	41	14
STAT	P.YQPVPEQPL.Y	535.7795	2	8.39	40	48	8
	P.EQPLYPQPY.Q	567.7769	2	8.34	46	56	10
	Y.GPYQPVPEQPL.Y	612.8166	2	7.30	38	48	10
	R.IGRFGYGYGPY.Q	625.3037	2	9.15	30	40	10
	P.VPEQPLYPQPY.Q	665.8375	2	9.19	43	53	10
	E.QPLYPQPYQPQ.Y	679.8406	2	7.81	46	56	10
	P.YQPVPEQPLYPQPYQPQ.Y	691.3423	3	9.89	40	56	16
	Y.QPVPEQPLYPQPY.Q	778.3932	2	9.69	41	53	12
	R.FGYGYGPYQPVPEQPL.Y	906.4356	2	11.07	33	48	15
	Y.QPVPEQPLYPQPYQPQ.Y	954.9782	2	9.22	41	56	15
	F.GYGYGPYQPVPEQPLYPQPY.Q	1157.0468	2	11.80	34	53	19

Table 6.11. Peptide markers for vaginal fluid. Cornulin (CRNN), involucrin (IVL), periplakin (PPL), and suprabasin (SBSN). RT = retention time, AA = amino acid.

Protein	Sequence	Mass (m/z)	Charge (z)	RT	Start AA	End AA	Peptide Length
CRNN	L.YSYLRSTKP.-	372.2012	3	4.76	487	495	8
	E.WVDDHSRET.V	382.1712	3	2.41	442	450	8
	A.DVIVKPHDPA.T	545.7982	2	5.19	43	52	9
	R.SQTSQAVTGGHTQIQAGSH.T	632.3053	3	3.62	336	354	18
	L.DEDHTGTVEFK.E	639.2859	2	4.6	62	72	10
	M.PQLLQNINGIIE.A	676.3826	2	13.12	2	13	11
	D.VIVKPHDPATVDE.V	710.3775	2	5.25	44	56	12
	F.ADVIVKPHDPATVDE.V	803.4096	2	6.29	42	56	14
IVL	V.ELPVEVPSKQEEKH.M	412.9690	4	5.98	50	63	13
PPL	L.KTENPGDASDLQGRQL.L	864.9292	2	5.71	492	507	15
SBSN	R.VVQGLHHGVSQAGR.E	722.8920	2	2.31	404	417	13
	N.NAAGQVGKEADKLIHHGVHHGAN.Q	787.4037	3	6.29	121	143	22

6.2.5 Performance Assessment

Reported below are results at the protein level for each sample evaluated. Peptide level results for the performance assessment are detailed in **Appendix N**.

6.2.5.1 Sensitivity Samples

Sensitivity replicates were prepared by diluting target body fluid with deionized water and applying the dilution to a cotton swab. Therefore, the dilution factor was inherently diluted a second time during the solubilization step of the developed workflow. Data for seminal fluid, saliva, and vaginal fluid dilutions are outlined in **Tables 6.12-6.14**. Proteins were positively identified by the presence of at least one peptide target from the inclusion list. Seminal fluid was reproducibly identified at the lowest dilution factor (10,000-fold) by both SEMG1 and SEMG2. However, the remaining seminal fluid proteins showed poor sensitivity, with little to no identifications at the 100-fold dilution. Salivary protein biomarkers demonstrated robust sensitivity, with a majority of proteins detected at the 100-fold dilution. Basic salivary proline-rich protein 1, salivary acidic proline-rich protein 1/2, and submaxillary gland androgen-regulated protein 3B were detected in at least one replicate at the lowest dilution factor. Vaginal fluid sensitivity was contingent on cornulin identification, which was positive through the 100-fold dilution. The remaining protein biomarkers demonstrated poor sensitivity.

Table 6.12. Sensitivity sample results for seminal fluid. Prostate specific antigen (KLK3), prolactin inducible protein (PIP), prostatic acid phosphatase (PPAP), semenogelin 1 (SEMG1), and semenogelin 2 (SEMG2). (+) indicates at least one peptide detected for the designated protein. (-) indicates a negative result.

Dilution	KLK3	PIP	PPAP	SEMG1	SEMG2
1:100	+ - -	+ + +	- - -	+ + +	+ + +
1:1000	- - -	- - -	- - -	+ + +	+ + +
1:2000	- - -	- - -	- - -	+ + +	+ + +
1:5000	- - -	- - -	- - -	+ + +	+ + +
1:10000	- - -	- - -	- - -	+ + +	+ + +

Table 6.13. Sensitivity sample results for saliva. (+) indicates at least one peptide detected for the designated protein. (-) indicates a negative result. Histatin-1 (HIS1), basic salivary proline-rich protein 1 (PRP_1), basic salivary proline-rich protein 2 (PRP_2), basic salivary proline-rich protein 4 (PRP_4), salivary acidic proline-rich protein 1/2 (PRPC), submaxillary androgen-regulated protein 3B (SRM3B), and statherin (STAT).

Dilution	HIS1	PRP_1	PRP_2	PRP_4	PRPC	SMR3B	STAT
1:2	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
1:10	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
1:50	+ - -	+ + +	+ + +	- - -	+ + +	+ + +	+ + -
1:100	+ - -	+ + +	+ + +	- - -	+ + +	+ + +	+ - -
1:500	- - -	+ - -	- - -	- - -	+ + -	+ - -	- - -

Table 6.14. Sensitivity sample results for vaginal fluid. (+) indicates at least one peptide detected for the designated protein. (-) indicates a negative result. Cornulin (CRNN), involucrin (IVL), periplakin (PPL), and suprabasin (SBSN).

Dilution	CRNN	IVL	PPL	SBSN
1:2	+ + +	- - -	- - -	+ + +
1:10	+ + +	- - -	- - -	- - -
1:50	+ + +	- - -	- - -	- - -
1:100	+ + -	- - -	- - -	- - -
1:500	- - -	- - -	- - -	- - -

6.2.5.2 Aged Samples

The effect of biomarker recovery and characterization from aged biological material was assessed using a two-pronged approach. Each target fluid was evaluated as a single source in addition to a vaginal swab matrix fortified with either semen or saliva. Samples were kept at room temperature for 2, 3, 7, and 30 days, with detailed results outlined in **Table 6.15**. Seminal fluid as a single source was positively characterized at every timepoint; however, PPAP exhibited complete

dropout. Consistency between replicates was observed, with a single replicate exhibiting a positive KLK3 result. Similar results were obtained for seminal fluid recovered from a vaginal swab matrix. However, complete dropout of PIP was recorded. It is hypothesized that a protease may be present in vaginal fluid that may degrade PIP past the point of characterization. A comparable observation was made for saliva and saliva recovered from a vaginal swab matrix. Although consistency in replicate characterization was recorded for saliva on its own, complete dropout of PRP_2, histatin-1, and statherin was exhibited when saliva was recovered from a vaginal swab. And lastly, as seen with sensitivity samples, vaginal fluid characterization relied on cornulin detection, which exhibited consistency with each replicate at all timepoints.

Table 6.15. Aged sample results. (+) indicates at least one peptide detected for the designated protein for each replicate. (-) indicates a negative result. NT = not tested. Prostate specific antigen (KLK3), prolactin inducible protein (PIP), prostatic acid phosphatase (PPAP), semenogelin 1 (SEMG1), and semenogelin 2 (SEMG2), histatin-1 (HIS1), basic salivary proline-rich protein 1 (PRP_1), basic salivary proline-rich protein 2 (PRP_2), basic salivary proline-rich protein 4 (PRP_4), salivary acidic proline-rich protein 1/2 (PRPC), submaxillary androgen-regulated protein 3B (SRM3B), and statherin (STAT), cornulin (CRNN), involucrin (IVL), periplakin (PPL), and suprabasin (SBSN).

Sample	Timepoint	KLK3	PIP	PPAP	SEMG1	SEMG2	HIS1	PRP_1	PRP_2	PRP_4	PRPC	SMR3B	STAT	CRNN	IVL	PPL	SBSN
Seminal Fluid	Day 2	+ - -	+ + +	- - -	+ + +	+ + +											
	Day 3	+ - -	+ + +	- - -	+ + +	+ + +											
	Day 7	+ - -	+ + +	- - -	+ + +	+ + +											
	Day 30	+ - -	+ + +	- - -	+ + +	+ + +											
Seminal Fluid on Vaginal Swab	Day 2	+ + -	- - -	- - -	+ + +	+ + +								+ + +	- - -	- - -	+ + -
	Day 3	+ - -	- - -	- - -	+ + +	+ + +								+ + +	- - -	- - -	+ - -
	Day 7	+ - -	- - -	- - -	+ + +	+ + +								+ + +	- - -	- - -	+ + +
	Day 30	+ + -	- - -	- - -	+ + +	+ + +								+ + +	- - -	- - -	+ - -
Saliva	Day 2						+ + +	+ + +	+ + +	+ - -	+ + +	+ + +	+ + -				
	Day 3						+ - -	+ + +	+ + +	- - -	+ + +	+ + +	+ + -				
	Day 7						+ + +	+ + +	+ + +	+ - -	+ + +	+ + +	+ + +				
	Day 30						+ + +	+ + +	+ + +	- - -	+ + +	+ + +	+ + +				
Saliva on Vaginal Swab	Day 2						- - -	- - -	- - -	- - -	+ + +	+ + +	- - -	+ + +	- - -	- - -	+ + +
	Day 3						- - -	+ + -	- - -	- - -	+ + +	+ + +	- - -	+ + +	+ - -	- - -	+ + +
	Day 7						- - -	+ - -	- - -	- - -	+ + +	+ + -	- - -	+ + +	- - -	- - -	+ + +
	Day 30						- - -	+ - -	- - -	- - -	+ + +	+ + +	- - -	+ + +	+ - -	- - -	+ + +
Vaginal Swab	Day 2													+ + +	+ - -	- - -	+ + -
	Day 3													+ + +	+ + -	+ - -	+ - -
	Day 7													+ + +	- - -	- - -	+ + +
	Day 30													+ + +	- - -	- - -	+ + +

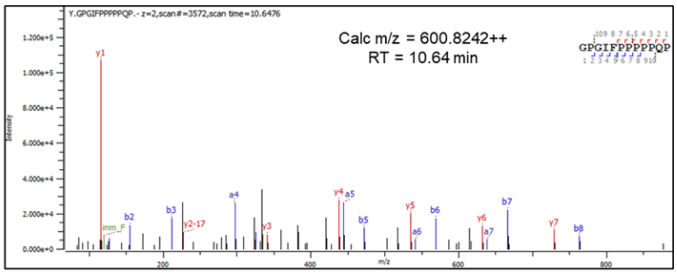
6.2.5.3 Sexual Assault Samples

Laboratory prepared sexual assault kit samples were generated to simulate authentic samples, with results outlined in **Table 6.16**. Of surprise was the ability of the developed method to positively characterize both seminal fluid and saliva when recovered from a rectal swab. Given the endogenous bacterial presence common with this sample type, the observation of reproducible peptide identifications demonstrates the robustness of a peptidomic strategy. Where previous studies have indicated the presence of fecal matter to inhibit the processivity of trypsin, the removal of proteolytic cleavage sites has eliminated this issue. For example, during validation of the bottom up LC-MS/MS method (Chapter 3), KLK3 was not detected on rectal swab samples, whereas this protein was consistently detected on replicates with the peptidomic technique. Furthermore, the recovery of saliva from rectal swabs was included in this portion of research. The presence of fecal matter exhibited no deleterious effects in the ability to identify saliva. For example, salivary protein SMR3B demonstrated excellent ion coverage and spectral quality (**Figure 6.13**).

Table 6.16. Sexual assault kit sample results. (+) indicates at least one peptide detected for the designated protein for each replicate. (-) indicates a negative result. NT = not tested. Prostate specific antigen (KLK3), prolactin inducible protein (PIP), prostatic acid phosphatase (PPAP), semenogelin 1 (SEMG1), and semenogelin 2 (SEMG2), histatin-1 (HIS1), basic salivary proline-rich protein 1 (PRP_1), basic salivary proline-rich protein 2 (PRP_2), basic salivary proline-rich protein 4 (PRP_4), salivary acidic proline-rich protein 1/2 (PRPC), submaxillary androgen-regulated protein 3B (SRM3B), and statherin (STAT), cornulin (CRNN), involucrin (IVL), periplakin (PPL), and suprabasin (SBSN).

Fluid	Protein	Vaginal Swab	Rectal Swab	Oral Swab	Breast Swab
Seminal Fluid	KLK3	- - -	+ + +	+ - -	NT
	PIP	- - -	+ - -	+ + +	NT
	PPAP	- - -	- - -	- - -	NT
	SEMG1	+ + +	+ + +	+ + +	NT
	SEMG2	+ + +	+ + +	+ + +	NT
Saliva	HIS1	- - -	+ + -	NT	- - -
	PRP_1	- - -	+ + +	NT	+ + +
	PRP_2	- - -	+ + -	NT	+ - -
	PRP_4	- - -	- - -	NT	+ - -
	PRPC	+ - -	+ + +	NT	+ + +
	SMR3B	+ - -	+ + +	NT	+ + +
	STAT	- - -	+ + +	NT	+ + -
Vaginal Fluid	CRNN	+ + +	NT	NT	NT
	IVL	+ + -	NT	NT	NT
	PPL	+ - -	NT	NT	NT
	SBSN	+ + +	NT	NT	NT

A.



B.

#	b. Calc	Seq	y. Calc	#
1	58.0287	G		12
2	**155.0815**	P	1143.6197	11
3	**212.1030**	G	1046.5669	10
4	**325.1870**	I	989.5455	9
5	**472.2554**	F	876.4614	8
6	**569.3082**	P	**729.3930**	7
7	**666.3609**	P	**632.3402**	6
8	**763.4137**	P	**535.2875**	5
9	860.4665	P	**438.2347**	4
10	957.5192	P	**341.1819**	3
11	1085.5778	Q	244.1292	2
12		P	**116.0706**	1

Figure 6.13. (A) Mass spectrum of saliva recovered from a rectal swab replicate. Depicted is peptide GPGIFPPPPPQP of protein submaxillary androgen-regulated protein 3B (SRM3B). (B) y and b ions characterized for the peptide sequence are shown in bold type.

6.2.5.4 Mixture Samples

Volume mixtures of seminal fluid and saliva were applied to vaginal swabs to assess ion suppression, particularly for a minor saliva contributor in a major seminal fluid contributor. Vaginal swabs were selected as the matrix in order to simulate sexual assault type samples more closely. In summary, all target fluids were positively identified (**Table 6.17**). Seminal fluid is protein rich, and despite the greater volumes, did not inhibit the ability to positively characterize saliva or vaginal fluid peptide targets. Saliva was consistently identified by the presence of PRPC, even when the amount of semen was greater (MIX03) or equivalent to (MIX04) the saliva contribution. As seen with previous performance samples, complete dropout of statherin was exhibited when in the presence of vaginal fluid.

Table 6.17. Mixture sample results. (+) indicates at least one peptide detected for the designated protein for each replicate. (-) indicates a negative result. Prostate specific antigen (KLK3), prolactin inducible protein (PIP), prostatic acid phosphatase (PPAP), semenogelin 1 (SEMG1), and semenogelin 2 (SEMG2), histatin-1 (HIS1), basic salivary proline-rich protein 1 (PRP_1), basic salivary proline-rich protein 2 (PRP_2), basic salivary proline-rich protein 4 (PRP_4), salivary acidic proline-rich protein 1/2 (PRPC), submaxillary androgen-regulated protein 3B (SRM3B), and statherin (STAT), cornulin (CRNN), involucrin (IVL), periplakin (PPL), and suprabasin (SBSN).

		MIX01	MIX02	MIX03	MIX04
Fluid	**Protein**	**10 µL SA + 2.5 µL SE**	**50 µL SA + 2.5 µL SE**	**25 µL SE + 10 µL SA**	**10 µL SE + 10 µL SA**
Seminal Fluid	KLK3	+ + -	+ - -	+ + +	+ + +
	PIP	- - -	+ - -	+ + +	+ + -
	PPAP	+ - -	+ - -	+ + +	+ + +
	SEMG1	+ + +	+ + +	+ + +	+ + +
	SEMG2	+ + +	+ + +	+ + +	+ + +
Saliva	HIS1	- - -	+ - -	- - -	- - -
	PRP_1	+ + -	+ + +	+ - -	+ - -
	PRP_2	- - -	+ - -	- - -	- - -
	PRP_4	+ + -	- - -	- - -	- - -
	PRPC	+ + +	+ + +	+ + +	+ + +
	SMR3B	+ - -	+ + +	- - -	+ - -
	STAT	- - -	- - -	- - -	- - -
Vaginal Fluid	CRNN	+ + +	+ + +	+ + +	+ + +
	IVL	+ + -	+ - -	+ + -	+ - -
	PPL	+ - -	+ - -	- - -	+ + -
	SBSN	+ + -	+ + +	+ - -	+ + -

6.2.5.5 Contaminant Samples

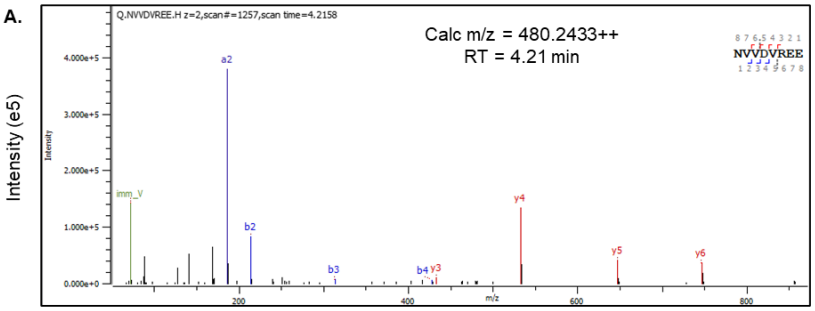
The recovery of target biomarkers when subjected to chemical insult and environmental contaminants was of interest to this research in order to more closely simulate authentic forensic evidence received in operational laboratories (**Table 6.18**). Based on data reported in Chapter 5, personal lubricants, in addition to bleach and dish soap, were identified as contaminants that have the potential to prevent the identification of seminal fluid. SEMG1 and SEMG2 were positively identified when in the presence of all contaminants. Uniform protein dropout PIP and PPAP was recorded. Overall, the presence of contaminants did not impede the ability to correctly characterize seminal fluid, particularly when in the presence of 10% bleach (**Figure 6.14**). SEMG2 peptide NVVDVREE exhibited a clean and clear spectrum, with characteristic b, y, and y++ ions detected.

Saliva characterization in the presence of chemical insults was not as robust as observed with seminal fluid biomarkers. Negative results for all peptide targets were recorded for saliva treated with 10% bleach. For the remaining samples, salivary acidic proline-rich protein 1/2 and SMR3B were identified by the presence of at least one target peptide. And lastly, vaginal fluid was identified from a menstrual swab based on the positive characterization of cornulin in all three replicates. Detergents and formulations containing halogen substances, such as bleach, present additional challenges with mass spectrometric analysis. Halogens, such as chlorine, have a characteristic isotope distribution on a mass spectra. This distribution is straightforward with small molecule analysis; however, with larger peptide masses, the chlorine isotope distribution can impact the quality of a mass spectrum.

Table 6.18. Contaminant samples results. (+) indicates at least one peptide detected for the designated protein for each replicate. (-) indicates a negative result. NT = not tested. Prostate specific antigen (KLK3), prolactin inducible protein (PIP), prostatic acid phosphatase (PPAP), semenogelin 1 (SEMG1), and semenogelin 2 (SEMG2), histatin-1 (HIS1), basic salivary proline-rich protein 1 (PRP_1), basic salivary proline-rich protein 2 (PRP_2), basic salivary proline-rich protein 4 (PRP_4), salivary acidic proline-rich protein 1/2 (PRPC), submaxillary androgen-regulated protein 3B (SRM3B), and statherin (STAT), cornulin (CRNN), involucrin (IVL), periplakin (PPL), and suprabasin (SBSN).

Fluid	Protein	Water-Based Lubricant	Natural Lubricant	Silicon-Based Lubricant	10% Bleach	Dish Soap	Menstrual Swab
Seminal Fluid	KLK3	+ - -	+ - -	+ + +	- - -	- - -	+ - -
	PIP	- - -	- - -	- - -	- - -	- - -	- - -
	PPAP	- - -	- - -	- - -	- - -	- - -	- - -
	SEMG1	+ + +	+ + +	+ + +	+ + +	+ + -	+ + +
	SEMG2	+ + +	+ + +	+ + +	+ + +	+ + -	+ + +
Saliva	HIS1	NT	NT	NT	- - -	- - -	- - -
	PRP_1	NT	NT	NT	- - -	- - -	+ - -
	PRP_2	NT	NT	NT	- - -	- - -	- - -
	PRP_4	NT	NT	NT	- - -	- - -	- - -
	PRPC	NT	NT	NT	- - -	+ + +	+ + +
	SMR3B	NT	NT	NT	- - -	+ + -	+ + -
	STAT	NT	NT	NT	- - -	- - -	- - -
Vaginal Fluid	CRNN	+ + +	+ + +	+ + +	NT	NT	+ + +
	IVL	+ - -	+ - -	+ - -	NT	NT	- - -
	PPL	- - -	- - -	- - -	NT	NT	- - -
	SBSN	+ + +	+ + -	+ + +	NT	NT	- - -

A.



B.

#	b. Calc	Seq	y. Calc	y++ calc	#
1	115.0502	N			8
2	**214.1186**	V	845.4363	423.2218	7
3	**313.1870**	V	**746.3679**	**373.6876**	6
4	**428.2140**	D	**647.2995**	324.1534	5
5	527.2824	V	**532.2725**	266.6399	4
6	683.3835	R	**433.2041**	217.1057	3
7	812.4261	E	277.1030	139.0551	2
8		E	148.0604	74.5339	1

Figure 6.14. (A) Mass spectrum of seminal fluid treated with 10% bleach. Depicted is peptide NVVDVREE of protein semenogelin 2 (SEMG2). (B) y and b ions characterized for the peptide sequence are shown in bold type.

6.2.5.6 Substrate Samples

In conjunction with contaminants, the ability to positively characterize biological fluids recovered from various substrates was important to consider. Four fabric types were selected for this portion of the study: 100% cotton, 100% polyester, polyester blend (60% polyester, 40% cotton), and denim. Each fabric had small quantities of biological fluid applied, with the 100% cotton underwear worn by a female subject prior to fluid application. Detailed results are outlined in **Table 6.19**. As with previous data sets, SEMG1 and SEMG2 outperformed the remaining seminal fluid protein targets. However, all protein targets were characterized when recovered from denim. It is hypothesized that, given the texture of denim fabric, the applied semen was retained over a smaller surface area and easily released back into solution once solubilized. In addition to these samples, semen stains were excised and recovered from bed sheets to assess biomarker loss during laundering. Seminal fluid was positively characterized on both bed sheets evaluated by the

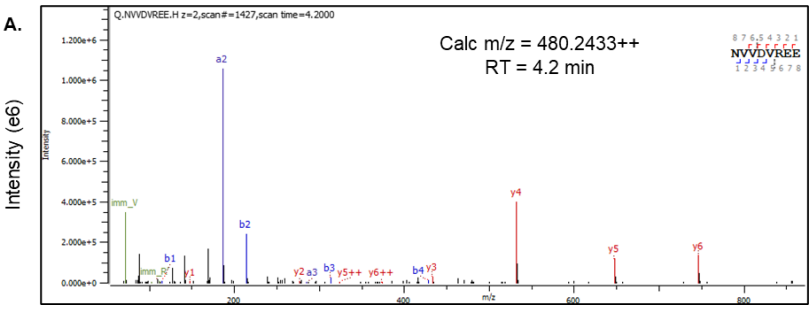
presence of SEMG1 and SEMG2, in addition to a single KLK3 peptide identification on the polyester blend bed sheet (**Figure 6.15**). Semenogelin 2 peptide NVVDVREE demonstrated great intensity from laundered items in comparison with the 10% bleach solution, further highlighting the lower sensitivity limits of the developed methodology.

Saliva protein characterization was substrate dependent. As with seminal fluid, recovery of saliva from denim was nearly complete, with protein dropout of basic salivary proline-rich protein 4 exhibited. However, both statherin and SMR3B, confirmatory saliva biomarkers, were identified. Additional protein dropout and greater inconsistency between replicates were reported for polyester blend and 100% polyester (**Table 6.19**).

Table 6.19. Contaminant samples results. (+) indicates at least one peptide detected for the designated protein for each replicate. (-) indicates a negative result. NT = not tested. Prostate specific antigen (KLK3), prolactin inducible protein (PIP), prostatic acid phosphatase (PPAP), semenogelin 1 (SEMG1), and semenogelin 2 (SEMG2), histatin-1 (HIS1), basic salivary proline-rich protein 1 (PRP_1), basic salivary proline-rich protein 2 (PRP_2), basic salivary proline-rich protein 4 (PRP_4), salivary acidic proline-rich protein 1/2 (PRPC), submaxillary androgen-regulated protein 3B (SRM3B), and statherin (STAT).

Fluid	Protein	100% Cotton Underwear	100% Polyester Sheet	Polyester Blend Sheet	Denim	Laundered 100% Polyester	Laundered Control (Poly)	Laundered Polyester Blend	Laundered Control (PB)
Seminal Fluid	KLK3	- - -	+ + +	+ + +	+ + +	-	-	+	-
	PIP	- - -	- - -	- - -	+ + +	-	-	-	-
	PPAP	- - -	- - -	- - -	+ + +	-	-	-	-
	SEMG1	+ + -	+ + +	+ + +	+ + +	+	-	+	-
	SEMG2	+ + -	+ + +	+ + +	+ + +	+	-	+	-
Saliva	HIS1	NT	- - -	+ - -	+ + -	NT	NT	NT	NT
	PRP_1	NT	+ + +	+ + +	+ + +	NT	NT	NT	NT
	PRP_2	NT	- - -	+ - -	+ + +	NT	NT	NT	NT
	PRP_4	NT	- - -	- - -	- - -	NT	NT	NT	NT
	PRPC	NT	+ + +	+ + +	+ + +	NT	NT	NT	NT
	SMR3B	NT	+ + +	+ + +	+ + +	NT	NT	NT	NT
	STAT	NT	- - -	- - -	+ + -	NT	NT	NT	NT

A.



B.

#	b. Calc	Seq	y. Calc	y++ calc	#
1	**115.0502**	N			8
2	**214.1186**	V	845.4363	423.2218	7
3	**313.1870**	V	**746.3679**	**373.6876**	6
4	**428.2140**	D	**647.2995**	**324.1534**	5
5	527.2824	V	**532.2725**	266.6399	4
6	683.3835	R	**433.2041**	217.1057	3
7	812.4261	E	**277.1030**	139.0551	2
8		E	**148.0604**	74.5339	1

Figure 6.15. (A) Mass spectrum of seminal fluid recovered from laundered polyester blend bed sheet. Depicted is peptide NVVDVREE of protein semenogelin 2 (SEMG2). (B) y and b ions characterized for the peptide sequence are shown in bold type.

6.3 Concluding Remarks

In summary, the use of a peptidomic approach for seminal fluid, saliva, and vaginal fluid identification revealed that multiple non-tryptic peptide fragments are produced through endogenous cleavage during analysis with high resolution mass spectrometry. The protein biomarkers identified with this technique were comparable to those detected with the validated bottom up LC-MS/MS assay. Therefore, the ability to prepare biological stains for proteomic analysis with an expedited, simple extraction prior to chromatographic separation eliminated the need for lengthy enzymatic digestion incubation periods. The results presented provide support for further development of peptidomic applications in the field of forensic serology. From a forensic biology standpoint, it is recommended that reporting of results be based on the identification of inclusion peptides. Although additional peptide fragments are identified by the proteomic software, the peptides included in the library have undergone quality control assessment during verification using purified reference material. Significant proteins include semenogelin 1 and 2 for seminal fluid

identification in addition to statherin and submaxillary gland androgen-regulated protein 3B for saliva identification.

Supplemental research is required for the identification of a vaginal fluid-specific protein biomarker. Although the four protein biomarkers described, particularly cornulin, demonstrated adequate sensitivity and robustness, the selected proteins do not exhibit specificity to vaginal fluid. Suprabasin, although expressed in vaginal, cervical, and uterine tissue, shares comparable expression patterns in esophagus tissue and keratinocytes of skin tissues. Similar protein expression patterns are evident for cornulin and involucrin. In addition, involucrin is expressed in tissues of the urinary bladder and epididymis [171].

The Protein Metrics Byonic™ application is a software tool used for identifying peptides and proteins, similar to more traditional applications such as Mascot and SEQUEST. However, this software provides additional functionalities that can contribute to areas of future work, such as advanced glycopeptide and crosslinking search capabilities.

Various challenges and limitations exist with the implementation of protein mass spectrometry techniques into an operational laboratory. First, the creation of training materials will be essential for onboarding molecular biologists in advanced proteomic techniques. Second, generating interest with stakeholders so that resources and capital investments are made to support the implementation of this screening technique, such as the purchase of the LC-MS/MS analytical system, will be crucial for a seamless implementation. And lastly, overcoming a Daubert admissibility hearing for using proteomic data in a court of law will impose significant challenges moving forward. The case findings from *Daubert v. Merrell Dow Pharmaceuticals, Inc.* is the documentation accepted by the federal government and a majority of state government for the admissibility of scientific evidence in court. The findings suggest that scientific methodology be testable, have a known or potential error rate, have applicable standards and controls, be subjected to peer review and publication, and be generally accepted in the scientific community. Sufficient gains have been made for use of protein mass spectrometry in forensic laboratories, specifically in the field of forensic toxicology, but additional research into drafting of interpretation guidelines,

applicable use of controls, and support within the greater community will be critical for admissibility of data in court. Furthermore, the addition of confidence level reporting would be beneficial in relaying the weight of data to the trier of fact.

In toto, the specific hypotheses outlined for completion of this research were successfully evaluated. Proteomic techniques in the form of bottom up and peptidomic methodologies were demonstrated to surpass the sensitivity and specificity capable of traditional serological screening techniques. The transferring of a multiplex mass spectrometry-based assay from a nanoflow to high performance LC-MS/MS analytical system sufficiently increased sample throughput. In addition, an automated sample processing procedure mitigated sources of human error and contributed to increased sample throughput without a loss in peptide intensity. The multiplex LC-MS/MS assay underwent rigorous validation and comparison, illustrating its robust performance and compatibilities with existing forensic biology workflows. It has been demonstrated that the ability to produce serological information at sensitivity levels consistent with STR/Y-STR typing is achievable with protein mass spectrometry technology. Body fluid identification of samples subjected to personal lubricants was achieved through specific sample preparation procedures and detection of surrogate peptides using the validated LC-MS/MS assay. And lastly, an expedited proteomic analysis of body fluids consistent with sexual assault evidence was developed using peptidomic techniques. The use of proteomics and advanced analytical instrumentation is a viable solution to meeting the sensitivity and specificity demands of the forensic biology community for the confirmation of biological origin, therefore contributing to the identification and individualization of evidentiary material.

6.4 Future Direction and Impact on the Criminal Justice System

The identification of biological stains has posed significant challenges since the onset and acceleration of DNA individualization. Currently, practitioners have no means of reliably confirming the presence of biological stains, especially the differentiation of saliva and vaginal fluid. The presumptive results obtained through serological screening prevent definitive statements regarding the origin of a biological stain by an expert witness during courtroom testimony. While implementing

a new technology such as protein mass spectrometry comes with significant onboarding actions, such as new analyst training and purchasing of instrumentation, the cost-to-benefit ratio of investing in this next generation technique is substantial.

Extensive research has demonstrated that protein mass spectrometry exhibits unmatched detection capabilities, particularly with trace quantities of biological stains in the presence of contaminants. The research presented in this dissertation contributes to the sufficient gains of the forensic subdiscipline that has fallen behind its counterpart. Detailed standard operating procedures for the validated proteomic methodology are written and presented for the effective transfer of this technology into an operational laboratory. However, a comparative assessment of proteomics with other next generation techniques, such as RNA-based assays and epigenetic profiling, would identify gaps in proposed methodologies and provide the forensic biology community with valuable information to aid in technology transfer and retirement of traditional serological screening protocols.

Additional research into characteristic protein biomarkers for vaginal fluid detection is necessary for the confirmatory identification of this biological matrix. Given its similarities in protein makeup to that of saliva, additional distinctive traits should be taken into consideration. The availability of a reliable and sensitive method for the confirmatory identification of vaginal fluid would have important forensic utility for the analysis of sexual assault evidence. While the presence of a victim's DNA profile on a suspect's genitals or underwear provides a strong indication of sexual contact on its own, a vaginal fluid assay would increase the probative value of a broader range of evidentiary items (*e.g.,* swabs of fingers, fingernail scrapings, outer clothing). Furthermore, items of evidence submitted in sexual assault cases involving vaginal rape with a foreign object would greatly benefit from such an assay.

This obstacle can be overcome by leveraging unique post translational modifications (PTM) that proteins undergo only in vaginal tissues. A common type of PTM includes glycosylation, and with the addition of carbohydrate moieties, glycosylation can have a significant effect on protein confirmation and antigenic properties. N-linked glycosylation occurs in the endoplasmic reticulum at

specific asparagine sequences (*i.e.,* Asn-X-Ser/Thr), where the addition of sugar residues result in unique structural modifications [172]. The process of N-linked glycosylation is highly conserved in eukaryotes, indicating the importance of carbohydrate labeling as a means of generating diversity within the proteome [41]. For instance, the protein glycodelin exhibits multiple isoforms with differential glycosylation that dictates body fluid specific functionalities. For example, the glycodelin-A (GdA) isoform in amniotic and vaginal fluids induces an immunosuppressive response by silencing maternal T cells, preventing an immune response toward an implanted embryo. In addition, this isoform also acts as a natural contraceptive by impeding spermatozoa binding to the oocyte outside the fertile window of ovulation [173]. In contrast, the glycodelin-S (GdS) isoform in seminal fluid blocks spermatozoa capacitation (*i.e.,* destabilization of the acrosomal membrane) prior to passing through the cervical mucus of the female reproductive tract [173].

Additional advancements in proteomic applications to forensic biology include the use of genetically variable peptides (GVPs) and the concept of proteomic genotyping. A single amino acid polymorphism can be correlated to a single nucleotide polymorphism (SNP), generating the link between identification and individualization of biological material. Research in this area has focused on fingermarks [174], bone [152], and hair [175], but the possibilities of expanding to other biological matrices are evident. For example, the detection of GVPs for the salivary protein statherin and subsequent SNP profiling could provide a confirmatory identification of saliva and the inclusion of a suspected individual for an evidentiary item associated with a sexual assault or rape.

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APPENDIX A: FINAL BIOMARKER LIST AND ASSOCIATED COLLISION ENERGIES

Body Fluid	Protein	Peptide Sequence	Precursor ion (m/z)	Product ions (m/z)	Collision Energy (V)	Retention Time (min)
Peripheral Blood	Alpha 1 Antitrypsin (P01009)	LSITGTYDLK	555.8057 ++	910.4880 +	15.2	4.9
				797.4040 +	18.2	
				696.3563 +	15.2	
		SVLGQLGITK	508.3109 ++	829.5142 +	19.8	5.7
				716.4301 +	13.8	
				531.3501 +	16.8	
	Hemopexin (P02790)	NFPSPVDAAFR	610.8066 ++	959.4945 +	22.9	6.1
				775.4097 +	25.9	
				480.2509 ++	16.9	
	Hemoglobin Subunit Beta (P68871)	LLVVYPWTQR	637.8664 ++	949.4890 +	17.8	7.6
				850.4206 +	23.8	
				687.3573 +	17.8	
		SAVTALWGK	466.7636 ++	774.4509 +	12.5	5.0
				675.3824 +	15.5	
				574.3348 +	18.5	
	Apolipoprotein A1 (P02647)	LLDNWDSVTSTFSK	806.8960 ++	971.4680 +	29.0	7.3
				670.3406 +	26.0	
				569.2930 +	26.0	
		VSFLSALEEYTK	693.8612 ++	1053.5463 +	22.5	9.3
				940.4622 +	22.5	
				600.8110 ++	19.5	
	Serotransferrin (P02787)	DGAGDVAFVK	489.7482 ++	735.4036 +	16.2	3.2
				678.3821 +	16.2	
				563.3552 +	19.2	
		SASDLTWDNLK	625.3066 ++	1091.5368 +	20.4	5.0
				776.3937 +	17.4	
				675.3461 +	20.4	

Body Fluid	Protein	Peptide Sequence	Precursor ion (m/z)	Product ions (m/z)	Collision Energy (V)	Retention Time (min)
Seminal Fluid	Semenogelin-2 (Q02383)	GSISIQTEEQIHGK	509.5985 +++	691.8673 ++	7.5	2.5
				635.3253 ++	19.5	
				461.5807 +++	10.5	
		DVSQSSISFQIEK	734.3699 ++	1038.5466 +	23.8	5
				951.5146 +	26.8	
				751.3985 +	20.8	
		GSISIQTEEK	546.2826 ++	834.4203 +	14.9	2
				747.3883 +	20.9	
				634.3042 +	13.9	
	Semenogelin-1 (P04279)	DIFSTQDELLVYNK	842.9251 ++	1309.6634 +	27.1	7.4
				523.2875 +	24.1	
				424.2191 +	24.1	
		QITIPSQEQEHSQK	551.6128 +++	706.3442 ++	12.1	
				599.2784 ++	18.1	
	Prostate Specific Antigen (P07288)	LSEPAELTDAVK	636.8677 ++	943.5095 +	20.7	4.4
				646.3770 +	23.7	
				472.2584 ++	20.7	
		FLRPGDDSSHDLMLLR	468.7413 ++++	760.4386 +	15.1	5.9
				532.3276 +	9.1	
				536.2844 ++	15.1	
				575.6298 +++	18.1	
	Prostatic Acid Phosphatase (P15309)	FQELESETLK	612.3113 ++	948.4884 +	20.0	3.5
				819.4458 +	23.0	
				276.1343 +	20.0	
		ELSELSLLSLYGIHK	567.9856 +++	930.5407 +	18.6	8.8
				817.4567 +	18.6	
				730.4246 +	18.6	
		FVTLVFR	441.2658 ++	635.3875 +	14.7	6.9
				247.1441 +	14.7	
				421.2558 +	14.7	
	Epididymal Secretory Protein E1 (P61916)	SGINCPIQK	508.7633 ++	759.3831 +	16.8	1.7
				645.3389 +	16.8	
				485.3082 +	16.8	
		DCGSVDGVIK	525.2502 ++	774.4356 +	14.3	2.4
				630.3821 +	14.3	
				276.0649 +	17.3	

Body Fluid	Protein	Peptide Sequence	Precursor ion (m/z)	Product ions (m/z)	Collision Energy (V)	Retention Time (min)
Saliva	Alpha-amylase 1 (P04745)	lAEYMNHLIDIGVAGFR	640.3330 +++	606.3358 +	18.3	8.7
				903.4538 ++	21.3	
				867.9352 ++	21.3	
		LSGLLDLALGK	550.3397 ++	986.5881 +	18.1	8.7
				899.5560 +	15.1	
				729.4505 +	15.1	
	Submaxillary Gland Androgen Regulated Protein 3B (P02814)	GPYPPGPLAPPQPFGPGFVPPPPPPPYGPGR	1034.5394 +++	1228.6473 +	29.4	9.2
				850.4459 +	29.4	
				1172.6099 +	29.4	
		lPPPPPAPYGPGIFPPPPPQP	710.7189 +++	729.3930 +	14.8	8.3
				535.2875 +	20.8	
				1141.604 +	23.8	
	Cystatin SA (P09228)	lIEGGIYDADLNDER	846.9074 ++	1466.6394 +	30.3	4.8
				1110.4698 +	30.3	
				947.4065 +	30.3	
		ALHFVISEYNK	440.9031 +++	640.2937 +	8.1	4.8
				424.2191 +	14.1	
				568.7904 ++	14.1	
	Statherin (P02808)	FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF	1215.2330 +++	1687.7751 +	30.9	9.5
				1074.4891 +	32.9	
				1229.5626 +	32.9	

Body Fluid	Protein	Peptide Sequence	Precursor ion (m/z)	Product ions (m/z)	Collision Energy (V)	Retention Time (min)
Vaginal Fluid/Menstrual Blood	Cornulin (Q9UBG3)	AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR	839.8888 ++++	1001.4647 ++	28.4	1.4
				886.9115 ++	22.4	
				851.3930 ++	25.4	
		ISPQIQLSGQTEQTQK	893.4707 ++	1247.6226 +	31.7	3.4
				1006.4800 +	31.7	
				793.4127 ++	31.7	
		LLDEDHTGTVEFK	501.9157 +++	294.1812 +	13.3	3.6
				695.8279 ++	16.3	
				639.2859 ++	16.3	
	Neutrophil Gelatinase Associated Lipocalin (P80188)	WYVVGLAGNAILR	716.4090 ++	884.5312 +	23.2	8.8
				350.1499 +	20.2	
				449.2183 +	23.2	
		MYATIYELK	566.2914 ++	1000.5350 +	18.6	5.4
				837.4716 +	21.6	
				295.1111 +	15.6	
	Ly6/PLAUR domain-containing protein 3 (O95274)	GCVQDEFCTR	636.2608 ++	1054.4622 +	23.7	2.3
				955.3938 +	20.7	
				827.3352 +	23.7	
		GLDLHGLLAFIQLQQCAQDR	766.0671 +++	905.3894 +	22.8	10.3
				649.2722 +	25.8	
				1037.5778 +	19.8	
	Suprabasin (Q6UWP8)	ALDGINSGITHAGR	461.2443 +++	599.3022 ++	11.8	2.8
				541.7887 ++	14.8	
		FGQGVHHGLSEGWK	513.5881 +++	696.3444 ++	13.7	2.3
				603.8044 ++	16.7	
	Involucrin (P07476)	GEVLLPVEHQQQK	502.2755 +++	659.8775 ++	10.3	3.3
				610.3433 ++	13.3	
				553.8013 ++	13.3	
		QEAQLELPEQQVGQPK	607.9829 +++	505.7669 ++	14.1	4.2
				699.3308 +	17.1	
				812.4149 +	14.1	
		HLVQQEGQLEQQER	574.6237 +++	689.3213 +	15.9	1.5
				560.2787 +	12.9	
				920.4585 +	12.9	
	Periplakin (O60437)	NQGPQESVVR	557.2860 ++	871.4632 +	24.3	1.4
				814.4417 +	18.3	
				243.1088 +	18.3	
		NLLDEIASR	515.7800 ++	803.4258 +	17.0	6.2
				690.3417 +	17.0	
				228.1343 +	14.0	
		AQSLQSAK	416.7298 ++	633.3566 +	10.9	0.9
				546.3246 +	16.9	
				200.1030 +	13.9	
	Small Proline Rich Protein 3 (Q9UBC9)	VPEPGCTK	444.2182 ++	788.3607+	17.8	1.1
				562.2654 +	17.8	
				394.6840 ++	17.8	
		VPVPGYTK	430.7475 ++	664.3665 +	17.4	2.3
				565.2980 +	14.4	
				381.2132 ++	14.4	

APPENDIX B: STANDARD OPERATING PROCEDURES FOR THE BOTTOM UP LC-MS/MS ASSAY

BODY FLUID IDENTIFICATION BY LC-MS/MS

TABLE OF CONTENTS

MATERIALS AND REAGENTS

Equipment

1. Analytical Balance
2. Refrigerated Microcentrifuge: Capable of speeds up to 12,000 RCF
3. Spectrophotometer: Capable of 562 nm reading
4. SpeedVac Concentrator set to 45 °C
5. Incubator set to 37ºC
6. Agilent AssayMAP Bravo Liquid Handler
7. Agilent 1290 Series Liquid Chromatography System
8. Agilent 6495 Triple Quadrupole LC/MS
9. Agilent AdvancedBio Peptide Map column, 3x100mm, 2.7 µM, #655950-302

General Plastics and Labware

1. Disposable scalpels
2. Clean Bench Paper
3. Deionized water
4. 10% Bleach
5. Microcentrifuge tubes Protein LoBind (Eppendorf 1.5 and 2.0 mL #022431081, 022431102)
6. Costar SpinX .45 µm spin filter (Corning #8163)
7. Costar SpinX spin baskets nofilter inserts (Corning #9301)
8. Pierce BSA Protein Assay Kit (Thermo #23225)
9. Corning Costar Assay Plate, clear, flat bottom (Corning #9017)

AssayMAP and Automation Labware

1. AssayMAP C18 5 µL Cartridge Rack (Agilent #5190-6532)
2. 250 µL sterile pipette tips for AssayMAP Bravo (Agilent #19477-012)
3. Eppendorf 96 well LoBind PCR plate (Eppendorf #0030129512)
4. 12 Column Reservoir Plate (SeahorseBio #201280-100)
5. Greiner U-Bottom White 96 well plate (Greiner #650207)
6. Greiner Universal Plate Lids (Grenier #656199)
7. Eppendorf Storage Film (Eppendorf #0030127870)

Chemicals

1. 100 mM Tris-HCl (Thermo #15568-025)
    a. Dilute 1M Tris-HCl 1:10 in LCMS grade water
2. 8 M Urea (Sigma BioUltra #51456)
    a. 3.123 grams in 4 mL 100 mM Tris-HCl
3. 1 M Tris(2-carboxyethyl)phosphine hydrochloride, TCEP (Thermo #20490)
    a. 0.286 grams in 1 mL LCMS grade water. 35 µL aliquots stored at -80 ºC.
4. 100 mM Iodoacetamide, IAA (Sigma BioUltra #I1149)
    a. 0.036 grams in 2.0 mL LCMS grade water

General Solvents

1. Acetonitrile , LCMS grade
2. Water, LCMS grade
3. Methanol, LCMS Grade
4. Isopropanol, LCMS Grade
5. Formic Acid (FA), LCMS Grade
6. Trifluoroacetic Acid (TFA), HPLC Grade

AssayMAP Solvents

1. Priming Solvent: 50% Acetonitrile in water with 0.5% TFA
    a. 500 mL acetonitrile, 500 mL water, 5 mL TFA
2. Equilibration and Wash Solvent: Water with 0.5% TFA
    a. 1,000 mL water, 5 mL TFA
3. Elution Solvent: 70% Acetonitrile in water with 0.1% FA
    a. 700 mL acetonitrile, 300 mL water, 1 mL formic acid
4. Cartridge Wash (60% Methanol)
    a. 600 mL Methanol, 400 mL water

Mass Spectrometry Solvents

1. Mobile Phase A: Water with 0.1% formic acid
    a. 1,000 mL LCMS grade water, 1 mL LCMS grade formic acid
2. Mobile Phase B: Acetonitrile with 0.1% formic acid
    a. 1,000 mL LCMS grade acetonitrile, 1 mL LCMS grade formic acid
3. Syringe Wash Solution: 1:1:1:1 Methanol:Acetonitrile:Isopropanol:Water
    a. 250 mL methanol, 250 mL acetonitrile, 250 mL isopropanol, 250 mL water
4. Seal Wash Solution: 10% Methanol in water
    a. 900 mL LCMS grade water, 100 mL LCMS grade methanol

Enzymes:

1. Sequencing Grade Trypsin (Promega #V511B)
    a. 100 µg sequencing grade trypsin reconstituted in 400 µL 50 mM acetic acid for a final concentration of 0.25 µg/µL
    b. 50 µL aliquots stored at -80 ºC for up to 1 year.

Myelin Internal Control Reagents:

1. Intact Bovine Myelin Basic Protein Stock Solution (Sigma #13-104)
    a. Reconstituted 10 mg in 20 mL 2% acetonitrile 0.1% FA for 0.5 mg/mL stock
    b. Aliquoted and freeze (-80°C) at stock concentration for up to 1 year
    c. Intact Bovine Myelin Basic Protein Working Solution
        i. Thawed and dilute 1:100 with 30% acetonitrile 0.1% FA for a final concentration of 5 ng/µL
        ii. Spike prior to digest (at dry down stage)
        iii. With a repeater pipette add 20 µL of 5 ng/µL for a 100 ng spike per sample
2. Myelin Isotopically Labelled Peptide Stock Solution (DTGILDSLG**R^**, New England Peptide)
    a. 2 nmol material from NEP (Molar equivalent = 2.11 µg)
    b. Reconstituted in 2.11 mL 30% acetonitrile 0.1% FA for 1 µg/mL stock
    c. Aliquoted and frozen (-80) at stock concentration for up to 1 year
    d. Myelin Isotopically Labelled Peptide Working Solution
        i. Thaw
        ii. Spiked prior to SPE cleanup
        iii. With a repeater pipette add 10 µL of 1 µg/mL stock for a 10 ng spike per sample

System Suitability:

1. HSA Peptide Standard Mix (Agilent #G2455-85001)
    a. To each vial of HSA standard (500 pmol) add 500 µL of 15% acetonitrile with 0.1% formic acid for a 1 pmol/µL stock.
    b. Vortex and mix vial for 30 seconds. Allow the vial to stand at room temperature for 5 minutes
2. Dilute 1 pmol/µL stock 1:10 for a final concentration of 100 fmol/µL.
    a. Aliquot 50 µL to 250 µL polypropylene snap caps vials.
3. Cap vials, store at -80°C for up to 1 year

CONTROLS

Positive Control (PC) – a known control sample (*e.g.* human semen or blood) is required for each analytical batch which undergoes extraction, digestion, and cleanup alongside questioned samples.

Reagent (negative) Control (RC) – a blank samples that undergoes the same extraction process as the samples to test the reagents for contaminants.

BODY FLUID EXTRACTION AND QUANTITATION

Initial preparations:

1. Clear off a workspace and lay a new, clean piece of bench paper. Check and replace any reagents that have expired.
2. Prepare one set of 2.0 mL microcentrifuge tubes for each sample marked with a unique sample identifier.

Extraction protocols:

1. Gather all utensils (*e.g.*, scalpel, scissors, forceps, weigh paper) needed for sample cutting.
2. Sterilize utensils with a 20% bleach solution followed by a water rinse.
3. If samples were not already collected and prepared during the evidence examination process (*i.e.* placed in a 2.0 mL microcentrifuge tube), carefully cut the stain (~1 x 1 cm, or as appropriate, depending on the nature of the stain) and place in the appropriately marked 2.0 mL microcentrifuge tube. If the sample is on a swab, use ½ of the swab or the stained area if the staining is uneven. In the case of loose flakes, use at the discretion of the analyst. In the case of cigarette butts, remove the paper around the filter. If the staining is light, a larger area may be taken.
4. Extract evidentiary material by soaking in 500 - 1,000 µL of deionized water for 30 minutes. Vortex the sample frequently to facilitate extraction of biological material from substrate. Pulse spin for 10 seconds to remove droplets from the lid, and transfer the sample substrate into a clean spin basket and centrifuge at 12,000 RPM for 10 minutes.
5. Transfer supernatant to a clean Costar 0.45 µM SpinX filter and reserve pelleted material for genetic testing. Filter the supernatant by centrifuging at 10,000 RPM for 2 minutes, or until all extract has passed through the filter. Remove the SpinX filter and discard.

    NOTES: Blood samples may not completely pass through the SpinX filter.

Quantitation protocols:

1. Prepare bovine serum albumin standards in 100 mM Tris-HCl using the following table:

Standard	Bovine Serum Albumin (µL)	100mM Tris-HCl (µL)	Protein Concentration (µg/mL)
A	2 mg/mL stock	None	2000
B	375 A	125	1500
C	325 A	325	1000
D	175 B	175	750
E	325 C	325	500
F	325 E	325	250
G	325 F	400	125
H	100 G	400	25
BLANK	None	All	N/A

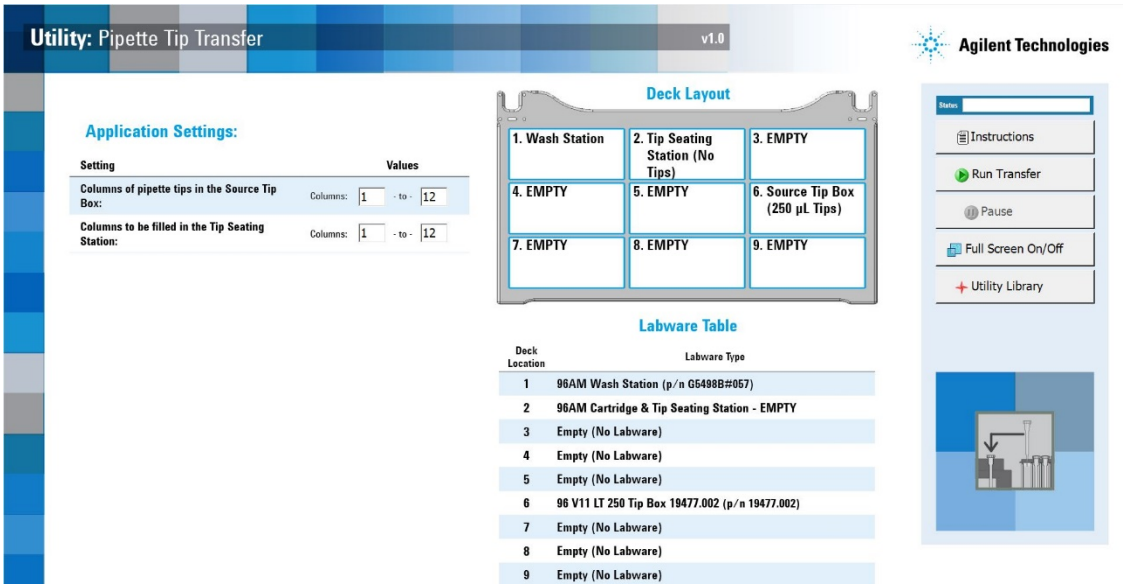
2. For sample preparation, dilute sample supernatant in 100mM Tris-HCl at the discretion of the analyst
    a. Blood and Semen Samples 1:100 dilution
    b. Saliva and Vaginal Fluid 1:50 dilution
    c. Dependent on the concentration and physical appearance of the extract
3. Prepare BCA Protein Assay working reagent according to the number of samples being prepared.
    a. Volume of Reagent A = (total # samples and standards + 2) x 200
    b. Volume of Reagent B = (volume of reagent A) / 50
4. Prepare a 96 well Corning square bottom plate. Aliquot 25 µL of standard or sample to the appropriate well. Add 200 µL of prepared working reagent to each well and seal using a protective film. Incubate the plat at 37 º C for 30 minutes.
5. Order the plate according to the following chart:

	1	2	3
A	BLANK	STD E	SAMPLE
B	BLANK	STD E	SAMPLE
C	STD B	STD F	SAMPLE
D	STD B	STD F	
E	STD C	STD G	
F	STD C	STD G	
G	STD D	STD H	
H	STD D	STD H	

6. Remove the protective film and analyze on a spectrophotometer at 562 nm. Ensure the spectrophotometer is programmed accordingly, with the standard concentrations in the appropriate well number.
7. Evaluate the results as follows:
    a. Standard Curve R^2 value: >0.98
8. Calculate the total protein for each question sample, targeting 100 µg total protein
    a. (100 µg) / (Reported protein concentration in µg/mL x Dilution Factor) x 1000
9. Prepare a 96 well Greiner U-bottom White plate, with a repeater pipette add 20 µL of 5 ng/µL for a 100 ng spike per sample of intact Bovine Myelin Basic Protein.
10. Add up to 100 µg total protein for each sample being analyzed.
11. Lyophilized to dryness in a SpeedVac concentrator at 45 º C

PROTEIN SAMPLE DIGESTION

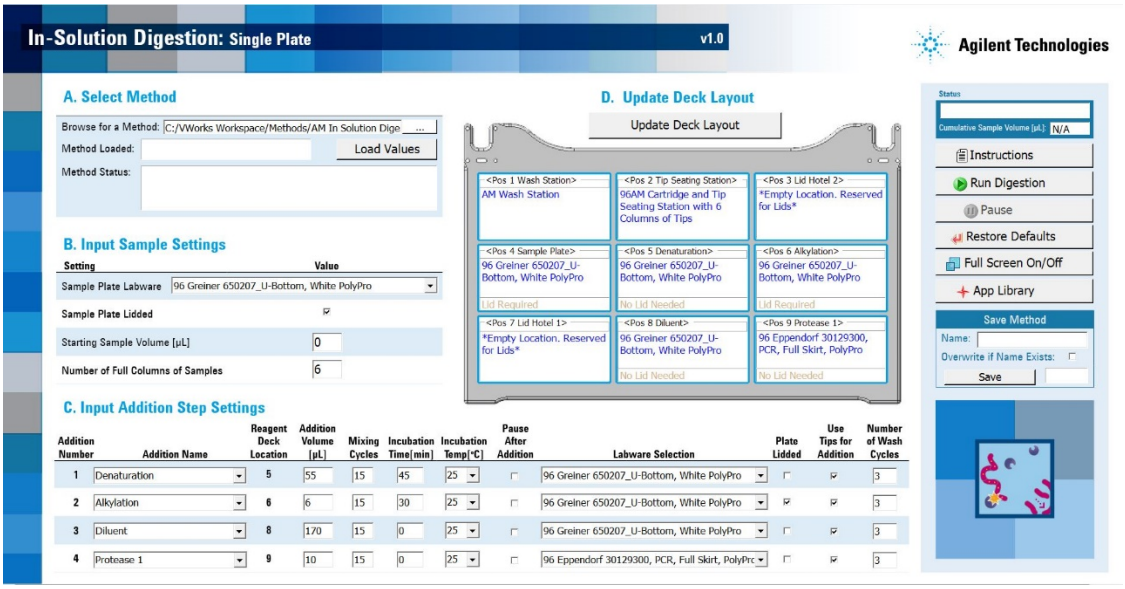
1. Run AssayMAP startup procedure found in Appendix I.
2. Open the Pipette Tip Transfer Utility Application in the VWorks Software Utility Library. Arrange the pipette tip rack according to the deck layout diagram displayed. Input the number of columns to transfer according to the number of samples being processed. A full row of tips should be transferred, regardless of whether the sample plate contains a complete row of samples to be analyzed. Run the Pipette Tip Transfer Application. Remove the pipette tip rack from the instrument.



3. Open the In-Solution Digest Single Plate application in the VWorks Software App Library.
4. Prepare the denaturant solution. Add 32.5 µL of 1 M TCEP stock solution to 8 M Urea in 100 mM Tris-HCl (for a 5 mM final concentration). Aliquot 100 µL of denaturant solution into each well of a Greiner U-Bottom White 96 well plate. Move the plate to deck position 5 on the AssayMAP.
5. Prepare the alkylant solution. Aliquot 50 µL of 100 mM IAA solution into each well of a Greiner U-Bottom White 96 well plate. Place a universal black lid on the plate. Check the corresponding box in the VWorks Software indicating the alkylant plate is lidded. Move the plate to deck position 6 on the AssayMAP.

6. Prepare the diluent solution. Aliquot 250 µL of 100 mM Tris-HCl into each well of a Greiner U-Bottom White 96 well plate. Move the plate to deck position 8 on the AssayMAP.
7. Prepare the trypsin solution. Aliquot 15 µL of 0.25 µg/µL sequencing grade trypsin into each well of an Eppendorf 96 well PCR plate. Move the plate to deck position 9 on the AssayMAP.
8. Move the sample plate containing the lyophilized sample to deck position 4 on the AssayMAP. Place a universal black lid on the sample plate. Check the corresponding box in the VWorks Software indicating the sample plate is lidded.
9. Input the correct number of columns to be digested according to the number of samples being processed in the sample plate. Verify the following parameters into the application:

Digest Solution	Addition Volume	# Mix Cycles	Incubation Time and Temperature
Denaturant	55 µL	15 mix cycles	45-minute incubation at 25 º C
Alkylant	6 µL	15 mix cycles	30-minute incubation at 25 º C
Diluent	170 µL	15 mix cycles	No incubation
Trypsin	10 µL	15 mix cycles	No incubation

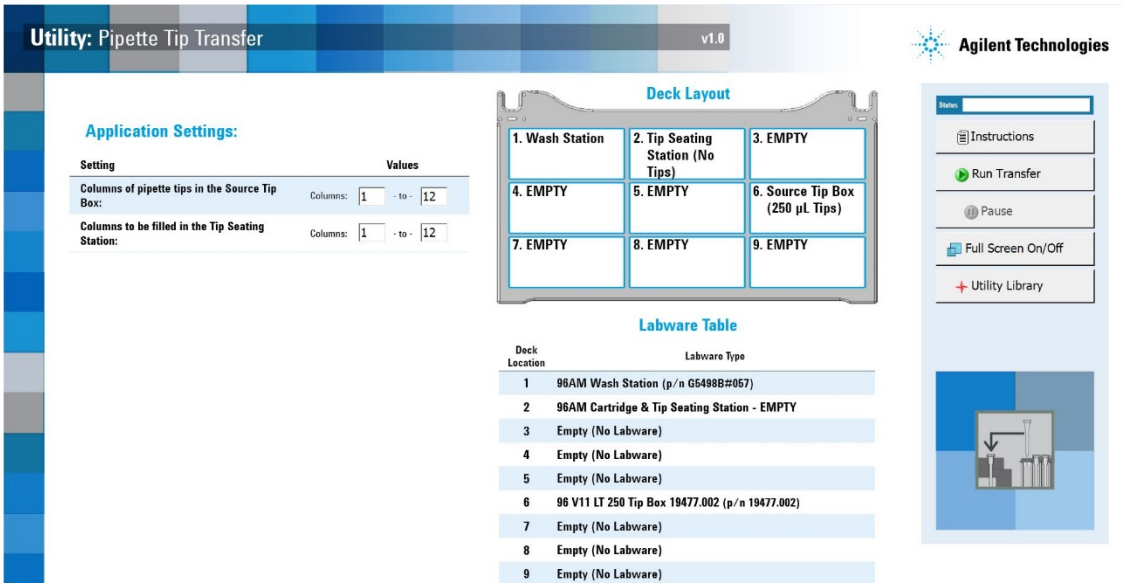


10. Run the digestion application.
11. With the completion of the digestion application, remove the sample plate and seal with an Eppendorf storage film. Place the sample plate in an incubator set at 37 ºC for 14-16 hours.
12. Run AssayMap shutdown procedure found in Appendix I.

PEPTIDE SAMPLE CLEANUP

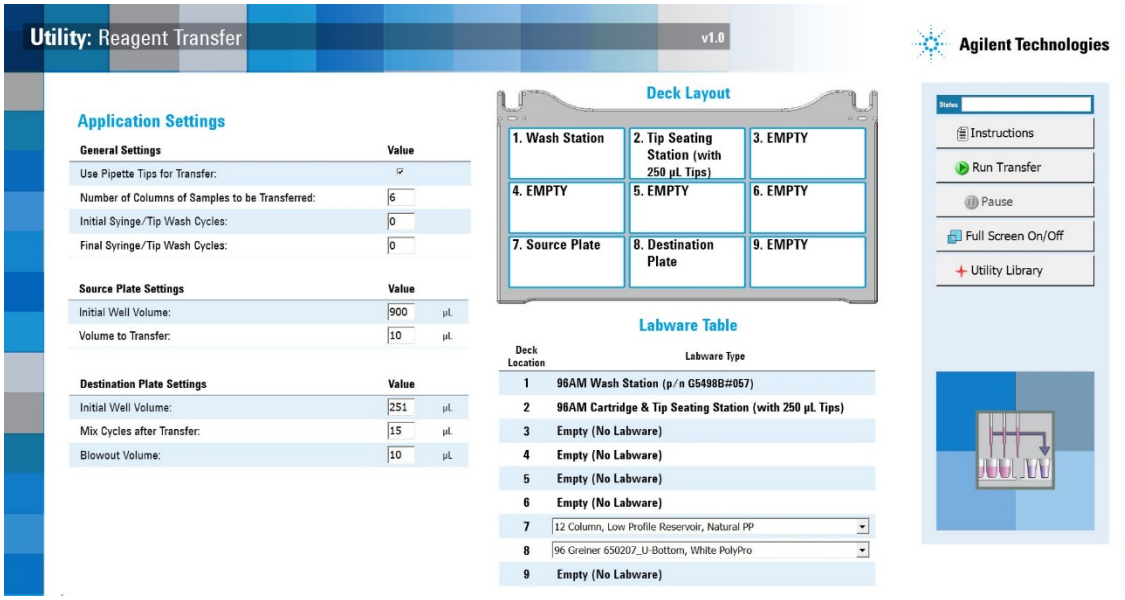
Sample Acidification:

1. Run AssayMap startup procedure found in Appendix I.
2. Allow the sample plate to come to room temperature. Remove Myelin Isotopically Labelled Peptide Stock Solution from freezer.
3. Remove storage film. With a repeater pipette, add 10 µL of Myelin Isotopically Labelled Peptide stock solution at1 µg/mL for a 10 ng spike per sample.
4. Open the Pipette Tip Transfer Utility Application in the VWorks Software Utility Library. Arrange the pipette tip rack according to the deck layout diagram displayed. Input the number of columns to transfer according to the number of samples being processed. A full row of tips should be transferred, regardless of whether the sample plate contains a complete row of samples to be analyzed. Run the Pipette Tip Transfer Application. Remove the pipette tip rack from the instrument.



5. Open the Reagent Transfer Utility application in the VWorks Software Utility library.
6. Fill the appropriate number of channels of a 12 Column Reservoir Plate with 25% TFA solution. Move the plate to the source location of the deck layout displayed.

7. Place the sample plate containing digested material to the destination location of the deck layout displayed.
8. In the Reagent Transfer Utility application, select the number of columns to be transferred, check the box indicating the use of pipette tips to transfer, and input 10 µL of 25% TFA to be transferred to each well.



9. Run the Reagent Transfer application.
10. Discard the pipette tips.
11. Discard the 25% TFA solution into solvent waste.

Cartridge Transfer:

1. Open the Cartridge Transfer Utility application in VWorks Software Utility library.
2. Arrange the cartridge rack according to the deck layout diagram displayed.
3. Input the number of columns to transfer according to the number of samples being processed. Note that full columns should be transferred.

4. Run the Cartridge Transfer application.
5. Remove the cartridge rack from the instrument.

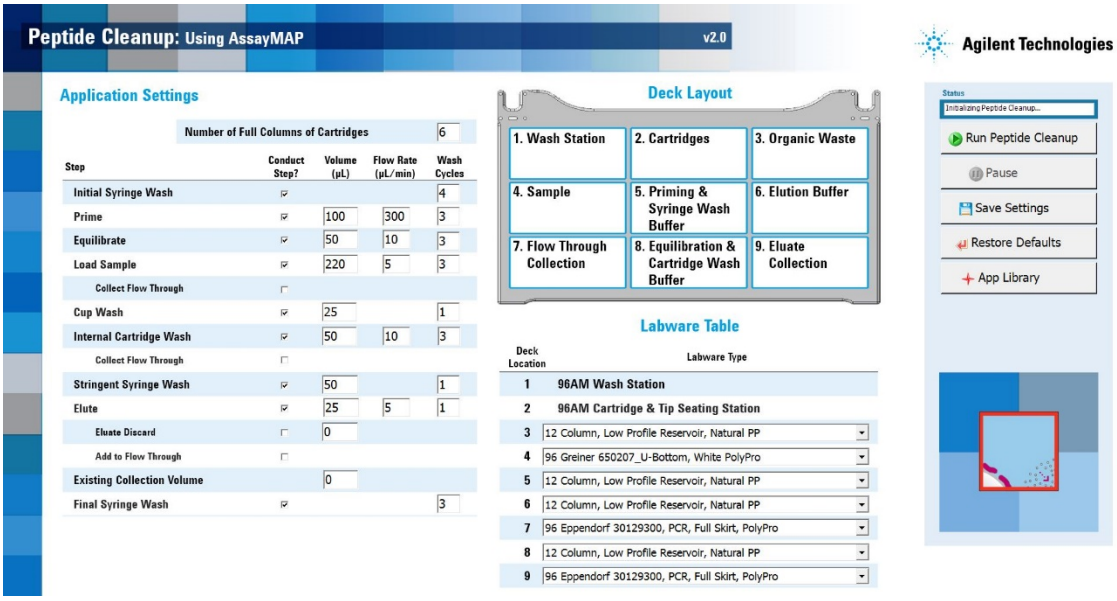
Peptide Cleanup Protocol:

1. Open the Peptide Cleanup application in VWorks Software application library.
2. Prepare the Prime solution. Fill the corresponding number of channels to capacity of a labeled 12 Column Reservoir Plate to the number of columns being processed. Move the plate position 5 on the AssayMAP deck.
3. Prepare the Equilibration solution. Fill the corresponding number of channels to capacity of a labeled 12 Column Reservoir Plate to the number of columns being processed. Move the plate to position 6 on the AssayMAP deck.
4. Prepare the Elution solution. Fill the corresponding number of channels to capacity of a labeled 12 Column Reservoir Plate to the number of columns being processed. Move the plate to position 8 on the AssayMAP deck.
5. Place the 96 well plate containing digested and acidified sample in the corresponding position to the displayed deck layout.
6. Place a 12 Column Reservoir plate in the Organic Waste/position 3 on the deck.
7. Place a 96 well PCR plate in the Eluate Collection/position 9 on the deck.

8. Input the appropriate number of columns being processed in the software.

9. Verify the following parameters in the software:

Cleanup Step	Addition Volume	Flow Rate	# Wash Cycles
Initial Syringe Wash	---	---	4 wash cycles
Prime	100 µL 50% ACN 0.5% TFA	300 µL/min	3 wash cycles
Equilibrate	50 µL of H2O 0.5% TFA	10 µL/min	3 wash cycles
Sample Load	220 µL	15 µL/min	3 wash cycles
Cup Wash	25 µL	---	1 wash cycle
Internal Cartridge Wash	50 µL	10 µL/min	3 wash cycles
Stringent Syringe Wash	50 µL	---	1 wash cycle
Elute	20 µL 70% ACN 0.1% FA	5 µL/min	3 wash cycles
Final Syringe Wash	---	---	3 wash cycles



10. Run the Peptide Cleanup application.

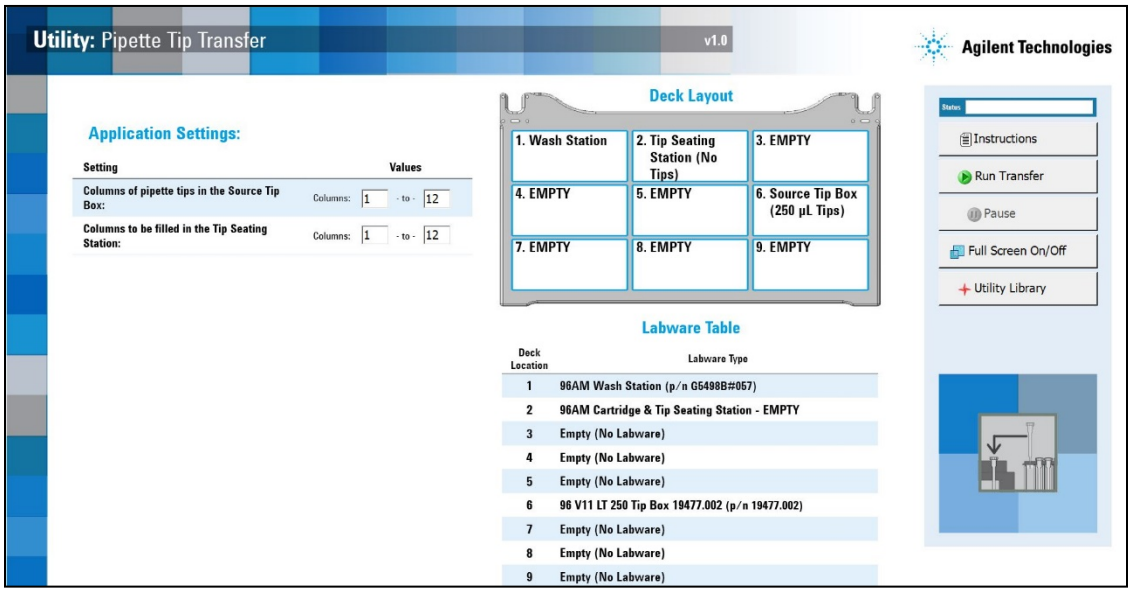
11. Upon completion, remove the eluate collection plate from the instrument.

12. Dry down in the SpeedVac concentrator at 45 ºC until lyophilized to dryness.

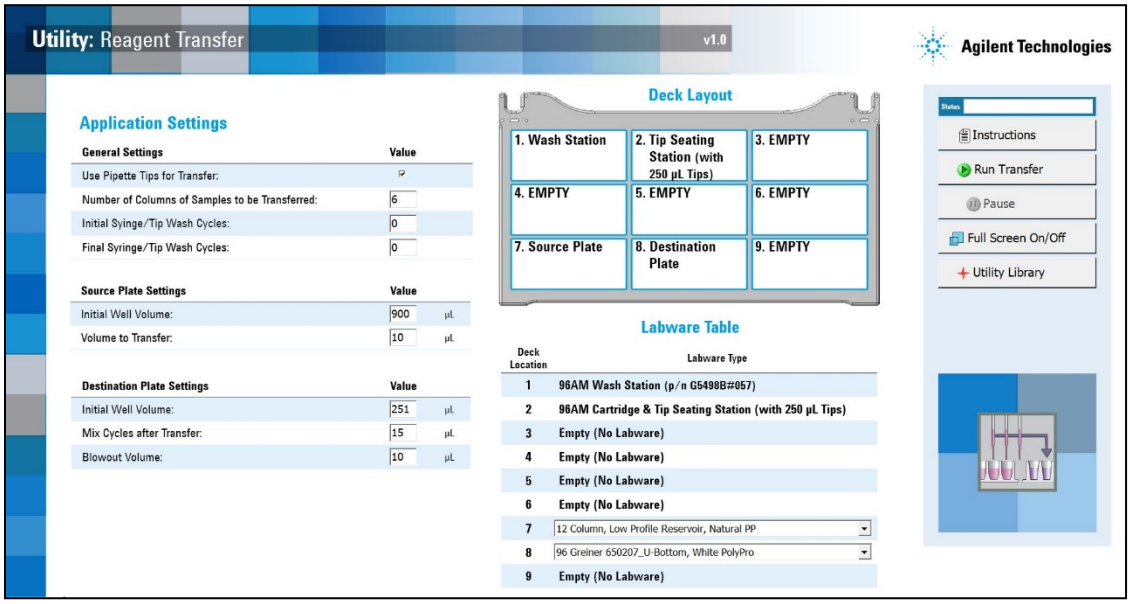
ANALYSIS BY MASS SPECTROMETRY

Initial Preparations:

1. Run AssayMap startup procedure found in Appendix I.
2. Open the Pipette Tip Transfer Utility Application in the VWorks Software Utility Library. Arrange the pipette tip rack according to the deck layout diagram displayed. Input the number of columns to transfer according to the number of samples being processed. A full row of tips should be transferred, regardless of if the sample plate contains a complete row of samples to be analyzed. Run the Pipette Tip Transfer Application. Remove the pipette tip rack from the instrument.



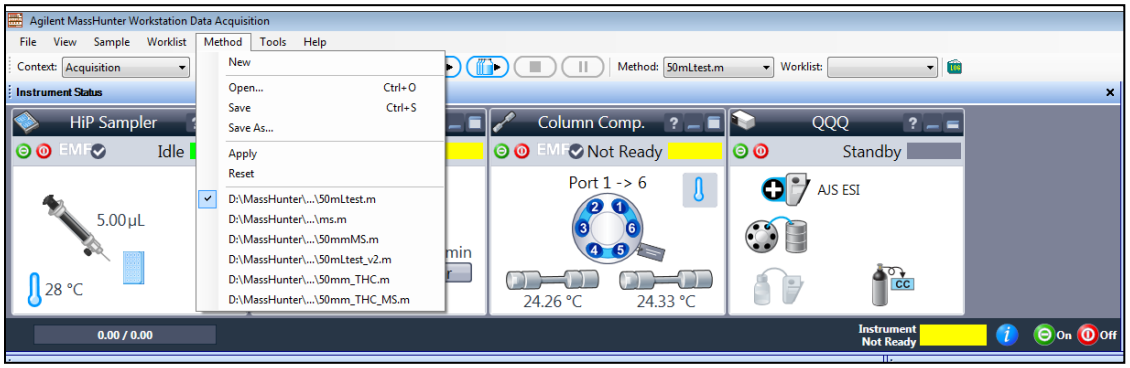
3. Open the Reagent Transfer Utility application in the VWorks Software Utility library.
4. Fill the appropriate number of channels of a 12 Column Reservoir Plate with 2% acetonitrile with 0.1% formic acid in LCMS grade water.
5. Move the plate to the source location of the deck layout displayed. Place the sample plate containing lyophilized, purified sample to the destination location of the deck layout displayed.



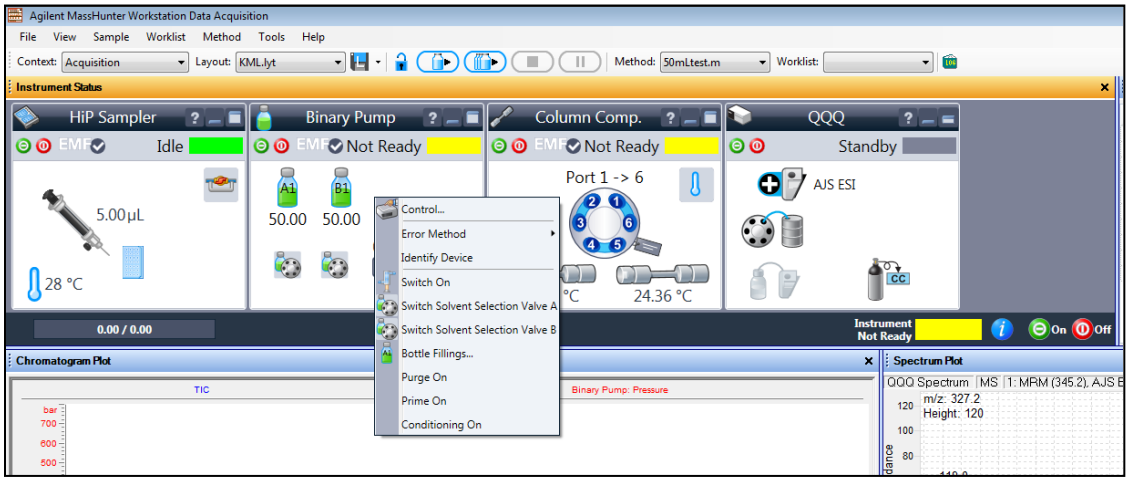
6. In the Reagent Transfer Utility application, select the number of columns to be transferred, check the box indicating the use of pipette tips to transfer, and input 100 µL of 2% ACN 0.1% FA to be transferred to each well.
7. Run the Reagent Transfer application.
8. Discard the pipette tips.
9. Discard the 2% ACN 0.1% FA solution into solvent waste.

Sample Plate Analysis:

1. Ensure all solvents are not expired.
2. Verify the Agilent Jet Stream source as been cleaned during the week of analysis or as necessary. For example, deterioration or material on the spray shield.
3. Verify the 6495 MS has been fully tuned within 1 month and a check tune has been performed during the week of analysis. If necessary, tune the instrument.
4. Open MassHunter Acquisition Software.
5. Under the Method Tab, open and load the HSA method.

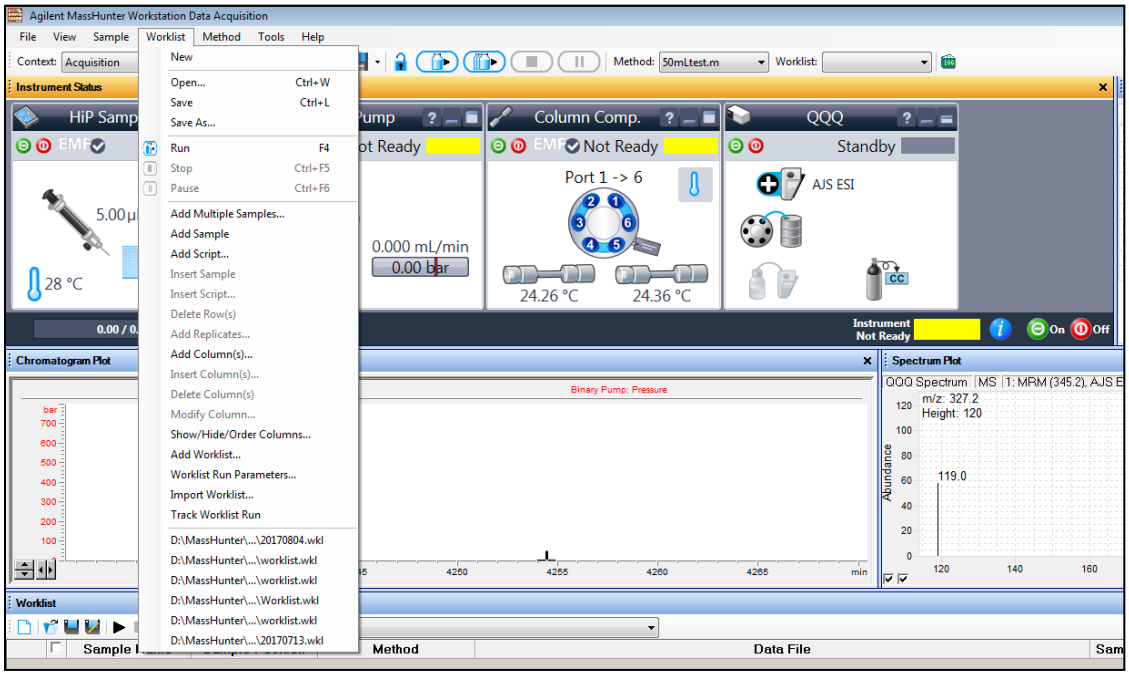


6. If fresh solvents were prepared, update the bottle fillings by right clicking the Binary Pump Module.

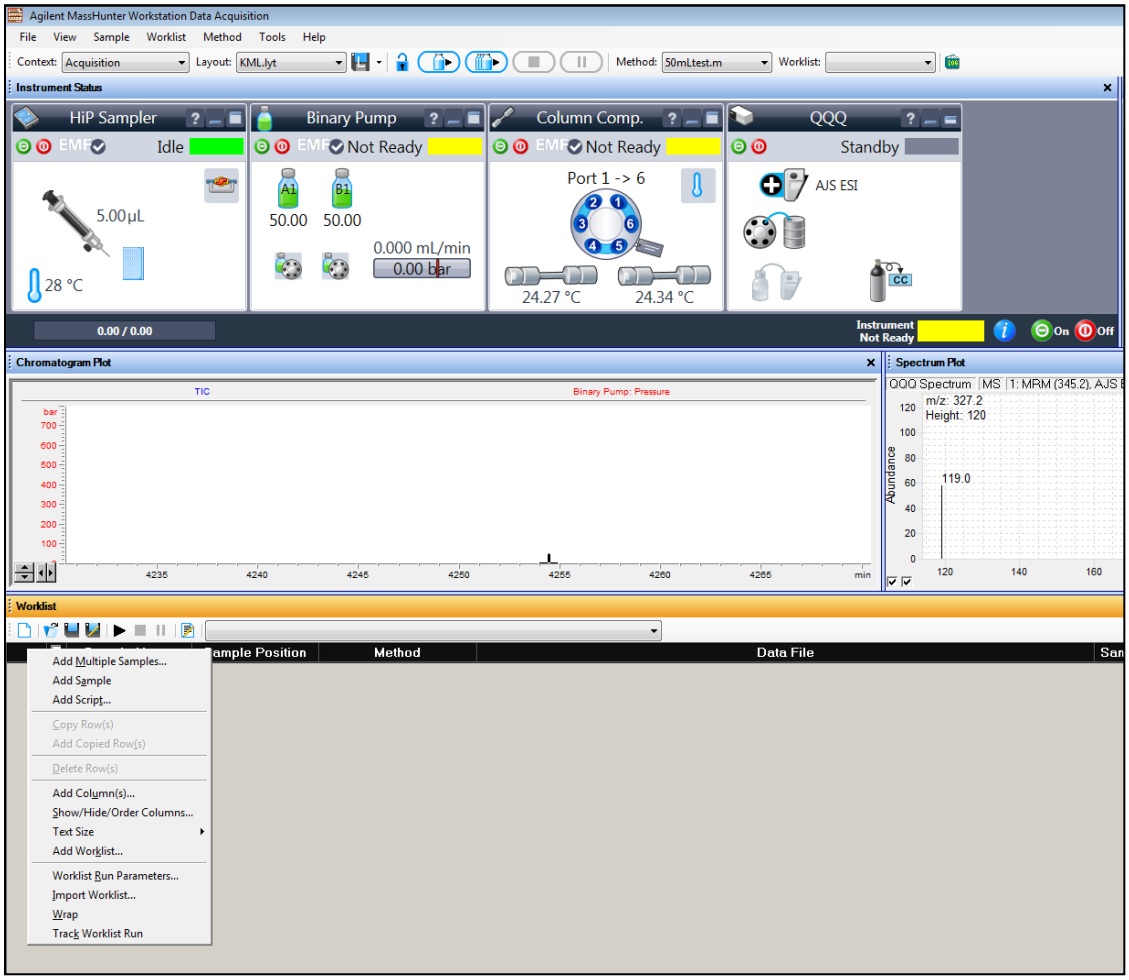


7. Purge the A and B Mobile Phase pump lines by right clicking the Binary Pump Module. Purge at 50:50 mobile phase A:B at 4 mL/min for 4 minutes.
8. Ensure the correct analytical column is connected in the proper position on the heat block. Agilent AdvancedBio Peptide Map column, 3x100mm, 2.7 µM, #655950-302.
9. Turn on the instrument by clicking the green ‘On’ button in the lower right corner of the Module bar. Allow the instrument to idly pump for approximately five minutes. Monitor the pressure listed on the Binary Pump Module. Pressures > 350 bar may indicate the column requires maintenance or replacement.
10. Create a new run folder on the computer hard drive, labeling appropriately according to laboratory protocol.

11. In MassHunter Acquisition Software, under the Worklist Tab, modify the Worklist Run Parameters. Input the new run folder and ensure the proper method and run folder is selected.

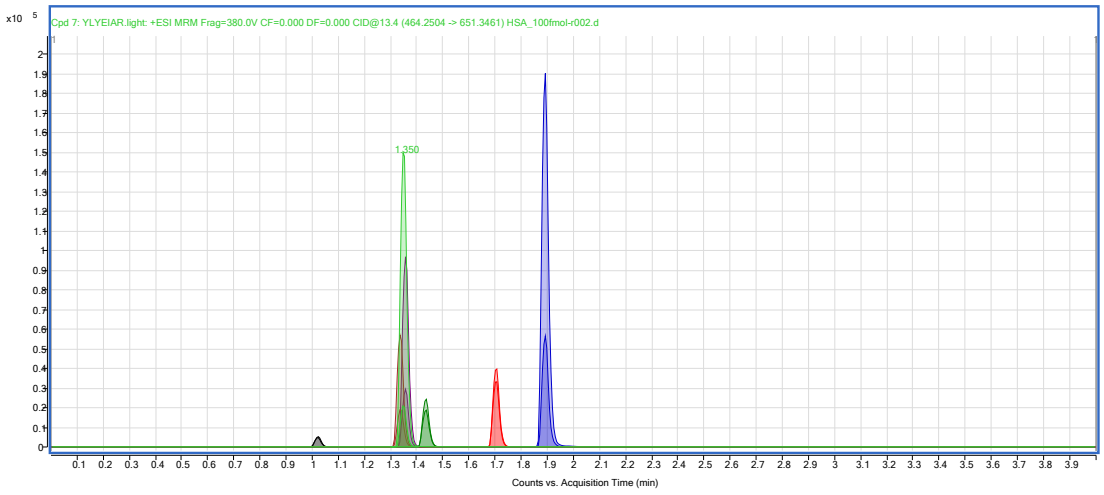


12. Click the Worklist tab in the bottom left corner of the screen. Begin a new worklist by right clicking the space and selecting Add Sample. Alternatively, open a previously made worklist by clicking the Worklist Tab at the top of the screen and opening the desired worklist. Label the samples appropriately and insert blanks where necessary. Always run HSA System Suitability samples in triplicate at the beginning of the worklist, to ensure proper instrument performance. Always include a shutdown script at the end of the worklist, communicating to the instrument to go into standby once the run is completed.



13. Once the worklist is prepared, place samples into the autosampler according to the plate/vial position.
14. Select all the samples in the worklist by checking the square box at the top left of the worklist. Hit the triangle “Play” button to start the worklist.
15. Review the results for the HSA System Suitability samples in the Qualitative Analysis Software.
16. Verify that all six peptides are present in the system suitability injections. Verify retention times, relative intensities, as well as peak shape for the peptides. If a problem is observed, troubleshoot the issue before running the full batch.
17. Refer to the table and representative chromatogram below:

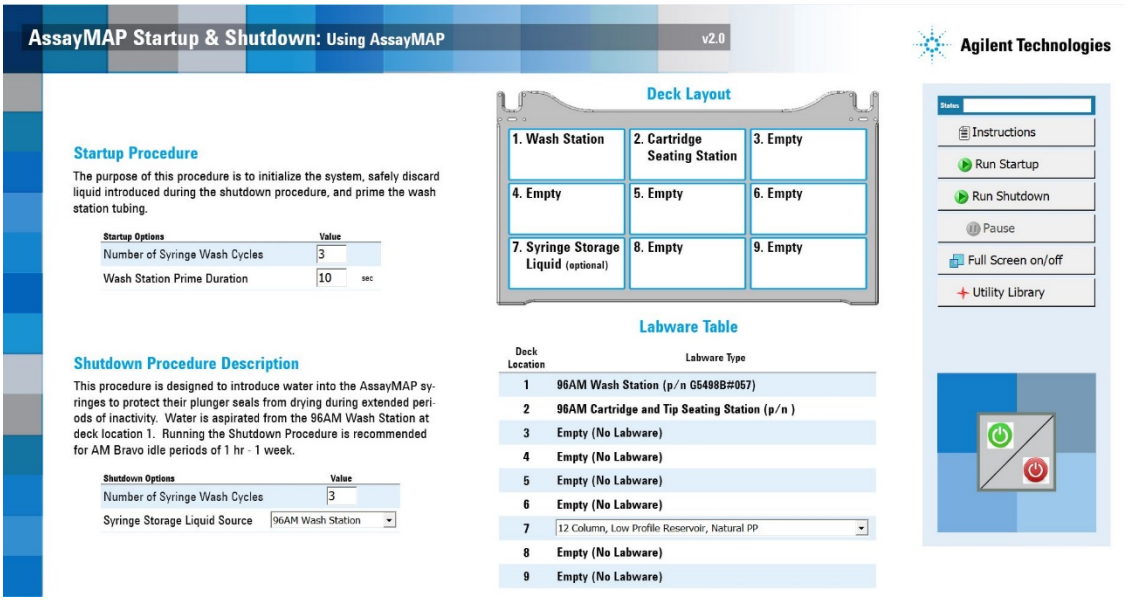
Peptide Sequence	Retention Time (min.)	Intensity (cts.)
AAFTEC[+57.0]C[+57.0]QAADK	1	5.5×10^3
AVMDDFAAFVEK	1.7	4.0×10^4
RPC[+57.0]FSALEVDETYVPK	1.4	2.5×10^4
HPYFYAPELLFFAK	1.8	2.0×10^5
LVNEVTEFAK	1.3	1.0×10^5
KVPQVSTPTLVEVSR	1.3	5.5×10^4
YLYEIAR	1.3	1.5×10^5



APPENDIX I ASSAYMAP BRAVO STARTUP AND SHUTDOWN

Startup

1. Turn on the AssayMAP Bravo automation system. Ensure the wash station carboy contains a sufficient amount of water and the waste carboy is empty. Open the Startup Utility Application in the VWorks Software. Run the application and follow the prompts on the computer.



Shutdown

1. Open the Startup & Shutdown Utility Application in the VWorks Software. Run the application and follow the prompts on the computer.

AssayMAP Startup & Shutdown: Using AssayMAP

v2.0

Agilent Technologies

Startup Procedure

The purpose of this procedure is to initialize the system, safely discard liquid introduced during the shutdown procedure, and prime the wash station tubing.

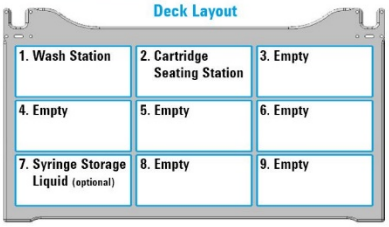
Startup Options	Value
Number of Syringe Wash Cycles	3
Wash Station Prime Duration	10 sec

Shutdown Procedure Description

This procedure is designed to introduce water into the AssayMAP syringes to protect their plunger seals from drying during extended periods of inactivity. Water is aspirated from the 96AM Wash Station at deck location 1. Running the Shutdown Procedure is recommended for AM Bravo idle periods of 1 hr - 1 week.

Shutdown Options	Value
Number of Syringe Wash Cycles	3
Syringe Storage Liquid Source	96AM Wash Station

Deck Layout



Labware Table

Deck Location	Labware Type
1	96AM Wash Station (p/n G5498B#057)
2	96AM Cartridge and Tip Seating Station (p/n)
3	Empty (No Labware)
4	Empty (No Labware)
5	Empty (No Labware)
6	Empty (No Labware)
7	12 Column, Low Profile Reservoir, Natural PP
8	Empty (No Labware)
9	Empty (No Labware)

Status

Instructions

Run Startup

Run Shutdown

Pause

Full Screen on/off

Utility Library

APPENDIX II HUMAN SERUM ALBUMIN ACQUISITION PARAMETERS

Instrument Parameters

- Instrumentation: Agilent 6495 Tandem Mass Spectrometer with an Agilent 1290 LC system.
    - Instrument Mode: MRM
- Agilent AdvancedBio Peptide Map column, 3x100mm, 2.7 µM
    - Mobile Phases:
        - A: Water with 0.2% Formic Acid
        - B: Acetonitrile with 0.2% Formic Acid
    - Needle Wash: 1:1:1:1 Methanol:Acetonitrile:Isopropanol:Water
    - Injection Volume: 2.5 -10 µL
- System Suitability: Agilent Human Serum Albumin peptide mix with 100 fmol on column.
- The ions monitored are listed below.

Compound Group	Compound Name	Precursor Ion	Product Ion
sp|P02768|ALBU_HUMAN	AAFTEC[+57.0]C[+57.0]QAADK.light	686.3	981.4
sp|P02768|ALBU_HUMAN	AAFTEC[+57.0]C[+57.0]QAADK.light	686.3	852.3
sp|P02768|ALBU_HUMAN	AVMDDFAAFVEK.light	671.8	1,172.5
sp|P02768|ALBU_HUMAN	AVMDDFAAFVEK.light	671.8	1,041.5
sp|P02768|ALBU_HUMAN	RPC[+57.0]FSALEVDETYVPK.light	637.6	961.5
sp|P02768|ALBU_HUMAN	RPC[+57.0]FSALEVDETYVPK.light	637.6	851.4
sp|P02768|ALBU_HUMAN	HPYFYAPELLFFAK.light	581.6	779.4
sp|P02768|ALBU_HUMAN	HPYFYAPELLFFAK.light	581.6	482.8
sp|P02768|ALBU_HUMAN	LVNEVTEFAK.light	575.3	937.5
sp|P02768|ALBU_HUMAN	LVNEVTEFAK.light	575.3	595.3
sp|P02768|ALBU_HUMAN	KVPQVSTPTLVEVSR.light	547.3	702.4
sp|P02768|ALBU_HUMAN	KVPQVSTPTLVEVSR.light	547.3	589.3
sp|P02768|ALBU_HUMAN	YLYEIAR.light	464.3	651.3
sp|P02768|ALBU_HUMAN	YLYEIAR.light	464.3	488.3

- The acquisition parameters are as follows:

Time Segment 1

Scan Segments

Cpd Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Dwell	Frag (V)	CE (V)	Cell Acc (V)	Polarity
AAFTEC[+57.0]C[+57.0]QAADK.light	No	686.287019	Unit/Enh (6490)	981.376442	Unit/Enh (6490)	20	380	24	4	Positive
AAFTEC[+57.0]C[+57.0]QAADK.light	No	686.287019	Unit/Enh (6490)	852.333849	Unit/Enh (6490)	20	380	26	4	Positive
AVMDDFAAFVEK.light	No	671.82102	Unit/Enh (6490)	1172.529237	Unit/Enh (6490)	20	380	21.8	4	Positive
AVMDDFAAFVEK.light	No	671.82102	Unit/Enh (6490)	1041.488752	Unit/Enh (6490)	20	380	23.8	4	Positive
RPC[+57.0]FSALEVDETYVPK.light	No	637.648743	Unit/Enh (6490)	961.456013	Unit/Enh (6490)	20	380	18.2	4	Positive
RPC[+57.0]FSALEVDETYVPK.light	No	637.648743	Unit/Enh (6490)	851.414525	Unit/Enh (6490)	20	380	18.2	4	Positive
HPYFYAPELLFFAK.light	No	581.636214	Unit/Enh (6490)	779.351137	Unit/Enh (6490)	20	380	12.1	4	Positive
HPYFYAPELLFFAK.light	No	581.636214	Unit/Enh (6490)	482.778753	Unit/Enh (6490)	20	380	12.1	4	Positive
LVNEVTEFAK.light	No	575.311146	Unit/Enh (6490)	937.462538	Unit/Enh (6490)	20	380	18.8	4	Positive
LVNEVTEFAK.light	No	575.311146	Unit/Enh (6490)	595.308603	Unit/Enh (6490)	20	380	18.8	4	Positive
KVPQVSTPTLVEVSR.light	No	547.317433	Unit/Enh (6490)	702.414465	Unit/Enh (6490)	20	380	18.9	4	Positive
KVPQVSTPTLVEVSR.light	No	547.317433	Unit/Enh (6490)	589.330401	Unit/Enh (6490)	20	380	18.9	4	Positive
YLYEIAR.light	No	464.250361	Unit/Enh (6490)	651.346051	Unit/Enh (6490)	20	380	13.4	4	Positive
YLYEIAR.light	No	464.250361	Unit/Enh (6490)	488.282723	Unit/Enh (6490)	20	380	15.4	4	Positive

Scan Parameters

Data Stg	Threshold
Centroid	0

Source Parameters

Parameter	Value (+)	Value (-)
Gas Temp (°C)	200	200
Gas Flow (l/min)	13	13
Nebulizer (psi)	35	35
SheathGasHeater	200	200
SheathGasFlow	12	12
Capillary (V)	3500	3000
VCharging	300	500

Ion Funnel Parameters

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

Chromatograms

Chrom Type	Label	Offset	Y-Range
TIC	TIC	0	10000000

Instrument Curves

Actual

Acquisition Method Info

Method Name	HSA.m
Method Path	D:\MassHunter\Methods\Proteomics_DOD\3mm_Final\HSA.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

Time Segments

Index	Start Time (min)	Scan Type	Ion Mode	Div Valve	Delta EMV	Store
1	0	MRM	ESI+Agilent Jet Stream	To MS	200	Yes

Name: HiP Sampler Model: G4226A

Auxiliary	
Draw Speed	100.0 µL/min
Eject Speed	100.0 µL/min
Draw Position Offset	0.0 mm
Wait Time After Drawing	2.0 s
Sample Flush Out Factor	5.0
Vial/Well bottom sensing	No
Injection	
Injection Mode	Injection with needle wash
Injection Volume	1.00 µL
Needle Wash	
Needle Wash Location	Flush Port
Wash Time	3.0 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	1.000 mL/min
Use Solvent Types	Yes
Stroke Mode	Synchronized
Low Pressure Limit	0.00 bar
High Pressure Limit	500.00 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	4.00 min
Post Time	
Posttime Mode	Time set
Posttime	3.00 min

Solvent Composition

	Channel	Ch. 1 Solv.	Name 1	Ch2 Solv.	Name 2	Selected	Used	Percent
1	A	100.0 % Water V.03		100.0 % Water V.03		Ch. 1	Yes	95.00 %
2	B	100.0 % Acetonitrile V.03		100.0 % Acetonitrile V.03		Ch. 1	Yes	5.00 %

Timetable

	Time	A	B	Flow	Pressure
1	2.40 min	40.00 %	60.00 %	--- mL/min	--- bar
2	3.10 min	20.00 %	80.00 %	--- mL/min	--- bar
3	3.80 min	20.00 %	80.00 %	--- mL/min	--- bar
4	3.90 min	95.00 %	5.00 %	--- mL/min	--- bar
5	4.00 min	95.00 %	5.00 %	--- mL/min	--- bar

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

APPENDIX III BODY FLUID IDENTIFICATION ACQUISITION PARAMETERS

Instrument Parameters:

- Instrumentation: Agilent 6495 Tandem Mass Spectrometer with an Agilent 1290 LC system.
    - Instrument Mode: MRM
- Agilent AdvancedBio Peptide Map column, 3x100mm, 2.7 µM
    - Mobile Phases:
        - A: Water with 0.2% Formic Acid
        - B: Acetonitrile with 0.2% Formic Acid
    - Needle Wash: 1:1:1:1 Methanol:Acetonitrile:Isopropanol:Water
    - Injection Volume: 2.5 -10 µL
- System Suitability: Agilent Human Serum Albumin peptide mix with 100 fmol on column.
- The ions monitored are listed below and with representative chromatograms for each protein target following.

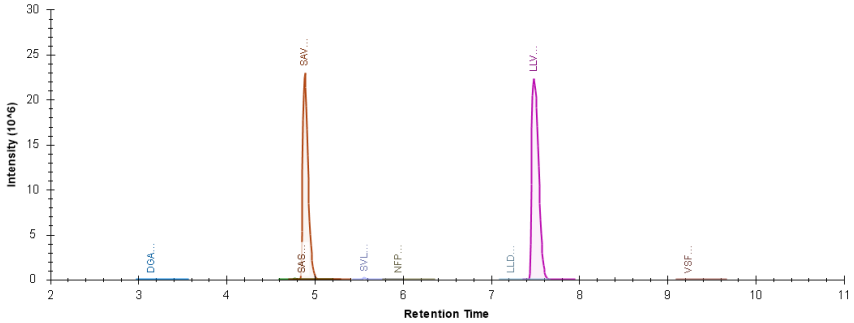
Compound Group	Compound Name	Precursor Ion	Product Ion
sp|O95274|LYPD3_HUMAN	GLDLHGLLAFIQLQQC[+57.0]AQDR.light	766.1	649.3
sp|O95274|LYPD3_HUMAN	GLDLHGLLAFIQLQQC[+57.0]AQDR.light	766.1	905.4
sp|O95274|LYPD3_HUMAN	GLDLHGLLAFIQLQQC[+57.0]AQDR.light	766.1	1037.6
sp|P02808|STAT_HUMAN	FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF.light	1215.2	1074.5
sp|P02808|STAT_HUMAN	FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF.light	1215.2	1229.6
sp|P02808|STAT_HUMAN	FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF.light	1215.2	1687.8
sp|P02647|APOA1_HUMAN	VSFLSALEEYTK.light	693.9	600.8
sp|P02647|APOA1_HUMAN	VSFLSALEEYTK.light	693.9	940.5
sp|P02647|APOA1_HUMAN	VSFLSALEEYTK.light	693.9	1053.5
sp|P02814|SMR3B_HUMAN	GPYPPGPLAPPQPFGPGFVPPPPPPPYGPGR.light	1034.5	850.4
sp|P02814|SMR3B_HUMAN	GPYPPGPLAPPQPFGPGFVPPPPPPPYGPGR.light	1034.5	1172.6
sp|P02814|SMR3B_HUMAN	GPYPPGPLAPPQPFGPGFVPPPPPPPYGPGR.light	1034.5	1228.6
sp|P80188|NGAL_HUMAN	WYVVGLAGNAILR.light	716.4	350.1
sp|P80188|NGAL_HUMAN	WYVVGLAGNAILR.light	716.4	449.2
sp|P80188|NGAL_HUMAN	WYVVGLAGNAILR.light	716.4	884.5
sp|P15309|PPAP_HUMAN	ELSELSLLSLYGIHK.light	568.0	730.4
sp|P15309|PPAP_HUMAN	ELSELSLLSLYGIHK.light	568.0	817.5
sp|P15309|PPAP_HUMAN	ELSELSLLSLYGIHK.light	568.0	930.5
sp|P04745|AMY1_HUMAN	IAEYMNHLIDIGVAGFR.light	640.3	606.3
sp|P04745|AMY1_HUMAN	IAEYMNHLIDIGVAGFR.light	640.3	867.9
sp|P04745|AMY1_HUMAN	IAEYMNHLIDIGVAGFR.light	640.3	903.5
sp|P04745|AMY1_HUMAN	LSGLLDLALGK.light	550.3	729.5
sp|P04745|AMY1_HUMAN	LSGLLDLALGK.light	550.3	899.6
sp|P04745|AMY1_HUMAN	LSGLLDLALGK.light	550.3	986.6
sp|P02814|SMR3B_HUMAN	IPPPPPAPYGPGIFPPPPPQP.light	710.7	535.3
sp|P02814|SMR3B_HUMAN	IPPPPPAPYGPGIFPPPPPQP.light	710.7	729.4
sp|P02814|SMR3B_HUMAN	IPPPPPAPYGPGIFPPPPPQP.light	710.7	1141.6
sp|P68871|HBB_HUMAN	LLVVYPWTQR.light	637.9	687.4
sp|P68871|HBB_HUMAN	LLVVYPWTQR.light	637.9	850.4
sp|P68871|HBB_HUMAN	LLVVYPWTQR.light	637.9	949.5
sp|P04279|SEMG1_HUMAN	DIFSTQDELLVYNK.light	842.9	424.2
sp|P04279|SEMG1_HUMAN	DIFSTQDELLVYNK.light	842.9	523.3
sp|P04279|SEMG1_HUMAN	DIFSTQDELLVYNK.light	842.9	1309.7
sp|P02647|APOA1_HUMAN	LLDNWDSVTSTFSK.light	806.9	569.3
sp|P02647|APOA1_HUMAN	LLDNWDSVTSTFSK.light	806.9	670.3
sp|P02647|APOA1_HUMAN	LLDNWDSVTSTFSK.light	806.9	971.5
sp|P15309|PPAP_HUMAN	FVTLVFR.light	441.3	247.1
sp|P15309|PPAP_HUMAN	FVTLVFR.light	441.3	421.3

sp|P15309|PPAP_HUMAN	FVTLVFR.light	441.3	635.4
sp|O60437|PEPL_HUMAN	NLLDEIASR.light	515.8	228.1
sp|O60437|PEPL_HUMAN	NLLDEIASR.light	515.8	690.3
sp|O60437|PEPL_HUMAN	NLLDEIASR.light	515.8	803.4
sp|P02790|HEMO_HUMAN	NFPSPVDAAFR.light	610.8	480.3
sp|P02790|HEMO_HUMAN	NFPSPVDAAFR.light	610.8	775.4
sp|P02790|HEMO_HUMAN	NFPSPVDAAFR.light	610.8	959.5
sp|P07288|KLK3_HUMAN	FLRPGDDSSHDLMLLR.light	468.7	536.3
sp|P07288|KLK3_HUMAN	FLRPGDDSSHDLMLLR.light	468.7	575.6
sp|P07288|KLK3_HUMAN	FLRPGDDSSHDLMLLR.light	468.7	760.4
sp|P01009|A1AT_HUMAN	SVLGQLGITK.light	508.3	531.4
sp|P01009|A1AT_HUMAN	SVLGQLGITK.light	508.3	716.4
sp|P01009|A1AT_HUMAN	SVLGQLGITK.light	508.3	829.5
sp|P02687|MBP_BOVIN	DTGILDSLGR.light	523.8	660.4
sp|P02687|MBP_BOVIN	DTGILDSLGR.light	523.8	830.5
sp|P02687|MBP_BOVIN	DTGILDSLGR.heavy	528.8	670.4
sp|P02687|MBP_BOVIN	DTGILDSLGR.heavy	528.8	840.5
sp|P80188|NGAL_HUMAN	MYATIYELK.light	566.3	295.1
sp|P80188|NGAL_HUMAN	MYATIYELK.light	566.3	837.5
sp|P80188|NGAL_HUMAN	MYATIYELK.light	566.3	1000.5
sp|P68871|HBB_HUMAN	SAVTALWGK.light	466.8	574.3
sp|P68871|HBB_HUMAN	SAVTALWGK.light	466.8	675.4
sp|P68871|HBB_HUMAN	SAVTALWGK.light	466.8	774.5
sp|Q02383|SEMG2_HUMAN	DVSQSSISFQIEK.light	734.4	751.4
sp|Q02383|SEMG2_HUMAN	DVSQSSISFQIEK.light	734.4	951.5
sp|Q02383|SEMG2_HUMAN	DVSQSSISFQIEK.light	734.4	1038.5
sp|P02787|TRFE_HUMAN	SASDLTWDNLK.light	625.3	675.3
sp|P02787|TRFE_HUMAN	SASDLTWDNLK.light	625.3	776.4
sp|P02787|TRFE_HUMAN	SASDLTWDNLK.light	625.3	1091.5
sp|P01009|A1AT_HUMAN	LSITGTYDLK.light	555.8	696.4
sp|P01009|A1AT_HUMAN	LSITGTYDLK.light	555.8	797.4
sp|P01009|A1AT_HUMAN	LSITGTYDLK.light	555.8	910.5
sp|P09228|CYTT_HUMAN	ALHFVISEYNK.light	440.9	424.2
sp|P09228|CYTT_HUMAN	ALHFVISEYNK.light	440.9	568.3
sp|P09228|CYTT_HUMAN	ALHFVISEYNK.light	440.9	640.3
sp|P09228|CYTT_HUMAN	IIEGGIYDADLNDER.light	846.9	947.4
sp|P09228|CYTT_HUMAN	IIEGGIYDADLNDER.light	846.9	1110.5
sp|P09228|CYTT_HUMAN	IIEGGIYDADLNDER.light	846.9	1466.6
sp|P07288|KLK3_HUMAN	LSEPAELTDAVK.light	636.8	472.3

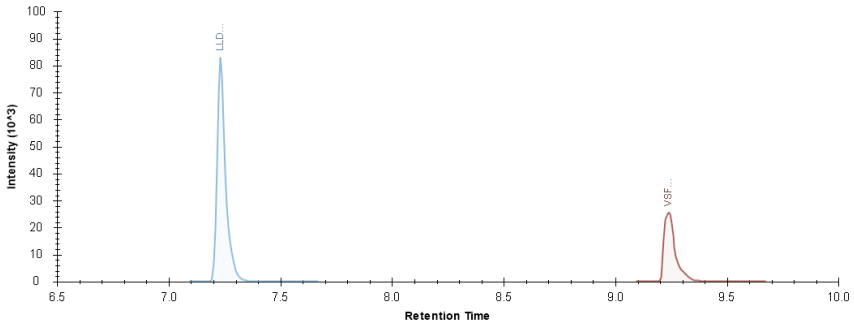
sp|P07288|KLK3_HUMAN	LSEPAELTDAVK.light	636.8	646.4
sp|P07288|KLK3_HUMAN	LSEPAELTDAVK.light	636.8	943.5
sp|P07476|INVO_HUMAN	QEAQLELPEQQVGQPK.light	608.0	505.8
sp|P07476|INVO_HUMAN	QEAQLELPEQQVGQPK.light	608.0	699.3
sp|P07476|INVO_HUMAN	QEAQLELPEQQVGQPK.light	608.0	812.4
sp|Q9UBG3|CRNN_HUMAN	LLDEDHTGTVEFK.light	501.9	294.2
sp|Q9UBG3|CRNN_HUMAN	LLDEDHTGTVEFK.light	501.9	639.3
sp|Q9UBG3|CRNN_HUMAN	LLDEDHTGTVEFK.light	501.9	695.8
sp|P15309|PPAP_HUMAN	FQELESETLK.light	612.3	276.1
sp|P15309|PPAP_HUMAN	FQELESETLK.light	612.3	819.4
sp|P15309|PPAP_HUMAN	FQELESETLK.light	612.3	948.5
sp|Q9UBG3|CRNN_HUMAN	ISPQIQLSGQTEQTQK.light	893.5	793.4
sp|Q9UBG3|CRNN_HUMAN	ISPQIQLSGQTEQTQK.light	893.5	1006.5
sp|Q9UBG3|CRNN_HUMAN	ISPQIQLSGQTEQTQK.light	893.5	1247.6
sp|P07476|INVO_HUMAN	GEVLLPVEHQQQK.light	502.3	553.8
sp|P07476|INVO_HUMAN	GEVLLPVEHQQQK.light	502.3	610.3
sp|P07476|INVO_HUMAN	GEVLLPVEHQQQK.light	502.3	659.9
sp|P02787|TRFE_HUMAN	DGAGDVAFVK.light	489.7	563.4
sp|P02787|TRFE_HUMAN	DGAGDVAFVK.light	489.7	678.4
sp|P02787|TRFE_HUMAN	DGAGDVAFVK.light	489.7	735.4
sp|Q6UWP8|SBSN_HUMAN	ALDGINSGITHAGR.light	461.2	541.8
sp|Q6UWP8|SBSN_HUMAN	ALDGINSGITHAGR.light	461.2	599.3
sp|Q02383|SEMG2_HUMAN	GSISIQTEEQIHGK.light	509.6	461.6
sp|Q02383|SEMG2_HUMAN	GSISIQTEEQIHGK.light	509.6	635.3
sp|Q02383|SEMG2_HUMAN	GSISIQTEEQIHGK.light	509.6	691.9
sp|P61916|NPC2_HUMAN	DC[+57.0]GSVDGVIK.light	525.3	276.1
sp|P61916|NPC2_HUMAN	DC[+57.0]GSVDGVIK.light	525.3	630.4
sp|P61916|NPC2_HUMAN	DC[+57.0]GSVDGVIK.light	525.3	774.4
sp|Q6UWP8|SBSN_HUMAN	FGQGVHHGLSEGWK.light	513.6	603.8
sp|Q6UWP8|SBSN_HUMAN	FGQGVHHGLSEGWK.light	513.6	696.3
sp|O95274|LYPD3_HUMAN	GC[+57.0]VQDEFC[+57.0]TR.light	636.3	827.3
sp|O95274|LYPD3_HUMAN	GC[+57.0]VQDEFC[+57.0]TR.light	636.3	955.4
sp|O95274|LYPD3_HUMAN	GC[+57.0]VQDEFC[+57.0]TR.light	636.3	1054.5
sp|Q9UBC9|SPRR3_HUMAN	VPVPGYTK.light	430.7	381.2
sp|Q9UBC9|SPRR3_HUMAN	VPVPGYTK.light	430.7	565.3
sp|Q9UBC9|SPRR3_HUMAN	VPVPGYTK.light	430.7	664.4
sp|Q02383|SEMG2_HUMAN	GSISIQTEEK.light	546.3	634.3
sp|Q02383|SEMG2_HUMAN	GSISIQTEEK.light	546.3	747.4
sp|Q02383|SEMG2_HUMAN	GSISIQTEEK.light	546.3	834.4

sp|P04279|SEMG1_HUMAN	QITIPSQEQEHSQK.light	551.6	599.3
sp|P04279|SEMG1_HUMAN	QITIPSQEQEHSQK.light	551.6	706.3
sp|P61916|NPC2_HUMAN	SGINC[+57.0]PIQK.light	508.8	485.3
sp|P61916|NPC2_HUMAN	SGINC[+57.0]PIQK.light	508.8	645.3
sp|P61916|NPC2_HUMAN	SGINC[+57.0]PIQK.light	508.8	759.4
sp|P07476|INVO_HUMAN	HLVQQEGQLEQQER.light	574.6	560.3
sp|P07476|INVO_HUMAN	HLVQQEGQLEQQER.light	574.6	689.3
sp|P07476|INVO_HUMAN	HLVQQEGQLEQQER.light	574.6	920.5
sp|Q9UBG3|CRNN_HUMAN	AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR.light	839.9	851.4
sp|Q9UBG3|CRNN_HUMAN	AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR.light	839.9	886.9
sp|Q9UBG3|CRNN_HUMAN	AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR.light	839.9	1001.5
sp|O60437|PEPL_HUMAN	NQGPQESVVR.light	557.3	243.1
sp|O60437|PEPL_HUMAN	NQGPQESVVR.light	557.3	814.4
sp|O60437|PEPL_HUMAN	NQGPQESVVR.light	557.3	871.5
sp|Q9UBC9|SPRR3_HUMAN	VPEPGC[+57.0]TK.light	444.2	394.7
sp|Q9UBC9|SPRR3_HUMAN	VPEPGC[+57.0]TK.light	444.2	562.3
sp|Q9UBC9|SPRR3_HUMAN	VPEPGC[+57.0]TK.light	444.2	788.4
sp|O60437|PEPL_HUMAN	AQSLQSAK.light	416.7	200.1
sp|O60437|PEPL_HUMAN	AQSLQSAK.light	416.7	546.3
sp|O60437|PEPL_HUMAN	AQSLQSAK.light	416.7	633.4

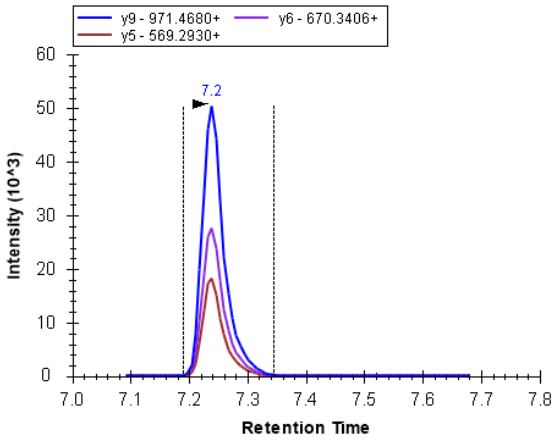
Peripheral Blood:



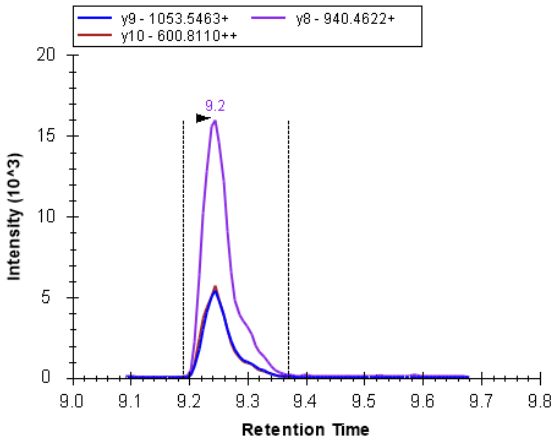
Apolipoprotein P02647|APOA1



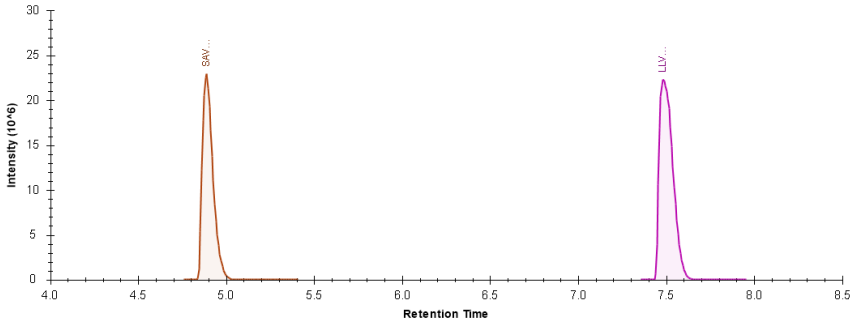
K.LLDNWDSVTSTFSK.L [69, 82]



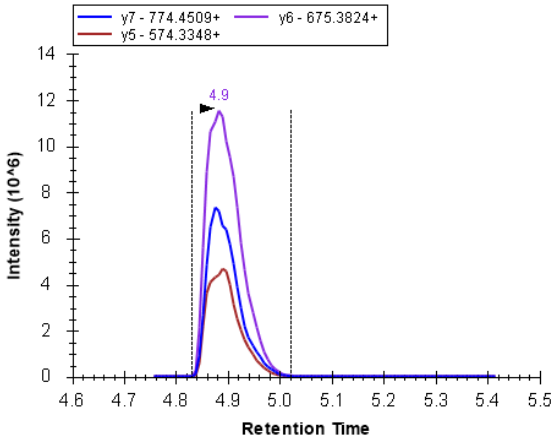
K.VSFLSALEEYTK.K [250, 261]



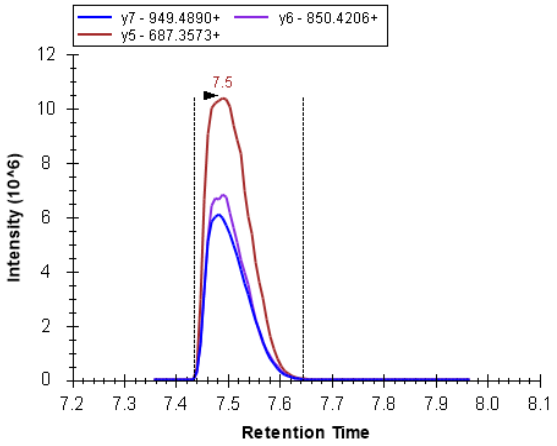
Hemoglobin Beta P68871|HBB



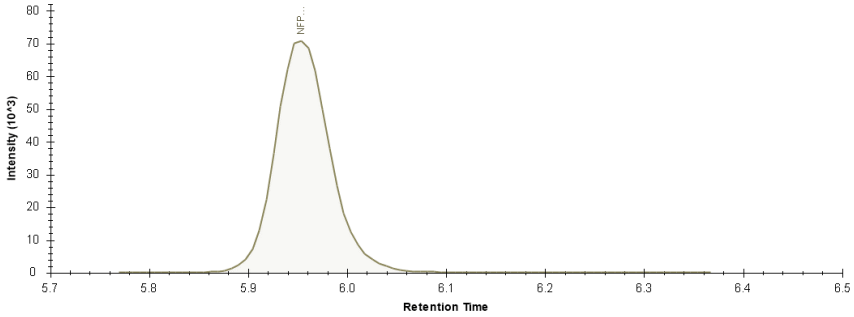
SAVTALWGK



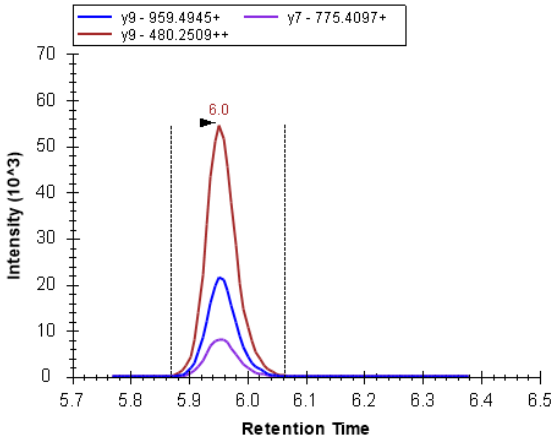
LLVVYPWTQR



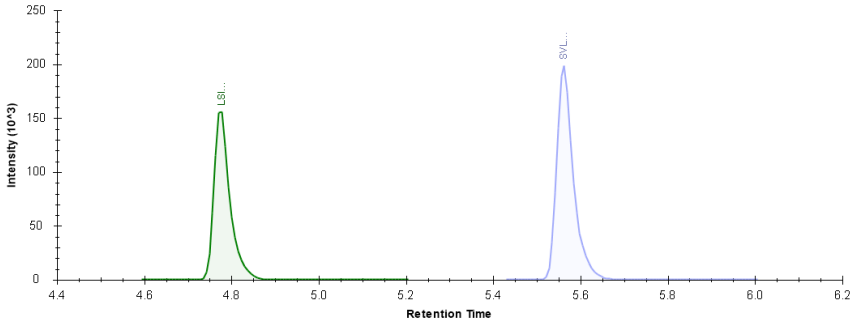
Hemopexin P02790|HEMO



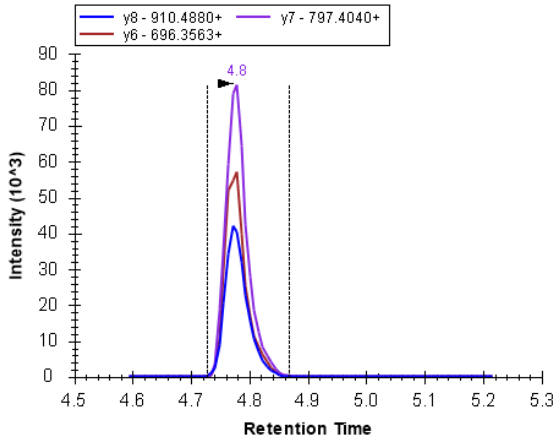
K.NFPSPVDAAFR.Q [91, 101]



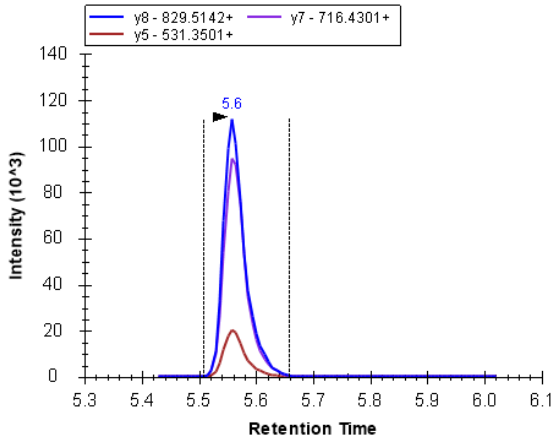
Alpha-1 Antitrypsin P01009|A1AT



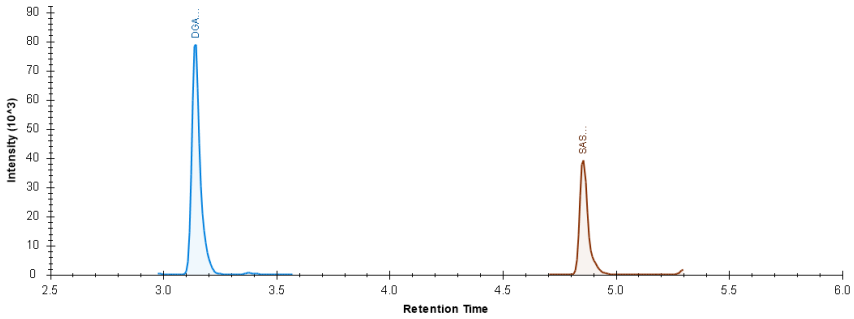
K.LSITGTYDLK.S [314, 323]



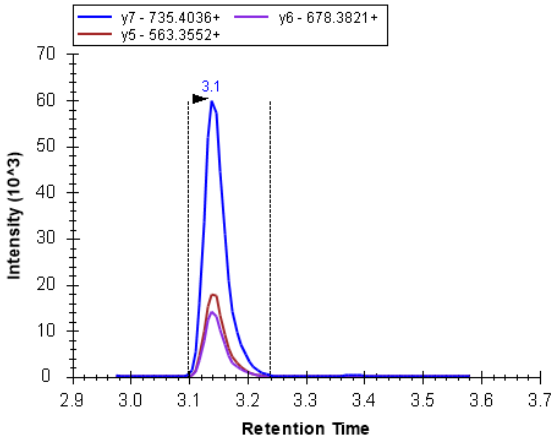
K.SVLGQLGITK.V [324, 333]



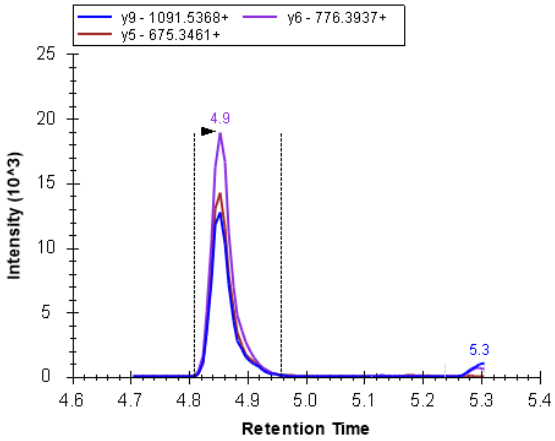
Serotransferrin P02787|TRFE



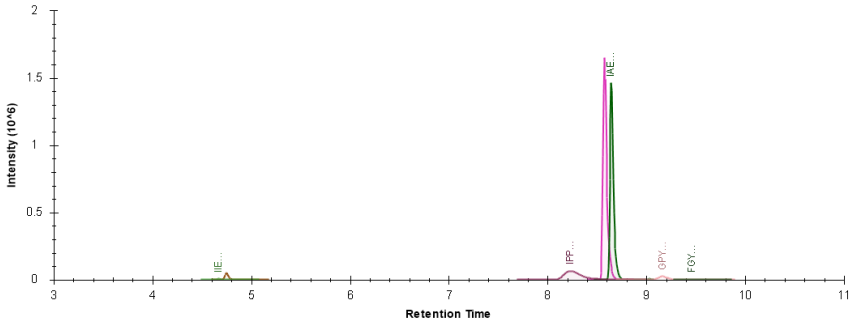
K.DGAGDVAFVK.H [215, 224]



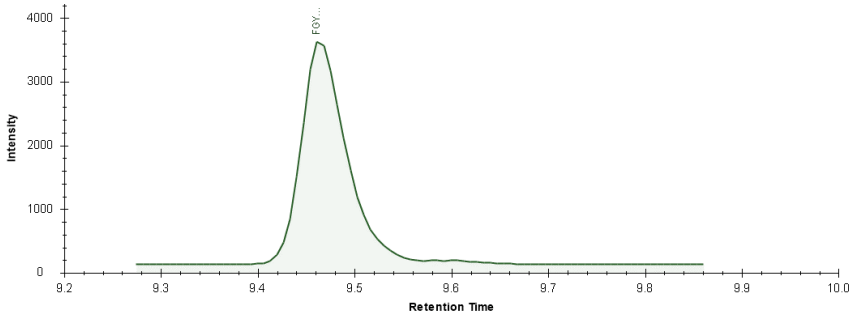
K.SASDLTWDNLK.G [453, 463]



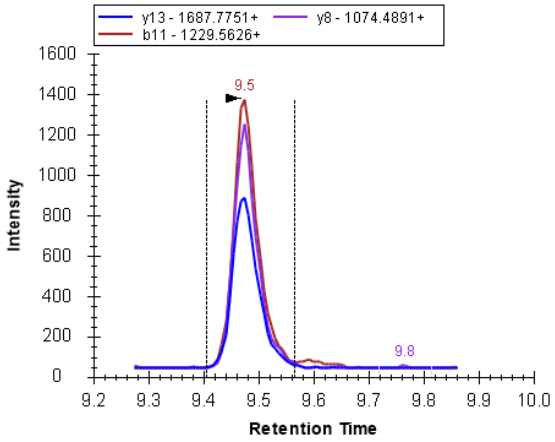
Saliva



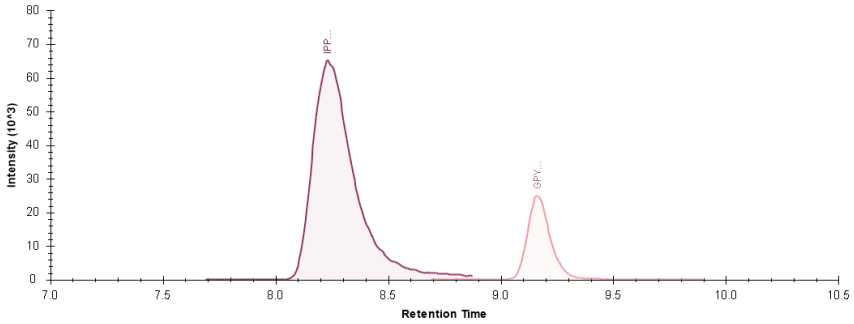
Statherin P02808 |STAT



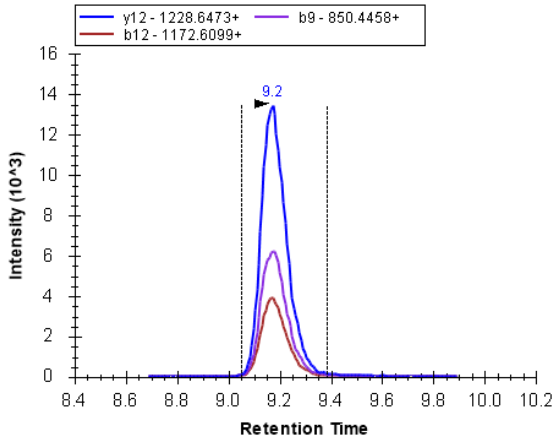
R.FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF - [32, 61]



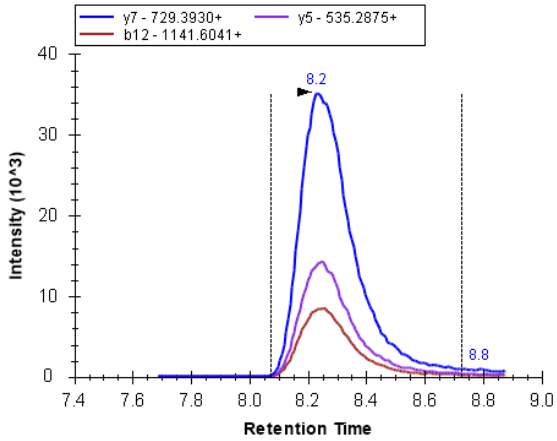
Submaxillary Gland Androgen-Regulated Protein 3B P02814|SMR3B



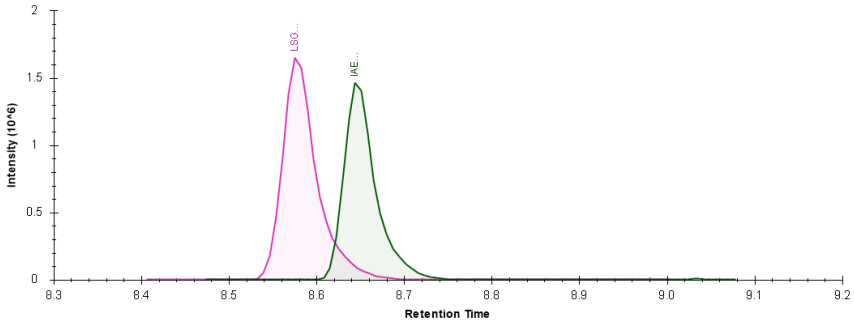
R.GPYPPGPLAPPQPFGPGFVPPPPPPPYGPGR.I [27, 57]



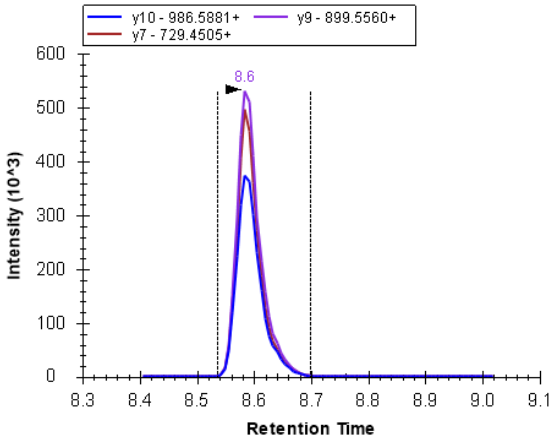
R.IPPPPPAPYGPGIFPPPPPQP - [58, 78]



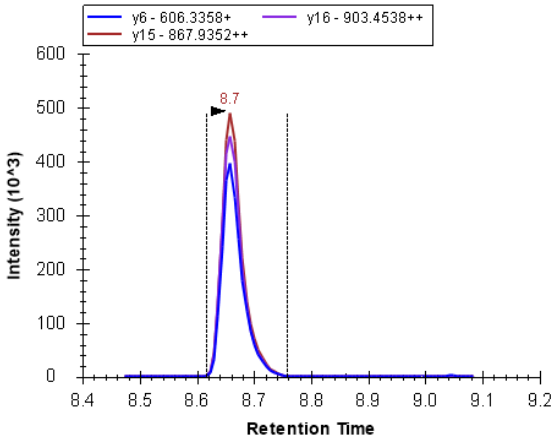
Alpha Amylase-1 P04745|AMY1



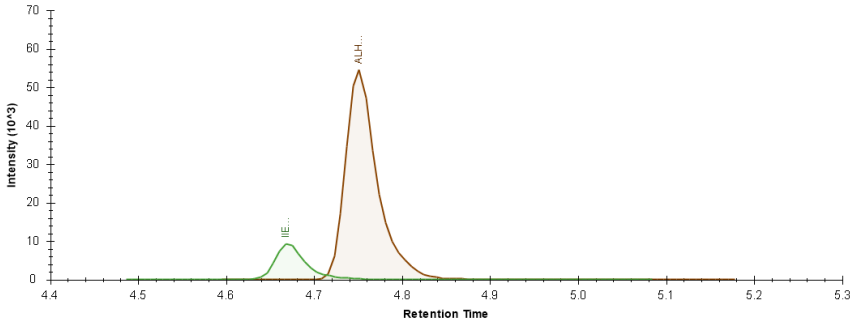
R.LSGLLDLALGK.D [176, 186]



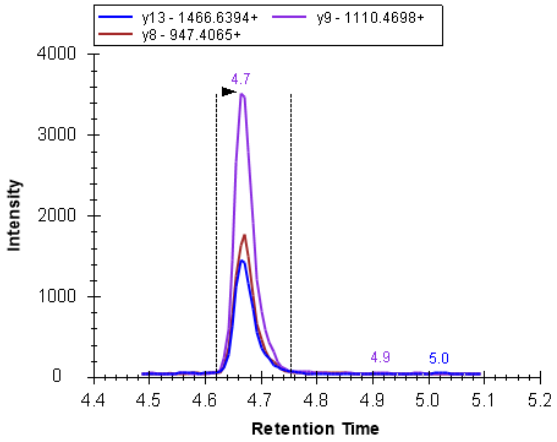
K.IAEYMNHLIDIGVAGFR.I [193, 209]



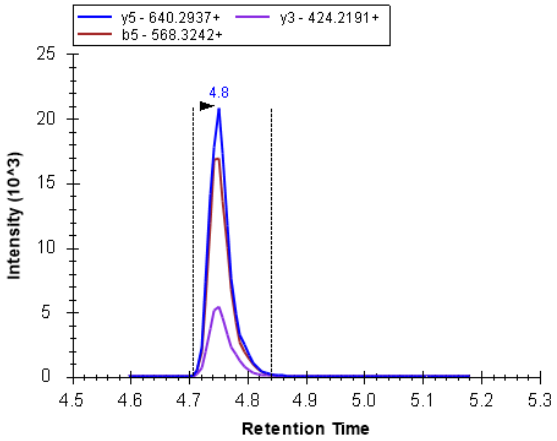
Cystatin SA P09228|CYTT



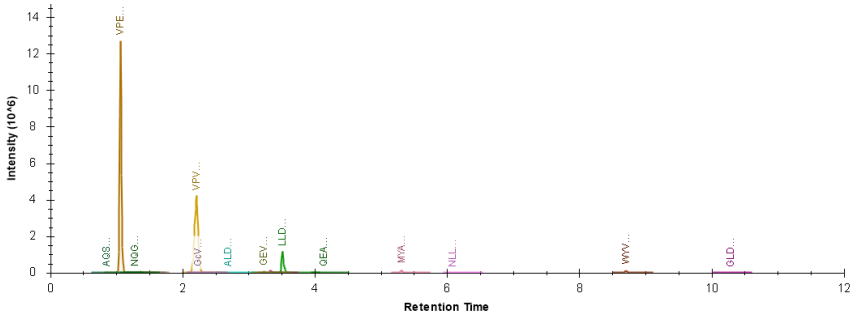
R.IIEGGIYDADLNDER.V [28, 42]



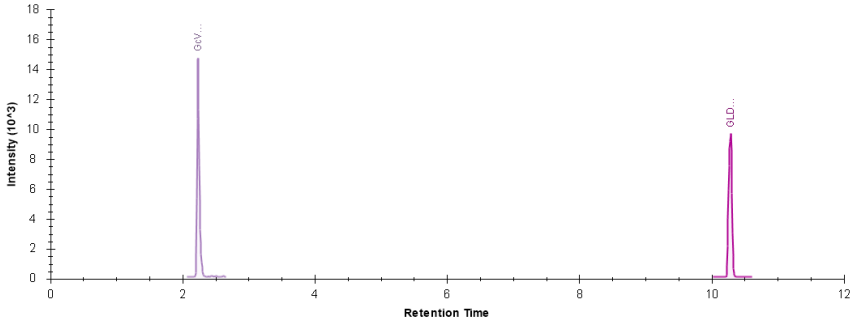
R.ALHFVISEYNK.A [46, 56]



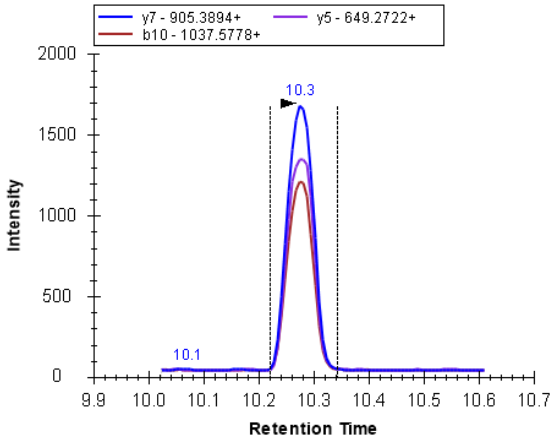
Vaginal Fluid



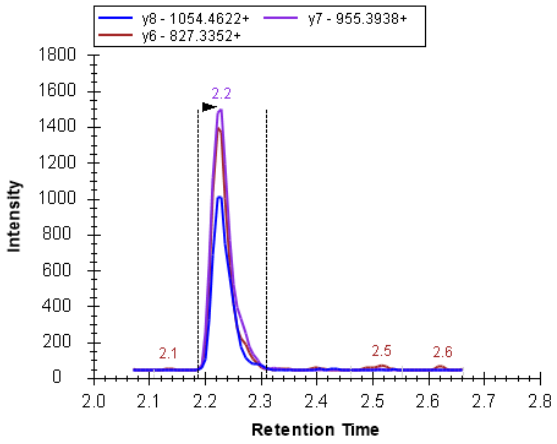
Ly6/PLAUR O95274|LYPD3



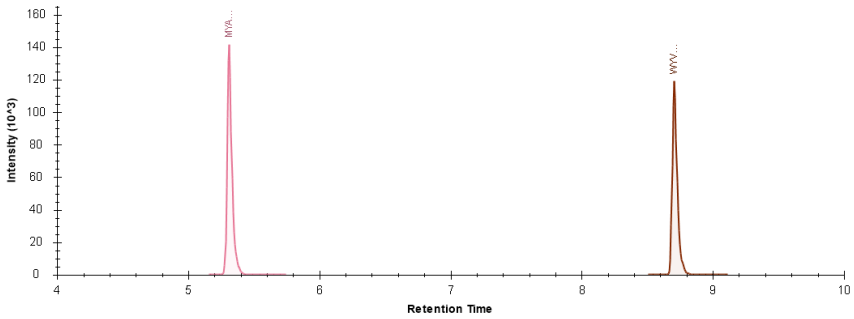
R.GLDLHGLLAFIQLQQCAQDR.C [92, 111]



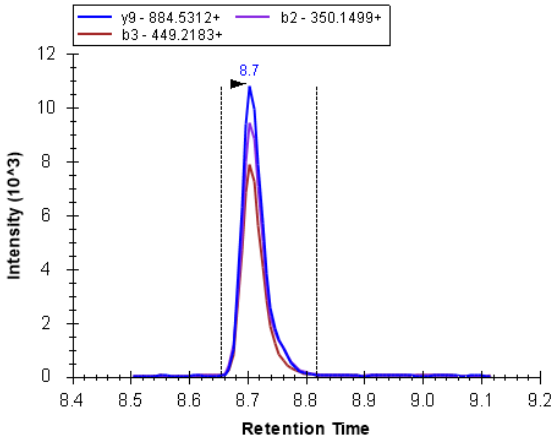
R.GCVQDEFCTR.D [191, 200]



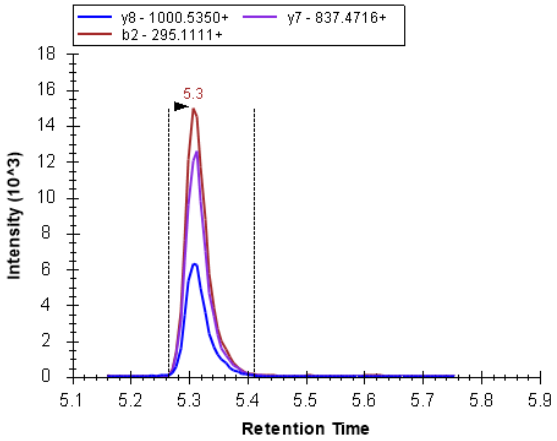
Neutrophil Gelatinase P80188|NGAL



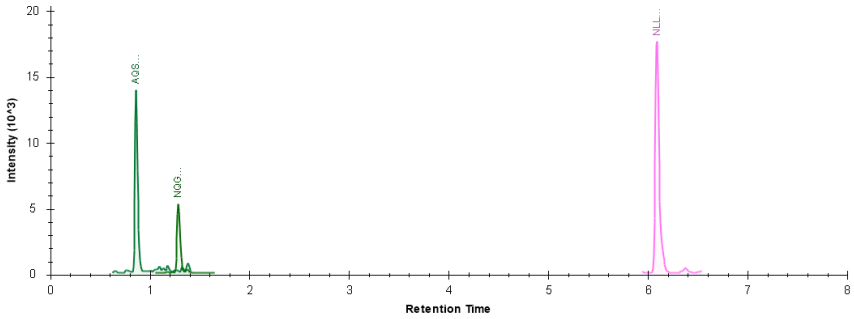
K.WYVVGLAGNAILR.E [50, 62]



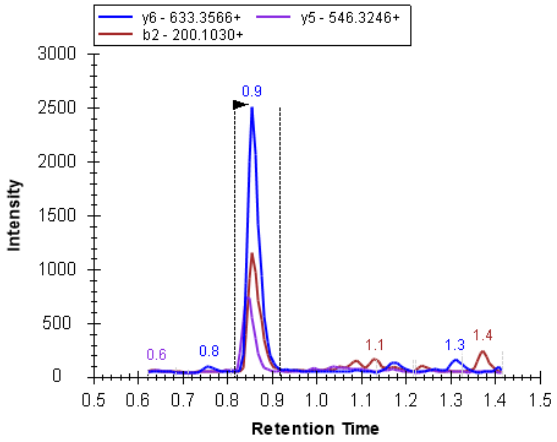
K.MYATIYELK.E [70, 78]



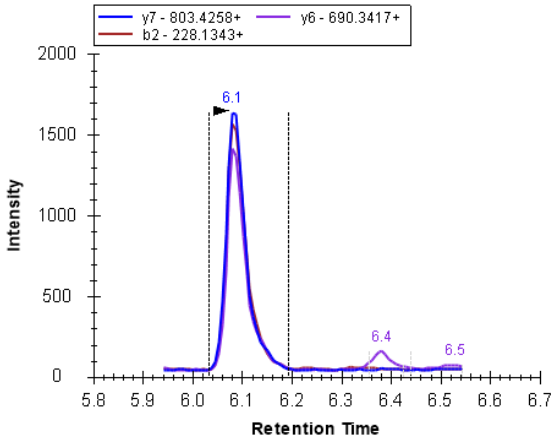
Periplakin O60437|PEPL



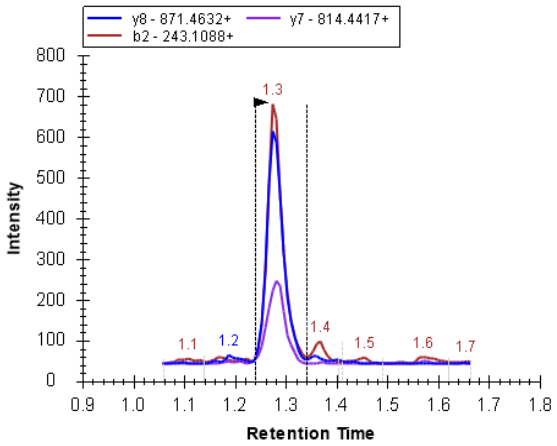
R.AQSLQSAK.A [721, 728]



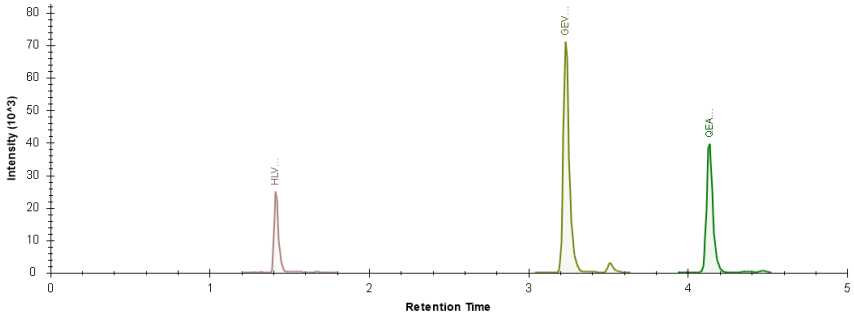
K.NLLDEIASR.E [771, 779]



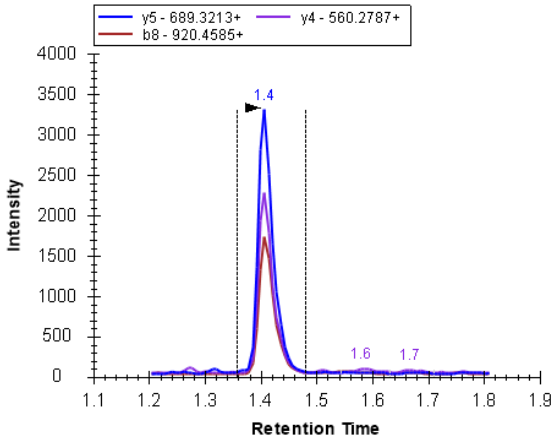
R.NQGPQESVVR.K [924, 933]



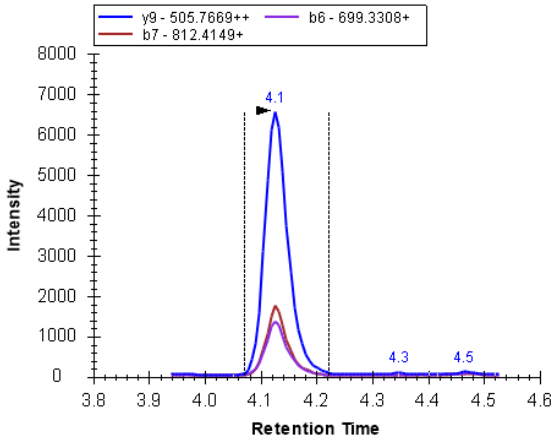
Involucrin P07476|INVO



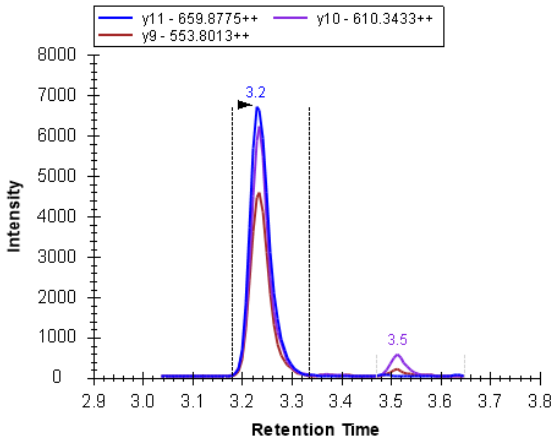
K.HLVQQEGQLEQQER.Q [404, 417]



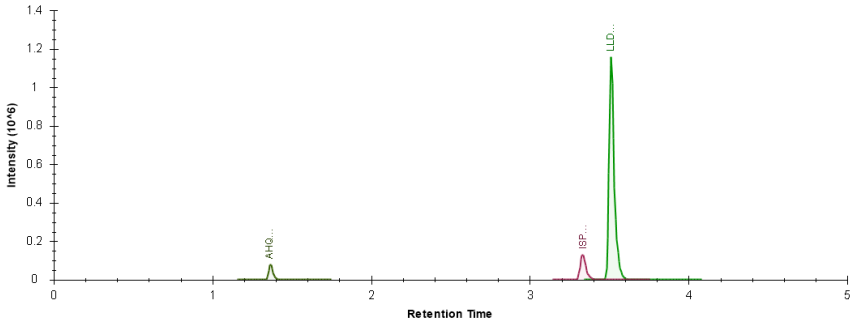
K.QEAQLELPEQQVGQPK.H [485, 500]



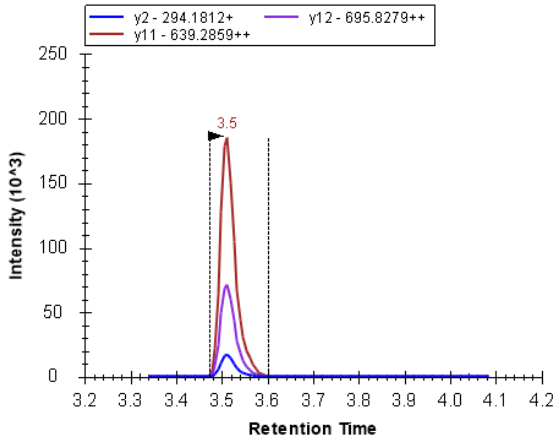
K.GEVLLPVEHQQQK.Q [562, 574]



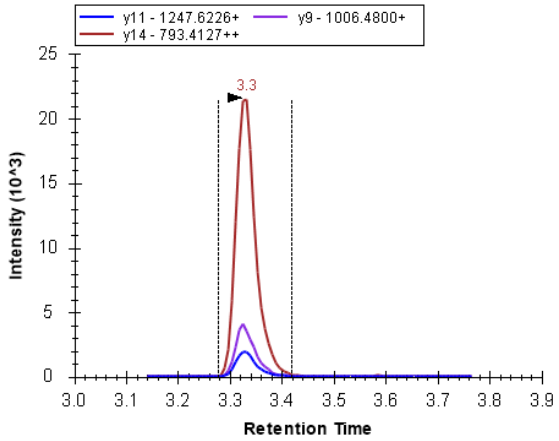
Cornulin Q9UBG3|CRNN



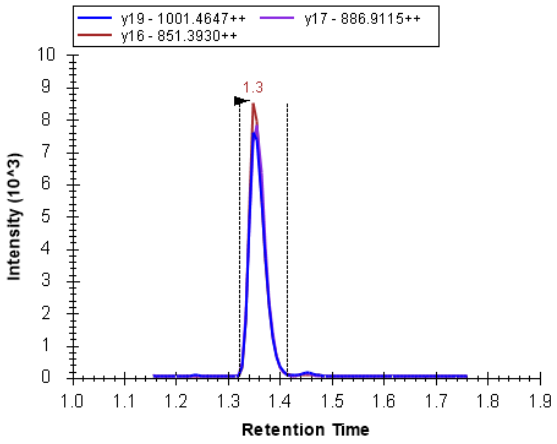
R.LLDEDHTGTVEFK.E [59, 71]



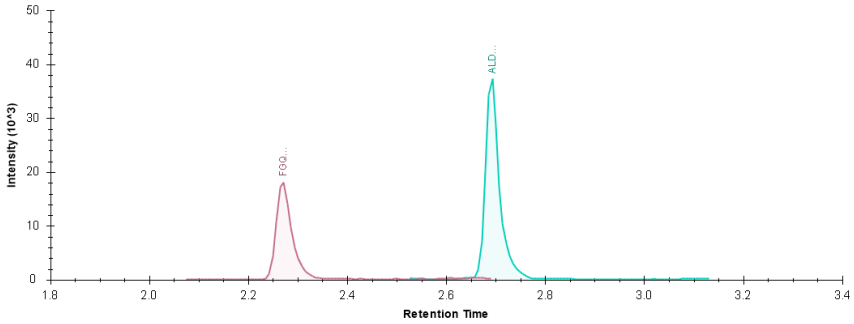
R.ISPQIQLSGQTEQTQK.A [178, 193]



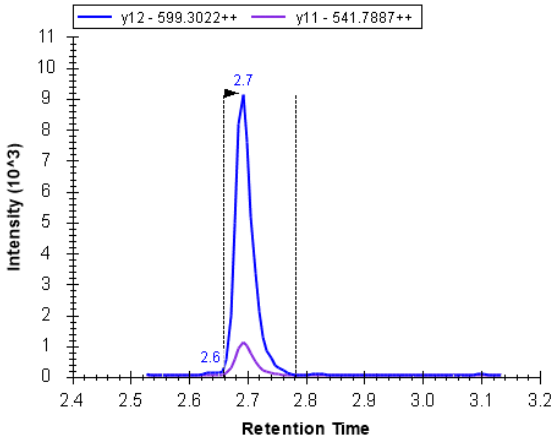
R.AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR.T [219, 251]



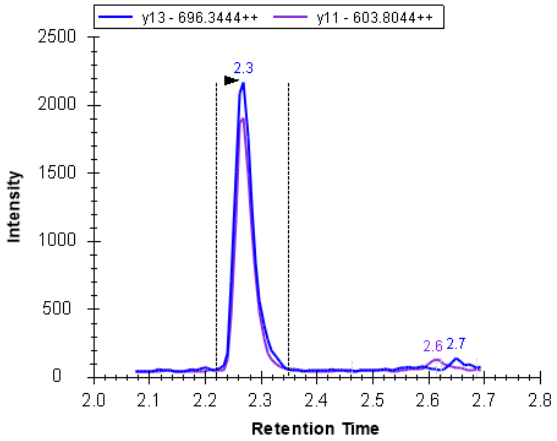
Suprabasin Q6UWP8|SBSN



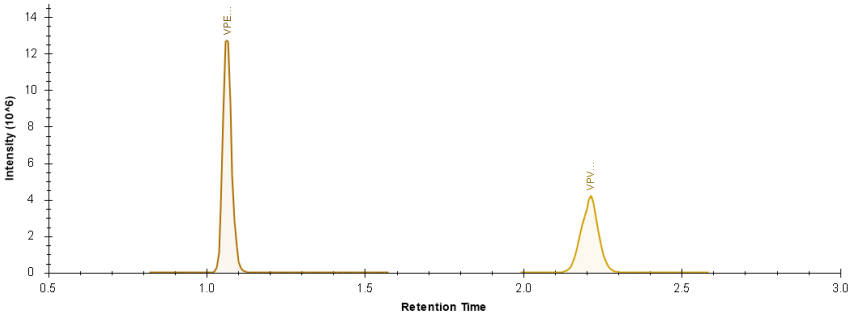
K.ALDGINSGITHAGR.E [49, 62]



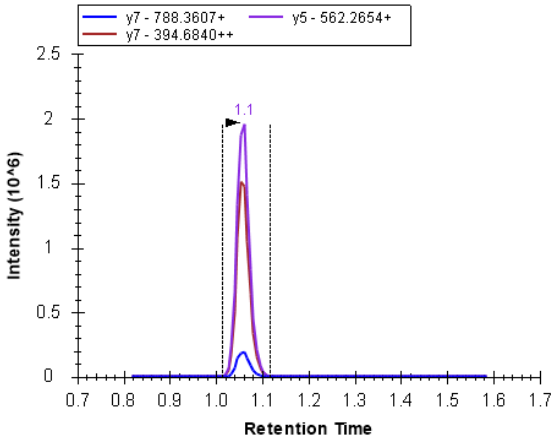
R.FGQGVHHGLSEGWK.E [331, 344]



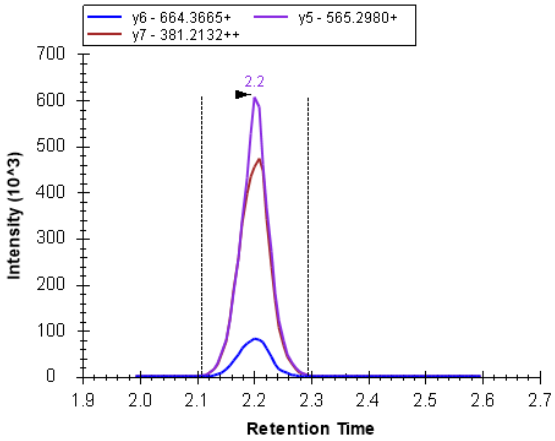
Small Proline Rich Protein 3 Q9UBC9|SPRR3



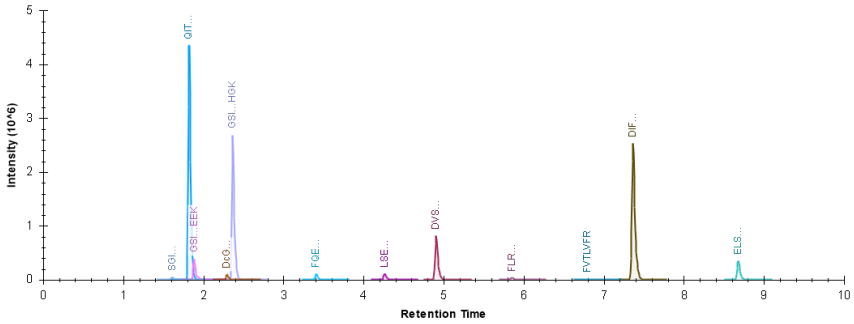
K.VPEPGCTK.V [60, 67]



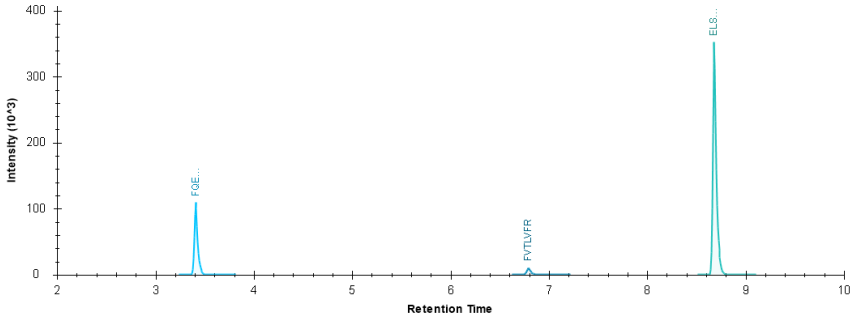
K.VPVPGYTK.L [140, 147]



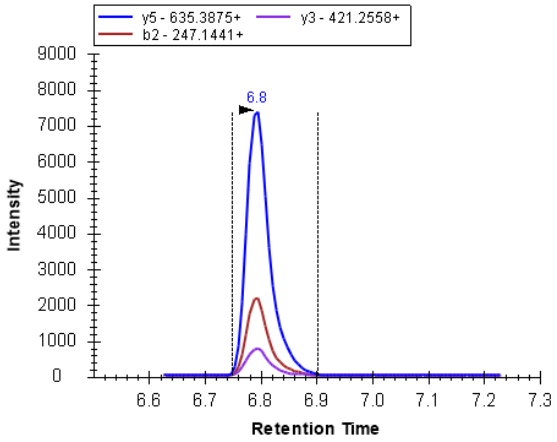
Seminal Fluid



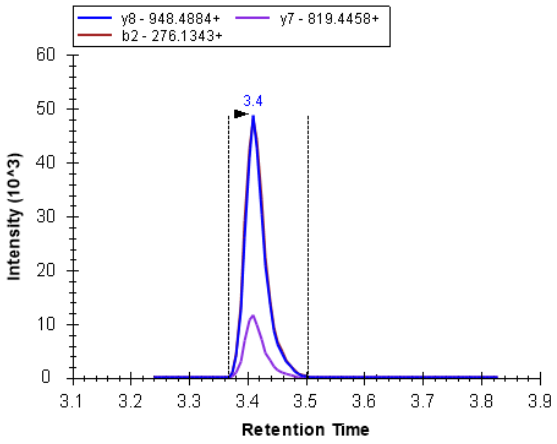
Prostatic Acid Phosphatase P15309|PPAP



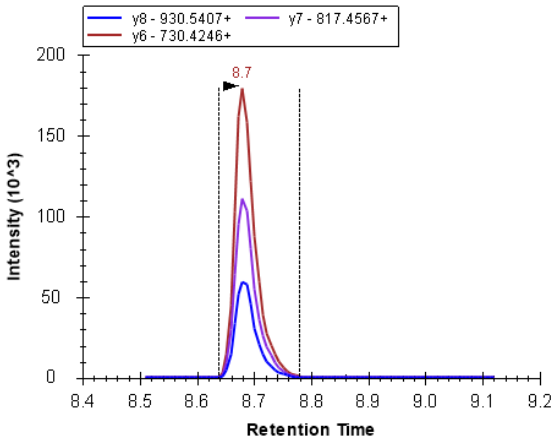
K.FVTLVFR.H [36, 42]



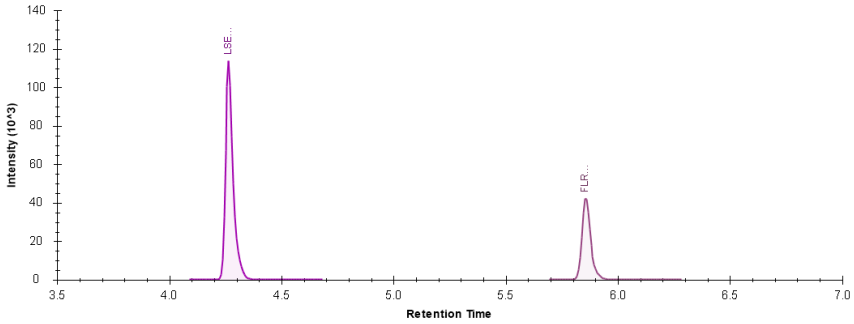
R.FQELESETLK.S [163, 172]



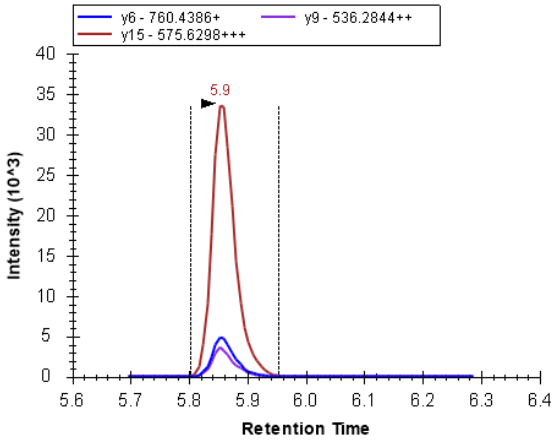
R.ELSELSLLSLYGIHK.Q [236, 250]



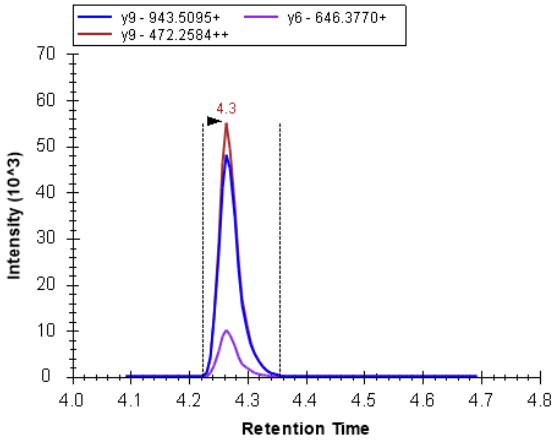
Prostate Specific Antigen P07288|KLK3



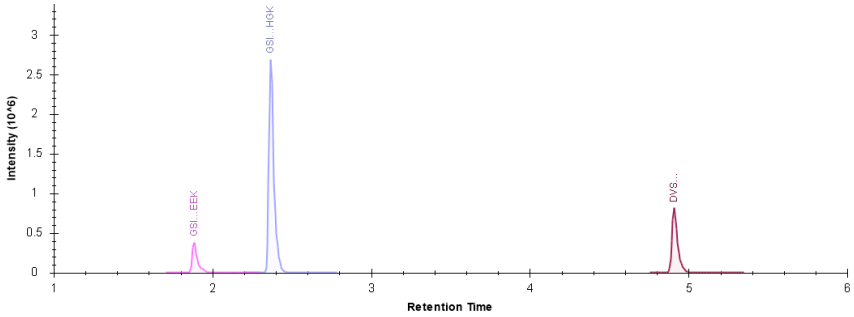
R.FLRPGDDSSHDLMLLR.L [109, 124]



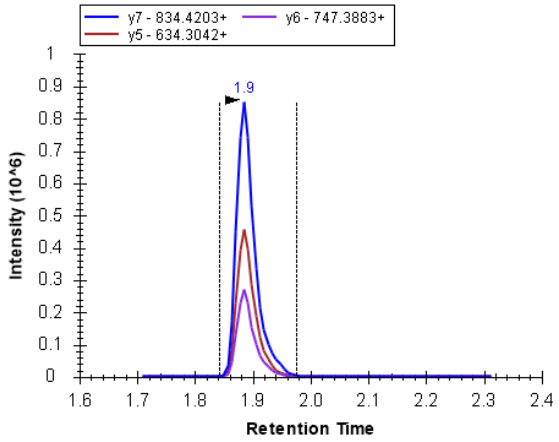
R.LSEPAELTDAVK.V [125, 136]



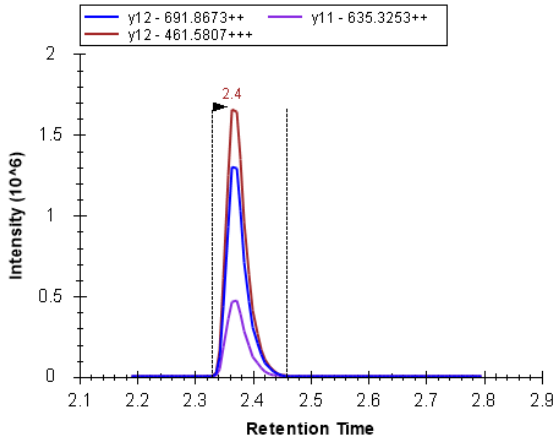
Semenogelin 2 Q02383|SEMG2



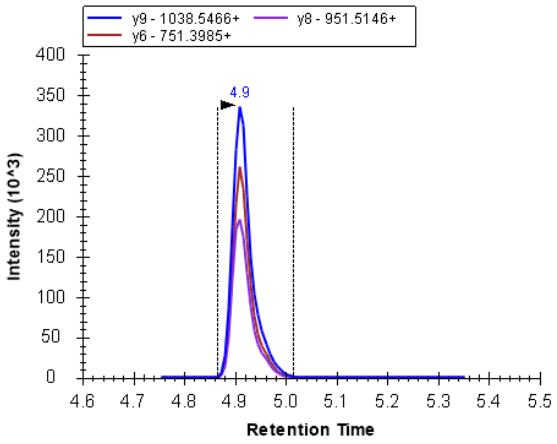
K.GSISIQTEEK.I [311, 320]



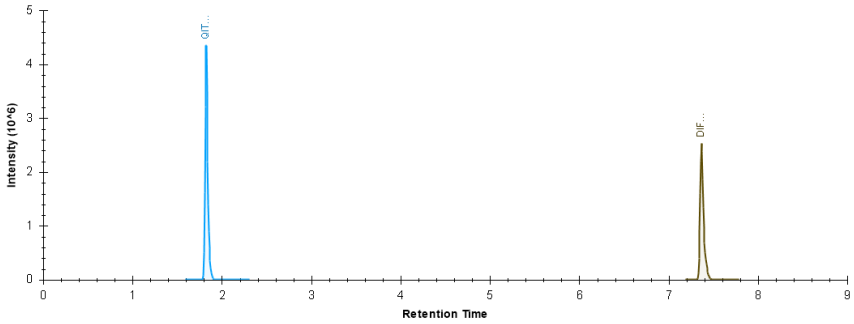
K.GSISIQTEEQIHGK.S [371, 384]



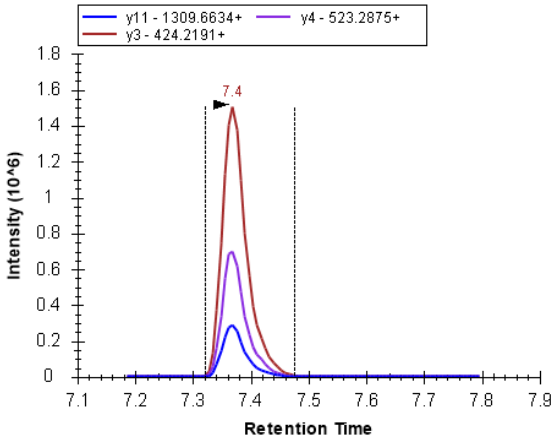
K.DVSQSSISFQIEK.L [487, 499]



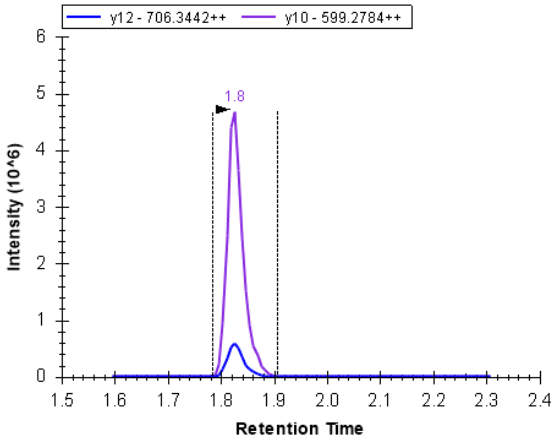
Semenogelin 1 P04279|SEMG1



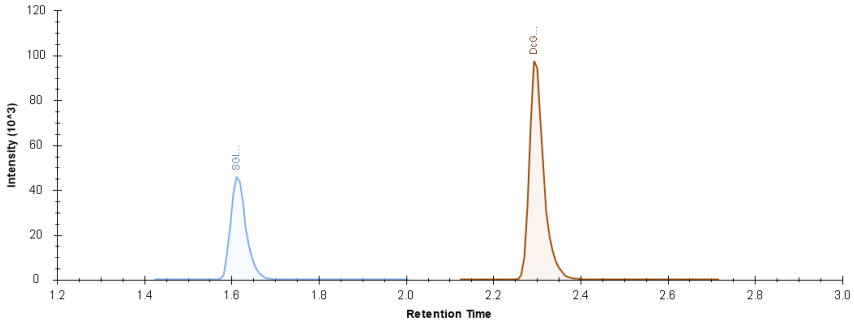
K.DIFSTQDELLVYNK.N [251, 264]



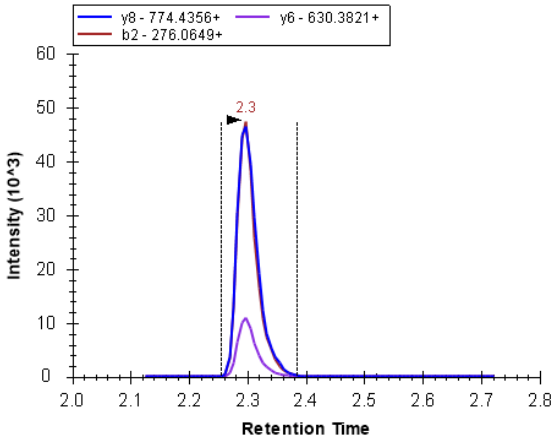
K.QITIPSQEQEHSQK.A [328, 341]



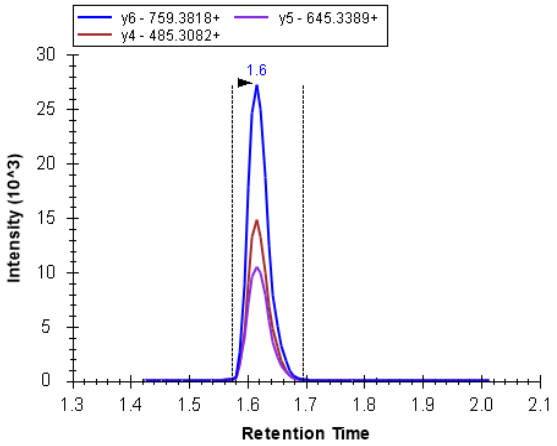
Epididymal Secretory Protein P61916|NPC2



K.DCGSVDGVIK.E [25, 34]



K.SGINCPIQK.D [94, 102]



- The acquisition parameters are as follows:

Acquisition Method Info

Method Name 11min_dMRM_FINAL.m

Method Path D:\MassHunter\methods\Proteomics_DOD\3mm_Final\11min_dMRM_FINAL.m

Method Description 11 min 3x100

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	400	No	3

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
AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR.light	No	839.8888	Unit/Enh (6490)	1001.464663	Unit/Enh (6490)	380	28	4	1.46	0.6	Positive
AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR.light	No	839.8888	Unit/Enh (6490)	886.911535	Unit/Enh (6490)	380	22	4	1.46	0.6	Positive
AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR.light	No	839.8888	Unit/Enh (6490)	851.392978	Unit/Enh (6490)	380	25.4	4	1.46	0.6	Positive
ALDGINSGITHAGR.light	No	461.244268	Unit/Enh (6490)	599.302175	Unit/Enh (6490)	380	11.8	4	2.83	0.6	Positive
ALDGINSGITHAGR.light	No	461.244268	Unit/Enh (6490)	541.788704	Unit/Enh (6490)	380	15	4	2.83	0.6	Positive
ALHFVISEYNK.light	No	440.903072	Unit/Enh (6490)	640.293681	Unit/Enh (6490)	380	8	4	4.89	0.6	Positive
ALHFVISEYNK.light	No	440.903072	Unit/Enh (6490)	568.324194	Unit/Enh (6490)	380	14	4	4.89	0.6	Positive
ALHFVISEYNK.light	No	440.903072	Unit/Enh (6490)	424.219066	Unit/Enh (6490)	380	14	4	4.89	0.6	Positive
AQSLQSAK.light	No	416.729792	Unit/Enh (6490)	633.356616	Unit/Enh (6490)	380	11	4	0.92	0.6	Positive
AQSLQSAK.light	No	416.729792	Unit/Enh (6490)	546.324588	Unit/Enh (6490)	380	17	4	0.92	0.6	Positive
AQSLQSAK.light	No	416.729792	Unit/Enh (6490)	200.102967	Unit/Enh (6490)	380	13.9	4	0.92	0.6	Positive
DC[+57.0]GSVDGVIK.light	No	525.250231	Unit/Enh (6490)	774.435595	Unit/Enh (6490)	380	14	4	2.43	0.6	Positive
DC[+57.0]GSVDGVIK.light	No	525.250231	Unit/Enh (6490)	630.382102	Unit/Enh (6490)	380	14	4	2.43	0.6	Positive
DC[+57.0]GSVDGVIK.light	No	525.250231	Unit/Enh (6490)	276.064868	Unit/Enh (6490)	380	17.3	4	2.43	0.6	Positive
DGAGDVAFVK.light	No	489.748181	Unit/Enh (6490)	735.403566	Unit/Enh (6490)	380	16.2	4	3.28	0.6	Positive
DGAGDVAFVK.light	No	489.748181	Unit/Enh (6490)	678.382102	Unit/Enh (6490)	380	16.2	4	3.28	0.6	Positive

Cpd Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Frag (V)	CE (V)	Cell Acc (V)	Ret Time (min)	Ret Window	Polarity
DGAGDVAFVK.light	No	489.748181	Unit/Enh (6490)	563.355159	Unit/Enh (6490)	380	19	4	3.28	0.6	Positive
DIFSTQDELLVYNK.light	No	842.92506	Unit/Enh (6490)	1309.663422	Unit/Enh (6490)	380	27.1	4	7.5	0.6	Positive
DIFSTQDELLVYNK.light	No	842.92506	Unit/Enh (6490)	523.287474	Unit/Enh (6490)	380	24	4	7.5	0.6	Positive
DIFSTQDELLVYNK.light	No	842.92506	Unit/Enh (6490)	424.21906	Unit/Enh (6490)	380	24	4	7.5	0.6	Positive
DTGILDSLGR.heavy	Yes	528.781605	Unit/Enh (6490)	840.481312	Unit/Enh (6490)	380	17.2	4	5.5	0.6	Positive
DTGILDSLGR.heavy	Yes	528.781605	Unit/Enh (6490)	670.375784	Unit/Enh (6490)	380	17.2	4	5.5	0.6	Positive
DTGILDSLGR.light	No	523.777474	Unit/Enh (6490)	830.473043	Unit/Enh (6490)	380	17.2	4	5.5	0.6	Positive
DTGILDSLGR.light	No	523.777474	Unit/Enh (6490)	660.367515	Unit/Enh (6490)	380	17.2	4	5.5	0.6	Positive
DVSQSSISFQIEK.light	No	734.369924	Unit/Enh (6490)	1038.54660202	Unit/Enh (6490)	380	23.8	4	5.06	0.6	Positive
DVSQSSISFQIEK.light	No	734.369924	Unit/Enh (6490)	951.514573	Unit/Enh (6490)	380	27	4	5.06	0.6	Positive
DVSQSSISFQIEK.light	No	734.369924	Unit/Enh (6490)	751.398481	Unit/Enh (6490)	380	21	4	5.06	0.6	Positive
ELSELSLLSLYGIHK.light	No	567.985572	Unit/Enh (6490)	930.540728	Unit/Enh (6490)	380	19	4	8.81	0.6	Positive
ELSELSLLSLYGIHK.light	No	567.985572	Unit/Enh (6490)	817.456664	Unit/Enh (6490)	380	19	4	8.81	0.6	Positive
ELSELSLLSLYGIHK.light	No	567.985572	Unit/Enh (6490)	730.424636	Unit/Enh (6490)	380	19	4	8.81	0.6	Positive
FGQGVHHGLSEGWK.light	No	513.588146	Unit/Enh (6490)	696.344374	Unit/Enh (6490)	380	13.7	4	2.4	0.6	Positive
FGQGVHHGLSEGWK.light	No	513.588146	Unit/Enh (6490)	603.804354	Unit/Enh (6490)	380	17	4	2.4	0.6	Positive
FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF.light	No	1215.233017	Unit/Enh (6490)	1687.775098	Unit/Enh (6490)	380	31	4	9.57	0.6	Positive
FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF.light	No	1215.233017	Unit/Enh (6490)	1229.562586	Unit/Enh (6490)	380	33	4	9.57	0.6	Positive
FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF.light	No	1215.233017	Unit/Enh (6490)	1074.489087	Unit/Enh (6490)	380	33	4	9.57	0.6	Positive
FLRPGDDSSHDLMLLR.light	No	468.741268	Unit/Enh (6490)	760.438572	Unit/Enh (6490)	380	15	4	5.99	0.6	Positive
FLRPGDDSSHDLMLLR.light	No	468.741268	Unit/Enh (6490)	575.629794	Unit/Enh (6490)	380	18	4	5.99	0.6	Positive
FLRPGDDSSHDLMLLR.light	No	468.741268	Unit/Enh (6490)	536.284408	Unit/Enh (6490)	380	15	4	5.99	0.6	Positive
FQELESETLK.light	No	612.311343	Unit/Enh (6490)	948.488418	Unit/Enh (6490)	380	20	4	3.54	0.6	Positive
FQELESETLK.light	No	612.311343	Unit/Enh (6490)	819.445825	Unit/Enh (6490)	380	23	4	3.54	0.6	Positive
FQELESETLK.light	No	612.311343	Unit/Enh (6490)	276.134267	Unit/Enh (6490)	380	20	4	3.54	0.6	Positive
FVTLVFR.light	No	441.265813	Unit/Enh (6490)	635.387522	Unit/Enh (6490)	380	14.7	4	6.93	0.6	Positive
FVTLVFR.light	No	441.265813	Unit/Enh (6490)	421.25578	Unit/Enh (6490)	380	14.7	4	6.93	0.6	Positive
FVTLVFR.light	No	441.265813	Unit/Enh (6490)	247.1441	Unit/Enh (6490)	380	14.7	4	6.93	0.6	Positive
GC[+57.0]VQDEFC[+57.0]TR.light	No	636.260804	Unit/Enh (6490)	1054.46222	Unit/Enh (6490)	380	24	4	2.37	0.6	Positive

Cpd Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Frag (V)	CE (V)	Cell Acc (V)	Ret Time (min)	Ret Window	Polarity
GC[+57.0]VQDEFC[+57.0]TR.light	No	636.260804	Unit/Enh (6490)	955.393806	Unit/Enh (6490)	380	20.7	4	2.37	0.6	Positive
GC[+57.0]VQDEFC[+57.0]TR.light	No	636.260804	Unit/Enh (6490)	827.335229	Unit/Enh (6490)	380	24	4	2.37	0.6	Positive
GEVLLPVEHQQQK.light	No	502.275457	Unit/Enh (6490)	659.877519	Unit/Enh (6490)	380	10	4	3.35	0.6	Positive
GEVLLPVEHQQQK.light	No	502.275457	Unit/Enh (6490)	610.343312	Unit/Enh (6490)	380	13.3	4	3.35	0.6	Positive
GEVLLPVEHQQQK.light	No	502.275457	Unit/Enh (6490)	553.80128	Unit/Enh (6490)	380	13.3	4	3.35	0.6	Positive
GLDLHGLLAFIQLQQC[+57.0]AQDR.light	No	766.067071	Unit/Enh (6490)	1037.577842	Unit/Enh (6490)	380	20	4	10.32	0.6	Positive
GLDLHGLLAFIQLQQC[+57.0]AQDR.light	No	766.067071	Unit/Enh (6490)	905.389395	Unit/Enh (6490)	380	22.8	4	10.32	0.6	Positive
GLDLHGLLAFIQLQQC[+57.0]AQDR.light	No	766.067071	Unit/Enh (6490)	649.272235	Unit/Enh (6490)	380	26	4	10.32	0.6	Positive
GPYPPGPLAPPQPFGPGFVPPPPPPYGPGR.light	No	1034.539388	Unit/Enh (6490)	1228.647319	Unit/Enh (6490)	380	29	4	9.3	1.2	Positive
GPYPPGPLAPPQPFGPGFVPPPPPPYGPGR.light	No	1034.539388	Unit/Enh (6490)	1172.609871	Unit/Enh (6490)	380	29	4	9.3	1.2	Positive
GPYPPGPLAPPQPFGPGFVPPPPPPYGPGR.light	No	1034.539388	Unit/Enh (6490)	850.445765	Unit/Enh (6490)	380	29	4	9.3	1.2	Positive
GSISIQTEEK.light	No	546.282585	Unit/Enh (6490)	834.420339	Unit/Enh (6490)	380	15	4	2.01	0.6	Positive
GSISIQTEEK.light	No	546.282585	Unit/Enh (6490)	747.388316	Unit/Enh (6490)	380	21	4	2.01	0.6	Positive
GSISIQTEEK.light	No	546.282585	Unit/Enh (6490)	634.304246	Unit/Enh (6490)	380	14	4	2.01	0.6	Positive
GSISIQTEQIHGK.light	No	509.598488	Unit/Enh (6490)	691.867348	Unit/Enh (6490)	380	8	4	2.5	0.6	Positive
GSISIQTEQIHGK.light	No	509.598488	Unit/Enh (6490)	635.325316	Unit/Enh (6490)	380	20	4	2.5	0.6	Positive
GSISIQTEQIHGK.light	No	509.598488	Unit/Enh (6490)	461.580657	Unit/Enh (6490)	380	11	4	2.5	0.6	Positive
HLVQQEGQLEQQER.light	No	574.623696	Unit/Enh (6490)	920.458455	Unit/Enh (6490)	380	13	4	1.51	0.6	Positive
HLVQQEGQLEQQER.light	No	574.623696	Unit/Enh (6490)	689.321293	Unit/Enh (6490)	380	15.9	4	1.51	0.6	Positive
HLVQQEGQLEQQER.light	No	574.623696	Unit/Enh (6490)	560.2787	Unit/Enh (6490)	380	13	4	1.51	0.6	Positive
IAEYMNHLIDIGVAGFR.light	No	640.332977	Unit/Enh (6490)	903.453796	Unit/Enh (6490)	380	21	4	8.78	0.6	Positive
IAEYMNHLIDIGVAGFR.light	No	640.332977	Unit/Enh (6490)	867.935239	Unit/Enh (6490)	380	21	4	8.78	0.6	Positive
IAEYMNHLIDIGVAGFR.light	No	640.332977	Unit/Enh (6490)	606.335821	Unit/Enh (6490)	380	18.3	4	8.78	0.6	Positive
IIEGGIYDADLNDER.light	No	846.907398	Unit/Enh (6490)	1466.639393	Unit/Enh (6490)	380	30	4	4.8	0.6	Positive
IIEGGIYDADLNDER.light	No	846.907398	Unit/Enh (6490)	1110.469808	Unit/Enh (6490)	380	30	4	4.8	0.6	Positive

Cpd Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Frag (V)	CE (V)	Cell Acc (V)	Ret Time (min)	Ret Window	Polarity
IIEGGIYD ADLNDER. light	No	846.90739 8	Unit/Enh (6490)	947.40647 9	Unit/Enh (6490)	380	30	4	4.8	0.6	Positive
IPPPPPAP YGPGIFP PPPPQP.li ght	No	710.71893 7	Unit/Enh (6490)	1141.6040 57	Unit/Enh (6490)	380	24	4	8.29	1.2	Positive
IPPPPPAP YGPGIFP PPPPQP.li ght	No	710.71893 7	Unit/Enh (6490)	729.39300 1	Unit/Enh (6490)	380	15	4	8.29	1.2	Positive
IPPPPPAP YGPGIFP PPPPQP.li ght	No	710.71893 7	Unit/Enh (6490)	535.28747 4	Unit/Enh (6490)	380	20.8	4	8.29	1.2	Positive
ISPQIQLS GQTEQTQ K.light	No	893.47069 7	Unit/Enh (6490)	1247.6226 2	Unit/Enh (6490)	380	32	4	3.46	0.6	Positive
ISPQIQLS GQTEQTQ K.light	No	893.47069 7	Unit/Enh (6490)	1006.4799 79	Unit/Enh (6490)	380	32	4	3.46	0.6	Positive
ISPQIQLS GQTEQTQ K.light	No	893.47069 7	Unit/Enh (6490)	793.41265 1	Unit/Enh (6490)	380	32	4	3.46	0.6	Positive
LLDEDHT GTVEFK.li ght	No	501.91570 6	Unit/Enh (6490)	695.82788 8	Unit/Enh (6490)	380	16	4	3.65	0.6	Positive
LLDEDHT GTVEFK.li ght	No	501.91570 6	Unit/Enh (6490)	639.28585 6	Unit/Enh (6490)	380	16	4	3.65	0.6	Positive
LLDEDHT GTVEFK.li ght	No	501.91570 6	Unit/Enh (6490)	294.18121 8	Unit/Enh (6490)	380	13.3	4	3.65	0.6	Positive
LLDNWDS VTSTFSK.l ight	No	806.89630 2	Unit/Enh (6490)	971.46801 7	Unit/Enh (6490)	380	29	4	7.39	0.6	Positive
LLDNWDS VTSTFSK.l ight	No	806.89630 2	Unit/Enh (6490)	670.34063 2	Unit/Enh (6490)	380	26	4	7.39	0.6	Positive
LLDNWDS VTSTFSK.l ight	No	806.89630 2	Unit/Enh (6490)	569.29295 3	Unit/Enh (6490)	380	26	4	7.39	0.6	Positive
LLVVYPW TQR.light	No	637.86642 3	Unit/Enh (6490)	949.48902 7	Unit/Enh (6490)	380	18	4	7.66	0.6	Positive
LLVVYPW TQR.light	No	637.86642 3	Unit/Enh (6490)	850.42061 3	Unit/Enh (6490)	380	24	4	7.66	0.6	Positive
LLVVYPW TQR.light	No	637.86642 3	Unit/Enh (6490)	687.35728 5	Unit/Enh (6490)	380	18	4	7.66	0.6	Positive
LSEPAELT DAVK.light	No	636.83772 5	Unit/Enh (6490)	943.50948 8	Unit/Enh (6490)	380	20.7	4	4.4	0.6	Positive
LSEPAELT DAVK.light	No	636.83772 5	Unit/Enh (6490)	646.37701 7	Unit/Enh (6490)	380	24	4	4.4	0.6	Positive
LSEPAELT DAVK.light	No	636.83772 5	Unit/Enh (6490)	472.25838 2	Unit/Enh (6490)	380	20.7	4	4.4	0.6	Positive
LSGLLDLA LGK.light	No	550.33970 6	Unit/Enh (6490)	986.58807 3	Unit/Enh (6490)	380	18.1	4	8.71	0.6	Positive
LSGLLDLA LGK.light	No	550.33970 6	Unit/Enh (6490)	899.55604 4	Unit/Enh (6490)	380	15	4	8.71	0.6	Positive
LSGLLDLA LGK.light	No	550.33970 6	Unit/Enh (6490)	729.45051 6	Unit/Enh (6490)	380	15	4	8.71	0.6	Positive
LSITGTYD LK.light	No	555.80569 6	Unit/Enh (6490)	910.48802 4	Unit/Enh (6490)	380	15	4	4.91	0.6	Positive
LSITGTYD LK.light	No	555.80569 6	Unit/Enh (6490)	797.40396	Unit/Enh (6490)	380	18.2	4	4.91	0.6	Positive
LSITGTYD LK.light	No	555.80569 6	Unit/Enh (6490)	696.35628 2	Unit/Enh (6490)	380	15	4	4.91	0.6	Positive
MYATIYEL K.light	No	566.29136 8	Unit/Enh (6490)	1000.5349 74	Unit/Enh (6490)	380	18.6	4	5.46	0.6	Positive
MYATIYEL K.light	No	566.29136 8	Unit/Enh (6490)	837.47164 6	Unit/Enh (6490)	380	22	4	5.46	0.6	Positive
MYATIYEL K.light	No	566.29136 8	Unit/Enh (6490)	295.11108 9	Unit/Enh (6490)	380	16	4	5.46	0.6	Positive
NFPSPVD AAFR.light	No	610.80656 2	Unit/Enh (6490)	959.49450 6	Unit/Enh (6490)	380	23	4	6.08	0.6	Positive
NFPSPVD AAFR.light	No	610.80656 2	Unit/Enh (6490)	775.40971 4	Unit/Enh (6490)	380	26	4	6.08	0.6	Positive
NFPSPVD AAFR.light	No	610.80656 2	Unit/Enh (6490)	480.25089 1	Unit/Enh (6490)	380	17	4	6.08	0.6	Positive
NLLDEIAS R.light	No	515.78001 3	Unit/Enh (6490)	803.42575 8	Unit/Enh (6490)	380	17	4	6.25	0.6	Positive
NLLDEIAS R.light	No	515.78001 3	Unit/Enh (6490)	690.34169 4	Unit/Enh (6490)	380	17	4	6.25	0.6	Positive
NLLDEIAS R.light	No	515.78001 3	Unit/Enh (6490)	228.13426 7	Unit/Enh (6490)	380	14	4	6.25	0.6	Positive

Cpd Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Frag (V)	CE (V)	Cell Acc (V)	Ret Time (min)	Ret Window	Polarity
NQGPQES VVR.light	No	557.28599 4	Unit/Enh (6490)	871.46320 6	Unit/Enh (6490)	380	24	4	1.36	0.6	Positive
NQGPQES VVR.light	No	557.28599 4	Unit/Enh (6490)	814.44174 3	Unit/Enh (6490)	380	18.3	4	1.36	0.6	Positive
NQGPQES VVR.light	No	557.28599 4	Unit/Enh (6490)	243.10878 1	Unit/Enh (6490)	380	18.3	4	1.36	0.6	Positive
QEAQLEL PEQQVG QPK.light	No	607.98289	Unit/Enh (6490)	812.41485 9	Unit/Enh (6490)	380	14	4	4.24	0.6	Positive
QEAQLEL PEQQVG QPK.light	No	607.98289	Unit/Enh (6490)	699.33079 5	Unit/Enh (6490)	380	17.1	4	4.24	0.6	Positive
QEAQLEL PEQQVG QPK.light	No	607.98289	Unit/Enh (6490)	505.76690 5	Unit/Enh (6490)	380	14	4	4.24	0.6	Positive
QITIPSQE QEHSQK.li ght	No	551.61279 7	Unit/Enh (6490)	706.34423 7	Unit/Enh (6490)	380	12	4	1.9	0.6	Positive
QITIPSQE QEHSQK.li ght	No	551.61279 7	Unit/Enh (6490)	599.27836 6	Unit/Enh (6490)	380	18	4	1.9	0.6	Positive
SASDLTW DNLK.light	No	625.30659 2	Unit/Enh (6490)	1091.5367 65	Unit/Enh (6490)	380	20.4	4	5.02	0.6	Positive
SASDLTW DNLK.light	No	625.30659 2	Unit/Enh (6490)	776.39373	Unit/Enh (6490)	380	17	4	5.02	0.6	Positive
SASDLTW DNLK.light	No	625.30659 2	Unit/Enh (6490)	675.34605 1	Unit/Enh (6490)	380	20.4	4	5.02	0.6	Positive
SAVTALW GK.light	No	466.76363 4	Unit/Enh (6490)	774.45085 1	Unit/Enh (6490)	380	13	4	5.08	0.6	Positive
SAVTALW GK.light	No	466.76363 4	Unit/Enh (6490)	675.38243 7	Unit/Enh (6490)	380	15.5	4	5.08	0.6	Positive
SAVTALW GK.light	No	466.76363 4	Unit/Enh (6490)	574.33475 8	Unit/Enh (6490)	380	19	4	5.08	0.6	Positive
SGINC [+57.0] PIQK.light	No	508.76330 9	Unit/Enh (6490)	759.38178 5	Unit/Enh (6490)	380	16.8	4	1.72	0.6	Positive
SGINC [+57.0] PIQK.light	No	508.76330 9	Unit/Enh (6490)	645.33885 8	Unit/Enh (6490)	380	16.8	4	1.72	0.6	Positive
SGINC [+57.0] PIQK.light	No	508.76330 9	Unit/Enh (6490)	485.30820 9	Unit/Enh (6490)	380	16.8	4	1.72	0.6	Positive
SVLGQLGI TK.light	No	508.31094 9	Unit/Enh (6490)	829.51417 9	Unit/Enh (6490)	380	20	4	5.72	0.6	Positive
SVLGQLGI TK.light	No	508.31094 9	Unit/Enh (6490)	716.43011 5	Unit/Enh (6490)	380	14	4	5.72	0.6	Positive
SVLGQLGI TK.light	No	508.31094 9	Unit/Enh (6490)	531.35007 4	Unit/Enh (6490)	380	16.8	4	5.72	0.6	Positive
VPEPGC [+57.0] TK.light	No	444.21820 3	Unit/Enh (6490)	788.36071 5	Unit/Enh (6490)	380	18	4	1.13	0.6	Positive
VPEPGC [+57.0] TK.light	No	444.21820 3	Unit/Enh (6490)	562.26535 8	Unit/Enh (6490)	380	18	4	1.13	0.6	Positive
VPEPGC [+57.0] TK.light	No	444.21820 3	Unit/Enh (6490)	394.68399 6	Unit/Enh (6490)	380	18	4	1.13	0.6	Positive
VPVPGYT K.light	No	430.74745 3	Unit/Enh (6490)	664.36645 2	Unit/Enh (6490)	380	17	4	2.3	0.6	Positive
VPVPGYT K.light	No	430.74745 3	Unit/Enh (6490)	565.29803 8	Unit/Enh (6490)	380	14.4	4	2.3	0.6	Positive
VPVPGYT K.light	No	430.74745 3	Unit/Enh (6490)	381.21324 6	Unit/Enh (6490)	380	14.4	4	2.3	0.6	Positive
VSFLSALE EYTK.light	No	693.8612	Unit/Enh (6490)	1053.5462 67	Unit/Enh (6490)	380	22.5	4	9.39	0.6	Positive
VSFLSALE EYTK.light	No	693.8612	Unit/Enh (6490)	940.46220 3	Unit/Enh (6490)	380	22.5	4	9.39	0.6	Positive
VSFLSALE EYTK.light	No	693.8612	Unit/Enh (6490)	600.81097 9	Unit/Enh (6490)	380	20	4	9.39	0.6	Positive
WYVVGLA GNAILR.lig ht	No	716.40898 6	Unit/Enh (6490)	884.53122 6	Unit/Enh (6490)	380	23.2	4	8.82	0.6	Positive
WYVVGLA GNAILR.lig ht	No	716.40898 6	Unit/Enh (6490)	449.21833 2	Unit/Enh (6490)	380	23.2	4	8.82	0.6	Positive
WYVVGLA GNAILR.lig ht	No	716.40898 6	Unit/Enh (6490)	350.14991 8	Unit/Enh (6490)	380	20	4	8.82	0.6	Positive

Scan Parameters

Data Stg	Threshold
Centroid	0

Source Parameters

Parameter	Value (+)	Value (-)
Gas Temp (°C)	200	200
Gas Flow (l/min)	13	13
Nebulizer (psi)	35	35
SheathGasHeater	200	200
SheathGasFlow	12	12
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	100.0 µL/min
Eject Speed	100.0 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	20.00 µL
Needle Wash	
Needle Wash Location	Flush Port
Wash Time	15.0 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	1.000 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	11.00 min
Post Time	
Posttime Mode	Time set
Posttime	3.00 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 % Acetonitrile V.03	IPA	Ch. 1	Yes	5.00 %

Timetable

	Time	A	B	Flow	Pressure
1	0.50 min	90.00 %	10.00 %	--- mL/min	--- bar
2	9.70 min	70.00 %	30.00 %	--- mL/min	--- bar
3	9.80 min	10.00 %	90.00 %	--- mL/min	--- bar
4	10.80 min	10.00 %	90.00 %	--- mL/min	--- bar
5	10.90 min	95.00 %	5.00 %	--- mL/min	--- bar
6	11.00 min	95.00 %	5.00 %	--- mL/min	--- bar

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

APPENDIX C: SAMPLE PREPARATION LIST FOR 3.1.6 CASEWORK

Descriptor	Identifier	Samples	Preparation
Aged	NPB_D#	Peripheral Blood	Neat fluid (single source) pipetted onto cotton swab. Incubated at room temperature for Day 0, 1, 3, 7, and 35 days in duplicate. Swab cut and placed in freezer at designated time, analyzed in a single batch.
	NMB_D#	Menstrual Blood	
	NSE_D#	Seminal Fluid	
	NVF50_D#	Vaginal Fluid	
	NSA_D#	Saliva	
Simulated Sexual Assault Incubation	SSA_ZERO	Day Zero	10 ul neat semen (single source) in 1 mL vaginal fluid (single source) incubated at 37 for the listed days. Tubes frozen at designated time, analyzed in a single batch.
	SSA_ONE	Day One	
	SSA_THREE	Day Three	
	SSA_FIVE	Day Five	
	SSA_SEVEN	Day Seven	
	SSA_NINE	Day Nine	
	SSA_ELEVEN	Day Eleven	
Substrates	SUB01.1 ; SUB01.2	Peripheral Blood on Cotton	50 ul neat peripheral blood on cutting of cotton Tshirt, dried at RT overnight
	SUB02.1 ; SUB02.2	Peripheral Blood on Denim	50 ul neat peripheral blood on cutting of denim, dried at RT overnight
	SUB03.1 ; SUB03.2	Peripheral Blood on Carpet	50 ul neat peripheral blood on cutting of carpet, dried at RT overnight
	SUB04.1 ; SUB04.2	Peripheral Blood on Leather	50 ul neat peripheral blood on cutting of leather, dried at RT overnight
	SUB05.1 ; SUB05.2	Peripheral Blood on Drywall	50 ul neat peripheral blood on cutting of drywall, cutting taken for analysis
	SUB06.1 ; SUB06.2	Semen on Cotton	50 ul neat semen on cutting of cotton Tshirt, dried at RT overnight
	SUB07.1 ; SUB07.2	Semen on Leather	50 ul neat semen on cutting of leather, dried at RT overnight, cutting taken for analysis
	SUB08.1 ; SUB08.2	Menstrual Blood on Cotton	50 ul neat menstrual blood on cutting of cotton T-shirt, dried at RT overnight
	SUB09.1 ; SUB09.2	Menstrual Blood on Denim	50 ul neat menstrual blood on cutting of denim, dried at RT overnight
	SUB10.1 ; SUB10.2	Menstrual Blood on Pad	50 ul neat menstrual blood on pad, dried at RT overnight, cutting taken for analysis
	SUB11.1 ; SUB11.2	Vaginal Fluid on Cotton	50 ul neat vaginal fluid on cutting of cotton T-shirt, dried at RT overnight
	SUB12.1 ; SUB12.2	Vaginal Fluid on Demin	50 ul neat vaginal fluid on cutting of denim, dried at RT overnight
	SUB13.1 ; SUB13.2	Vaginal Fluid on Leather	50 ul neat vaginal fluid on cutting of leather, dried at RT overnight
	SUB14.1 ; SUB14.2	Saliva on Plastic Bottle	50 ul on bottle, dried at RT overnight
	SUB15.1 ; SUB15.2	Saliva on Aluminum Can	50 ul on bottle, dried at RT overnight

Descriptor	Identifier	Samples	Preparation
Environmental Contaminants	CON01.1 ; CON01.2	Peripheral Blood & Dirt	50 ul neat peripheral blood & 50 ul dirt slurry on 1/2 swab, dried at RT overnight
	CON02.1 ; CON02.2	Peripheral Blood & Rust	50 ul neat peripheral blood & 50 ul rust slurry on 1/2 swab, dried at RT overnight
	CON03.1 ; CON03.2	Peripheral Blood & 10% Bleach	50 ul neat peripheral blood & 50 ul 10% bleach on 1/2 swab, dried at RT overnight
	CON04.1 ; CON04.2	Menstrual Blood & Lube	50 ul neat menstrual blood & 50 ul lube on 1/2 swab, dried at RT overnight
	CON05.1 ; CON05.2	Menstrual Blood & Spermicide Condom	50 ul neat menstrual blood on cutting of condom, dried at RT overnight
	CON06.1 ; CON06.2	Vaginal Fluid & Spermicide Condom	50 ul neat vaginal fluid on condom cutting of , dried at RT overnight
	CON07.1 ; CON07.2	Vaginal Fluid & Lube	50 ul neat vaginal fluid & 50 ul lube on 1/2 swab, dried at RT overnight
	CON08.1 ; CON08.2	Semen & Lube	50 ul neat semen & 50 ul lube on 1/2 swab, dried at RT overnight
	CON09.1 ; CON09.2	Semen & Spermicide Condom	50 ul neat semen on condom cutting of , dried at RT overnight
	CON10.1 ; CON10.2	Semen & 10% Bleach	50 ul neat semen & 50 ul 10% bleach on 1/2 swab, dried at RT overnight
	CON11.1 ; CON11.2	Saliva & Tobacco	50 ul saliva/tobacco slurry on 1/2 swab, dried at RT overnight
	CON12.1 ; CON12.2	Saliva & Cigarette	50 ul saliva on cutting of filter, dried at RT overnight
Mixtures	MIX01	Saliva (minor) & Vaginal Fluid (major)	10 ul minor fluid mixed with 500 ul major fluid, 100 ul spotted on full swab. Dried at RT overnight, resuspended in 1 ml deionized water. Immunochromatographic comparison. Single Source.
	MIX02	Semen (minor) & Vaginal Fluid (major)	
	MIX03	Semen (minor) & Menstural Blood (major)	
	MIX04	Saliva (minor) & Menstrual Blood (major)	
	MIX05	Vaginal Fluid (minor) & Urine (major)	

Descriptor	Identifier	Samples	Preparation
Dilutions on Swabs	SEN01	Peripheral Blood 1:100	Dilutions prepared in deionized water, 150 ul spotted on full swabs. Dried at RT overnight, resuspended in 1 ml deionized water. Immunochromatographic. Single Source. Injected 20 uL on column.
	SEN02	Peripheral Blood 1:1,000	
	SEN03	Peripheral Blood 1:2,000	
	SEN04	Peripheral Blood 1:5,000	
	SEN05	Peripheral Blood 1:10,000	
	SEN06	Peripheral Blood 1:20,000	
	SEN07	Peripheral Blood 1:40,000	
	SEN08	Semen 1:100	
	SEN09	Semen 1:1,000	
	SEN10	Semen 1:2,000	
	SEN11	Semen 1:5,000	
	SEN12	Semen 1:10,000	
	SEN13	Semen 1:20,000	
	SEN14	Semen 1:40,000	
	SEN15	Saliva 1:10	
	SEN16	Saliva 1:100	
	SEN17	Saliva 1:500	
	SEN18	Saliva 1:1,000	
	SEN19	Saliva 1:2,000	
	SEN20	Saliva 1:5,000	
	SEN21	Saliva 1:10,000	
	SEN22	Menstrual Blood 1:100	
	SEN23	Menstrual Blood 1:1,000	
	SEN24	Menstrual Blood 1:2,000	
	SEN25	Menstrual Blood 1:5,000	
	SEN26	Menstrual Blood 1:10,000	
	SEN27	Menstrual Blood 1:20,000	
	SEN28	Menstrual Blood 1:40,000	
	SEN29	Vaginal Fluid 1:10	
	SEN30	Vaginal Fluid 1:100	
	SEN31	Vaginal Fluid 1:500	
	SEN32	Vaginal Fluid 1:1,000	
	SEN33	Vaginal Fluid 1:2,000	
	SEN34	Vaginal Fluid 1:5,000	
	SEN35	Vaginal Fluid 1:10,000	
Authentic	**Sexual Assault Kit**		Swabs taken from a single female individual in duplicate immediately before preparation. 10 ul of 1:100 semen dilution spotted on swabs, dried at RT overnight. Full swab resuspended in 1 mL deionized water. Immunochromatographic comparison. Single source male and female donors.
	SA01_01.1; SA01_01.2	Vaginal Swab	
	SA01_02.1; SA01_02.2	Oral Swab	
	SA01_03.1; SA01_03.2	Rectal Swab	
	Sexual Assault Kit-Vasectomized Suspect		
	SA02_01.1; SA02_01.2	Vaginal Swab	
	SA02_02.1; SA02_02.2	Oral Swab	
	SA02_03.1; SA02_03.2	Rectal Swab	
	Laundered Samples		1 mL of fluid 'squirted' on substrate, outlined with marker. Substrates washed separately (by fluid) on regular cycle with detergent and dried completely in dryer. Cutting of stain and control region taken for analysis.
	LS01	Semen on Bed Sheet	
	LS02	Semen on Underwear	
	LS03	Peripheral Blood on Towel	
	LS04	Peripheral Blood on Denim	
	Digital Swabs		Dry swab of finger, dried at RT overnight prior to analysis. Full swab resuspended in 1 mL of deionized water. Single Source.
	DS01.1; DS01.2	Saliva	
	DS02.1; DS02.2	Vaginal Fluid	
	DS03.1; DS03.2	Menstrual Blood	

APPENDIX D: REPEATABILITY AND REPRODUCIBILITY CALCULATED COEFFICIENT OF VARIATION FOR PEAK AREA RESPONSE

Fluid	Protein	Peptide	Analyst 1	Analyst 2	Combined
Peripheral Blood	Alpha-1 Antitrypsin	LSITGTYDLK	11.845	10.800	11.530
		SVLGQLGITK	9.366	9.210	9.336
	Hemoglobin	SAVTALWGK	7.054	6.768	6.792
		LLVVYPWTQR	7.457	5.374	6.463
	Hemopexin	NFPSPVDAAFR	10.990	9.664	10.756
	Apolipoprotein	LLDNWDSVTSTFSK	13.664	9.755	11.945
		VSFLSALEEYTK	7.843	15.313	12.289
	Serotransferrin	DGAGDVAFVK	12.350	10.574	11.615
		SASDLTWDNLK	6.351	7.044	7.045
Saliva	Alpha Amylase	LSGLLDLALGK	6.434	7.201	9.067
		IAEYMNHLIDIGVAGFR	3.954	9.962	9.079
	Statherin	FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF	12.736	11.058	13.988
	Submaxillary Protein	GPYPPGPLAPPQPFGPGFVPPPPPPPTGPGR	7.189	9.944	10.139
		IPPPPPAPYGPGIFPPPPPQP	5.941	8.414	9.873
	Cystatin	IIEGGIYDADLNDER	5.811	10.255	12.909
		ALHFVISEYNK	5.991	8.555	10.719
Vaginal Fluid	Cornulin	LLDEDHTGTVEFK	6.647	15.880	13.902
		ISPQIQLSGQTEQTQK	6.635	17.521	14.595
		AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR	4.567	10.743	8.064
	Neutrophil Gelatinase	WYVVGLAGNAILR	7.926	11.252	10.790
		MYATIYELK	9.313	12.546	12.265
	Ly6/PLAUR	GLDHGLLAFIQLQQCAQDR	10.027	7.458	8.874
		GCVQDEFCTR	7.244	16.604	14.520
	Suprabasin	ALGDINSGITHAGR	4.790	12.886	11.096
		FGQGVHHGLSEGWK	10.922	13.427	13.655
	Periplakin	AQSLQSAK	5.933	11.403	11.056
		NLLDEIASR	9.246	15.735	16.460
		NQGPQESVVR	7.738	13.654	12.771
	Small Proline Rich Protein 3	VPEPGCTK	8.700	7.030	8.070
		VPVPGYTK	9.925	11.226	10.284
	Involucrin	HLVQQEGQLEQQER	5.846	18.955	15.417
		QEAQLELPEQQVGQPK	4.357	15.600	12.361
		GEVLLPVEHQQQK	9.393	10.984	10.677

Fluid	Protein	Peptide	Analyst 1	Analyst 2	Combined
Seminal Fluid	Acid Phosphatase	FVTLVFR	87.638	41.252	95.688
		FQELESETLK	19.405	14.717	26.483
		ELSELSLLSLYGIHK	5.787	10.988	8.773
	Prostate Specific Antigen	FLRPGDDSSHDLMLLR	54.820	21.388	62.553
		LSEPAELTDAVK	34.849	17.358	45.393
	Semenogelin 2	GSISIQTEEK	38.678	19.467	51.764
		GSISIQTEEQIHGK	10.208	10.259	15.247
		DVSQSSISFQIEK	2.677	11.744	8.304
	Semenogelin 1	DIFSTQDELLVYNK	5.340	10.368	8.063
		QITIPSQEQEHSQK	6.822	7.261	7.747
	Epididymal Secretory	DCGSVDGVIK	6.243	11.758	9.125
		SGINCPIQK	9.318	9.948	9.425
Menstrual Blood	Cornulin	LLDEDHTGTVEFK	14.904	12.570	14.500
		ISPQIQLSGQTEQTQK	N/D	N/D	N/D
		AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR	N/D	N/D	N/D
	Neutrophil Gelatinase	WYVVGLAGNAILR	24.821	15.549	20.181
		MYATIYELK	22.655	24.441	23.949
	Ly6/PLAUR	GLDHGLLAFIQLQQCAQDR	N/D	N/D	N/D
		GCVQDEFCTR	N/D	N/D	N/D
	Suprabasin	ALGDINSGITHAGR	N/D	N/D	N/D
		FGQGVHHGLSEGWK	N/D	N/D	N/D
	Periplakin	AQSLQSAK	N/D	N/D	N/D
		NLLDEIASR	N/D	N/D	N/D
		NQGPQESVVR	N/D	N/D	N/D
	Involucrin	HLVQQEGQLEQQER	N/D	N/D	N/D
		QEAQLELPEQQVGQPK	N/D	N/D	N/D
		GEVLLPVEHQQQK	N/D	N/D	N/D
	Small Proline Rich Protein 3	VPEPGCTK	8.312	10.234	10.224
		VPVPGYTK	8.304	4.714	8.635
	Alpha-1 Antitrypsin	LSITGTYDLK	19.303	11.382	16.432
		SVLGQLGITK	13.169	11.973	12.729
	Hemoglobin	SAVTALWGK	9.186	6.718	8.131
		LLVVYPWTQR	8.966	5.508	7.739
	Hemopexin	NFPSPVDAAFR	15.337	12.862	14.875
	Apolipoprotein	LLDNWDSVTSTFSK	21.725	13.442	19.061
		VSFLSALEEYTK	22.803	17.151	21.686
	Serotransferrin	DGAGDVAFVK	15.868	11.908	14.349
		SASDLTWDNLK	12.424	11.779	12.704

APPENDIX E: REPEATABILITY AND REPRODUCIBILITY CALCULATED COEFFICIENT OF VARIATION FOR RELATIVE RETENTION TIME

Fluid	Protein	Peptide	Analyst 1	Analyst 2	Combined
Peripheral Blood	Alpha-1 Antitrypsin	LSITGTYDLK	0.093	0.093	0.090
		SVLGQLGITK	0.077	0.043	0.060
	Hemoglobin	SAVTALWGK	0.106	0.132	0.118
		LLVVYPWTQR	0.184	0.124	0.157
	Hemopexin	NFPSPVDAAFR	0.062	0.059	0.059
	Apolipoprotein	LLDNWDSVTSTFSK	0.109	0.070	0.089
		VSFLSALEEYTK	0.106	0.025	0.075
	Serotransferrin	DGAGDVAFVK	0.000	0.000	0.000
		SASDLTWDNLK	0.102	0.076	0.088
Saliva	Alpha Amylase	LSGLLDLALGK	0.044	0.036	0.040
		IAEYMNHLIDIGVAGFR	0.058	0.036	0.047
	Statherin	FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF	0.044	0.032	0.039
	Submaxillary Protein	GPYPPGPLAPPQPFGPGFVPPPPPPPTGPGR	0.076	0.055	0.064
		IPPPPPAPYGPGIFPPPPPQP	0.072	0.073	0.074
	Cystatin	IIEGGIYDADLNDER	0.053	0.053	0.051
		ALHFVISEYNK	0.000	0.000	0.000
Vaginal Fluid	Cornulin	LLDEDHTGTVEFK	0.118	0.065	0.093
		ISPQIQLSGQTEQTQK	0.128	0.107	0.115
		AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR	0.176	0.264	0.226
	Neutrophil Gelatinase	WYVVGLAGNAILR	0.064	0.036	0.052
		MYATIYELK	0.066	0.066	0.064
	Ly6/PLAUR	GLDHGLLAFIQLQQCAQDR	0.029	0.022	0.025
		GCVQDEFCTR	0.193	0.170	0.185
	Suprabasin	ALGDINSGITHAGR	0.135	0.171	0.152
		FGQGVHHGLSEGWK	0.248	0.159	0.215
	Periplakin	AQSLQSAK	0.000	0.269	0.190
		NLLDEIASR	0.038	0.061	0.053
		NQGPQESVVR	0.186	0.295	0.258
	Small Proline Rich Protein 3	VPEPGCTK	0.324	0.342	0.331
		VPVPGYTK	0.172	0.163	0.163
	Involucrin	HLVQQEGQLEQQER	0.000	0.225	0.165
		QEAQLELPEQQVGQPK	0.100	0.118	0.107
		GEVLLPVEHQQQK	0.155	0.110	0.136

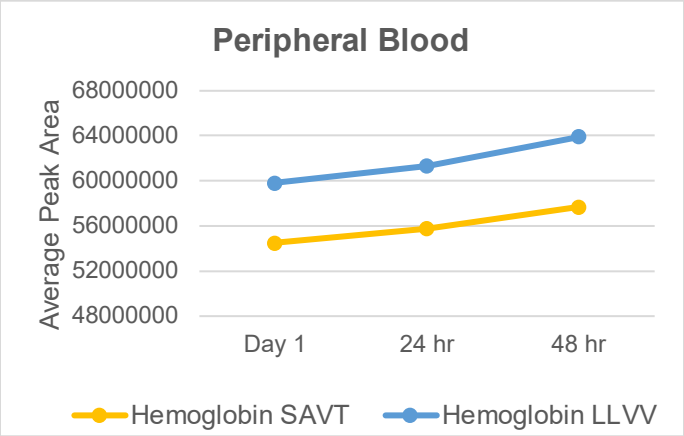
Fluid	Protein	Peptide	Analyst 1	Analyst 2	Combined
Seminal Fluid	Acid Phosphatase	FVTLVFR	0.049	0.059	0.054
		FQELESETLK	0.069	0.069	0.067
		ELSELSLLSLYGIHK	0.058	0.059	0.058
	Prostate Specific Antigen	FLRPGDDSSHDLMLLR	0.081	0.081	0.078
		LSEPAELTDAVK	0.085	0.071	0.078
	Semenogelin 2	GSISIQTEEK	0.124	0.124	0.120
		GSISIQTEEQIHGK	0.134	0.160	0.147
		DVSQSSISFQIEK	0.075	0.079	0.076
	Semenogelin 1	DIFSTQDELLVYNK	0.063	0.042	0.052
		QITIPSQEQEHSQK	0.174	0.132	0.151
	Epididymal Secretory	DCGSVDGVIK	0.138	0.157	0.148
		SGINCPIQK	0.147	0.000	0.104
Menstrual Blood	Cornulin	LLDEDHTGTVEFK	0.104	0.118	0.113
		ISPQIQLSGQTEQTQK	N/D	N/D	N/D
		AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR	N/D	N/D	N/D
	Neutrophil Gelatinase	WYVVGLAGNAILR	0.089	0.083	0.084
		MYATIYELK	0.066	0.114	0.093
	Ly6/PLAUR	GLDHGLLAFIQLQQCAQDR	N/D	N/D	N/D
		GCVQDEFCTR	N/D	N/D	N/D
	Suprabasin	ALGDINSGITHAGR	N/D	N/D	N/D
		FGQGVHHGLSEGWK	N/D	N/D	N/D
	Periplakin	AQSLQSAK	N/D	N/D	N/D
		NLLDEIASR	N/D	N/D	N/D
		NQGPQESVVR	N/D	N/D	N/D
	Involucrin	HLVQQEGQLEQQER	N/D	N/D	N/D
		QEAQLELPEQQVGQPK	N/D	N/D	N/D
		GEVLLPVEHQQQK	N/D	N/D	N/D
	Small Proline Rich Protein 3	VPEPGCTK	0.000	0.000	0.000
		VPVPGYTK	0.231	0.163	0.202
	Alpha-1 Antitrypsin	LSITGTYDLK	0.078	0.082	0.078
		SVLGQLGITK	0.064	0.085	0.073
	Hemoglobin	SAVTALWGK	0.066	0.128	0.099
		LLVVYPWTQR	0.176	0.094	0.137
	Hemopexin	NFPSPVDAAFR	0.114	0.071	0.094
	Apolipoprotein	LLDNWDSVTSTFSK	0.106	0.043	0.078
		VSFLSALEEYTK	0.054	0.062	0.057
	Serotransferrin	DGAGDVAFVK	0.100	0.136	0.122
		SASDLTWDNLK	0.102	0.051	0.078

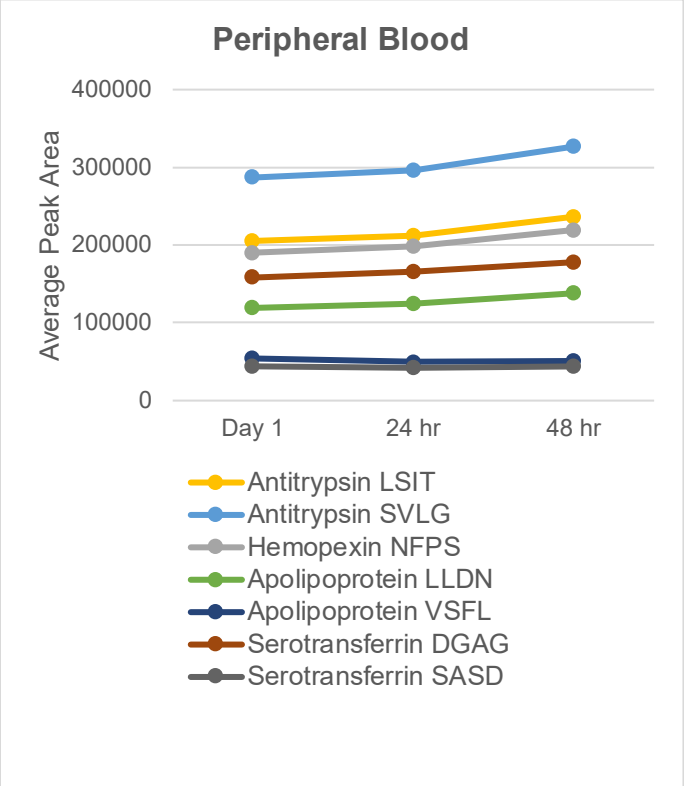
APPENDIX F: REPEATABILITY AND REPRODUCIBILITY CALCULATED COEFFICIENT OF VARIATION FOR ION RATIO RESPONSE

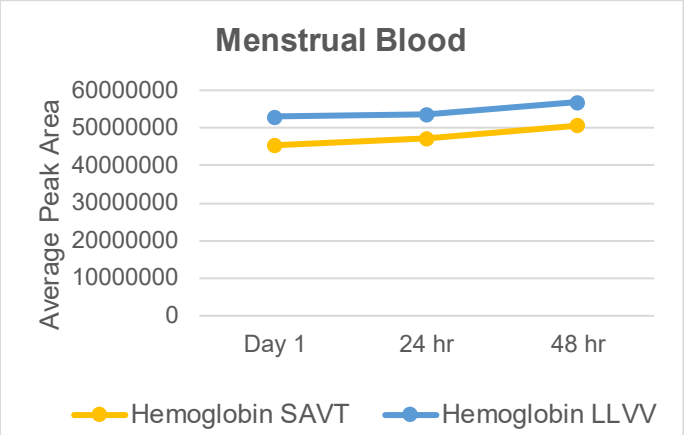
Fluid	Protein	Peptide	Analyst 1	Analyst 2	Combined
Peripheral Blood	Alpha-1 Antitrypsin	LSITGTYDLK	7.520	5.011	6.315
		SVLGQLGITK	3.103	3.745	3.341
	Hemoglobin	SAVTALWGK	4.820	4.842	4.743
		LLVVYPWTQR	1.963	1.824	1.969
	Hemopexin	NFPSPVDAAFR	3.156	3.490	3.496
	Apolipoprotein	LLDNWDSVTSTFSK	1.830	3.817	3.088
		VSFLSALEEYTK	2.973	2.785	2.796
	Serotransferrin	DGAGDVAFVK	3.095	2.746	2.853
		SASDLTWDNLK	4.843	5.640	5.469
Saliva	Alpha Amylase	LSGLLDLALGK	3.647	2.038	3.099
		IAEYMNHLIDIGVAGFR	4.826	4.782	4.867
	Statherin	FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF	3.617	6.615	5.362
	Submaxillary Protein	GPYPPGPLAPPQPFGPGFVPPPPPPPTGPGR	0.864	1.209	1.044
		IPPPPPAPYGPGIFPPPPPQP	0.511	0.588	0.586
	Cystatin	IIEGGIYDADLNDER	4.112	4.217	4.541
		ALHFVISEYNK	2.928	4.286	3.695
Vaginal Fluid	Cornulin	LLDEDHTGTVEFK	4.193	3.883	4.534
		ISPQIQLSGQTEQTQK	2.287	2.340	2.333
		AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR	7.412	6.244	7.175
	Neutrophil Gelatinase	WYVVGLAGNAILR	6.804	5.743	6.108
		MYATIYELK	4.088	3.308	3.753
	Ly6/PLAUR	GLDHGLLAFIQLQQCAQDR	2.913	1.805	2.364
		GCVQDEFCTR	5.220	4.690	5.500
	Suprabasin	ALGDINSGITHAGR	3.605	5.569	4.568
		FGQGVHHGLSEGWK	6.678	6.419	6.370
	Periplakin	AQSLQSAK	2.477	2.819	2.896
		NLLDEIASR	4.053	3.911	4.113
		NQGPQESVVR	8.886	3.902	7.232
	Small Proline Rich Protein 3	VPEPGCTK	4.396	3.693	3.954
		VPVPGYTK	5.035	2.408	4.044
	Involucrin	HLVQQEGQLEQQER	5.630	7.285	6.368
		QEAQLELPEQQVGQPK	1.731	2.578	2.309
		GEVLLPVEHQQQK	2.940	2.489	2.651

Fluid	Protein	Peptide	Analyst 1	Analyst 2	Combined
Seminal Fluid	Acid Phosphatase	FVTLVFR	1.503	1.240	1.342
		FQELESETLK	4.248	3.127	3.636
		ELSELSLLSLYGIHK	4.844	2.577	4.455
	Prostate Specific Antigen	FLRPGDDSSHDLMLLR	3.828	3.360	3.677
		LSEPAELTDAVK	1.918	1.032	1.601
	Semenogelin 2	GSISIQTEEK	4.360	3.249	4.025
		GSISIQTEEQIHGK	3.749	3.127	3.505
		DVSQSSISFQIEK	6.209	3.842	5.066
	Semenogelin 1	DIFSTQDELLVYNK	3.455	4.174	3.801
		QITIPSQEQEHSQK	2.578	3.036	2.810
	Epididymal Secretory	DCGSVDGVIK	5.835	5.021	5.501
		SGINCPIQK	3.971	3.225	3.863
Menstrual Blood	Cornulin	LLDEDHTGTVEFK	5.145	7.970	6.519
		ISPQIQLSGQTEQTQK	N/D	N/D	N/D
		AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR	N/D	N/D	N/D
	Neutrophil Gelatinase	WYVVGLAGNAILR	11.812	14.081	12.682
		MYATIYELK	8.859	29.230	20.998
	Ly6/PLAUR	GLDHGLLAFIQLQQCAQDR	N/D	N/D	N/D
		GCVQDEFCTR	N/D	N/D	N/D
	Suprabasin	ALGDINSGITHAGR	N/D	N/D	N/D
		FGQGVHHGLSEGWK	N/D	N/D	N/D
	Periplakin	AQSLQSAK	N/D	N/D	N/D
		NLLDEIASR	N/D	N/D	N/D
		NQGPQESVVR	N/D	N/D	N/D
	Involucrin	HLVQQEGQLEQQER	N/D	N/D	N/D
		QEAQLELPEQQVGQPK	N/D	N/D	N/D
		GEVLLPVEHQQQK	N/D	N/D	N/D
	Small Proline Rich Protein 3	VPEPGCTK	4.830	1.963	3.579
		VPVPGYTK	5.886	7.259	6.432
	Alpha-1 Antitrypsin	LSITGTYDLK	4.598	4.323	4.418
		SVLGQLGITK	3.147	2.257	2.661
	Hemoglobin	SAVTALWGK	4.661	3.597	4.200
		LLVVYPWTQR	3.446	2.150	2.820
	Hemopexin	NFPSPVDAAFR	3.964	2.758	3.320
	Apolipoprotein	LLDNWDSVTSTFSK	3.863	2.246	3.070
		VSFLSALEEYTK	3.316	3.242	3.805
	Serotransferrin	DGAGDVAFVK	2.730	3.418	3.001
		SASDLTWDNLK	3.875	6.952	5.488

APPENDIX G: AUTOSAMPLER STABILITY COMPREHENSIVE RESULTS



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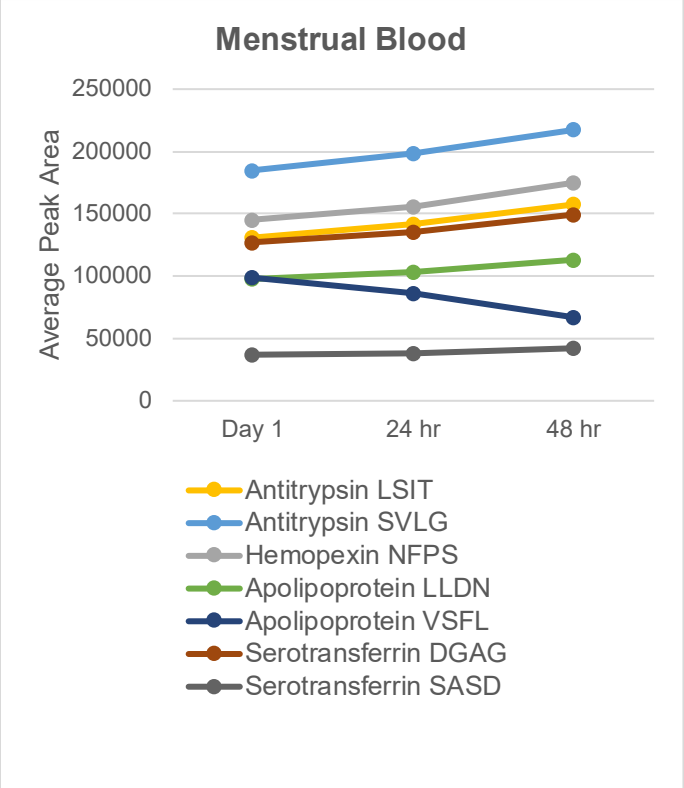
48 hr
Hemoglobin SAVT
Hemoglobin LLVV


Menstrual Blood
Average Peak Area
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150000
100000
50000
0
Day 1
24 hr
48 hr
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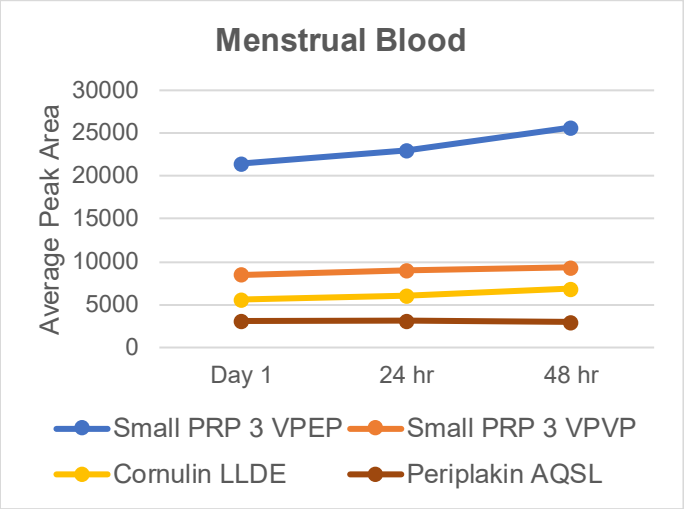
Antitrypsin SVLG
Hemopexin NFPS
Apolipoprotein LLDN
Apolipoprotein VSFL
Serotransferrin DGAG
Serotransferrin SASD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Average Peak Area
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20000
15000
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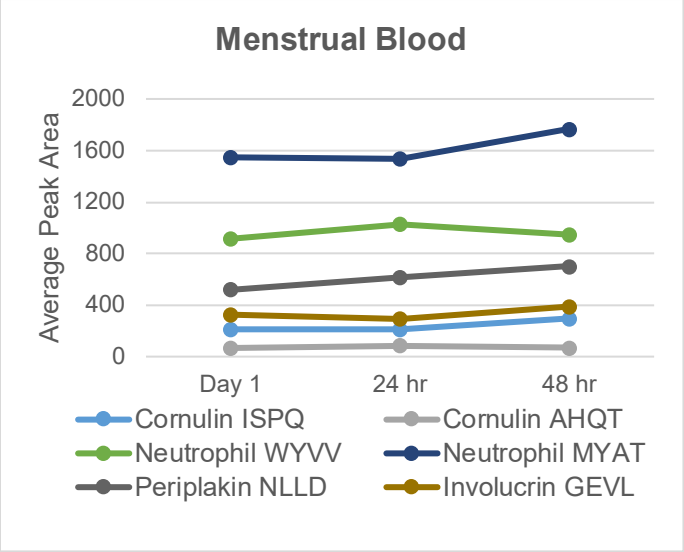
5000
0
Day 1
24 hr
48 hr
Small PRP 3 VPEP
Small PRP 3 VPVP
Cornulin LLDE
Periplakin AQSL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Average Peak Area
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1600
1200
800
400
0
Day 1
24 hr
48 hr
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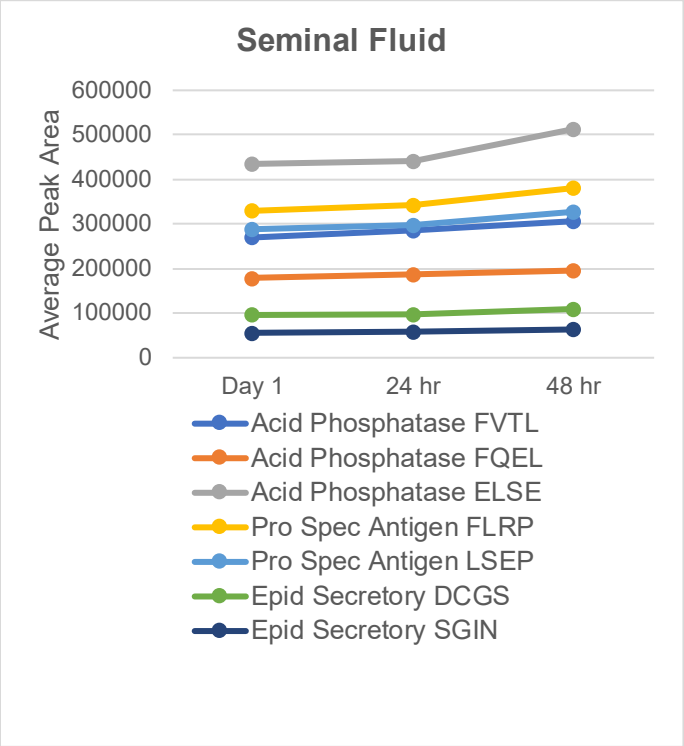
Cornulin AHQT
Neutrophil WYVV
Neutrophil MYAT
Periplakin NLLD
Involucrin GEVL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Average Peak Area
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400000
300000
200000
100000
0
Day 1
24 hr
48 hr
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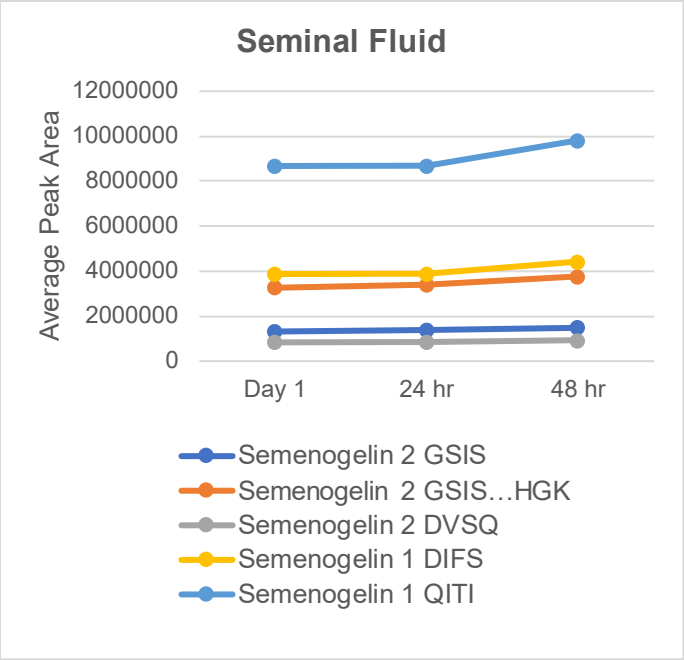
Acid Phosphatase FQEL
Acid Phosphatase ELSE
Pro Spec Antigen FLRP
Pro Spec Antigen LSEP
Epid Secretory DCGS
Epid Secretory SGIN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Average Peak Area
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4000000
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Day 1
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48 hr
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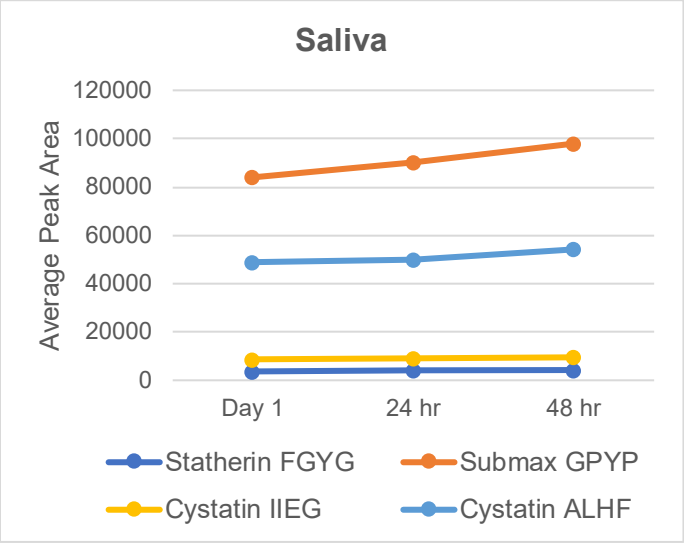
Semenogelin 2 GSIS…HGK
Semenogelin 2 DVSQ
Semenogelin 1 DIFS
Semenogelin 1 QITI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Average Peak Area
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60000
40000
20000
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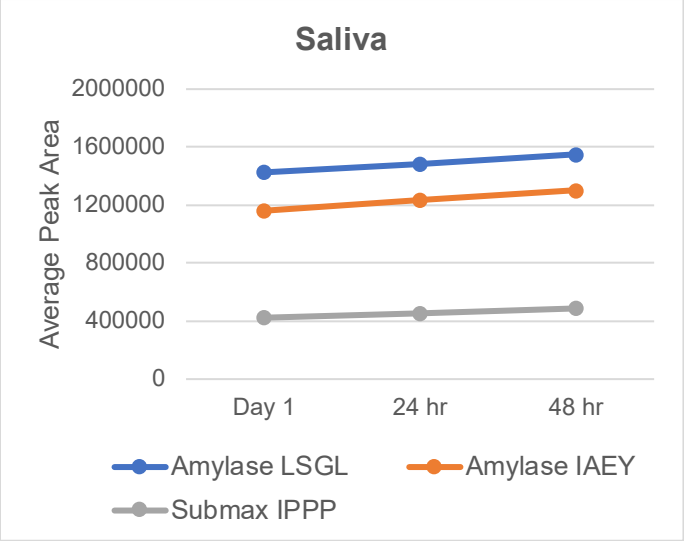
Day 1
24 hr
48 hr
Statherin FGYG
Submax GPYP
Cystatin IIEG
Cystatin ALHF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Day 1
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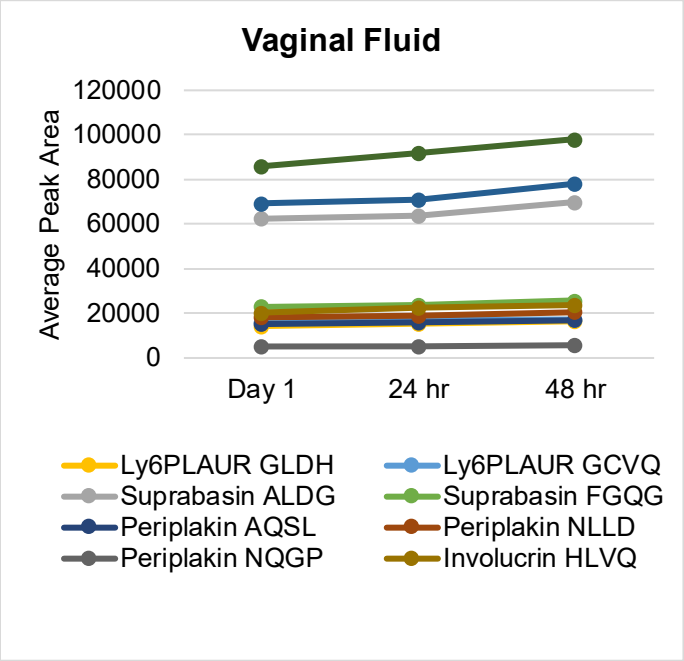
48 hr
Amylase LSGL
Amylase IAEY
Submax IPPP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Average Peak Area
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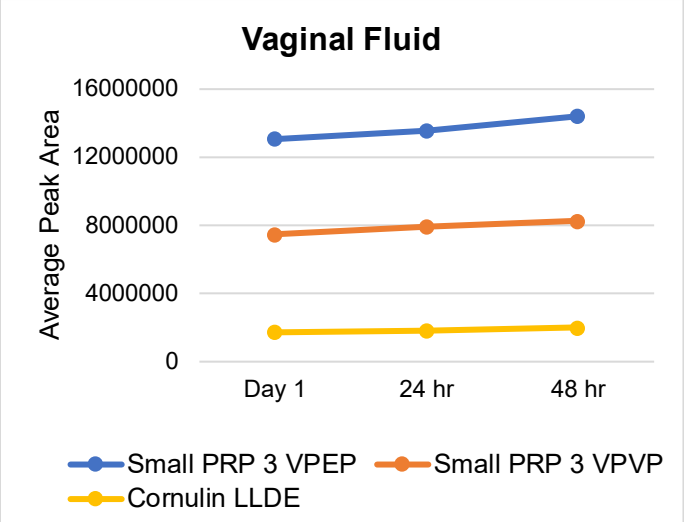
80000
60000
40000
20000
0
Day 1
24 hr
48 hr
Ly6PLAUR GLDH
Ly6PLAUR GCVQ
Suprabasin ALDG
Suprabasin FGQG
Periplakin AQSL
Periplakin NLLD
Periplakin NQGP
Involucrin HLVQ


Vaginal Fluid
Average Peak Area
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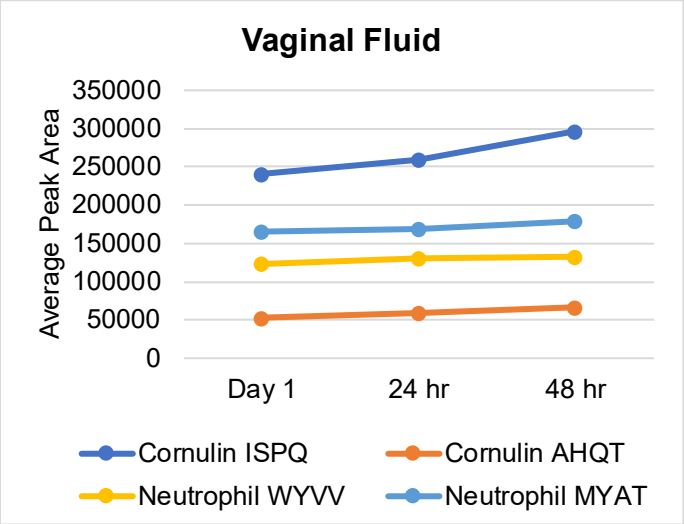
12000000
8000000
4000000
0
Day 1
24 hr
48 hr
Small PRP 3 VPEP
Small PRP 3 VPVP
Cornulin LLDE


Vaginal Fluid
Average Peak Area
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300000
250000
200000
150000
100000
50000
0
Day 1
24 hr
48 hr
Cornulin ISPQ
Cornulin AHQT
Neutrophil WYVV
Neutrophil MYAT

APPENDIX H: BLIND SAMPLE ANALYSIS RESULTS

Sample Number	Sample Description	Quant Value (µg/mL)	Experimental Fluid Identification	True Fluid Identification	Supplemental Info	Points of Discrepancy
1	Cloth swatch No visible staining	ND	Peripheral Blood	Peripheral Blood	100 µL of neat blood, laundered (w/o bleach)	NTR
2	Condom	29.318	ND	ND	Blank	NTR
		25.381	ND	ND	Blank	NTR
3	Cotton swab (1/2) No visible staining	454.335	Seminal Fluid Saliva Vaginal Fluid	Vaginal Fluid Seminal Fluid Saliva	10 µL each semen & saliva in 100 µL V_T on pre-collected and dried vaginal swab	NTR
4	Cloth swatch No visible staining	ND	Seminal Fluid	Seminal Fluid	100 µL neat semen, laundered (w/ bleach)	NTR
5	Cotton swab (1/2) Pale yellow staining	420.607	Seminal Fluid Vaginal Fluid	Vaginal Fluid Seminal Fluid	100 µL of 1:100 diluted semen on a pre-collected and dried vaginal swab	NTR
6	Cotton swab (1/2) No visible staining	382.187	Saliva Vaginal Fluid	Vaginal Fluid Saliva	100 µL 1:10 diluted saliva on a pre-collected and dried vaginal swab	NTR
7	Cloth swatch No visible staining	130.684	Seminal Fluid Saliva	Saliva Seminal Fluid	100 µL 10:1 mixture of saliva:semen on cloth	NTR
8	Cotton swab (1/2) Red-brown staining	607.767	Peripheral Blood Vaginal Fluid	Menstrual Blood	Single-source swab collected from donor, no associated volume	NTR
9	Cotton swab (1/2) No visible staining	ND	ND	ND	Blank	NTR
10	Cloth swatch No visible staining	68.632	Seminal Fluid	Seminal Fluid	100 µL neat semen on cloth, laundered (w/o bleach)	NTR

Sample Number	Sample Description	Quant Value (µg/mL)	Experimental Fluid Identification	True Fluid Identification	Supplemental Info	Points of Discrepancy
11	Cotton swab (1/2) No visible staining	1120.000	Seminal Fluid Saliva	Seminal Fluid Saliva	100 µL 1:1 mixture of saliva and semen on cotton swab	NTR
12	Cotton swab (1/2) Pale yellow staining	4144.280	Seminal Fluid	Seminal Fluid	100 µL neat fluid per swab	NTR
13	Cotton swab (1/2) Red-brown staining	953.931	Peripheral Blood Seminal Fluid Vaginal Fluid	Menstrual Blood Seminal Fluid	100 µL of 1:10 diluted semen on a pre-collected and dried menstrual blood swab	NTR
14	Cloth swatch No visible staining	51.953	Seminal Fluid Saliva	Saliva Seminal Fluid	100 µL of 100:1 mixture of saliva:semen on cloth	NTR
15	Cotton swab (1/2) Pale yellow staining	778.717	Seminal Fluid Vaginal Fluid	Vaginal Fluid Seminal Fluid Saliva	100 µL of mixture including 10 µL semen, 1 µL saliva, pipetted onto a previously collected and dried vaginal swab.	False Negative Missed Saliva
16	Cloth swatch No visible staining	1027.320	Seminal Fluid	Seminal Fluid	100 µL neat fluid per swab	NTR
17	Cotton swab (1/2) Red-brown staining	1215.418	Peripheral Blood Seminal Fluid Vaginal Fluid	Menstrual Blood	Single-source swab collected from donor, no associated volume	False Positive False Semen
18	Cloth swatch No visible staining	38.773	Saliva	Saliva	100 µL neat fluid per swab	NTR
19	Cotton swab (1/2) Red-brown staining	3941.220	Peripheral Blood Seminal Fluid Vaginal Fluid	Menstrual Blood Seminal Fluid	100 µL of 1:100 semen dilution on a previously collected and dried menstrual swab	NTR
20	Cotton swab (1/2) Pale yellow staining	331.883	Peripheral Blood Seminal Fluid Vaginal Fluid	Vaginal Fluid Seminal Fluid	100 µL of a 1:100 semen dilution on a previously collected and dried vaginal swab	NTR

Sample Number	Sample Description	Quant Value (µg/mL)	Experimental Fluid Identification	True Fluid Identification	Supplemental Info	Points of Discrepancy
21	Cotton swab (1/2) No visible staining	440.115	Seminal Fluid Vaginal Fluid	Vaginal Fluid Seminal Fluid	100 µL of a 1:10 semen dilution on a previously collected and dried vaginal swab	NTR
22	Cotton swab (1/2) No visible staining	110.124	Saliva Vaginal Fluid	Saliva	100 µL neat fluid per swab	False Positive: Incorrect Vaginal Fluid Identification
23	Cotton swab (1/2) No visible staining	221.366	ND	Urine	100 µL neat fluid per swab	NTR
24	Cotton swab (1/2) No visible staining	219.389	ND	Urine	100 µL neat fluid per swab	NTR
25	Cotton swab (1/2) No visible staining	386.845	Vaginal Fluid	Vaginal Fluid	Swab collected from donor, no associated volume	NTR
26	Condom	33.993	ND	ND	Sample on outside	NTR
		33.295	Seminal Fluid	Seminal Fluid Saliva	100 µL of a 1:1 mixture of semen and saliva on a lubricated condom	False Negative: Missed Saliva Identification
27	Cotton swab (1/2) Faint Red-brown staining	368.870	Peripheral Blood Vaginal Fluid	Rectal Swab	Swab collected from donor, no associated volume	False Positive: Incorrect Vaginal Fluid Identification
28	Cloth swatch Red-brown staining	4367.860	Peripheral Blood	Peripheral Blood	100 µL neat fluid per swab	NTR
29	Cotton swab (1/2) Pale yellow staining	398.438	Vaginal Fluid	Vaginal Fluid	Swab collected from donor, no associated volume	NTR
30	Cotton swab (1/2) No visible staining	267.377	Peripheral Blood Saliva	Nasal Secretions	100 µL neat fluid per swab	Analyst Note: Consistent with Nasal Secretions

Sample Number	Sample Description	Quant Value (µg/mL)	Experimental Fluid Identification	True Fluid Identification	Supplemental Info	Points of Discrepancy
31	Cotton swab (1/2) Pale yellow staining	578.119	Seminal Fluid Vaginal Fluid	Vaginal Fluid Seminal Fluid	100 µL of a 1:10 semen dilution on a previously collected and dried vaginal swab	NTR
32	Cotton swab (1/2) Yellow staining	1159.981	Peripheral Blood Seminal Fluid Vaginal Fluid	Vaginal Fluid Seminal Fluid	100 µL neat semen on a previously collected and dried vaginal swab.	NTR
33	Cotton swab (1/2) No visible staining	2997.140	Seminal Fluid Saliva	Seminal Fluid Saliva	100 µL of a 1:10 mixture of semen:saliva on a cotton swab	NTR
34	Condom	ND	ND	ND	Sample on outside	NTR
		4861.345	Seminal Fluid Saliva	Seminal Fluid Saliva	100 µL of a 1:10 mixture of saliva:semen on a lubricated condom	NTR
35	Cloth swatch No visible staining	564.706	Seminal Fluid	Seminal Fluid	100 µL neat fluid per swab	NTR
36	Cotton swab (1/2) Pale yellow staining	724.519	Saliva Vaginal Fluid	Vaginal Fluid Saliva	100 µL neat saliva on a previously collected and dried vaginal swab	NTR
37	Cotton swab (1/2) No visible staining	367.790	Vaginal Fluid	Vaginal Fluid Saliva	100 µL of a 1:100 saliva dilution on a previously collected and dried vaginal swab.	False Negative: Missed Saliva Identification
38	Cotton swab (1/2) Pale yellow staining	3511.905	Seminal Fluid Vaginal Fluid	Vaginal Fluid Seminal Fluid	100 µL neat semen on a previously collected and dried vaginal swab.	NTR
39	Cotton swab (1/2) No visible staining	524.051	ND	Urine	100 µL neat fluid per swab	NTR
40	Cotton swab (1/2) Yellow staining	577.667	Seminal Fluid Saliva Vaginal Fluid	Vaginal Fluid Saliva Seminal Fluid	100 µL containing 1 µL semen/ 10 µL saliva pipetted onto a previously collected and dried vaginal swab	NTR

Sample Number	Sample Description	Quant Value (µg/mL)	Experimental Fluid Identification	True Fluid Identification	Supplemental Info	Points of Discrepancy
41	Cotton swab (1/2) No visible staining	1473.296	Seminal Fluid	Seminal Fluid Saliva	100 µL of a 1:100 mixture of semen:saliva on a cotton swab	False Negative: Missed Saliva Identification
42	Cloth swatch No visible staining	ND	ND	ND	Blank	NTR
43	Condom	ND	ND	ND	Sample on outside	NTR
		340.854	Seminal Fluid Saliva	Seminal Fluid Saliva	100 µL of a 1:10 mixture of saliva:semen on a lubricated condom	NTR
44	Cotton swab (1/2) Red-brown staining, saturated	7414.495	Peripheral Blood Seminal Fluid	Menstrual Blood Seminal Fluid	100 µL neat semen on a previously collected and dried menstrual swab	False Negative: Missed Vaginal Fluid Identification
45	Cotton swab (1/2) Red-brown staining, saturated	10172.420	Peripheral Blood	Peripheral Blood	100 µL neat fluid per swab	NTR
46	Cotton swab (1/2) No visible staining	2301.795	Seminal Fluid	Seminal Fluid	100 µL neat fluid per swab	NTR
47	Condom	25.050	ND	ND	Sample on outside	NTR
		1314.222	Seminal Fluid Saliva	Saliva Seminal Fluid	100 µL of a 1:1 mixture of semen and saliva on a non-lubricated condom	NTR
48	Cloth swatch No visible staining	ND	Peripheral Blood	Peripheral Blood	100 µL on cloth, laundered (w/ bleach)	NTR
49	Cotton swab (1/2) No visible staining	843.944	Undetermined	Breast Milk	100 µL neat fluid per swab	NTR
50	Cotton swab (1/2) No visible staining	50.705	ND	Sweat	100 µL neat fluid per swab	NTR

APPENDIX I: INTERPRETATION GUIDELINES

INTERPRETATION GUIDELINES

Introduction:

The interpretation of data from LC-MS/MS analyses is a matter of professional training and expertise. The following objective criteria are to be used by analysts to guide most routine data interpretation scenarios. Not every situation, however, may be fully covered by these interpretation guidelines. The treatment of samples that appear to fall outside of these guidelines should be addressed through discussion with the Technical Reviewer in order to reach agreement on a reportable opinion. In the event that agreement on a reportable opinion cannot be reached, the laboratory's Technical Leader should be consulted to issue a final decision on a reportable opinion or other course of action. These interpretation guidelines are based upon validation studies, the peer-reviewed scientific literature, and professional training and expertise. These interpretation guidelines establish a solid framework of quality criteria to ensure that:

- Conclusions in the casework report are scientifically supported by the analytical data, including that obtained from appropriate standards and controls;
- Interpretations are made objectively; and
- Interpretations are consistent and accurate from analyst to analyst and case to case.

Evaluation of Controls:

Internal Positive Control: This control (*e.g.*, bovine myelin basic protein) serves to demonstrate that the sample digest (including digestion, denaturation, reduction and alkylation) performed successfully for each sample in the batch. The internal positive control should be evaluated for each sample to determine if it meets the laboratory's established quality criteria. Specifically, both the natural targeted peptide peak as well as the heavy labeled peak should be identified based on the appearance of two MRM transitions per peptide. Additionally, peaks should fall within two peak widths of the acceptable retention times outlined in the table below (± 0.2 min). The area ratio of the heavy labeled peptide to the natural peptide should be 15.0 ± 30%. Area ratios falling outside

of this range may indicate the presence of digestion inhibition. Notes: As the required heavy labelled peptide is not currently commercially available, it should be custom synthesized. In addition, the purity of commercially available myelin basic protein can vary. As such, the area ratio of the heavy labeled peptide to the natural peptide may fluctuate between reagents lots and should be monitored by the analyst. However, within a single preparation of these standards, the ratio will remain stable.

1. If there appears to be an injection or other chromatographic problem, the sample should be re-injected.
2. If the internal positive control fails to generate a peak for the natural/light peptide, or if the ratio falls to meet the response criteria indicated above, the sample should be considered for re-extraction.

Protein	Peptide Sequence	MRM transitions	Retention time (min)
Myelin	DTGILDSLGR (Light)	523.7 → 660.3, 830.4	5.5
	DTGILDSLGR (Heavy)	528.7 → 670.3, 840.4	5.5

Reagent (Negative) Control: This control (*e.g.*, a sample processed in parallel with the casework samples of a batch but to which no protein source material was added) serves to demonstrate that the protein extraction and processing reagents do not contain targeted protein. The negative reagent control should be evaluated and meet the laboratory's quality criteria. Specifically, the negative reagent control should be free of detectible target protein upon analysis. The occurrence of more than one targeted peptide peak should be considered an indication of protein contamination. Such findings should be considered necessary but not sufficient for failing the negative reagent control. The official designation of a failure of the negative reagent control should be reviewed and documented by the laboratory's supervisor or Technical Leader.

1. If protein contamination is observed in a negative reagent control, acknowledgment of the contaminant and subsequent actions should be documented in the case file. In addition, the analyst should endeavor to determine the point at which the contamination was introduced, and the scope of the samples affected by the contamination.

2. If the contaminating source affects all samples in the extraction set, the analysis should be redone from the point at which the contamination was introduced.
3. If it is unclear at what point the contamination was introduced, the analysis should be repeated from the protein sample preparation step forward.
4. If additional negative reagent controls were prepared with the batch and these show no sign of contamination AND the associated samples show no sign of contamination, the incident may be considered tube-specific. The data already derived from these samples can be used for fluid identification purposes.

Additional Considerations:

Carryover – Carryover was observed for Semenogelin-1 and Hemoglobin during the validation studies of the analytical method. Accordingly, any elevated signal from these proteins should be evaluated in subsequent injections. A Blank (*i.e.*, neat methanol) can be run after every sample in order to wash the column and prevent sample carryover.

Peak Designation and Peptide Identification

Proteins are composed of a sequence of amino acids arranged in a linear order. This allows for the prediction, to a given degree of confidence, of the fragmentation pattern and MS/MS spectra that will be produced. To enhance the specificity of the method, up to three MRM transitions for each peptide are employed. Detectable peptide peaks are those that meet the following criteria:

- The peak has a signal to noise ratio greater than 3
- The peak height is greater than 1,000 counts
- The peak for the peptide should fall within two peak widths (± 0.2 min) from the target retention time
- The response ratio for the qualifier ions should be within ± 20% of the target. Note: extreme low or high signal intensity can result in a deviation from expected ion ratios.

Body Fluid Identification and Reporting Language

Confirmatory Identification: The presence of at least one confirmatory protein (see Body Fluid Specificity Table below) for a body fluid of interest provides a confirmatory indication of the

presence of the corresponding targeted biological fluid. This will be reported as “A confirmatory identification of (blood/saliva/semen) was obtained for item…”

Presumptive Detection: The presence of at least one presumptive protein (see Specificity Table below) for a body fluid of interest provides a presumptive indication of the presence of the corresponding targeted biological fluid. This will be reported as “A presumptive indication of (vaginal fluid) was obtained for item…”

Not Detected: In all cases, a failure to detect a minimum of one targeted peptide for any body fluid represents a negative result. This will be reported as “No targeted biological fluids were detected”.

Statements Regarding Human Specificity: Within the context of a confirmatory result, reporting of the result as human specific requires the detection of a peptide target unique to humans (see Species Specificity Table below). This will be reported as “the confirmatory identification of human (blood/vaginal fluid/saliva/semen) was obtained”

Body Fluid Specificity

Fluid	Protein	Confirmatory	Presumptive	Non-Specific	Notes:
Peripheral Blood	P02647|APOA1	Y			
	P68871|HBB	Y			Trace levels in some saliva/seminal fluid (<1,000 cts.)
	P02790|HEMO	Y			
	P01009|A1AT	Y			Trace levels in some saliva/seminal fluid (<2,000 cts.)
	P02787|TRFE		Y		Detectable in seminal fluid
Saliva	P02808 |STAT	Y			
	P02814|SMR3B	Y			
	P04745|AMY1		Y		Trace levels in some vaginal fluid (<1,000 cts.)
	P09228|CYTT	Y			
Vaginal Fluid	O95274|LYPD3		Y		Consistent trace levels in saliva (<1,000 cts.)
	P80188|NGAL		Y		Consistent trace levels in saliva (<1,000 cts.)
	O60437|PEPL		Y		
	P07476|INVO		Y		Consistent trace levels in saliva (<2,000 cts.)
	Q9UBG3|CRNN		Y		Consistent trace levels in saliva (<1,000 cts.)
	Q6UWP8|SBSN			Y	Detectable in saliva
	Q9UBC9|SPRR3			Y	Detectable in saliva
Seminal Fluid	P15309|PPAP		Y		Detectable in vaginal fluid
	P07288|KLK3		Y		Consistent Trace levels in vaginal fluid
	Q02383|SEMG2	Y			
	P04279|SEMG1	Y			
	P61916|NPC2		Y		Consistent Trace levels in vaginal fluid (<1,000 cts.)

Human/Species Specificity

Fluid	Protein	Peptide	Human Specific	Species Cross Reactivity
Peripheral Blood	P02647|APOA1	LLDNWDSVTSTFSK	N	Chimpanzee
	P02647|APOA1	VSFLSALEEYTK	N	Primates
	P68871|HBB	LLVVYPWTQR	N	Primates / mammals
	P68871|HBB	SAVTALWGK	N	Primates / mammals
	P02790|HEMO	NFPSPVDAAFR	N	Orangutan
	P01009|A1AT	SVLGQLGITK	Y	
	P01009|A1AT	LSITGTYDLK	N	Orangutan
	P02787|TRFE	SASDLTWDNLK	N	Chimpanzee
	P02787|TRFE	DGAGDVAFVK	N	Primates
Saliva	P02808 |STAT	FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF	Y	
	P02814|SMR3B	GPYPPGPLAPPQPFGPGFVPPPPPPPYGPG R	Y	
	P02814|SMR3B	IPPPPPAPYGPGIFPPPPPQP	Y	
	P04745|AMY1	IAEYMNHLIDIGVAGFR	Y	
	P04745|AMY1	LSGLLDLALGK	Y	
	P09228|CYTT	ALHFVISEYNK	Y	
	P09228|CYTT	IIEGGIYDADLNDER	Y	
Seminal Fluid	P15309|PPAP	ELSELSLLSLYGIHK	N	Mouse
	P15309|PPAP	FVTLVFR	N	Mouse
	P15309|PPAP	FQELESETLK	Y	
	P07288|KLK3	FLRPGDDSSHDLMLLR	Y	
	P07288|KLK3	LSEPAELTDAVK	Y	
	Q02383|SEMG2	GSISIQTEEK	N	Primates
	Q02383|SEMG2	DVSQSSISFQIEK	Y	
	Q02383|SEMG2	GSISIQTEEQIHGK	N	Primates
	P04279|SEMG1	QITIPSQEQEHSQK	Y	
	P04279|SEMG1	DIFSTQDELLVYNK	Y	
	P61916|NPC2	DC[+57.0]GSVDGVIK	Y	
	P61916|NPC2	SGINC[+57.0]PIQK	N	Mammals

Fluid	Protein	Peptide	Human Specific	Species Cross Reactivity
Vaginal Fluid	O95274|LYPD3	GC[+57.0]VQDEFC[+57.0]TR	Y	
	O95274|LYPD3	GLDLHGLLAFIQLQQC[+57.0]AQDR	Y	
	P80188|NGAL	WYVVGLAGNAILR	Y	
	P80188|NGAL	MYATIYELK	Y	
	O60437|PEPL	NQGPQESVVR	Y	
	O60437|PEPL	NLLDEIASR	Y	
	O60437|PEPL	AQSLQSAK	Y	
	P07476|INVO	GEVLLPVEHQQQK	Y	
	P07476|INVO	QEAQLELPEQQVGQPK	N	Chimpanzee
	P07476|INVO	HLVQQEGQLEQQER	Y	
	Q9UBG3|CRNN	LLDEDHTGTVEFK	Y	
	Q9UBG3|CRNN	ISPQIQLSGQTEQTQK	Y	
	Q9UBG3|CRNN	AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR	Y	
	Q6UWP8|SBSN	ALDGINSGITHAGR	Y	
	Q6UWP8|SBSN	FGQGVHHGLSEGWK	Y	
	Q9UBC9|SPRR3	VPVPGYTK	Y	
	Q9UBC9|SPRR3	VPEPGC[+57.0]TK	Y	

*Data Searched using NCBI Algorithm PHI-BLAST (Pattern Hit Initiated BLAST), Animalia (taxid: 33208), Non-redundant UniProt KB/SwissProt sequences database, Molecule Type: Protein Update date: 2019/03/26, Number of sequences: 471513.

NOTES:

Body fluid identification is confirmed through the mass spectral identification of multiple protein markers. Those protein markers, in turn, are confirmed through the detection of multiple tryptic peptides per protein. Below is a brief description of the biological function each targeted protein.

Peripheral Blood is identified through the detection of α-1-antitrypsin, hemopexin, apolipoprotein A1, Serotransferrin, and hemoglobin subunit beta. α-1-antitrypsin is a non-specific serine protease inhibitor found in human plasma. This protein's primary role is as an inhibitor of neutrophil elastase which protects tissues from proteolytic damage (Kolarich et al.; Parfrey et al.). Hemopexin is produced in the liver and found in plasma. This protein is responsible for trapping free heme in

plasma as well as iron recycling in the liver (Ascenzi et al.; Liebert). Apolipoprotein A1 is a component of HDL particles and is involved with the transport of cholesterol from tissues (Breslow et al.). Serotransferrin is an iron binding protein responsible for the transport of iron from sites of absorption and heme degradation to sites of storage and utilization (Aisen et al.; Yang et al.). Hemoglobin subunit beta - The metalloprotein hemoglobin is responsible for oxygen transport and is the major protein contained within erythrocytes. Hemoglobin exists as a tetramer containing two beta chains and two alpha chains (Berg et al.).

Vaginal Fluid is identified through the detection of neutrophil gelatinase-associated lipocalin, cornulin, ly6/PLAUR domain-containing protein 3, periplakin, involucrin, and suprabasin. Neutrophil gelatinase-associated lipocalin belongs to the lipocalin family of transport proteins which have been associated with innate immunity though iron sequestration (Goetz et al.). As such, this protein can be found in tissues prone to exposure to bacterial and other microorganisms including the respiratory tract, salivary glands, uterus, and prostate (Goetz et al.; Cowland and Borregaard). Cornulin is also expressed in squamous cells where it plays a role in epithelial cell differentiation. It may also play a role in mucosal-epithelial immune response. The protein has been characterized in the cervix and in esophageal tissues (Contzler et al.; Arnouk et al.). Ly6/PLAUR domain-containing protein 3 is involved in the regulation between extracellular structural support scaffolding and epithelial cell layers (Smith et al.). Suprabasin is expressed in keratinocytes and plays a role in epidermal differentiation. It has been reported to be expressed in the uterus as well as the esophagus (Park et al.). Involucrin is a component of cornified cell envelope (CE) of stratified squamous epithelia and is involved with membrane protein cross-linking (Djian et al.). Periplakin is a component of the cornified envelope of keratinocytes. May link the cornified cell envelope to desmosomes and intermediate filaments (Ruhrberg et al.).

Saliva is confirmed through the detection of statherin, submaxillary gland androgen-regulated protein 3B, Cystatin SA, and alpha amylase. Statherin, as well as submaxillary gland androgen-regulated protein 3B, assist in inhibiting potentially harmful calcium phosphate precipitation in saliva (Schlesinger and Hay; Hay et al.). Alpha amylase is the most abundant protein found in saliva

where it digests starches into glucose and maltose (Ramasubbu et al.). While highly abundant in saliva, this protein can be found in a number of alternate body fluids including vaginal fluid, breast milk, fecal matter, urine, blood and semen. Cystatin SA is a cysteine protease inhibitor located in the cystatin locus. It is found at high levels in saliva and tears.

Seminal Fluid identification is based off the detection of Epididymal secretory protein E1, prostatic acid phosphatase, semenogelin-I/II, and prostate-specific antigen. Epididymal secretory protein E1 is involved with intracellular cholesterol transporters. It acts in concert with NPC1 and plays an important role in the egress of cholesterol from the lysosomal compartment. Prostatic Acid Phosphatase (also known as Seminal Acid Phosphatase or SAP) is a glycoprotein secreted by the epithelial cells of the prostate gland. It is capable of hydrolyzing phosphate groups from substrate molecules (Yam). SAP is a seminal fluid protein which has seen utility as a clinical marker for prostate cancer (Taira et al.). While largely replaced by PSA/p30 for screening purposes, the combination of low expression in non-target tissues and assay detection limits makes this protein useful as a potential marker of seminal fluid. Semenogelin-I/II are the most abundant proteins in seminal plasma and are responsible for the gel-like matrix of human semen. Both isoforms act as substrates for prostate specific antigen (p30), where upon lysis, sperm are able to move freely through the seminal matrix (Kise et al.; Malm and Hellman). Prostate-Specific Antigen (also known as PSA or p30) is a serine protease produced by epithelial cells located in the prostate (Ward et al.). The primary function of prostate-specific antigen is to cleave semenogelin-I/II thus creating a soluble, liquid medium, for spermatozoa movement (Ward et al.). Prostate-specific antigen has been well studied as an indicator for prostate cancer when serum levels reach approximately 4-10 ng/mL (Basch et al.). While this protein is not absolutely seminal fluid-specific, the detection limits of most assays make it difficult to detect it in whole blood (Keshishian et al.). As a result, this protein may have utility, in combination with other biomarkers, for the detection of seminal fluid.

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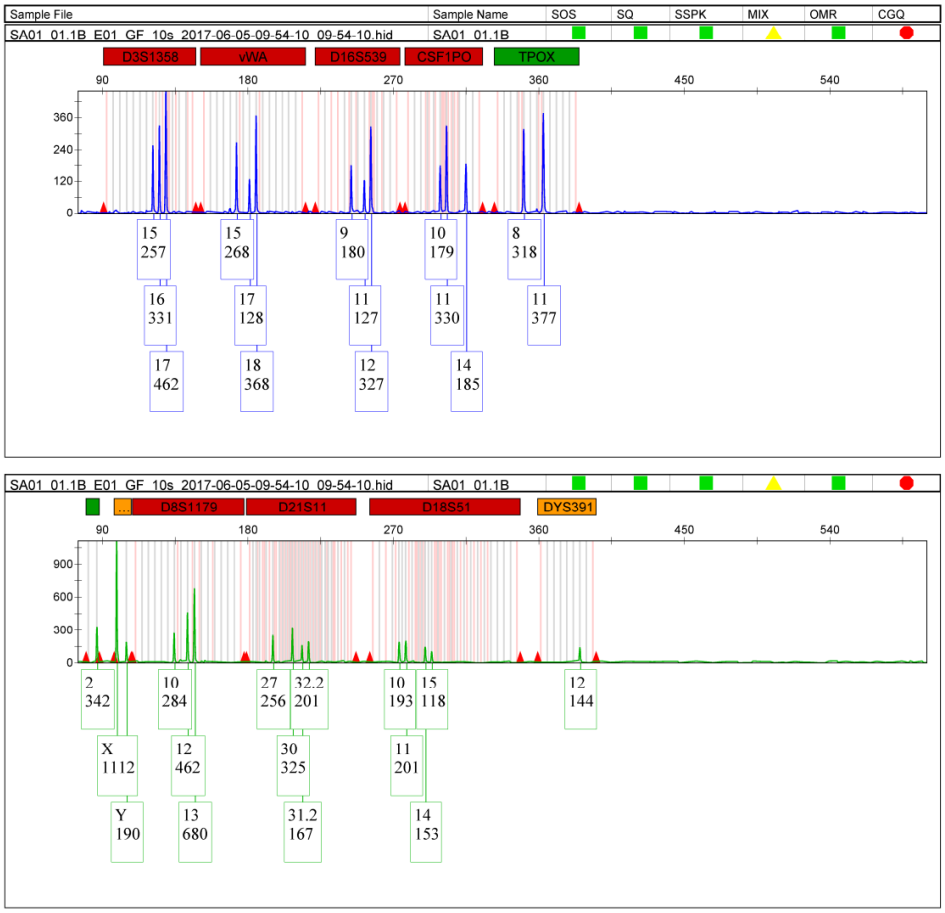
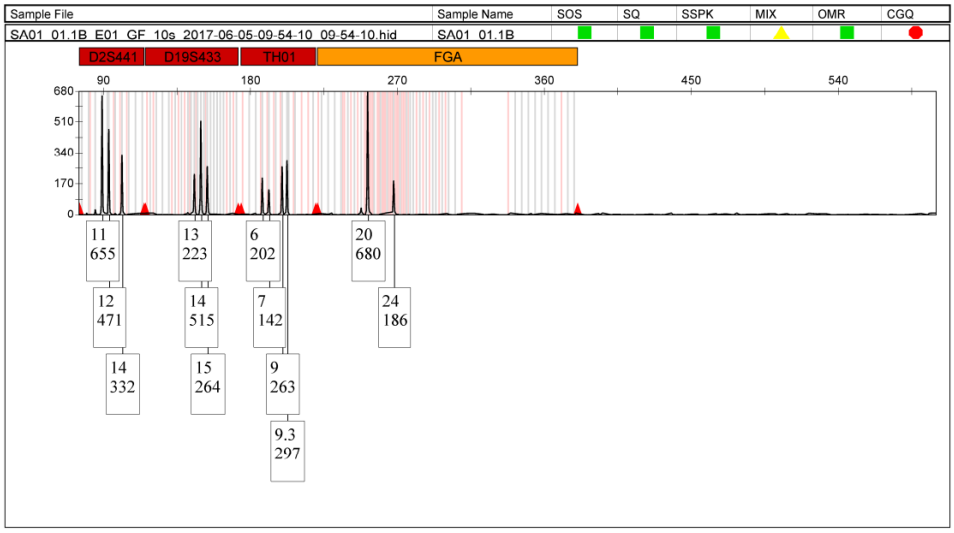
APPENDIX J: GENETIC PROFILES OF CASEWORK SAMPLES

Sample ID: SA01_01.1 Vaginal swab with semen from a non-vasectomized donor (sperm fraction)

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D2S441
D19S433
TH01
FGA
90
180
270
360
450
540
680
510
340
170
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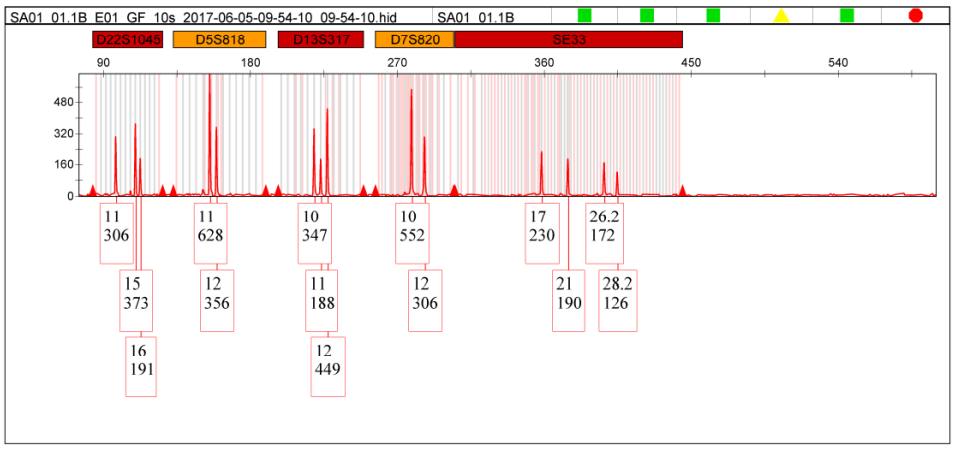
12 471
14 332
13 223
14 515
15 264
6 202
7 142
9 263
9.3 297
20 680
24 186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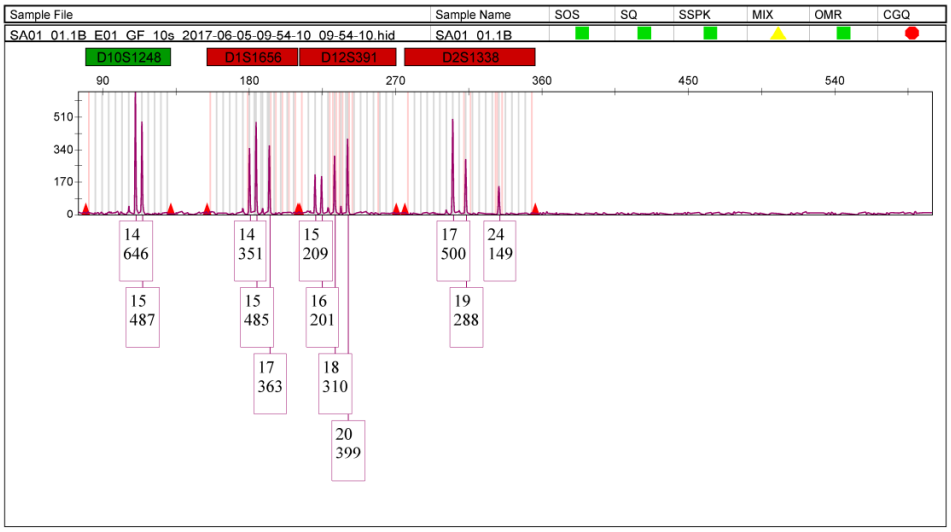
D22S1045
D5S818
D13S317
D7S820
SE33
90
180
270
360
450
540
480
320
160
0
11 306
15 373
16 191
11 628
12 356
10 347
11 188
12 449
10 552
12 306
17 230
21 190
26.2 172
28.2 126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D1S1656
D12S391
D2S1338
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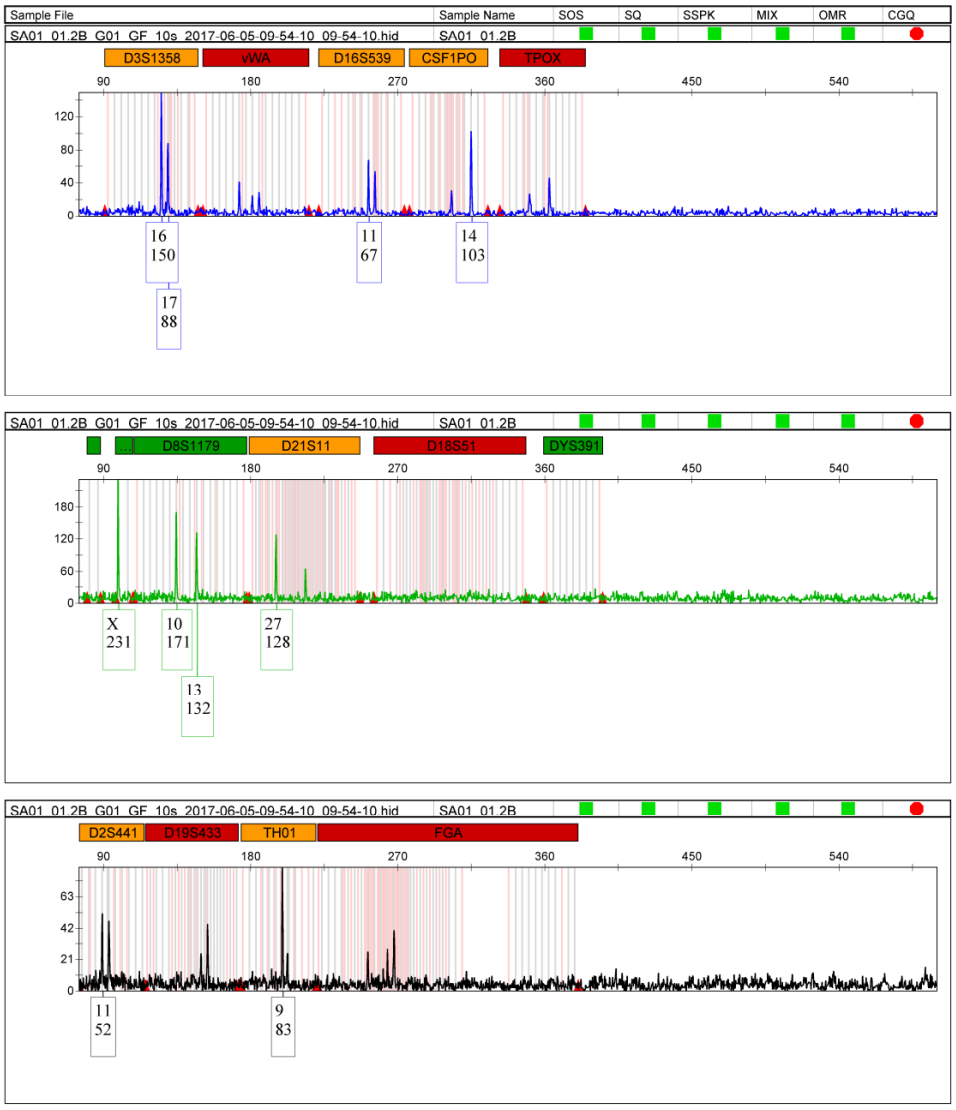
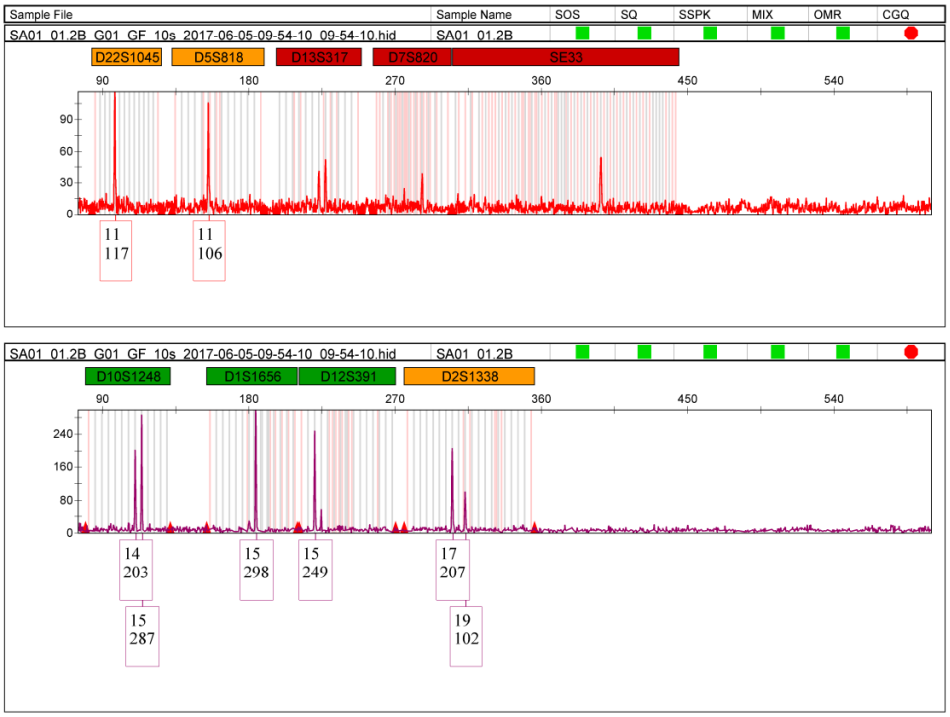
180
270
360
450
540
510
340
170
0
14 646
15 487
14 351
15 485
17 363
15 209
16 201
18 310
20 399
17 500
19 288
24 149

Sample ID: SA01_01.2 Vaginal swab with semen from a non-vasectomized donor (sperm fraction)

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D5S818
D13S317
D7S820
SE33
90
180
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360
450
540
90
60
30
0
11
117
11
106
SA01_01.2B_G01_GF_10s_2017-06-05-09-54-10_09-54-10.hid
SA01_01.2B
D10S1248
D1S1656
D12S391
D2S1338
90
180
270
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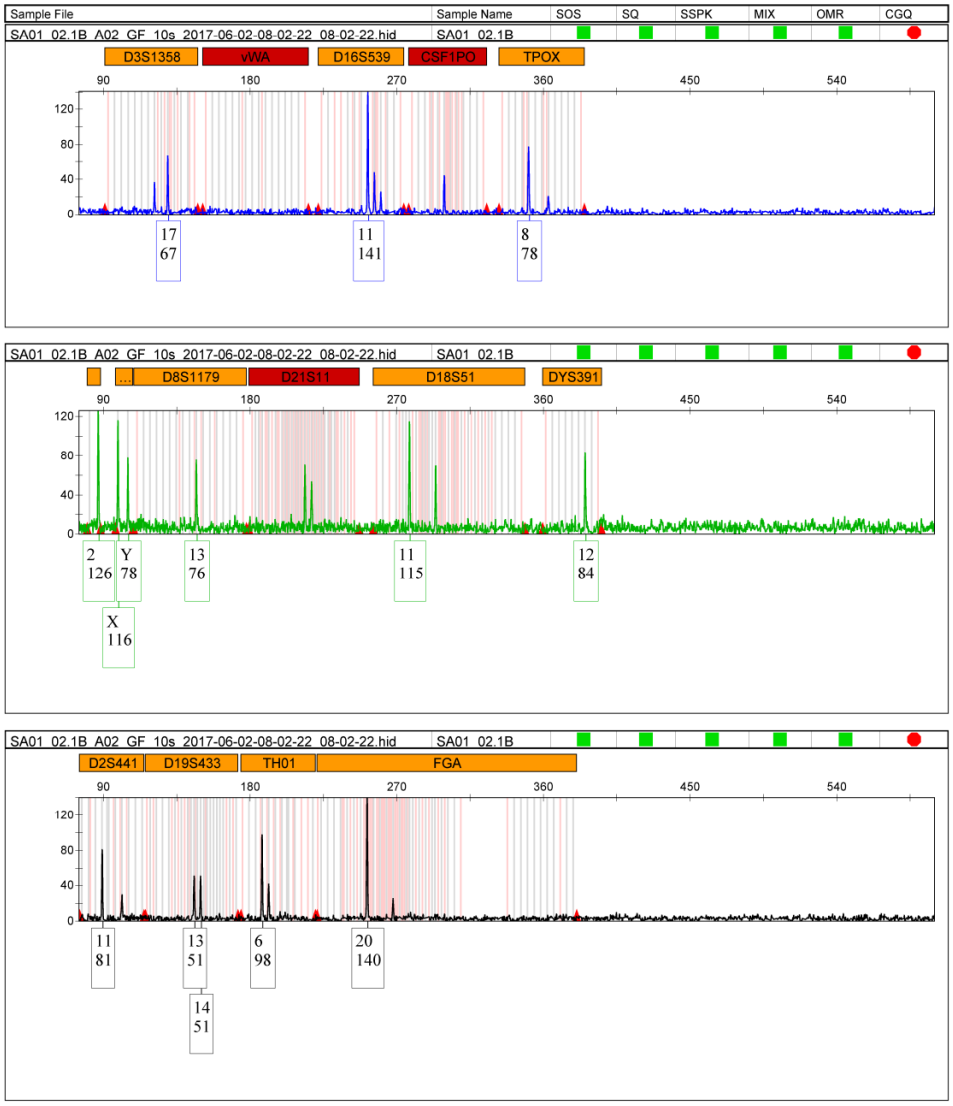
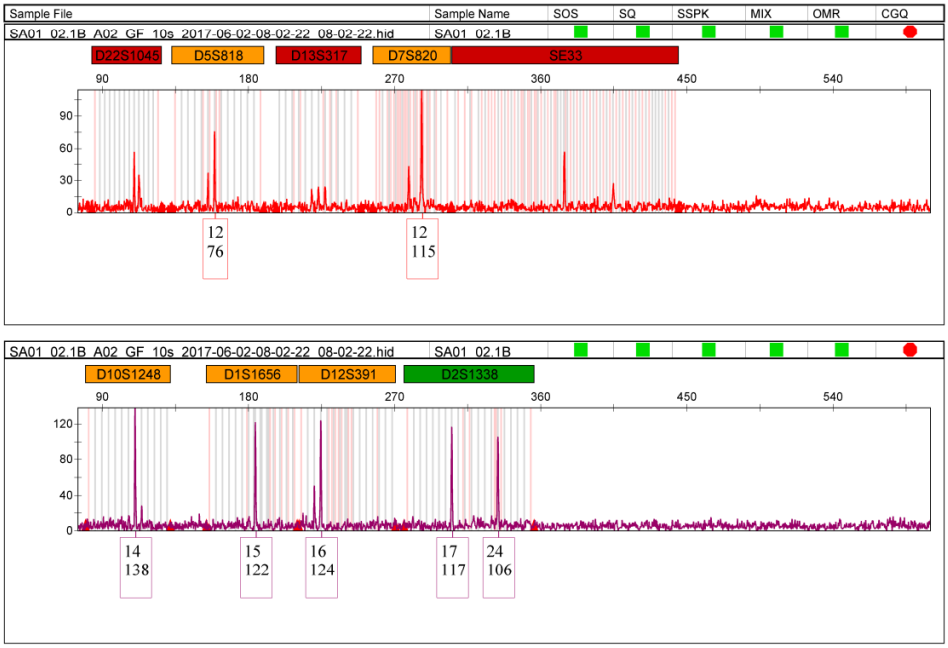
240
160
80
0
14
203
15
298
15
249
17
207
15
287
19
102

Sample ID: SA01_02.1 Oral swab with semen from a non-vasectomized donor (sperm fraction)

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D7S820
SE33
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450
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30
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12
76
12
115
SA01_02.1B_A02_GF_10s_2017-06-02-08-02-22_08-02-22.hid
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D12S391
D2S1338
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180
270
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540
120
80
40
0
14
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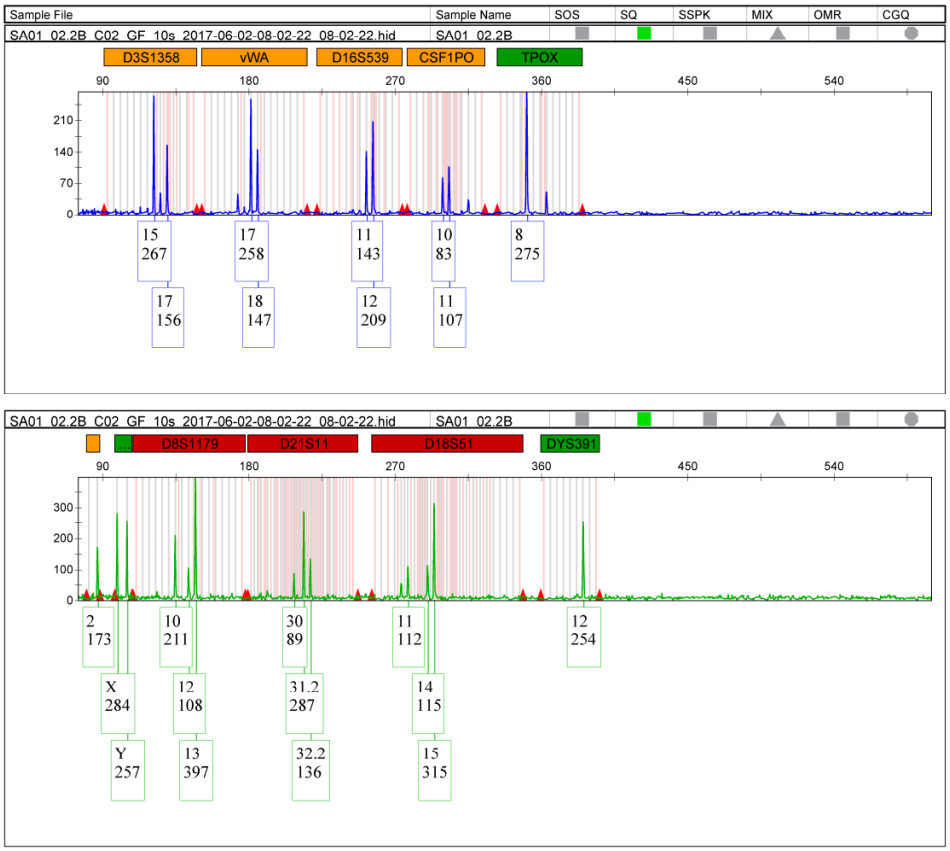
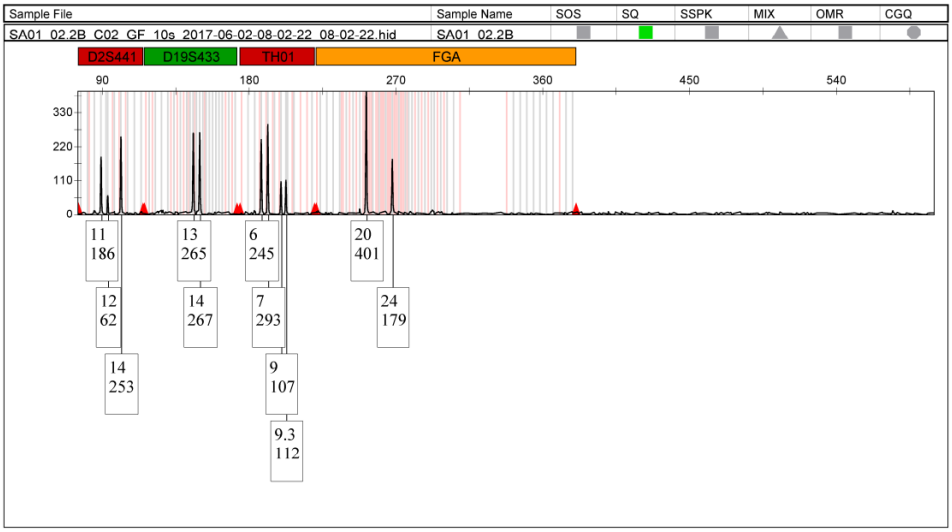
16
124
17
117
24
106

Sample ID: SA01_02.2 Oral swab with semen from a non-vasectomized donor (sperm fraction)

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D2S441
D19S433
TH01
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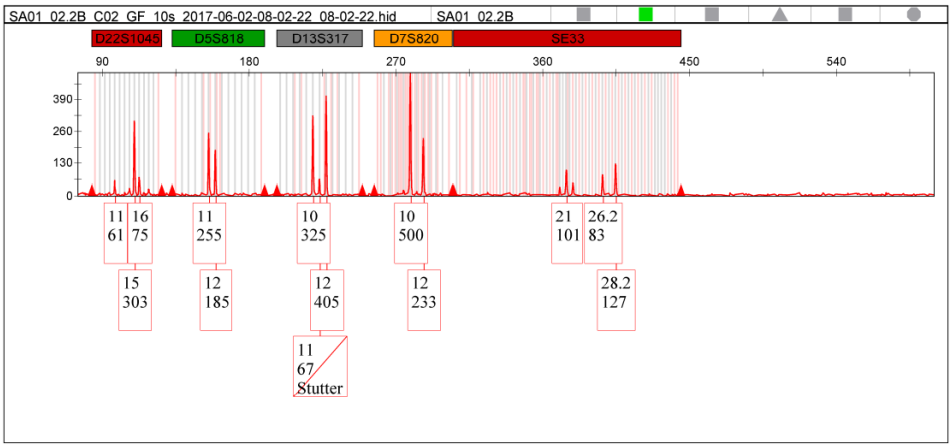
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D13S317
D7S820
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180
270
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130
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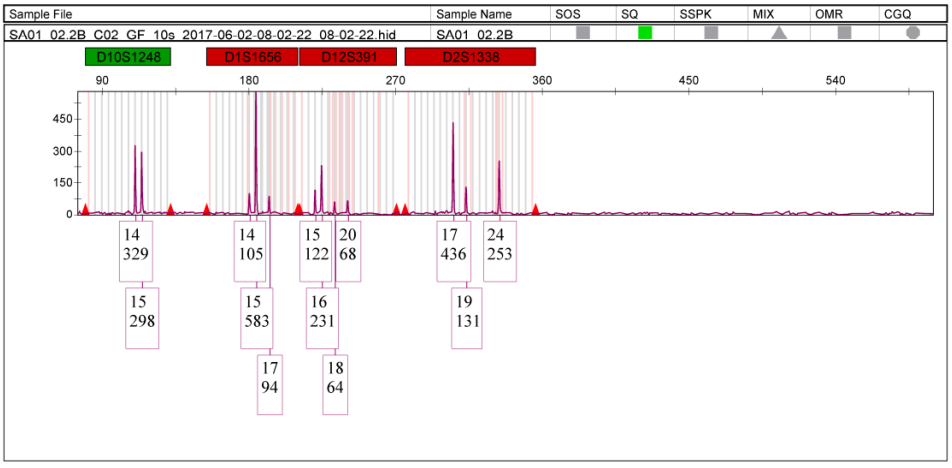
28.2 127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150
0
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15 298
14 105
15 583
17 94
15 122
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17 436
19 131
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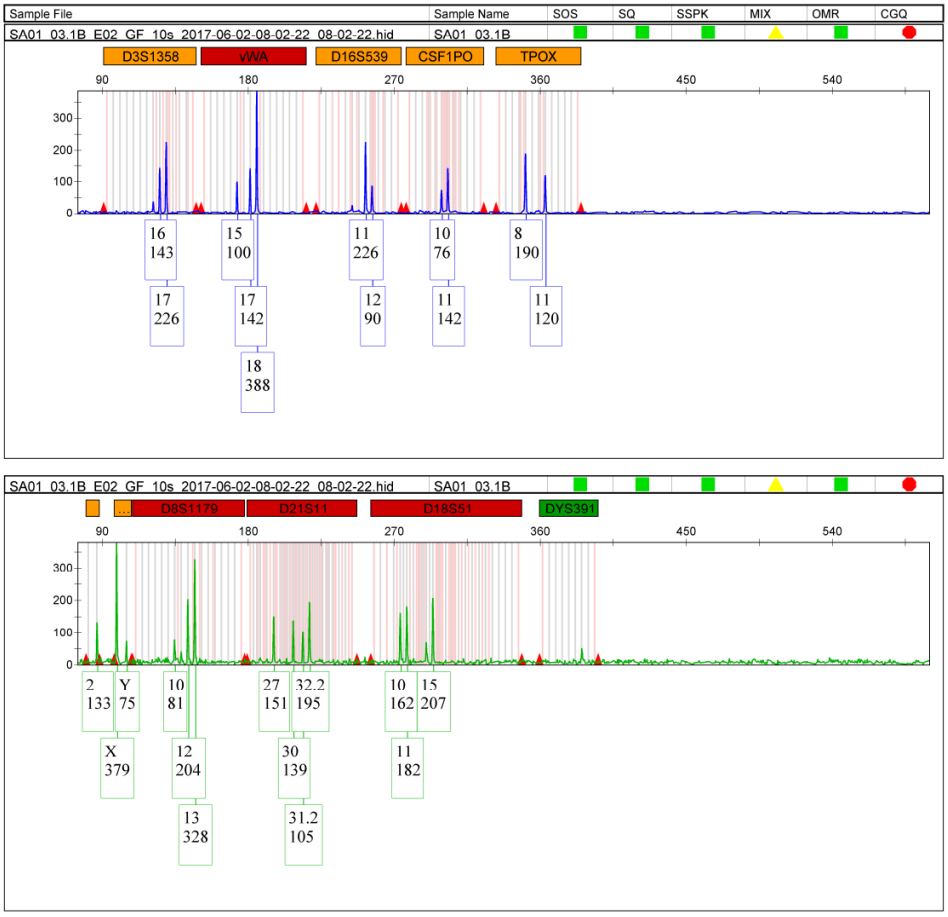
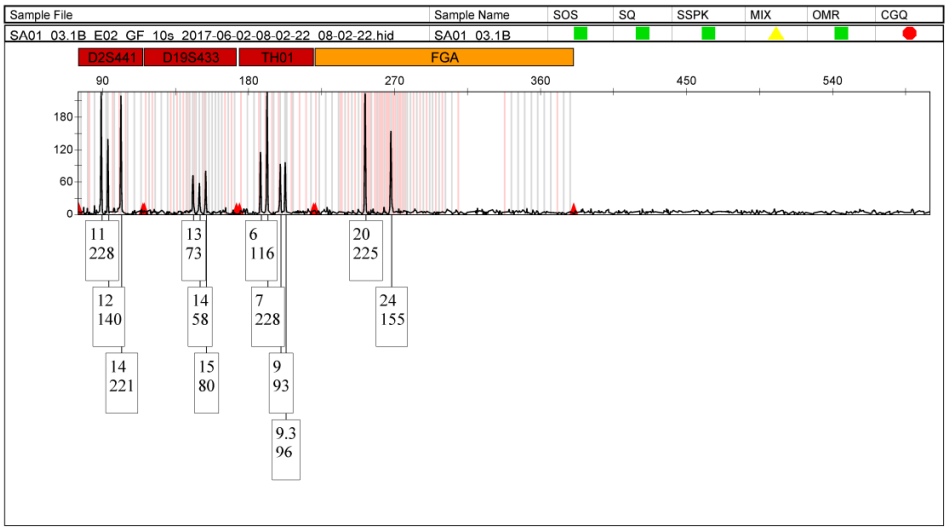
Sample ID: SA01_03.1 Rectal swab with semen from a non-vasectomized donor (sperm fraction)

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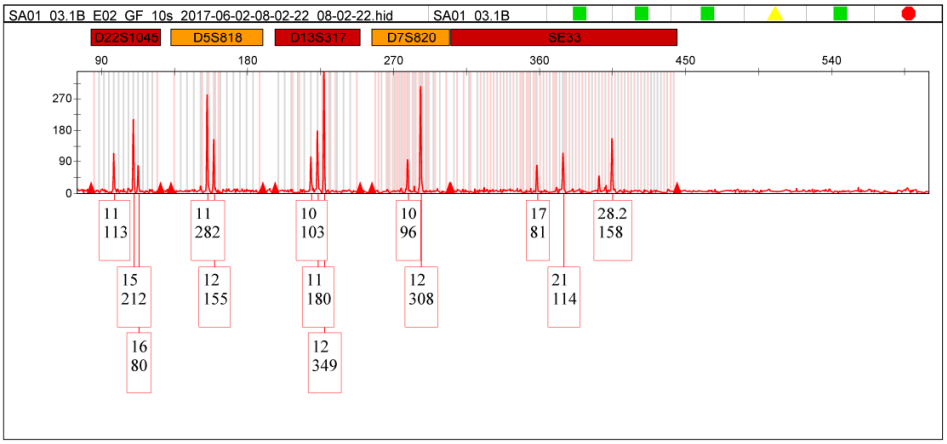
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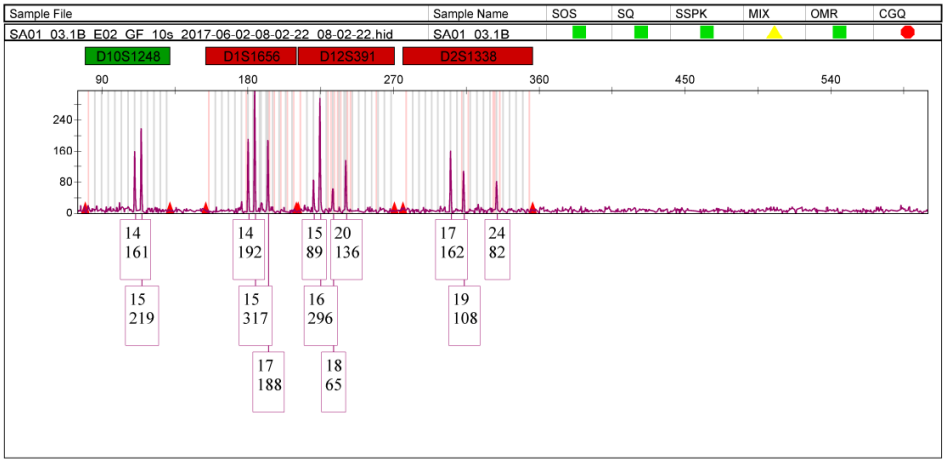
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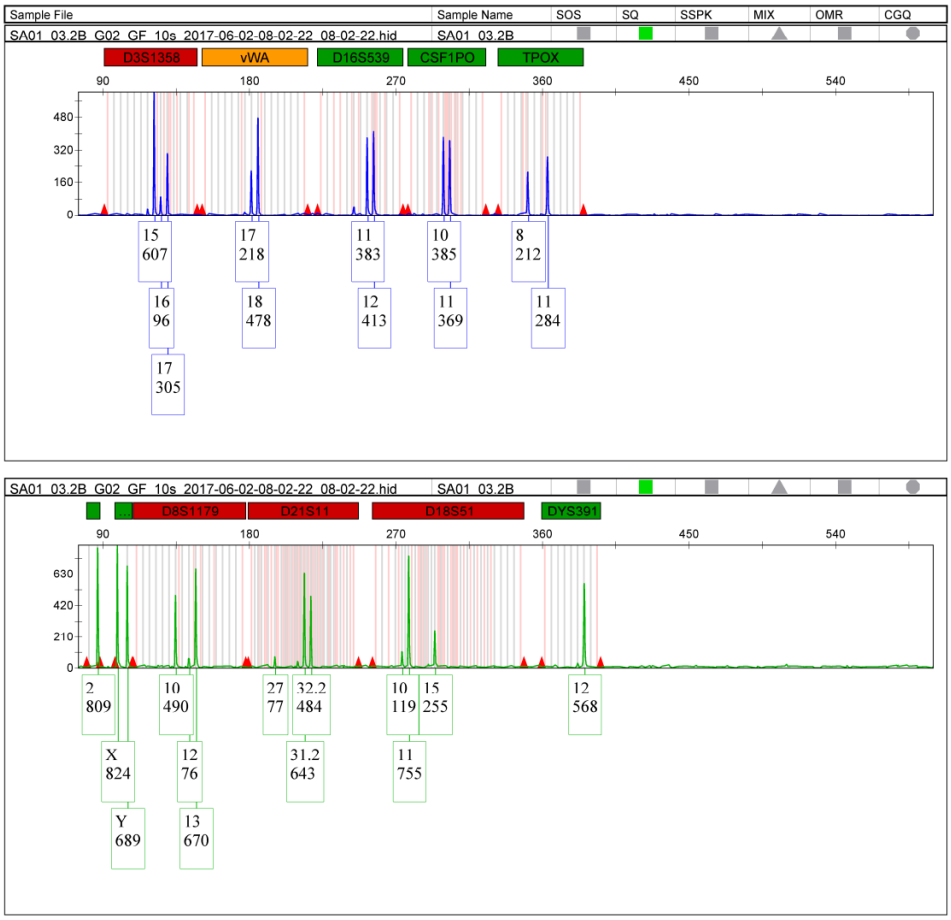
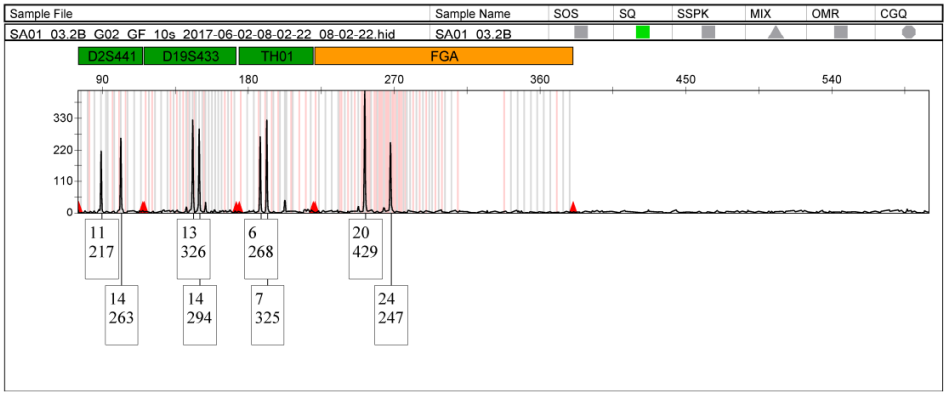
24 82

Sample ID: SA01_03.2 Rectal swab with semen from a non-vasectomized donor (sperm fraction)

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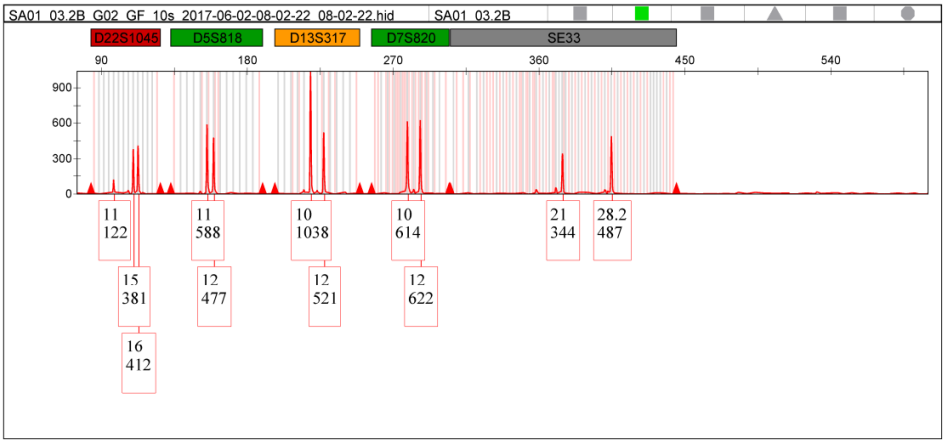
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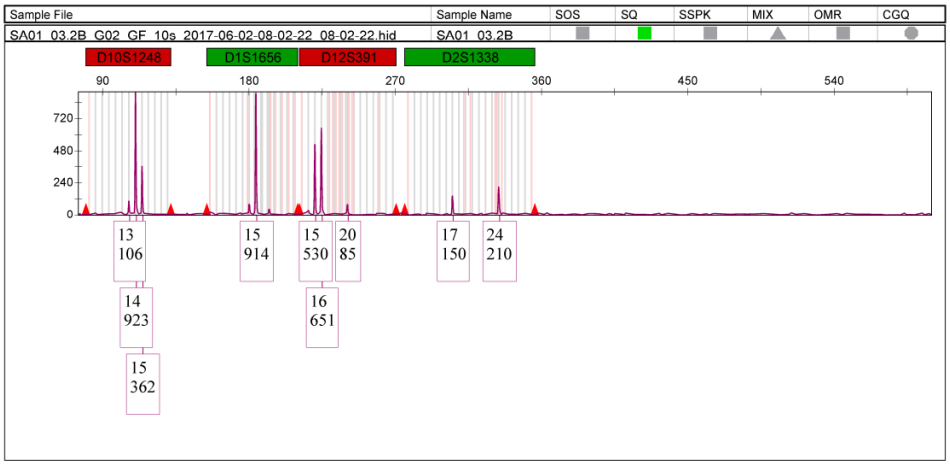
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923
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362
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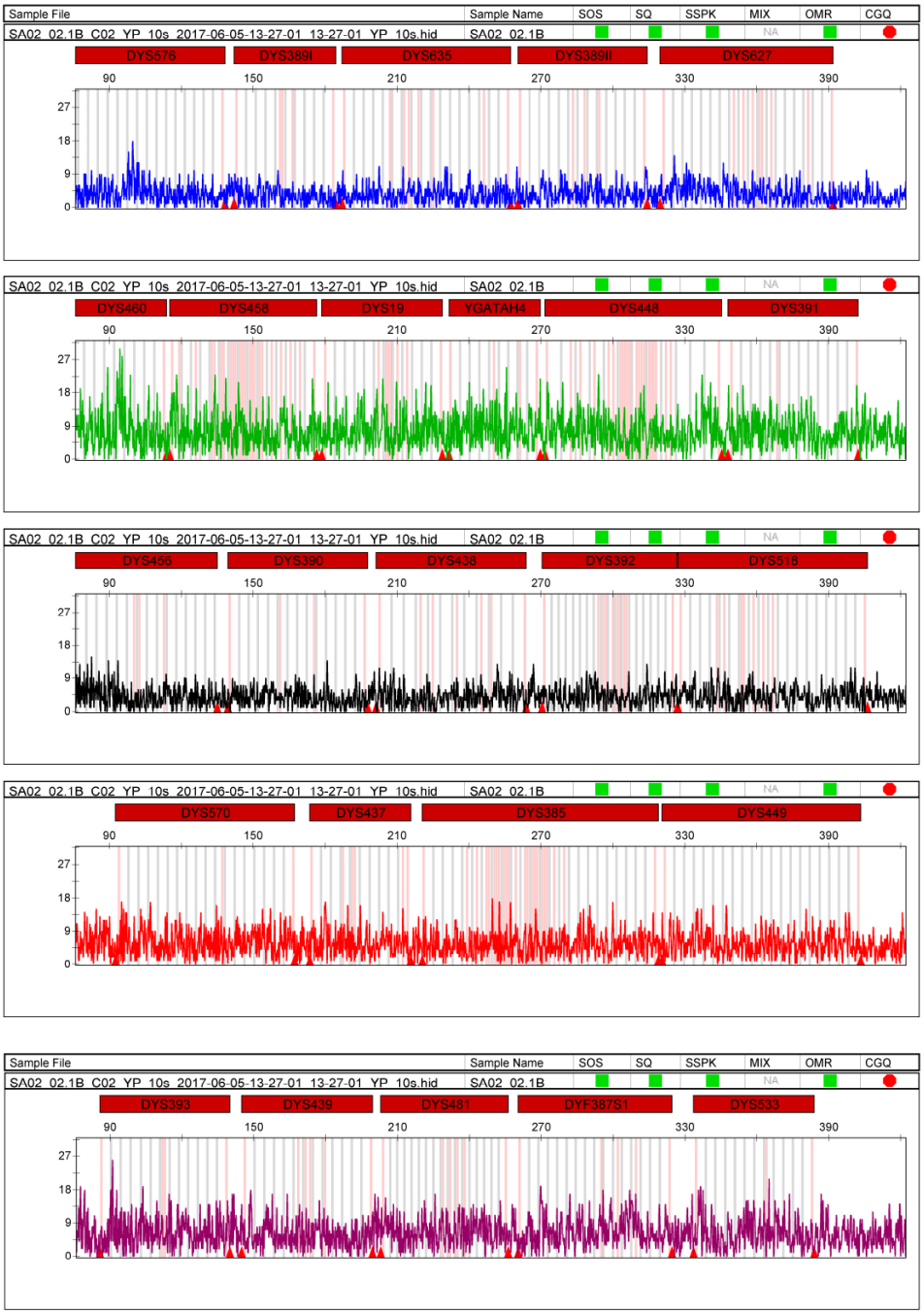
651
20
85
17
150
24
210

Sample ID: SA02_02.1 Oral swab with semen from a vasectomized donor (sperm fraction)



APPENDIX K: PROTEIN IDENTIFICATION OF SEMEN POPULATION SAMPLES

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
06C6979	SEMG1	85.71	383	4.30E+09	1.68E+09	1141.90	801.85
	SEMG2	78.69	301	2.39E+09		1350.90	
	KLK3	30.65	8	9.74E+06		296.30	
	PPAP	20.98	9	3.45E+07		418.30	
06C7294	SEMG1	90.04	399	5.05E+09	2.08E+09	1087.90	811.03
	SEMG2	80.58	295	3.24E+09		1404.20	
	KLK3	34.87	7	7.17E+06		330.50	
	PPAP	16.58	6	1.64E+07		421.50	
06C7352	SEMG1	78.57	376	2.86E+09	1.30E+09	1146.30	912.28
	SEMG2	75.94	290	2.04E+09		1321.70	
	KLK3	40.61	22	1.35E+08		482.70	
	PPAP	48.45	64	1.82E+08		698.40	
06C9228	SEMG1	86.80	425	4.00E+09	1.78E+09	1167.50	880.45
	SEMG2	81.27	322	3.00E+09		1366.40	
	KLK3	40.61	19	6.87E+07		542.20	
	PPAP	36.27	18	6.41E+07		445.70	
06C9237	SEMG1	71.43	465	3.66E+09	1.32E+09	1102.90	860.20
	SEMG2	69.42	255	1.57E+09		1301.00	
	KLK3	39.08	15	2.12E+07		496.90	
	PPAP	28.24	24	2.59E+07		540.00	
07C3133	SEMG1	86.36	346	2.92E+09	1.29E+09	1125.00	868.58
	SEMG2	74.57	222	2.14E+09		1368.50	
	KLK3	40.61	16	2.87E+07		484.00	
	PPAP	29.27	17	7.84E+07		496.80	
07C3212	SEMG1	87.01	366	2.60E+09	1.17E+09	1075.90	804.13
	SEMG2	79.21	304	2.06E+09		1364.20	
	KLK3	27.97	6	3.78E+06		445.10	
	PPAP	12.18	3	3.21E+06		331.30	
08C3217	SEMG1	90.26	358	3.48E+09	1.41E+09	1113.70	831.98
	SEMG2	76.46	246	2.12E+09		1373.50	
	KLK3	34.87	9	1.44E+07		456.70	
	PPAP	32.12	10	1.42E+07		384.00	
09C2531	SEMG1	88.31	245	1.65E+09	6.79E+08	1276.10	851.13
	SEMG2	71.65	153	1.05E+09		1236.90	
	KLK3	19.54	4	2.48E+06		478.80	
	PPAP	22.28	8	9.01E+06		412.70	
17_08_505	SEMG1	83.12	245	7.41E+08	3.46E+08	1016.10	729.13
	SEMG2	72.16	188	6.38E+08		1209.20	
	KLK3	21.07	5	2.88E+06		385.80	
	PPAP	15.28	5	3.16E+06		305.40	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
17_08_506	SEMG1	86.15	365	2.62E+09	8.16E+08	1067.30	765.45
	SEMG2	65.81	172	5.57E+08		1112.30	
	KLK3	40.61	18	4.07E+07		469.80	
	PPAP	20.98	16	4.46E+07		412.40	
17_12_513	SEMG1	83.33	400	3.48E+09	1.48E+09	1103.50	818.48
	SEMG2	79.38	265	2.39E+09		1283.00	
	KLK3	30.27	10	1.64E+07		469.00	
	PPAP	20.98	9	3.29E+07		418.40	
18_01_691	SEMG1	77.27	339	2.35E+09	1.17E+09	1145.90	824.23
	SEMG2	77.66	243	2.19E+09		1189.60	
	KLK3	40.61	16	4.91E+07		439.40	
	PPAP	39.38	24	8.46E+07		522.00	
18_03_543	SEMG1	83.55	385	3.64E+09	1.54E+09	1184.40	890.90
	SEMG2	74.23	283	2.48E+09		1345.20	
	KLK3	30.27	12	1.99E+07		460.20	
	PPAP	27.72	26	3.08E+07		573.80	
18_03_579	SEMG1	82.47	354	4.60E+09	1.84E+09	1136.40	913.00
	SEMG2	78.52	253	2.49E+09		1362.80	
	KLK3	40.61	23	1.38E+08		619.50	
	PPAP	36.79	28	1.21E+08		533.30	
18_03_579	SEMG1	92.86	314	4.57E+09	1.84E+09	1345.90	906.78
	SEMG2	77.49	219	2.77E+09		1417.90	
	KLK3	14.94	4	5.54E+06		453.50	
	PPAP	20.98	7	7.11E+06		409.80	
18_03_615	SEMG1	92.42	452	5.98E+09	2.47E+09	1108.50	857.23
	SEMG2	83.33	319	3.82E+09		1400.10	
	KLK3	31.80	12	2.74E+07		479.60	
	PPAP	28.24	17	5.61E+07		440.70	
18_09_594	SEMG1	89.83	376	5.51E+09	2.26E+09	1181.60	861.70
	SEMG2	80.58	287	3.43E+09		1461.10	
	KLK3	40.61	14	3.83E+07		354.10	
	PPAP	25.39	14	5.19E+07		450.00	
19_02_507	SEMG1	83.12	305	2.26E+09	1.14E+09	1128.60	864.40
	SEMG2	82.47	249	2.16E+09		1409.10	
	KLK3	37.93	15	8.07E+07		478.90	
	PPAP	44.04	20	4.97E+07		441.00	
19_03_518	SEMG1	82.90	347	3.02E+09	1.41E+09	1157.50	833.18
	SEMG2	77.49	285	2.61E+09		1298.90	
	KLK3	40.23	8	1.12E+07		480.00	
	PPAP	13.47	4	6.17E+06		396.30	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
19_06_629	SEMG1	86.15	325	2.44E+09	1.21E+09	1149.90	850.25
	SEMG2	74.40	202	2.29E+09		1326.60	
	KLK3	33.33	11	4.13E+07		457.90	
	PPAP	31.09	25	6.81E+07		466.60	
19_07_577	SEMG1	89.39	362	3.43E+09	1.42E+09	1121.00	853.40
	SEMG2	76.98	255	2.22E+09		1390.40	
	KLK3	31.80	11	2.74E+07		490.40	
	PPAP	20.98	11	2.78E+07		411.80	
19_09_609	SEMG1	86.58	395	4.05E+09	1.82E+09	1145.90	822.53
	SEMG2	79.04	243	3.10E+09		1383.80	
	KLK3	40.61	16	5.68E+07		318.90	
	PPAP	38.60	20	7.09E+07		441.50	
19_10_584	SEMG1	88.96	285	1.50E+09	6.58E+08	1075.80	765.08
	SEMG2	74.40	191	1.11E+09		1270.00	
	KLK3	21.84	6	3.25E+06		379.90	
	PPAP	23.83	13	1.23E+07		334.60	
19_11_614	SEMG1	94.16	311	3.87E+09	1.62E+09	1234.90	806.78
	SEMG2	73.20	231	2.59E+09		1371.80	
	KLK3	23.37	6	8.93E+06		237.90	
	PPAP	12.18	4	3.72E+06		382.50	
19_12_573	SEMG1	95.02	398	5.83E+09	2.17E+09	1288.00	884.88
	SEMG2	80.24	264	2.80E+09		1375.90	
	KLK3	31.80	8	1.82E+07		470.60	
	PPAP	20.98	7	2.02E+07		405.00	
20_02_628	SEMG1	88.53	334	4.30E+09	1.72E+09	1242.10	892.28
	SEMG2	75.43	258	2.52E+09		1427.50	
	KLK3	39.08	15	4.04E+07		499.10	
	PPAP	20.98	7	2.15E+07		400.40	
20_02_628	SEMG1	89.18	322	2.83E+09	1.08E+09	1277.20	861.25
	SEMG2	75.43	209	1.44E+09		1303.00	
	KLK3	38.70	11	1.91E+07		462.70	
	PPAP	24.87	14	2.75E+07		402.10	
20_02_694	SEMG1	94.59	278	3.09E+09	1.29E+09	1205.70	691.90
	SEMG2	78.35	209	2.07E+09		1247.70	
	KLK3	11.49	2	2.26E+05		68.80	
	PPAP	13.47	4	1.70E+06		245.40	
20_04_559	SEMG1	91.56	367	5.21E+09	2.09E+09	1250.10	920.75
	SEMG2	79.72	262	3.08E+09		1491.40	
	KLK3	33.33	10	2.66E+07		469.40	
	PPAP	20.98	8	3.14E+07		472.10	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
31978_01	SEMG1	83.55	374	3.74E+09	1.78E+09	1125.10	953.08
	SEMG2	80.24	250	2.70E+09		1417.20	
	KLK3	41.38	33	2.25E+08		600.60	
	PPAP	48.45	54	4.32E+08		669.40	
31978_02	SEMG1	90.04	397	5.76E+09	2.42E+09	1198.10	919.63
	SEMG2	82.30	295	3.56E+09		1391.60	
	KLK3	40.61	21	1.59E+08		503.70	
	PPAP	43.78	30	1.84E+08		585.10	
31978_03	SEMG1	83.98	387	5.71E+09	2.16E+09	1098.60	903.23
	SEMG2	79.72	247	2.67E+09		1360.90	
	KLK3	39.08	20	1.32E+08		577.20	
	PPAP	48.19	40	1.42E+08		576.20	
31978_04	SEMG1	86.58	415	4.22E+09	1.79E+09	1166.70	850.18
	SEMG2	82.30	290	2.84E+09		1353.90	
	KLK3	37.93	12	1.70E+07		343.50	
	PPAP	28.24	12	7.01E+07		536.60	
31978_05	SEMG1	87.88	396	7.31E+09	3.00E+09	1176.00	870.73
	SEMG2	78.35	301	4.64E+09		1395.00	
	KLK3	27.20	8	1.69E+07		478.60	
	PPAP	16.58	5	1.93E+07		433.30	
31978-06	SEMG1	90.9	234	1.58E+09	6.24E+08	1268.5	863.10
	SEMG2	70.6	163	9.09E+08		1359.8	
	KLK3	14.2	4	3.77E+06		443.7	
	PPAP	4.4	2	1.94E+06		380.4	
31978-07	SEMG1	78.8	375	1.80E+09	7.50E+08	1123.1	650.80
	SEMG2	76.6	305	1.20E+09		1273.6	
	KLK3	0	0	0		0	
	PPAP	3.1	2	1.43E+05		206.5	
31978-08	SEMG1	84	390	6.53E+09	2.62E+09	1243.2	813.63
	SEMG2	80.8	272	3.93E+09		1351.5	
	KLK3	8.8	2	3.08E+06		286.4	
	PPAP	25.6	9	8.28E+06		373.4	
31978-09	SEMG1	89.8	418	7.20E+09	2.90E+09	1171.6	843.28
	SEMG2	84.4	327	4.36E+09		1315.8	
	KLK3	27.2	9	1.94E+07		423.4	
	PPAP	25.4	10	2.40E+07		462.3	
31978-10	SEMG1	86.8	330	2.49E+09	1.12E+09	1102.9	831.25
	SEMG2	74.1	289	1.97E+09		1350.6	
	KLK3	31.8	10	1.25E+07		432.5	
	PPAP	21	8	1.11E+07		439	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
31978-11	SEMG1	87.4	373	4.47E+09	1.79E+09	1233.4	816.65
	SEMG2	80.1	272	2.66E+09		1349.2	
	KLK3	31.8	9	1.38E+07		297.3	
	PPAP	21	8	1.47E+07		386.7	
31978-12	SEMG1	83.8	391	3.04E+09	1.37E+09	1095.3	815.05
	SEMG2	78.2	310	2.42E+09		1265.8	
	KLK3	33.3	14	2.30E+07		493.8	
	PPAP	16.6	7	9.46E+06		405.3	
31978-13	SEMG1	90	401	6.14E+09	2.69E+09	1146.9	831.90
	SEMG2	82.1	282	4.61E+09		1332.5	
	KLK3	36	9	1.02E+07		409.4	
	PPAP	21	6	1.27E+07		438.8	
31978-14	SEMG1	90.7	439	9.33E+09	3.60E+09	1172.5	879.85
	SEMG2	79.2	323	4.93E+09		1342.4	
	KLK3	33.3	16	7.47E+07		542.6	
	PPAP	25.4	11	8.03E+07		461.9	
31978-15	SEMG1	89.2	374	4.49E+09	1.99E+09	1217.8	860.88
	SEMG2	80.2	258	3.38E+09		1354.2	
	KLK3	40.2	12	2.47E+07		406.3	
	PPAP	25.4	10	5.91E+07		465.2	
31978-16	SEMG1	83.5	376	2.94E+09	1.25E+09	1136.8	829.40
	SEMG2	77.7	295	2.03E+09		1332.4	
	KLK3	37.9	11	1.04E+07		470.1	
	PPAP	16.6	5	2.97E+06		378.3	
31978-17	SEMG1	90.9	360	3.70E+09	1.57E+09	1097.2	784.13
	SEMG2	79.6	266	2.57E+09		1417.2	
	KLK3	20.7	6	6.00E+06		309	
	PPAP	11.4	2	3.17E+06		313.1	
31978-18	SEMG1	78.6	312	2.70E+09	1.35E+09	1171.3	911.98
	SEMG2	85.6	331	2.59E+09		1354.2	
	KLK3	33.4	30	5.85E+07		605.3	
	PPAP	37.2	11	3.61E+07		517.1	
31978_19	SEMG1	86.58	465	4.56E+09	2.08E+09	1158.20	963.53
	SEMG2	82.13	321	3.37E+09		1443.80	
	KLK3	40.61	23	1.42E+08		564.90	
	PPAP	53.63	61	2.28E+08		687.20	
31978_20	SEMG1	86.80	342	4.73E+09	2.01E+09	1217.90	923.63
	SEMG2	83.16	260	3.08E+09		1359.00	
	KLK3	39.46	21	8.12E+07		543.60	
	PPAP	45.08	31	1.49E+08		574.00	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
20_04_566	SEMG1	81.17	411	2.47E+09	1.18E+09	1112.60	882.85
	SEMG2	79.38	322	2.14E+09		1381.20	
	KLK3	40.23	13	5.41E+07		470.60	
	PPAP	46.63	29	6.43E+07		567.00	
20_05_510	SEMG1	92.21	305	2.96E+09	1.35E+09	1235.10	908.08
	SEMG2	76.80	225	2.26E+09		1300.60	
	KLK3	40.23	17	6.67E+07		502.90	
	PPAP	40.41	27	1.03E+08		593.70	
20_05_515	SEMG1	87.45	236	1.62E+09	6.48E+08	1232.20	817.33
	SEMG2	69.76	166	9.70E+08		1370.80	
	KLK3	9.96	5	3.50E+06		434.40	
	PPAP	3.63	1	7.58E+05		231.90	
20_05_522	SEMG1	89.83	321	3.60E+09	1.62E+09	1173.10	924.98
	SEMG2	79.38	246	2.60E+09		1396.60	
	KLK3	41.76	24	1.57E+08		584.10	
	PPAP	39.12	27	1.09E+08		546.10	
20_05_523	SEMG1	89.39	357	5.89E+09	2.66E+09	1200.10	989.08
	SEMG2	76.12	271	4.42E+09		1424.40	
	KLK3	44.83	27	1.61E+08		705.00	
	PPAP	48.45	43	1.69E+08		626.80	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
18_03_552 (Vasectomized)	SEMG1	87.88	323	2.53E+09	1.08E+09	1171.50	808.35
	SEMG2	74.57	227	1.78E+09		1360.80	
	KLK3	14.18	5	3.64E+06		460.40	
	PPAP	3.63	1	3.76E+05		240.70	
19_09_537 (Vasectomized)	SEMG1	87.88	411	3.99E+09	1.68E+09	1126.10	816.98
	SEMG2	79.38	299	2.72E+09		1391.20	
	KLK3	18.39	7	1.07E+07		443.00	
	PPAP	16.58	6	7.01E+06		307.60	
19_10_565 (Vasectomized)	SEMG1	90.26	334	2.42E+09	1.02E+09	1114.90	852.65
	SEMG2	76.46	210	1.63E+09		1383.20	
	KLK3	23.75	8	1.27E+07		483.60	
	PPAP	20.98	9	1.08E+07		428.90	
17_04_533 (Vasectomized)	SEMG1	90.69	438	4.48E+09	1.78E+09	1146.50	839.95
	SEMG2	76.29	308	2.65E+09		1375.90	
	KLK3	16.86	5	5.24E+06		479.30	
	PPAP	11.66	3	3.17E+06		358.10	
17_02_589 (Vasectomized)	SEMG1	89.83	356	3.21E+09	1.48E+09	1180.30	710.65
	SEMG2	82.65	302	2.71E+09		1350.80	
	KLK3	12.26	2	7.74E+05		137.50	
	PPAP	4.66	2	4.04E+05		174.00	

APPENDIX L: PROTEIN IDENTIFICATION OF SALIVA POPULATION SAMPLES

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
1866	Basic Salivary 1	95.41	181	7.17E+08	2.24E+08	1024.30	667.77
	Basic Salivary 2	45.43	31	1.05E+08		757.10	
	Basic Salivary 3	64.10	78	3.61E+08		976.40	
	Basic Salivary 4	45.16	42	7.03E+07		616.60	
	Salivary Acidic 1/2	90.36	156	7.62E+08		928.70	
	Statherin	54.84	145	2.09E+08		557.80	
	Submaxillary Gland	72.15	76	1.43E+08		496.10	
	Histatin 1	66.67	71	6.83E+07		482.60	
	Histatin 3	62.74	15	1.66E+07		578.90	
	Cystatin-SN	30.50	13	1.26E+07		424.60	
	Alpha-Amylase 1	9.59	6	1.86E+06		502.40	
1995	Basic Salivary 1	18.11	7	8.74E+06	1.83E+08	501.40	532.11
	Basic Salivary 2	85.10	97	2.21E+08		735.90	
	Basic Salivary 3	81.55	96	4.82E+08		824.20	
	Basic Salivary 4	64.52	61	2.32E+08		595.30	
	Salivary Acidic 1/2	90.36	126	7.98E+08		798.10	
	Statherin	45.16	53	4.42E+07		397.60	
	Submaxillary Gland	72.15	80	1.84E+08		500.00	
	Histatin 1	43.86	45	3.62E+07		564.90	
	Histatin 3	31.37	4	1.76E+06		319.50	
	Cystatin-SN	11.35	6	2.74E+06		382.30	
	Alpha-Amylase 1	5.28	4	6.03E+05		234.00	
2407	Basic Salivary 1	93.62	158	6.61E+08	3.22E+08	874.40	601.41
	Basic Salivary 2	45.91	19	4.88E+07		536.50	
	Basic Salivary 3	73.79	76	5.14E+08		927.20	
	Basic Salivary 4	26.45	12	1.63E+07		560.60	
	Salivary Acidic 1/2	90.36	146	1.36E+09		809.90	
	Statherin	54.84	119	2.81E+08		544.90	
	Submaxillary Gland	72.15	98	4.70E+08		609.00	
	Histatin 1	47.37	104	1.87E+08		567.70	
	Histatin 3	60.78	7	4.63E+06		384.00	
	Cystatin-SN	19.86	4	9.64E+05		424.70	
	Alpha-Amylase 1	4.89	3	7.63E+05		376.60	
3955	Basic Salivary 1	94.90	204	1.40E+09	4.06E+08	1032.80	662.20
	Basic Salivary 2	45.43	26	1.23E+08		674.50	
	Basic Salivary 3	70.87	61	3.03E+08		872.50	
	Basic Salivary 4	51.29	51	2.15E+08		1024.80	
	Salivary Acidic 1/2	90.36	149	1.33E+09		923.30	
	Statherin	66.13	96	2.07E+08		482.40	
	Submaxillary Gland	72.15	107	7.15E+08		587.70	
	Histatin 1	45.61	90	1.58E+08		555.00	
	Histatin 3	21.57	3	1.06E+06		338.50	
	Cystatin-SN	10.64	4	1.60E+06		408.90	
	Alpha-Amylase 1	4.89	2	7.41E+05		383.80	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
4646	Basic Salivary 1	93.88	134	6.69E+08	3.72E+08	1050.90	657.98
	Basic Salivary 2	38.70	14	4.16E+07		631.70	
	Basic Salivary 3	76.70	79	6.55E+08		1008.40	
	Basic Salivary 4	34.52	22	8.05E+07		658.80	
	Salivary Acidic 1/2	90.36	131	1.50E+09		961.50	
	Statherin	54.84	109	3.37E+08		623.00	
	Submaxillary Gland	72.15	80	5.42E+08		617.30	
	Histatin 1	66.67	105	2.51E+08		602.10	
	Histatin 3	62.74	14	1.58E+07		405.20	
	Cystatin-SN	5.67	1	2.18E+05		307.50	
	Alpha-Amylase 1	2.15	1	8.82E+05		371.40	
4970	Basic Salivary 1	53.57	26	2.84E+07	1.37E+08	637.90	514.24
	Basic Salivary 2	93.03	140	5.78E+08		1068.00	
	Basic Salivary 3	6.84	1	2.79E+05		125.50	
	Basic Salivary 4	63.23	65	1.49E+08		674.50	
	Salivary Acidic 1/2	81.32	91	3.01E+08		823.90	
	Statherin	54.84	62	7.11E+07		395.70	
	Submaxillary Gland	72.15	91	2.47E+08		504.00	
	Histatin 1	64.91	89	1.09E+08		502.80	
	Histatin 3	62.74	17	1.65E+07		559.10	
	Cystatin-SN	13.48	3	7.40E+05		365.20	
	Alpha-Amylase 1	0.00	0	0.00E+00		0.00	
5711	Basic Salivary 1	93.37	152	6.73E+08	3.98E+08	921.10	631.76
	Basic Salivary 2	42.31	20	5.01E+07		685.00	
	Basic Salivary 3	71.20	77	4.72E+08		927.00	
	Basic Salivary 4	41.61	17	6.32E+07		513.40	
	Salivary Acidic 1/2	90.36	161	1.65E+09		881.30	
	Statherin	54.84	118	4.10E+08		537.60	
	Submaxillary Gland	72.15	100	8.47E+08		633.40	
	Histatin 1	47.37	107	2.05E+08		582.40	
	Histatin 3	41.18	5	1.88E+06		377.30	
	Cystatin-SN	13.48	3	2.25E+06		440.40	
	Alpha-Amylase 1	2.15	1	6.11E+05		450.50	
6003	Basic Salivary 1	47.70	24	4.15E+07	2.17E+08	571.60	582.05
	Basic Salivary 2	90.14	103	3.56E+08		786.40	
	Basic Salivary 3	73.79	54	1.98E+08		783.50	
	Basic Salivary 4	53.87	58	1.25E+08		635.20	
	Salivary Acidic 1/2	90.36	122	8.03E+08		701.00	
	Statherin	54.84	114	1.94E+08		506.30	
	Submaxillary Gland	72.15	94	5.19E+08		543.90	
	Histatin 1	47.37	91	1.36E+08		568.60	
	Histatin 3	60.78	13	9.74E+06		470.30	
	Cystatin-SN	30.50	13	8.03E+06		482.50	
	Alpha-Amylase 1	8.41	4	1.26E+06		353.20	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
6014	Basic Salivary 1	92.35	168	1.22E+09	4.19E+08	1044.50	670.97
	Basic Salivary 2	44.71	22	1.23E+08		597.30	
	Basic Salivary 3	58.90	62	4.93E+08		1051.10	
	Basic Salivary 4	74.84	89	8.56E+08		1027.20	
	Salivary Acidic 1/2	90.36	142	9.72E+08		744.30	
	Statherin	66.13	106	2.63E+08		527.70	
	Submaxillary Gland	67.09	107	5.36E+08		537.30	
	Histatin 1	45.61	69	1.31E+08		532.40	
	Histatin 3	21.57	4	2.12E+06		299.50	
	Cystatin-SN	13.48	9	1.22E+07		520.10	
	Alpha-Amylase 1	4.50	2	8.99E+05		499.30	
6066	Basic Salivary 1	93.37	152	8.45E+08	2.42E+08	1016.50	570.11
	Basic Salivary 2	43.75	21	1.11E+08		592.30	
	Basic Salivary 3	19.94	4	6.27E+06		425.80	
	Basic Salivary 4	67.10	80	3.95E+08		839.80	
	Salivary Acidic 1/2	18.07	5	1.53E+07		529.50	
	Statherin	66.13	102	2.70E+08		535.70	
	Submaxillary Gland	72.15	113	7.10E+08		546.50	
	Histatin 1	54.39	117	3.03E+08		550.70	
	Histatin 3	60.78	12	7.08E+06		353.10	
	Cystatin-SN	10.64	4	1.64E+06		395.90	
	Alpha-Amylase 1	2.15	1	7.39E+05		485.40	
6163	Basic Salivary 1	95.15	196	1.82E+09	3.45E+08	1068.90	647.77
	Basic Salivary 2	54.09	51	3.09E+08		867.80	
	Basic Salivary 3	46.72	34	1.70E+08		806.90	
	Basic Salivary 4	58.39	54	2.72E+08		714.50	
	Salivary Acidic 1/2	90.36	83	6.72E+08		735.90	
	Statherin	53.22	56	8.83E+07		493.10	
	Submaxillary Gland	72.15	94	2.88E+08		635.20	
	Histatin 1	66.67	96	1.47E+08		550.80	
	Histatin 3	60.78	9	1.88E+07		554.70	
	Cystatin-SN	11.35	2	8.45E+05		395.40	
	Alpha-Amylase 1	2.15	1	4.00E+05		302.30	
6166	Basic Salivary 1	94.90	213	1.16E+09	2.62E+08	997.70	597.95
	Basic Salivary 2	50.96	34	1.02E+08		651.30	
	Basic Salivary 3	73.79	67	3.06E+08		788.40	
	Basic Salivary 4	37.74	36	9.86E+07		584.90	
	Salivary Acidic 1/2	90.36	118	6.54E+08		744.70	
	Statherin	54.84	100	1.30E+08		545.90	
	Submaxillary Gland	72.15	93	3.12E+08		521.60	
	Histatin 1	45.61	68	1.07E+08		509.30	
	Histatin 3	58.82	6	1.98E+06		450.60	
	Cystatin-SN	18.44	7	1.32E+06		328.90	
	Alpha-Amylase 1	4.89	2	1.03E+06		454.10	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
6171	Basic Salivary 1	36.67	14	5.80E+07	2.29E+08	637.50	525.11
	Basic Salivary 2	83.41	127	5.17E+08		867.20	
	Basic Salivary 3	54.42	20	9.01E+07		767.90	
	Basic Salivary 4	77.10	94	6.91E+08		895.80	
	Salivary Acidic 1/2	90.36	91	4.43E+08		685.20	
	Statherin	50.00	76	1.90E+08		510.60	
	Submaxillary Gland	72.15	78	4.53E+08		546.00	
	Histatin 1	45.61	49	7.33E+07		480.40	
	Histatin 3	0.00	0.00	0.00E+00		0.00	
	Cystatin-SN	0.00	0.00	0.00E+00		0.00	
	Alpha-Amylase 1	4.89	2	6.38E+05		385.60	
6238	Basic Salivary 1	93.88	162	9.99E+08	2.78E+08	1016.80	606.36
	Basic Salivary 2	44.23	18	9.76E+07		699.00	
	Basic Salivary 3	73.46	65	5.06E+08		823.90	
	Basic Salivary 4	57.10	61	3.59E+08		1068.20	
	Salivary Acidic 1/2	18.07	5	9.13E+06		558.50	
	Statherin	54.84	87	2.49E+08		528.30	
	Submaxillary Gland	72.15	87	7.00E+08		578.80	
	Histatin 1	47.37	82	1.34E+08		565.90	
	Histatin 3	0	0	0.00E+00		0	
	Cystatin-SN	18.44	3	1.44E+06		411.30	
	Alpha-Amylase 1	4.89	2	7.70E+05		419.30	
6239	Basic Salivary 1	37.24	34	6.61E+07	1.28E+08	541.50	459.75
	Basic Salivary 2	2.40	1	5.99E+06		443.70	
	Basic Salivary 3	62.39	39	1.48E+08		765.50	
	Basic Salivary 4	55.47	36	1.00E+08		525.50	
	Salivary Acidic 1/2	81.93	99	5.29E+08		662.80	
	Statherin	61.29	70	7.26E+07		425.70	
	Submaxillary Gland	67.09	56	4.63E+08		500.00	
	Histatin 1	50.88	24	1.55E+07		418.90	
	Histatin 3	0	0	0.00E+00		0	
	Cystatin-SN	24.11	6	1.49E+06		403.30	
	Alpha-Amylase 1	2.74	2	6.76E+05		370.30	
6260	Basic Salivary 1	23.47	7	5.98E+06	1.24E+08	333.10	520.52
	Basic Salivary 2	88.46	77	1.57E+08		715.20	
	Basic Salivary 3	70.87	67	2.43E+08		841.50	
	Basic Salivary 4	49.03	42	6.76E+07		628.90	
	Salivary Acidic 1/2	21.08	7	5.33E+06		283.40	
	Statherin	69.35	127	2.86E+08		491.00	
	Submaxillary Gland	72.15	106	4.79E+08		583.40	
	Histatin 1	47.37	96	1.10E+08		550.70	
	Histatin 3	60.78	9	1.03E+07		473.80	
	Cystatin-SN	12.06	2	2.75E+06		443.30	
	Alpha-Amylase 1	2.15	1	4.18E+05		381.40	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
6310	Basic Salivary 1	0	0	0.00E+00	1.58E+08	0	359.75
	Basic Salivary 2	18.75	10	4.00E+06		299.10	
	Basic Salivary 3	68.95	49	3.36E+08		752.90	
	Basic Salivary 4	30.00	21	2.39E+07		438.60	
	Salivary Acidic 1/2	87.35	94	5.36E+08		707.80	
	Statherin	43.55	55	7.17E+07		460.50	
	Submaxillary Gland	67.09	79	7.37E+08		500.40	
	Histatin 1	42.10	29	2.89E+07		441.10	
	Histatin 3	0	0	0.00E+00		0	
	Cystatin-SN	0	0	0.00E+00		0	
	Alpha-Amylase 1	2.74	2	9.66E+05		356.80	
6343	Basic Salivary 1	95.92	194	2.01E+09	4.18E+08	1115.80	744.31
	Basic Salivary 2	59.62	47	1.77E+08		921.80	
	Basic Salivary 3	55.66	38	2.03E+08		792.00	
	Basic Salivary 4	63.55	48	2.70E+08		961.20	
	Salivary Acidic 1/2	90.36	94	9.83E+08		1105.80	
	Statherin	67.74	101	1.52E+08		527.40	
	Submaxillary Gland	72.15	93	4.47E+08		559.50	
	Histatin 1	66.67	99	2.87E+08		569.90	
	Histatin 3	60.78	15	7.26E+07		670.20	
	Cystatin-SN	18.44	6	3.09E+06		466.10	
	Alpha-Amylase 1	7.04	3	1.18E+06		497.70	
6462	Basic Salivary 1	89.39	101	5.34E+08	3.45E+08	1033.60	599.55
	Basic Salivary 2	25.48	10	2.19E+07		449.20	
	Basic Salivary 3	67.96	70	7.75E+08		924.40	
	Basic Salivary 4	53.87	45	3.74E+08		867.70	
	Salivary Acidic 1/2	90.36	120	1.42E+09		734.20	
	Statherin	51.61	75	1.28E+08		502.10	
	Submaxillary Gland	72.15	92	4.31E+08		600.30	
	Histatin 1	66.67	80	1.08E+08		535.10	
	Histatin 3	58.82	7	2.46E+06		446.70	
	Cystatin-SN	0.00	0.00	0.00E+00		0	
	Alpha-Amylase 1	4.31	2	1.14E+06		501.80	
6472	Basic Salivary 1	4.08	1	1.09E+06	1.39E+08	221.90	453.25
	Basic Salivary 2	51.44	34	3.71E+07		583.20	
	Basic Salivary 3	60.52	47	1.86E+08		741.00	
	Basic Salivary 4	38.71	34	8.53E+07		546.50	
	Salivary Acidic 1/2	86.75	147	6.83E+08		836.70	
	Statherin	50.00	114	2.56E+08		565.20	
	Submaxillary Gland	67.09	95	2.45E+08		494.80	
	Histatin 1	42.10	16	2.98E+07		421.10	
	Histatin 3	0	0	0.00E+00		0	
	Cystatin-SN	30.50	9	4.05E+06		309.40	
	Alpha-Amylase 1	2.15	1	3.97E+05		266.00	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
6503	Basic Salivary 1	92.35	136	3.13E+08	2.07E+08	746.90	511.56
	Basic Salivary 2	21.63	11	1.98E+07		471.90	
	Basic Salivary 3	69.58	54	2.91E+08		768.30	
	Basic Salivary 4	65.16	57	1.62E+08		598.30	
	Salivary Acidic 1/2	21.08	12	7.36E+07		623.40	
	Statherin	66.13	105	2.53E+08		523.70	
	Submaxillary Gland	67.09	134	1.08E+09		540.80	
	Histatin 1	50.88	50	7.65E+07		557.30	
	Histatin 3	0	0	0.00E+00		0	
	Cystatin-SN	15.60	5	6.60E+06		518.30	
	Alpha-Amylase 1	2.15	1	3.51E+05		278.30	
6510	Basic Salivary 1	95.66	163	5.53E+08	1.59E+08	865.50	573.24
	Basic Salivary 2	47.84	30	4.51E+07		624.60	
	Basic Salivary 3	67.64	36	8.87E+07		760.50	
	Basic Salivary 4	47.74	21	3.13E+07		472.80	
	Salivary Acidic 1/2	90.36	85	4.21E+08		903.00	
	Statherin	58.06	100	1.81E+08		412.90	
	Submaxillary Gland	72.15	88	3.36E+08		542.50	
	Histatin 1	43.86	23	9.27E+07		605.20	
	Histatin 3	31.37	3	1.19E+06		394.50	
	Cystatin-SN	24.82	5	2.52E+06		414.30	
	Alpha-Amylase 1	2.15	1	3.44E+05		309.80	
6554	Basic Salivary 1	91.82	150	7.49E+08	3.11E+08	1023.30	638.67
	Basic Salivary 2	51.20	22	5.44E+07		589.70	
	Basic Salivary 3	73.14	68	3.75E+08		859.20	
	Basic Salivary 4	46.45	39	1.47E+08		708.10	
	Salivary Acidic 1/2	90.36	117	1.04E+09		966.20	
	Statherin	69.35	120	1.76E+08		447.40	
	Submaxillary Gland	72.15	89	6.97E+08		557.90	
	Histatin 1	45.61	100	1.77E+08		515.70	
	Histatin 3	58.82	12	6.98E+06		588.50	
	Cystatin-SN	13.48	3	9.48E+05		393.90	
	Alpha-Amylase 1	2.15	1	4.57E+05		375.50	
6557	Basic Salivary 1	10.97	3	2.72E+06	2.42E+08	366.90	480.94
	Basic Salivary 2	71.39	41	3.16E+07		506.10	
	Basic Salivary 3	73.79	73	4.35E+08		787.40	
	Basic Salivary 4	41.61	29	8.25E+07		516.30	
	Salivary Acidic 1/2	90.36	154	1.06E+09		801.10	
	Statherin	54.84	115	2.36E+08		529.10	
	Submaxillary Gland	72.15	108	6.68E+08		523.40	
	Histatin 1	66.67	92	1.47E+08		505.30	
	Histatin 3	45.10	3	5.44E+05		276.70	
	Cystatin-SN	0.00	0.00	0.00E+00		0.00	
	Alpha-Amylase 1	2.15	1	9.23E+05		478.00	
SA_01	Basic Salivary 1	95.92	127	5.92E+08	1.75E+08	921.70	578.40
	Basic Salivary 2	50.48	29	5.34E+07		651.60	
	Basic Salivary 3	75.73	41	1.25E+08		776.10	
	Basic Salivary 4	51.94	26	7.05E+07		521.30	
	Salivary Acidic 1/2	82.53	80	4.50E+08		964.30	
	Statherin	66.13	102	1.47E+08		477.40	
	Submaxillary Gland	72.15	63	2.45E+08		507.50	
	Histatin 1	64.91	62	1.14E+08		527.70	
	Histatin 3	62.74	31	1.30E+08		683.30	
	Cystatin-SN	13.48	5	1.93E+06		331.50	
	Alpha-Amylase 1	0.00	0	0.00E+00		0.00	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
IR1	Basic Salivary 1	87.50	48	1.42E+08	4.89E+07	1042.00	412.48
	Basic Salivary 2	7.21	2	1.19E+06		132.00	
	Basic Salivary 3	27.07	13	3.16E+07		555.00	
	Basic Salivary 4	0.00	0	0.00E+00		0.00	
	Salivary Acidic 1/2	62.65	29	1.62E+08		646.60	
	Statherin	53.22	51	6.16E+07		542.10	
	Submaxillary Gland	72.15	53	9.14E+07		493.70	
	Histatin 1	63.16	13	5.14E+06		277.60	
	Histatin 3	62.74	17	4.17E+07		597.90	
	Cystatin-SN	0.00	0	0.00E+00		0.00	
	Alpha-Amylase 1	2.15	1	2.51E+05		250.40	
IR2	Basic Salivary 1	91.82	101	7.59E+08	3.26E+08	1063.60	590.71
	Basic Salivary 2	55.29	26	9.50E+07		791.20	
	Basic Salivary 3	70.55	51	3.01E+08		959.80	
	Basic Salivary 4	51.61	38	7.10E+07		597.80	
	Salivary Acidic 1/2	90.36	100	8.33E+08		999.10	
	Statherin	67.74	176	5.48E+08		535.50	
	Submaxillary Gland	72.15	116	9.29E+08		580.00	
	Histatin 1	63.16	31	4.62E+07		529.20	
	Histatin 3	0.00	0	0.00E+00		0.0	
	Cystatin-SN	12.06	3	1.25E+06		441.60	
	Alpha-Amylase 1	0.00	0	0.00E+00		0.0	
IR3	Basic Salivary 1	52.55	28	3.50E+07	1.34E+08	537.60	445.27
	Basic Salivary 2	6.01	3	2.97E+06		445.70	
	Basic Salivary 3	75.40	52	1.67E+08		815.50	
	Basic Salivary 4	11.29	3	1.06E+06		239.30	
	Salivary Acidic 1/2	90.36	95	6.05E+08		743.80	
	Statherin	69.35	93	1.07E+08		424.70	
	Submaxillary Gland	72.15	75	5.31E+08		496.40	
	Histatin 1	45.61	40	2.62E+07		468.40	
	Histatin 3	54.90	3	5.81E+05		374.70	
	Cystatin-SN	0.00	0	0.00E+00		0.00	
	Alpha-Amylase 1	2.15	1	4.53E+05		351.90	
IR4	Basic Salivary 1	3.83	1	1.47E+05	9.20E+07	203.80	423.75
	Basic Salivary 2	57.93	24	2.28E+07		463.30	
	Basic Salivary 3	66.67	39	8.67E+07		563.10	
	Basic Salivary 4	50.00	21	1.69E+07		547.90	
	Salivary Acidic 1/2	90.36	74	3.77E+08		737.00	
	Statherin	69.35	94	7.75E+07		370.20	
	Submaxillary Gland	72.15	78	3.82E+08		545.80	
	Histatin 1	64.91	40	3.93E+07		492.70	
	Histatin 3	60.78	9	8.94E+06		520.60	
	Cystatin-SN	0.00	0	0.00E+00		0.00	
	Alpha-Amylase 1	2.15	1	1.65E+05		216.80	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
IR5	Basic Salivary 1	21.68	8	4.46E+06	2.75E+07	403.00	334.59
	Basic Salivary 2	51.68	29	2.81E+07		485.20	
	Basic Salivary 3	68.95	31	7.42E+07		768.30	
	Basic Salivary 4	38.39	18	1.75E+07		419.00	
	Salivary Acidic 1/2	8.43	4	2.02E+07		274.80	
	Statherin	66.13	55	2.54E+07		412.60	
	Submaxillary Gland	72.15	54	1.31E+08		543.50	
	Histatin 1	33.33	5	1.10E+06		291.30	
	Histatin 3	17.65	1	6.24E+04		82.80	
	Cystatin-SN	0.00	0	0.00E+00		0.00	
	Alpha-Amylase 1	0.00	0	0.00E+00		0.00	
IR6	Basic Salivary 1	95.66	172	1.53E+09	3.94E+08	978.30	489.52
	Basic Salivary 2	47.84	18	4.32E+07		430.50	
	Basic Salivary 3	55.66	34	2.97E+08		815.40	
	Basic Salivary 4	85.42	77	6.00E+08		888.90	
	Salivary Acidic 1/2	89.76	108	6.06E+08		692.30	
	Statherin	62.90	85	1.34E+08		536.30	
	Submaxillary Gland	69.62	130	1.11E+09		620.20	
	Histatin 1	24.56	7	1.10E+07		422.80	
	Histatin 3	0.00	0	0.00E+00		0.00	
	Cystatin-SN	0.00	0	0.00E+00		0.00	
	Alpha-Amylase 1	0.00	0	0.00E+00		0.00	
IR7	Basic Salivary 1	90.61	91	6.77E+08	1.87E+08	1052.50	621.54
	Basic Salivary 2	46.15	24	3.50E+07		727.40	
	Basic Salivary 3	44.44	27	1.04E+08		834.50	
	Basic Salivary 4	29.68	14	3.14E+07		549.10	
	Salivary Acidic 1/2	90.36	91	4.58E+08		802.30	
	Statherin	61.29	139	3.17E+08		545.00	
	Submaxillary Gland	72.15	79	3.39E+08		517.90	
	Histatin 1	66.67	53	7.43E+07		535.40	
	Histatin 3	62.74	8	1.63E+07		568.00	
	Cystatin-SN	5.67	2	1.61E+06		414.50	
	Alpha-Amylase 1	2.15	1	2.02E+05		290.30	
IR8	Basic Salivary 1	93.03	57	1.01E+08	9.76E+07	941.00	508.85
	Basic Salivary 2	0	0	0.00E+00		0	
	Basic Salivary 3	41.60	22	4.68E+07		815.90	
	Basic Salivary 4	25.81	10	8.11E+06		529.40	
	Salivary Acidic 1/2	90.36	78	4.75E+08		904.30	
	Statherin	58.06	96	1.58E+08		480.80	
	Submaxillary Gland	72.15	65	2.26E+08		568.40	
	Histatin 1	63.16	29	2.26E+07		453.10	
	Histatin 3	62.74	15	3.45E+07		567.20	
	Cystatin-SN	5.67	2	9.82E+05		337.20	
	Alpha-Amylase 1	0.00	0	0.00E+00		0.00	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
IR9	Basic Salivary 1	66.07	20	1.46E+07	4.28E+07	410.00	350.85
	Basic Salivary 2	0	0	0.00E+00		0	
	Basic Salivary 3	40.74	15	2.33E+07		576.80	
	Basic Salivary 4	12.90	3	1.05E+06		145.00	
	Salivary Acidic 1/2	90.36	76	2.53E+08		679.30	
	Statherin	58.06	55	1.77E+07		309.70	
	Submaxillary Gland	72.15	49	1.54E+08		539.50	
	Histatin 1	66.67	16	3.89E+06		395.10	
	Histatin 3	49.02	5	3.21E+06		496.50	
	Cystatin-SN	0.00	0	0.00E+00		0.00	
	Alpha-Amylase 1	2.15	1	2.38E+05		307.50	
IR10	Basic Salivary 1	13.52	5	1.58E+06	1.75E+08	319.60	430.98
	Basic Salivary 2	30.05	19	2.59E+07		458.80	
	Basic Salivary 3	54.99	32	7.78E+07		549.40	
	Basic Salivary 4	41.70	27	5.73E+07		436.40	
	Salivary Acidic 1/2	83.73	127	5.30E+08		720.90	
	Statherin	61.29	65	4.53E+07		418.30	
	Submaxillary Gland	72.15	140	1.18E+09		581.10	
	Histatin 1	36.84	10	7.23E+06		415.00	
	Histatin 3	0.00	0	0.00E+00		0.00	
	Cystatin-SN	29.79	8	1.33E+06		286.30	
	Alpha-Amylase 1	3.13	2	1.95E+06		555.00	
IR11	Basic Salivary 1	28.57	10	3.96E+06	2.32E+07	360.90	442.00
	Basic Salivary 2	78.37	33	5.22E+07		695.40	
	Basic Salivary 3	21.37	14	8.63E+06		839.40	
	Basic Salivary 4	0.00	0	0.00E+00		0.00	
	Salivary Acidic 1/2	69.28	34	7.56E+07		665.90	
	Statherin	67.74	58	6.92E+07		439.50	
	Submaxillary Gland	72.15	29	2.08E+07		429.80	
	Histatin 1	61.40	19	7.75E+06		443.40	
	Histatin 3	62.74	17	1.67E+07		626.70	
	Cystatin-SN	5.67	1	7.77E+04		222.80	
	Alpha-Amylase 1	2.15	1	1.74E+05		138.20	
IR12	Basic Salivary 1	12.76	5	1.94E+06	1.19E+08	347.80	447.31
	Basic Salivary 2	87.98	49	3.90E+07		572.70	
	Basic Salivary 3	66.95	45	1.43E+08		738.70	
	Basic Salivary 4	43.87	26	1.90E+07		425.90	
	Salivary Acidic 1/2	90.36	87	4.24E+08		712.80	
	Statherin	69.35	158	3.37E+08		480.40	
	Submaxillary Gland	72.15	79	3.10E+08		570.80	
	Histatin 1	61.40	56	3.63E+07		439.90	
	Histatin 3	0.00	0	0.00E+00		0.00	
	Cystatin-SN	5.67	1	1.71E+05		333.20	
	Alpha-Amylase 1	2.15	1	3.03E+05		298.20	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
IR13	Basic Salivary 1	95.41	116	3.28E+08	8.28E+07	914.70	459.74
	Basic Salivary 2	54.81	19	2.48E+07		460.70	
	Basic Salivary 3	74.76	44	1.45E+08		806.70	
	Basic Salivary 4	51.94	22	7.46E+07		573.40	
	Salivary Acidic 1/2	83.13	70	1.91E+08		713.50	
	Statherin	51.61	52	3.40E+07		401.40	
	Submaxillary Gland	65.82	59	1.09E+08		501.20	
	Histatin 1	35.09	10	4.54E+06		414.90	
	Histatin 3	0.00	0	0.00E+00		0.00	
	Cystatin-SN	0.00	0	0.00E+00		0.00	
	Alpha-Amylase 1	2.15	1	2.37E+05		270.60	
IR14	Basic Salivary 1	23.21	10	6.26E+06	1.58E+08	217.10	365.89
	Basic Salivary 2	2.40	1	2.70E+05		283.40	
	Basic Salivary 3	57.60	24	4.88E+07		533.80	
	Basic Salivary 4	18.71	8	7.22E+06		328.70	
	Salivary Acidic 1/2	83.73	139	6.06E+08		737.40	
	Statherin	64.52	80	7.60E+07		384.40	
	Submaxillary Gland	72.15	103	9.82E+08		499.30	
	Histatin 1	43.86	10	7.60E+06		439.10	
	Histatin 3	0.00	0	0.00E+00		0.00	
	Cystatin-SN	24.82	6	1.41E+06		232.80	
	Alpha-Amylase 1	21.33	16	3.64E+06		368.80	
IR15	Basic Salivary 1	95.92	110	7.24E+08	2.13E+08	872.60	579.25
	Basic Salivary 2	64.90	28	6.04E+07		575.60	
	Basic Salivary 3	72.49	56	2.44E+08		794.00	
	Basic Salivary 4	59.68	52	1.58E+08		989.60	
	Salivary Acidic 1/2	89.76	78	5.84E+08		709.70	
	Statherin	66.13	104	2.07E+08		507.60	
	Submaxillary Gland	72.15	58	2.98E+08		527.40	
	Histatin 1	45.61	46	6.41E+07		477.20	
	Histatin 3	0.00	0	0.00E+00		0.00	
	Cystatin-SN	13.48	4	2.94E+06		439.50	
	Alpha-Amylase 1	2.15	1	7.86E+05		478.60	
IR16	Basic Salivary 1	92.42	114	7.02E+08	2.03E+08	995.50	640.42
	Basic Salivary 2	47.84	23	4.31E+07		602.90	
	Basic Salivary 3	77.67	48	1.61E+08		810.60	
	Basic Salivary 4	54.52	38	9.46E+07		741.30	
	Salivary Acidic 1/2	90.36	77	6.44E+08		882.50	
	Statherin	54.84	78	9.33E+07		459.10	
	Submaxillary Gland	72.15	64	3.70E+08		555.00	
	Histatin 1	45.61	58	9.51E+07		596.20	
	Histatin 3	45.10	8	2.77E+07		655.90	
	Cystatin-SN	13.48	2	4.79E+05		367.00	
	Alpha-Amylase 1	2.15	1	3.36E+05		378.60	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
IR17	Basic Salivary 1	58.93	44	1.02E+08	1.24E+08	544.40	468.24
	Basic Salivary 2	11.06	4	4.15E+06		416.30	
	Basic Salivary 3	60.68	33	1.08E+08		552.60	
	Basic Salivary 4	45.48	25	4.13E+07		521.10	
	Salivary Acidic 1/2	87.35	66	4.96E+08		734.50	
	Statherin	58.06	158	1.58E+08		393.60	
	Submaxillary Gland	72.15	78	4.43E+08		532.00	
	Histatin 1	63.16	13	5.77E+06		497.50	
	Histatin 3	39.21	3	1.68E+06		429.20	
	Cystatin-SN	12.06	3	1.09E+06		201.10	
	Alpha-Amylase 1	2.15	1	3.17E+05		328.30	
IR18	Basic Salivary 1	80.61	77	4.80E+08	1.87E+08	728.00	472.75
	Basic Salivary 2	10.82	6	1.43E+07		449.10	
	Basic Salivary 3	69.26	39	2.12E+08		564.70	
	Basic Salivary 4	44.84	23	2.80E+07		432.20	
	Salivary Acidic 1/2	90.36	74	5.57E+08		719.70	
	Statherin	59.68	105	9.68E+07		461.70	
	Submaxillary Gland	72.15	69	6.42E+08		543.20	
	Histatin 1	63.16	29	2.84E+07		505.60	
	Histatin 3	47.06	4	3.48E+06		527.00	
	Cystatin-SN	0.00	0	0.00E+00		0.00	
	Alpha-Amylase 1	2.15	1	2.21E+05		269.00	
IR19	Basic Salivary 1	56.38	34	8.93E+07	1.17E+08	704.90	526.48
	Basic Salivary 2	95.43	98	5.78E+08		1014.00	
	Basic Salivary 3	56.13	32	7.63E+07		772.90	
	Basic Salivary 4	39.03	20	4.06E+07		575.30	
	Salivary Acidic 1/2	90.36	66	2.76E+08		684.30	
	Statherin	67.74	130	1.17E+08		372.40	
	Submaxillary Gland	72.15	49	9.52E+07		525.80	
	Histatin 1	36.84	20	5.87E+06		404.60	
	Histatin 3	62.74	9	6.87E+06		525.10	
	Cystatin-SN	0.00	0	0.00E+00		0.00	
	Alpha-Amylase 1	5.28	2	2.15E+05		212.00	
IR20	Basic Salivary 1	62.24	54	1.61E+08	1.25E+08	547.80	415.86
	Basic Salivary 2	11.78	8	2.76E+07		468.90	
	Basic Salivary 3	65.24	43	1.73E+08		574.50	
	Basic Salivary 4	56.45	38	1.03E+08		550.40	
	Salivary Acidic 1/2	86.75	105	5.53E+08		664.90	
	Statherin	61.29	92	8.76E+07		394.10	
	Submaxillary Gland	69.62	64	2.71E+08		494.40	
	Histatin 1	31.58	4	9.17E+05		273.20	
	Histatin 3	0.00	0	0.00E+00		0.00	
	Cystatin-SN	19.15	3	4.64E+05		254.90	
	Alpha-Amylase 1	2.15	1	3.09E+05		351.40	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
IR21	Basic Salivary 1	44.90	29	4.49E+07	8.41E+07	682.50	480.65
	Basic Salivary 2	95.67	95	4.95E+08		1022.60	
	Basic Salivary 3	38.46	29	1.02E+08		524.20	
	Basic Salivary 4	37.42	25	4.89E+07		575.70	
	Salivary Acidic 1/2	74.10	27	7.83E+07		609.40	
	Statherin	67.74	27	1.44E+07		246.80	
	Submaxillary Gland	72.15	37	1.19E+08		491.10	
	Histatin 1	64.91	44	1.81E+07		347.40	
	Histatin 3	62.74	4	4.17E+06		516.50	
	Cystatin-SN	0.00	0	0.00E+00		0.00	
	Alpha-Amylase 1	2.15	1	1.93E+05		271.00	
IR22	Basic Salivary 1	68.62	56	1.75E+08	2.54E+08	703.90	484.06
	Basic Salivary 2	12.74	7	1.04E+07		493.90	
	Basic Salivary 3	75.73	46	2.38E+08		632.80	
	Basic Salivary 4	38.39	20	1.36E+08		508.10	
	Salivary Acidic 1/2	89.76	140	9.88E+08		717.10	
	Statherin	51.61	90	1.62E+08		470.80	
	Submaxillary Gland	72.15	89	1.07E+09		507.00	
	Histatin 1	31.58	13	7.40E+06		423.50	
	Histatin 3	0.00	0	0.00E+00		0.00	
	Cystatin-SN	16.31	7	3.74E+06		507.40	
	Alpha-Amylase 1	2.15	1	4.40E+05		360.20	
IR23	Basic Salivary 1	92.73	152	1.42E+09	2.29E+08	1066.30	566.65
	Basic Salivary 2	52.16	36	8.21E+07		859.80	
	Basic Salivary 3	64.10	42	1.68E+08		783.10	
	Basic Salivary 4	7.10	2	1.69E+07		559.20	
	Salivary Acidic 1/2	83.73	73	4.67E+08		740.00	
	Statherin	69.35	111	1.41E+08		447.00	
	Submaxillary Gland	72.15	70	1.96E+08		537.00	
	Histatin 1	56.14	31	2.67E+07		474.50	
	Histatin 3	0.00	0	0.00E+00		0.00	
	Cystatin-SN	10.64	1	3.35E+05		295.10	
	Alpha-Amylase 1	2.15	1	6.03E+05		471.20	
IR24	Basic Salivary 1	10.97	6	7.90E+06	1.10E+08	554.40	431.72
	Basic Salivary 2	85.34	60	1.59E+08		700.50	
	Basic Salivary 3	70.87	49	3.16E+08		653.80	
	Basic Salivary 4	53.55	42	1.63E+08		579.00	
	Salivary Acidic 1/2	89.76	73	3.57E+08		717.00	
	Statherin	54.84	83	6.36E+07		424.10	
	Submaxillary Gland	72.15	51	1.45E+08		503.70	
	Histatin 1	45.61	10	2.47E+06		339.00	
	Histatin 3	0.00	0.00	0.00E+00		0.00	
	Cystatin-SN	0.00	0.00	0.00E+00		0.00	
	Alpha-Amylase 1	2.15	1	2.71E+05		277.40	
IR25	Basic Salivary 1	92.09	99	3.91E+08	1.50E+08	755.00	446.95
	Basic Salivary 2	22.84	10	1.11E+07		438.00	
	Basic Salivary 3	73.79	47	3.06E+08		778.50	
	Basic Salivary 4	42.58	32	1.40E+08		566.30	
	Salivary Acidic 1/2	86.75	82	4.72E+08		714.80	
	Statherin	61.29	63	5.06E+07		426.50	
	Submaxillary Gland	67.09	56	2.64E+08		496.20	
	Histatin 1	45.61	23	1.21E+07		431.40	
	Histatin 3	0.00	0	0.00E+00		0.00	
	Cystatin-SN	0.00	0	0.00E+00		0.00	
	Alpha-Amylase 1	2.15	1	4.49E+05		309.70	

APPENDIX M: PROTEIN IDENTIFICATION OF VAGINAL FLUID POPULATION SAMPLES

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
3714	CRNN	75.2	90	3.85E+08	1.02E+08	1099.50	605.93
	SBSN	32.5	17	1.52E+07		647.60	
	IVL	23.1	8	8.24E+06		476.70	
	PPL	0.9	1	2.42E+05		199.90	
4669	CRNN	29.1	17	3.04E+06	1.01E+06	201.40	121.10
	SBSN	2.7	2	1.89E+05		67.30	
	IVL	1.9	2	8.06E+05		215.70	
	PPL	0.0	0	0.00E+00		0.00	
5488	CRNN	67.5	93	1.91E+08	5.01E+07	1105.70	434.68
	SBSN	25.8	14	8.49E+06		345.80	
	IVL	2.4	1	7.58E+05		287.20	
	PPL	0.0	0	0.00E+00		0.00	
5560	CRNN	40.0	28	9.83E+06	2.56E+06	629.50	189.83
	SBSN	0.0	0	0.00E+00		0.00	
	IVL	1.9	2	4.26E+05		129.80	
	PPL	0.0	0	0.00E+00		0.00	
5701	CRNN	69.1	68	8.39E+07	2.11E+07	1007.20	313.00
	SBSN	9.8	3	5.23E+05		244.80	
	IVL	0.0	0	0.00E+00		0.00	
	PPL	0.0	0	0.00E+00		0.00	
5854	CRNN	54.3	49	9.91E+07	2.80E+07	956.00	444.35
	SBSN	12.5	5	2.91E+06		146.50	
	IVL	22.4	7	9.34E+06		516.80	
	PPL	0.9	1	5.62E+05		158.10	
6239	CRNN	62.0	69	9.54E+07	2.83E+07	952.80	424.98
	SBSN	15.4	7	4.17E+06		178.60	
	IVL	45.5	33	1.38E+07		568.50	
	PPL	0.0	0	0.00E+00		0.00	
6241	CRNN	63.2	68	1.01E+08	2.65E+07	1022.00	407.98
	SBSN	5.4	3	9.27E+05		75.40	
	IVL	20.7	8	3.34E+06		260.60	
	PPL	0.9	1	5.36E+05		273.90	
6260	CRNN	62.6	42	2.22E+07	5.86E+06	557.30	171.03
	SBSN	15.4	5	1.26E+06		126.80	
	IVL	0.0	0	0.00E+00		0.00	
	PPL	0.0	0	0.00E+00		0.00	
6291	CRNN	51.5	39	4.49E+07	1.29E+07	652.10	284.73
	SBSN	11.7	5	1.72E+06		177.70	
	IVL	6.7	5	5.11E+06		309.10	
	PPL	0.0	0	0.00E+00		0.00	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
6303	CRNN	45.1	46	5.61E+07	1.51E+07	751.50	382.08
	SBSN	10.5	5	4.13E+06		666.10	
	IVL	0.0	0	0.00E+00		0.00	
	PPL	1.1	1	3.04E+05		110.70	
6310	CRNN	45.3	62	1.33E+08	3.50E+07	971.80	438.08
	SBSN	14.1	8	3.01E+06		298.20	
	IVL	10.1	5	3.29E+06		354.80	
	PPL	0.9	1	6.69E+05		127.50	
6311	CRNN	66.3	85	1.11E+08	3.18E+07	766.70	463.78
	SBSN	31.2	16	1.03E+07		415.00	
	IVL	13.9	6	3.33E+06		305.10	
	PPL	2.5	2	2.63E+06		368.30	
6320	CRNN	75.8	94	2.87E+08	8.75E+07	1054.30	596.90
	SBSN	57.3	35	3.85E+07		678.10	
	IVL	24.1	11	1.79E+07		356.10	
	PPL	5.0	6	6.38E+06		299.10	
6343	CRNN	3.2	1	8.44E+04	2.11E+04	36.60	9.15
	SBSN	0.0	0	0.00E+00		0.00	
	IVL	0.0	0	0.00E+00		0.00	
	PPL	0.0	0	0.00E+00		0.00	
6460	CRNN	42.8	41	8.54E+07	2.22E+07	1033.80	307.95
	SBSN	0.0	0	0.00E+00		0.00	
	IVL	10.6	2	3.01E+06		160.00	
	PPL	2.1	2	2.40E+05		38.00	
6551	CRNN	43.6	38	5.54E+07	1.40E+07	936.90	286.93
	SBSN	0.0	0	0.00E+00		0.00	
	IVL	7.4	2	7.40E+05		210.80	
	PPL	0.0	0	0.00E+00		0.00	
6557	CRNN	57.6	59	1.62E+08	5.02E+07	1044.20	514.98
	SBSN	32.7	27	3.73E+07		683.60	
	IVL	2.1	2	6.67E+05		271.80	
	PPL	1.6	2	9.20E+05		60.30	
6587	CRNN	59.6	70	1.53E+08	4.69E+07	690.20	441.00
	SBSN	35.1	26	3.28E+07		604.90	
	IVL	13.9	4	1.60E+06		277.40	
	PPL	0.9	1	4.26E+05		191.50	
6635	CRNN	40.6	38	1.60E+07	9.41E+06	424.40	288.25
	SBSN	15.6	12	3.03E+06		279.60	
	IVL	39.7	42	1.86E+07		449.00	
	PPL	0.0	0	0.00E+00		0.00	
6636	CRNN	52.7	48	3.13E+07	1.23E+07	465.50	226.10
	SBSN	3.4	2	2.47E+05		106.10	
	IVL	44.8	45	1.79E+07		332.80	
	PPL	0.0	0	0.00E+00		0.00	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
6654	CRNN	56.6	53	1.95E+08	5.11E+07	1023.70	401.23
	SBSN	12.0	7	3.29E+06		215.90	
	IVL	18.0	5	6.05E+06		365.30	
	PPL	0.0	0	0.00E+00		0.00	
6661	CRNN	68.7	95	2.69E+08	7.34E+07	1059.10	566.78
	SBSN	22.7	12	9.44E+06		382.70	
	IVL	19.5	11	1.49E+07		508.60	
	PPL	1.0	1	6.64E+05		316.70	
6674	CRNN	67.1	84	1.02E+08	2.61E+07	976.80	367.68
	SBSN	13.1	5	1.64E+06		223.80	
	IVL	4.4	2	2.42E+05		270.10	
	PPL	0.0	0	0.00E+00		0.00	
6681	CRNN	58.6	87	1.71E+08	4.48E+07	753.70	382.98
	SBSN	12.0	5	2.05E+06		225.90	
	IVL	13.9	8	5.03E+06		272.50	
	PPL	1.7	2	1.35E+06		279.80	
A1	CRNN	69.9	79	2.44E+08	7.99E+07	990.50	664.75
	SBSN	11.2	7	7.76E+06		519.80	
	IVL	41.2	28	6.61E+07		675.40	
	PPL	2.1	2	1.16E+06		473.30	
B1	CRNN	49.1	32	4.85E+07	1.29E+07	883.30	289.08
	SBSN	21.2	5	3.14E+06		271.90	
	IVL	3.1	1	1.22E+05		1.10	
	PPL	0.0	0	0.00E+00		0.00	
C1	CRNN	0.0	0	0.00E+00	0.00E+00	0.00	0.00
	SBSN	0.0	0	0.00E+00		0.00	
	IVL	0.0	0	0.00E+00		0.00	
	PPL	0.0	0	0.00E+00		0.00	
D1	CRNN	39.4	48	4.34E+07	1.10E+07	760.80	232.90
	SBSN	0.0	0	0.00E+00		0.00	
	IVL	4.6	2	5.87E+05		170.80	
	PPL	0.0	0	0.00E+00		0.00	
E1	CRNN	72.1	79	3.14E+08	8.62E+07	1104.70	646.35
	SBSN	13.9	9	8.66E+06		621.60	
	IVL	23.3	11	2.11E+07		376.90	
	PPL	1.1	1	7.97E+05		482.20	
F1	CRNN	14.6	7	1.33E+06	3.33E+05	357.90	89.48
	SBSN	0.0	0	0.00E+00		0.00	
	IVL	0.0	0	0.00E+00		0.00	
	PPL	0.0	0	0.00E+00		0.00	
G1	CRNN	62.4	62	1.19E+08	3.13E+07	1081.40	529.28
	SBSN	10.2	3	3.57E+06		349.50	
	IVL	6.5	3	2.10E+06		421.10	
	PPL	2.0	2	7.29E+05		265.10	

APPENDIX N: PEPTIDE IDENTIFICATION OF PERFORMANCE ASSESSMENT SAMPLES

TABLE LEGEND:

Green Box = Positive target peptide identification

Grey Box = Negative target peptide identification, positive protein identification

Red Box = Negative protein identification

NT = Not Tested

Seminal Fluid Sensitivity					Dilution Factor														
Protein	Sequence	Mass (m/z)	Charge (z)	RT	100	100	100	1000	1000	1000	2000	2000	2000	5000	5000	5000	10000	10000	10000
KLK3	F.LRPGDDSSHDLML.L	485.9014	3	8.80															
	R.FLRPGDDSSHDLML.L	534.9242	3	10.60															
	L.LRLSEPAELTD.A	622.3301	2	8.70															
	L.LRLSEPAELTDA.V	657.8486	2	9.20															
	W.GSIEPEEFLTPK.K	673.8456	2	10.50															
PIP	Y.TIEILKVE.-	472.7868	2	6.60															
	F.YTIEILKVE.-	554.3184	2	11.70															
PPAP	F.AELVGPVIPQDW.S	662.3508	2	13.85															
	F.GIWSKVYDPLY.C	670.8479	2	12.65															
	F.GQLTQLGMEQHYEL.G	823.8958	2	10.55															
	F.GQLTQLGMEQHYELGEY.I	998.4595	2	10.70															
SEMG1	Y.DLNALHKTTKSQRH.L	412.9774	4	1.50															
	Q.HGSHGGLDIVIIE.Q	449.5736	2	11.30															
	Y.GENGVQKDVSQRS.I	468.5673	3	1.60															
	V.VEVREEHS.S	492.7409	2	2.85															
	T.NREQDLLSHEQKGRHQ.H	494.5018	4	2.55															
	I.TIPSQEQEHSQKA.N	494.9110	3	1.30															
	Q.TEKLVAGKSQ.I	530.8035	2	1.70															
	Q.NVVEVREEH.S	555.7805	2	3.05															
	N.TEERLWVHG.L	563.7856	2	6.35															
	R.EQDLLSHEQKGRHQ.H	568.9519	3	2.50															
	Q.NVVEVREEHS.S	599.2966	2	3.05															
	Y.SQTEKLVAGKSQ.I	638.3488	2	2.40															
	Q.NVVEVREEHSSK.V	706.8601	2	2.05															
	Q.STNREQDLLSHEQKGRHQ.H	721.6932	3	2.65															
SEMG2	V.DINDHDWTR.K	391.1757	3	4.75															
	Q.NVVDVREEHS.S	395.1949	3	3.30															
	V.DINDHDWTRK.S	433.8741	3	3.85															
	Q.NVVDVREE.H	480.2433	2	4.30															
	Y.NEDRNPIST.-	523.2491	2	3.50															
	Y.DLNALHKATKSKQH.L	530.9610	3	2.05															
	Q.NVVDVREEHSSKLQ.T	547.2848	3	4.30															
	K.DVSQSSISFQIEKLVEGKSQ.I	553.0391	4	13.35															
	S.SISFQIEKLVEGKSQ.I	564.9771	3	11.40															
	Q.IEKLVEGKSQ.I	565.8244	2	3.15															
	Y.HVDINDHDWTRK.S	768.3711	2	4.60															
	K.DVSQSSISFQIEKLVEGKSQIQ.T	817.4306	3	13.80															
	Y.VLQTEELVVNKQQRETK.N	1021.5657	2	6.05															

Saliva Sensitivity					Dilution Factor														
Protein	Sequence	Mass (m/z)	Charge (z)	RT	2	2	2	10	10	10	50	50	50	100	100	100	500	500	500
HIS1	H.SHREFPF.Y	460.2247	2	8.25															
	H.REFPFYGDY.G	597.2682	2	10.58															
	K.HHSHREFPF.Y	597.2836	2	6.35															
	S.HREFPFYGDY.G	665.7962	2	9.85															
	F.YGDYGSNYLYDN.-	722.2886	2	7.33															
PRP_1	Q.GGNKPQGPPPPPGKPQ.G	518.2792	3	3.09															
	Q.GPPQQGGNRPQ.G	568.2828	2	0.99															
	A.GNPQGPSPQGGNKPQ.G	731.8553	2	1.42															
PRP_2	Q.GPPSPPGKPQ.G	481.2587	2	2.85															
	Q.GGNKPQGPPSPPGKPQ.G	514.9390	3	3.24															
	A.GNPQGAPPQGGNKPQ.G	723.8578	2	1.52															
PRP_4	Q.RPPPPPGKPQ.G	535.8089	2	1.39															
	Q.GPPPPPQGGRPP.R	577.3093	2	3.77															
	Q.SHRPPPPPGKPE.R	648.3469	2	1.26															
PRPC	Q.GPPQGQSPQ.-	448.2170	2	1.23															
	Q.GPPPPPPGKPQ.G	534.7955	2	2.76															
	Q.QGPPPPQGKPQ.G	565.8013	2	1.50															
	Q.GPPQQGGHPPPPQGRPQ.G	577.9606	3	2.07															
	Q.GPPQQGGHPRPP.R	612.8153	2	2.13															
SMR3B	P.RGPYPPGPL.A	477.2648	2	7.98															
	P.GIFPPPPPQP.-	523.7877	2	10.65															
	G.RIPPPPPAPY.G	552.8137	2	7.69															
	G.FVPPPPPPPY.G	554.2973	2	9.58															
	P.GRIPPPPPAPY.G	581.3244	2	7.55															
	P.GFVPPPPPPPY.G	582.8080	2	10.66															
	Y.GPGIFPPPPPQP.-	600.8242	2	10.74															
	P.YPPGPLAPPQPF.G	640.8373	2	12.45															
	L.APPQPFGPGFVPPPPPPPY.G	652.6733	3	13.54															
	Y.GPGRIPPPPPAPY.G	658.3615	2	8.13															
	F.GPGFVPPPPPPPY.G	659.8458	2	11.06															
	R.GPYPPGPLAPPQPF.G	717.8744	2	12.78															
	F.GPGFVPPPPPPPYGPG.R	765.3930	2	10.82															
	P.RGPYPPGPLAPPQPF.G	795.9250	2	11.89															
STAT	P.YQPVPEQPL.Y	535.7795	2	8.39															
	P.EQPLYPQPY.Q	567.7769	2	8.34															
	Y.GPYQPVPEQPL.Y	612.8166	2	7.30															
	R.IGRFGYGYGPY.Q	625.3037	2	9.15															
	P.VPEQPLYPQPY.Q	665.8375	2	9.19															
	E.QPLYPQPYQPQ.Y	679.8406	2	7.81															
	P.YQPVPEQPLYPQPYQPQ.Y	691.3423	3	9.89															
	Y.QPVPEQPLYPQPY.Q	778.3932	2	9.69															
	R.FGYGYGPYQPVPEQPL.Y	906.4356	2	11.07															
	Y.QPVPEQPLYPQPYQPQ.Y	954.9782	2	9.22															
	F.GYGYGPYQPVPEQPLYPQPY.Q	1157.0468	2	11.80															

Vaginal Fluid Sensitivity					Dilution Factor														
Protein	Sequence	Mass (m/z)	Charge (z)	RT	2	2	2	10	10	10	50	50	50	100	100	100	500	500	500
CRNN	L.YSYLRSTKP.-	372.2012	3	4.76															
	E.WVDDHSRET.V	382.1712	3	2.41															
	A.DVIVKPHDPA.T	545.7982	2	5.19															
	R.SQTSQAVTGGHTQIQAGSH.T	632.3053	3	3.62															
	L.DEDHTGTVEFK.E	639.2859	2	4.6															
	M.PQLLQNINGIIE.A	676.3826	2	13.12															
	D.VIVKPHDPATVDE.V	710.3775	2	5.25															
	F.ADVIVKPHDPATVDE.V	803.4096	2	6.29															
IVL	V.ELPVEVPSKQEEKH.M	412.9690	4	5.98															
PPL	L.KTENPGDASDLQGRQL.L	864.9292	2	5.71															
SBSN	R.VVQGLHHGVSQAGR.E	722.8920	2	2.31															
	N.NAAGQVGKEADKLIHHGVHHGAN.Q	787.4037	3	6.29															

Sexual Assault Samples - Semen					Oral Swab			Rectal Swab			Vaginal Swab		
Protein	Sequence	Mass (m/z)	Charge (z)	RT	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3
KLK3	F.LRPGDDSSHDLML.L	485.9014	3	8.80									
	R.FLRPGDDSSHDLML.L	534.9242	3	10.60									
	L.LRLSEPAELTD.A	622.3301	2	8.70									
	L.LRLSEPAELTDA.V	657.8498	2	9.20									
	W.GSIEPEEFLTPK.K	673.8456	2	10.50									
PIP	Y.TIEILKVE.-	472.7868	2	6.60									
	F.YTIEILKVE.-	554.3184	2	11.70									
PPAP	F.AELVGPVIPQDW.S	662.3508	2	13.85									
	F.GIWSKVYDPLY.C	670.8479	2	12.65									
	F.GQLTQLGMEQIHYELGEY.I	823.8958	2	10.55									
	F.GQLTQLGMEQIHYELGEY.I	998.4595	2	10.70									
SEMG1	Y.DLNALIHKTTKSQRH.L	412.9774	4	1.50									
	Q.HGSHGGLDIVIIE.Q	449.5736	2	11.30									
	Y.GENGVQKDVSQRS.I	468.5673	3	1.60									
	V.VEVREEHS.S	492.7409	2	2.85									
	T.NREQDLLSHEQKGRHQ.H	494.5018	4	2.55									
	I.TIPSQEQEHSQKA.N	494.9110	3	1.30									
	Q.TEKLVAGKSQ.I	530.8035	2	1.70									
	Q.NVVEVREEH.S	555.7805	2	3.05									
	N.TEERLWVHG.L	563.7856	2	6.35									
	R.EQDLLSHEQKGRHQ.H	568.9519	3	2.50									
	Q.NVVEVREEHS.S	599.2966	2	3.05									
	Y.SQTEKLVAGKSQ.I	638.3488	2	2.40									
	Q.NVVEVREEHSSK.V	706.8601	2	2.05									
	Q.STNREQDLLSHEQKGRHQ.H	721.6932	3	2.65									
SEMG2	V.DINDHDWTR.K	391.1757	3	4.75									
	Q.NVVDVREEHS.S	395.1949	3	3.30									
	V.DINDHDWTRK.S	433.8741	3	3.85									
	Q.NVVDVREE.H	480.2433	2	4.30									
	Y.NEDRNPIST.-	523.2491	2	3.50									
	Y.DLNALIHKATKSKQH.L	530.9610	3	2.05									
	Q.NVVDVREEHSSKLQ.T	547.2848	3	4.30									
	K.DVSQSSISFQIEKLVEGKSQ.I	553.0391	4	13.35									
	S.SISFQIEKLVEGKSQ.I	564.9771	3	11.40									
	Q.IEKLVEGKSQ.I	565.8244	2	3.15									
	Y.HVDINDHDWTRK.S	768.3711	2	4.60									
	K.DVSQSSISFQIEKLVEGKSQIQ.T	817.4306	3	13.80									
	Y.VLQTEELVVNKQQRETK.N	1021.5657	2	6.05									
CRNN	L.YSYLRSTKP.-	372.2012	3	4.76									
	E.WVDDHSRET.V	382.1712	3	2.41									
	A.DVIVKPHDPA.T	545.7982	2	5.19									
	R.SQTSQAVTGGHTQIQAGSH.T	632.3053	3	3.62									
	L.DEDHTGTVEFK.E	839.2859	2	4.6									
	M.PQLLQNINGIIE.A	676.3826	2	13.12									
	D.VIVKPHDPATVDE.V	710.3775	2	5.25									
	F.ADVIVKPHDPATVDE.V	803.4096	2	6.29									
IVL	V.ELPVEVPSKQEEKH.M	412.9690	4	5.98									
PPL	L.KTENPGDASDLQGRQL.L	864.9292	2	5.71									
SBSN	R.VVQGLHHGVSQAGR.E	722.8920	2	2.31									
	N.NAAGQVGKEADKLIHHGVHHGAN.Q	787.4037	3	6.29									

Sexual Assault Samples - Saliva					Breast Swab			Oral Swab			Rectal Swab			Vaginal Swab		
Protein	Sequence	Mass (m/z)	Charge (z)	RT	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3
HIS1	H.SHREFPF.Y	460.2247	2	8.25												
	H.REFPFYGDY.G	597.2682	2	10.58												
	K.HHSHREFPF.Y	597.2636	2	6.35												
	S.HREFPFYGDY.G	665.7962	2	9.85												
	F.YGDYGSNYLYDN.-	722.2886	2	7.33												
PRP_1	Q.GGNKPQGPPPPPGKPQ.G	518.2792	3	3.09												
	Q.GPPQQGGNRPQ.G	568.2828	2	0.99												
	A.GNPQGPSPQGGNKPQ.G	731.8553	2	1.42												
PRP_2	Q.GPPSPPGKPQ.G	481.2587	2	2.85												
	Q.GGNKPQGPPSPPGKPQ.G	514.9390	3	3.24												
	A.GNPQGAPPQGGNKPQ.G	723.8578	2	1.52												
PRP_4	Q.RPPPPPGKPQ.G	535.8089	2	1.39												
	Q.GPPPPPQGGRPPR	577.3093	2	3.77												
	Q.SHRPPPPPGKPER	648.3489	2	1.26												
PRPC	Q.GPPQGQSPQ.-	448.2170	2	1.23												
	Q.GPPPPPPGKPQ.G	534.7955	2	2.76												
	Q.QGPPPPQGKPQ.G	585.8013	2	1.50												
	Q.GPPQQGGHPPPPQGRPQ.G	577.9606	3	2.07												
	Q.GPPQQGGHPRPPR	612.8153	2	2.13												
SMR3B	P.RGPYPPGPL.A	477.2648	2	7.98												
	P.GIFPPPPPQP.-	523.7877	2	10.65												
	G.RIPPPPPAPY.G	552.8137	2	7.69												
	G.FVPPPPPPPY.G	554.2973	2	9.58												
	P.GRIPPPPPAPY.G	581.3244	2	7.55												
	P.GFVPPPPPPPY.G	582.8080	2	10.66												
	Y.GPGIFPPPPPQP.-	600.8242	2	10.74												
	P.YPPGPLAPPQPF.G	640.8373	2	12.45												
	L.APPQPFGPGFVPPPPPPPY.G	652.6733	3	13.54												
	Y.GPGRIPPPPPAPY.G	658.3615	2	8.13												
	F.GPGFVPPPPPPPY.G	659.8458	2	11.06												
	R.GPYPPGPLAPPQPF.G	717.8744	2	12.78												
	F.GPGFVPPPPPPPYGPG.R	765.3930	2	10.82												
	P.RGPYPPGPLAPPQPF.G	795.9250	2	11.89												
STAT	P.YQPVPEQPL.Y	535.7795	2	8.39												
	P.EQPLYPQPY.Q	567.7769	2	8.34												
	Y.GPYQPVPEQPL.Y	612.8166	2	7.30												
	R.IGRFGYGYGPY.Q	625.3037	2	9.15												
	P.VPEQPLYPQPY.Q	665.8375	2	9.19												
	E.QPLYPQPYQPQ.Y	679.8406	2	7.81												
	P.YQPVPEQPLYPQPYQPQ.Y	691.3423	3	9.89												
	Y.QPVPEQPLYPQPY.Q	778.3932	2	9.69												
	R.FGYGYGPYQPVPEQPL.Y	908.4356	2	11.07												
	Y.QPVPEQPLYPQPYQPQ.Y	954.9782	2	9.22												
	F.GYGYGPYQPVPEQPLYPQPY.Q	1157.0468	2	11.80												
CRNN	L.YSYLRSTKP.-	372.2012	3	4.76												
	E.WVDDIHSRET.V	382.1712	3	2.41												
	A.DVIVKPHDPA.T	545.7982	2	5.19												
	R.SQTSQAVTGGHTQIQAGSH.T	632.3053	3	3.62												
	L.DEDHTGTVEFK.E	639.2859	2	4.6												
	M.PQLLQNINGIIE.A	676.3826	2	13.12												
	D.VIVKPHDPATVDE.V	710.3775	2	5.25												
	F.ADVIVKPHDPATVDE.V	803.4096	2	6.29												
IVL	V.ELPVEVPSKQEEKH.M	412.9690	4	5.98												
PPL	L.KTENPGDASDLQGRQL.L	864.9292	2	5.71												
SBSN	R.VVQGLHHGVSQAGR.E	722.8920	2	2.31												
	N.NAAGQVGKEADKLIHHGVHHGAN.Q	787.4037	3	6.29												

Seminal Fluid Aged Samples					Day 2			Day 3			Day 7			Day 30		
Protein	Sequence	Mass (m/z)	Charge (z)	RT	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3
KLK3	F.LRPGDDSSHDLML.L	485.9014	3	8.80												
	R.FLRPGDDSSHDLML.L	534.9242	3	10.60												
	L.LRLSEPAELTD.A	622.3301	2	8.70												
	L.LRLSEPAELTDA.V	657.8486	2	9.20												
	W.GSIEPEEFLTPK.K	673.8456	2	10.50												
PIP	Y.TIEILKVE.-	472.7868	2	6.60												
	F.YTIEILKVE.-	554.3184	2	11.70												
PPAP	F.AELVGPVIPQDW.S	662.3508	2	13.85												
	F.GIWSKVYDPLY.C	670.8479	2	12.65												
	F.GQLTQLGMEQHYEL.G	823.8958	2	10.55												
	F.GQLTQLGMEQHYELGEY.I	998.4595	2	10.70												
SEMG1	Y.DLNALHKTTKSQRH.L	412.9774	4	1.50												
	Q.HGSHGGLDIVIIE.Q	449.5736	2	11.30												
	Y.GENGVQKDVSQRS.I	468.5673	3	1.60												
	V.VEVREEHS.S	492.7409	2	2.85												
	T.NREQDLLSHEQKGRHQ.H	494.5018	4	2.55												
	I.TIPSQEQEHSQKA.N	494.9110	3	1.30												
	Q.TEKLVAGKSQ.I	530.8035	2	1.70												
	Q.NVVEVREEH.S	555.7805	2	3.05												
	N.TEERLWVHG.L	563.7856	2	6.35												
	R.EQDLLSHEQKGRHQ.H	568.9519	3	2.50												
	Q.NVVEVREEHS.S	599.2966	2	3.05												
	Y.SQTEKLVAGKSQ.I	638.3488	2	2.40												
	Q.NVVEVREEHSSK.V	706.8601	2	2.05												
	Q.STNREQDLLSHEQKGRHQ.H	721.6932	3	2.65												
SEMG2	V.DINDHDWTR.K	391.1757	3	4.75												
	Q.NVVDVREEHS.S	395.1949	3	3.30												
	V.DINDHDWTRK.S	433.8741	3	3.85												
	Q.NVVDVREE.H	480.2433	2	4.30												
	Y.NEDRNPIST.-	523.2491	2	3.50												
	Y.DLNALHKATKSKQH.L	530.9610	3	2.05												
	Q.NVVDVREEHSSKLQ.T	547.2848	3	4.30												
	K.DVSQSSISFQIEKLVEGKSQ.I	553.0391	4	13.35												
	S.SISFQIEKLVEGKSQ.I	564.9771	3	11.40												
	Q.IEKLVEGKSQ.I	565.8244	2	3.15												
	Y.HVDINDHDWTRK.S	768.3711	2	4.60												
	K.DVSQSSISFQIEKLVEGKSQIQ.T	817.4306	3	13.80												
	Y.VLQTEELVVNKQQRETK.N	1021.5657	2	6.05												

Saliva Aged Samples					Day 2			Day 3			Day 7			Day 30		
Protein	Sequence	Mass (m/z)	Charge (z)	RT	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3
HIS1	H.SHREFPF.Y	460.2247	2	8.25												
	H.REFPFYGDY.G	597.2682	2	10.58												
	K.HHSHREFPF.Y	597.2836	2	6.35												
	S.HREFPFYGDY.G	665.7962	2	9.85												
	F.YGDYGSNYLYDN.-	722.2886	2	7.33												
PRP_1	Q.GGNKPQGPPPPPGKPQ.G	518.2792	3	3.09												
	Q.GPPQQGGNRPQ.G	568.2828	2	0.99												
	A.GNPQGPSPQGGNKPQ.G	731.8553	2	1.42												
PRP_2	Q.GPPSPPGKPQ.G	481.2587	2	2.85												
	Q.GGNKPQGPPSPPGKPQ.G	514.9390	3	3.24												
	A.GNPQGAPPQGGNKPQ.G	723.8578	2	1.52												
PRP_4	Q.RPPPPPGKPQ.G	535.8089	2	1.39												
	Q.GPPPPPQGGRPP.R	577.3093	2	3.77												
	Q.SHRPPPPPGKPE.R	648.3469	2	1.26												
PRPC	Q.GPPQGQSPQ.-	448.2170	2	1.23												
	Q.GPPPPPPGKPQ.G	534.7955	2	2.76												
	Q.QGPPPPQGKPQ.G	565.8013	2	1.50												
	Q.GPPQQGGHPPPPQGRPQ.G	577.9606	3	2.07												
	Q.GPPQQGGHPRPP.R	612.8153	2	2.13												
SMR3B	P.RGPYPPGPL.A	477.2648	2	7.98												
	P.GIFPPPPPQP.-	523.7877	2	10.65												
	G.RIPPPPPAPY.G	552.8137	2	7.69												
	G.FVPPPPPPPY.G	554.2973	2	9.58												
	P.GRIPPPPPAPY.G	581.3244	2	7.55												
	P.GFVPPPPPPPY.G	582.8080	2	10.66												
	Y.GPGIFPPPPPQP.-	600.8242	2	10.74												
	P.YPPGPLAPPQPF.G	640.8373	2	12.45												
	L.APPQPFGPGFVPPPPPPPY.G	652.6733	3	13.54												
	Y.GPGRIPPPPPAPY.G	658.3615	2	8.13												
	F.GPGFVPPPPPPPY.G	659.8458	2	11.06												
	R.GPYPPGPLAPPQPF.G	717.8744	2	12.78												
	F.GPGFVPPPPPPPYGPG.R	765.3930	2	10.82												
	P.RGPYPPGPLAPPQPF.G	795.9250	2	11.89												
STAT	P.YQPVPEQPL.Y	535.7795	2	8.39												
	P.EQPLYPQPY.Q	567.7769	2	8.34												
	Y.GPYQPVPEQPL.Y	612.8166	2	7.30												
	R.IGRFGYGYGPY.Q	625.3037	2	9.15												
	P.VPEQPLYPQPY.Q	665.8375	2	9.19												
	E.QPLYPQPYQPQ.Y	679.8406	2	7.81												
	P.YQPVPEQPLYPQPYQPQ.Y	691.3423	3	9.89												
	Y.QPVPEQPLYPQPY.Q	778.3932	2	9.69												
	R.FGYGYGPYQPVPEQPL.Y	906.4356	2	11.07												
	Y.QPVPEQPLYPQPYQPQ.Y	954.9782	2	9.22												
	F.GYGYGPYQPVPEQPLYPQPY.Q	1157.0468	2	11.80												

Vaginal Swab Aged Samples					Day 2			Day 3			Day 7			Day 30		
Protein	Sequence	Mass (m/z)	Charge (z)	RT	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3
CRNN	L.YSYLRSTKP.-	372.2012	3	4.76												
	E.WVDDHSRET.V	382.1712	3	2.41												
	A.DVIVKPHDPA.T	545.7982	2	5.19												
	R.SQTSQAVTGGHTQIQAGSH.T	632.3053	3	3.62												
	L.DEDHTGTVEFK.E	639.2859	2	4.6												
	M.PQLLQNINGIIE.A	676.3826	2	13.12												
	D.VIVKPHDPATVDE.V	710.3775	2	5.25												
	F.ADVIVKPHDPATVDE.V	803.4096	2	6.29												
IVL	V.ELPVEVPSKQEEKH.M	412.9690	4	5.98												
PPL	L.KTENPGDASDLQGRQL.L	864.9292	2	5.71												
SBSN	R.VVQGLHHGVSQAGR.E	722.8920	2	2.31												
	N.NAAGQVGKEADKLIHHGVHHGAN.Q	787.4037	3	6.29												

Seminal Fluid on Vaginal Swab Aged Samples					Day 2			Day 3			Day 7			Day 30		
Protein	Sequence	Mass (m/z)	Charge (z)	RT	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3
KLK3	F.LRPGDDSSHDLML.L	485.9014	3	8.8												
	R.FLRPGDDSSHDLML.L	534.9242	3	10.6												
	L.LRLSEPAELTD.A	622.3301	2	8.7												
	L.LRLSEPAELTDA.V	657.8486	2	9.2												
	W.GSIEPEEFLTPK.K	673.8456	2	10.5												
PIP	Y.TIEILKVE.-	472.7868	2	6.6												
	F.YTIEILKVE.-	554.3184	2	11.7												
PPAP	F.AELVGPVIPQDW.S	662.3508	2	13.85												
	F.GIWSKVYDPLY.C	670.8479	2	12.65												
	F.GQLTQLGMEQHYEL.G	823.8958	2	10.55												
	F.GQLTQLGMEQHYELGEY.I	998.4595	2	10.7												
SEMG1	Y.DLNALHKTTKSQRH.L	412.9774	4	1.5												
	Q.HGSHGGLDIVIIE.Q	449.5736	2	11.3												
	Y.GENGVQKDVSQRS.I	468.5673	3	1.6												
	V.VEVREEHS.S	492.7409	2	2.85												
	T.NREQDLLSHEQKGRHQ.H	494.5018	4	2.55												
	I.TIPSQEQEHSQKA.N	494.9110	3	1.3												
	Q.TEKLVAGKSQ.I	530.8035	2	1.7												
	Q.NVVEVREEH.S	555.7805	2	3.05												
	N.TEERLWVHG.L	563.7856	2	6.35												
	R.EQDLLSHEQKGRHQ.H	568.9519	3	2.5												
	Q.NVVEVREEHS.S	599.2966	2	3.05												
	Y.SQTEKLVAGKSQ.I	638.3488	2	2.4												
	Q.NVVEVREEHSSK.V	706.8601	2	2.05												
	Q.STNREQDLLSHEQKGRHQ.H	721.6932	3	2.65												
SEMG2	V.DINDHDWTR.K	391.1757	3	4.75												
	Q.NVVDVREEHS.S	395.1949	3	3.3												
	V.DINDHDWTRK.S	433.8741	3	3.85												
	Q.NVVDVREE.H	480.2433	2	4.3												
	Y.NEDRNPIST.-	523.2491	2	3.5												
	Y.DLNALHKATKSKQH.L	530.9610	3	2.05												
	Q.NVVDVREEHSSKLQ.T	547.2848	3	4.3												
	K.DVSQSSISFQIEKLVEGKSQ.I	553.0391	4	13.35												
	S.SISFQIEKLVEGKSQ.I	564.9771	3	11.4												
	Q.IEKLVEGKSQ.I	565.8244	2	3.15												
	Y.HVDINDHDWTRK.S	768.3711	2	4.6												
	K.DVSQSSISFQIEKLVEGKSQIQ.T	817.4306	3	13.8												
	Y.VLQTEELVVNKQQRETK.N	1021.5657	2	6.05												
CRNN	L.YSYLRSTKP.-	372.2012	3	4.76												
	E.WVDDHSRET.V	382.1712	3	2.41												
	A.DVIVKPHDPA.T	545.7982	2	5.19												
	R.SQTSQAVTGGHTQIQAGSH.T	632.3053	3	3.62												
	L.DEDHTGTVEFK.E	639.2859	2	4.60												
	M.PQLLQNINGIIE.A	676.3826	2	13.12												
	D.VIVKPHDPATVDE.V	710.3775	2	5.25												
	F.ADVIVKPHDPATVDE.V	803.4096	2	6.29												
IVL	V.ELPVEVPSKQEEKH.M	412.9690	4	5.98												
PPL	L.KTENPGDASDLQGRQL.L	864.9292	2	5.71												
SBSN	R.VVQGLHHGVSQAGR.E	722.8920	2	2.31												
	N.NAAGQVGKEADKLIHHGVHHGAN.Q	787.4037	3	6.29												

Saliva on Vaginal Swab Aged Samples					Day 2			Day 3			Day 7			Day 30		
Protein	Sequence	Mass (m/z)	Charge (z)	RT	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3
HIS1	H.SHREFPF.Y	460.2247	2	8.25												
	H.REFPFYGDY.G	597.2682	2	10.58												
	K.HHSHREFPF.Y	597.2836	2	6.35												
	S.HREFPFYGDY.G	665.7962	2	9.85												
	F.YGDYGSNYLYDN.-	722.2886	2	7.33												
PRP_1	Q.GGNKPQGPPPPPGKPQ.G	518.2792	3	3.09												
	Q.GPPQQGGNRPQ.G	568.2828	2	0.99												
	A.GNPQGPSPQGGNKPQ.G	731.8553	2	1.42												
PRP_2	Q.GPPSPPGKPQ.G	481.2587	2	2.85												
	Q.GGNKPQGPPSPPGKPQ.G	514.9390	3	3.24												
	A.GNPQGAPPQGGNKPQ.G	723.8578	2	1.52												
PRP_4	Q.RPPPPPGKPQ.G	535.8089	2	1.39												
	Q.GPPPPPQGGRPP.R	577.3093	2	3.77												
	Q.SHRPPPPPGKPE.R	648.3469	2	1.26												
PRPC	Q.GPPQGQSPQ.-	448.2170	2	1.23												
	Q.GPPPPPPGKPQ.G	534.7955	2	2.76												
	Q.QGPPPPQGKPQ.G	565.8013	2	1.50												
	Q.GPPQQGGHPPPPQGRPQ.G	577.9606	3	2.07												
	Q.GPPQQGGHPRPP.R	612.8153	2	2.13												
SMR3B	P.RGPYPPGPL.A	477.2648	2	7.98												
	P.GIFPPPPPQP.-	523.7877	2	10.65												
	G.RIPPPPPAPY.G	552.8137	2	7.69												
	G.FVPPPPPPPY.G	554.2973	2	9.58												
	P.GRIPPPPPAPY.G	581.3244	2	7.55												
	P.GFVPPPPPPPY.G	582.8080	2	10.66												
	Y.GPGIFPPPPPQP.-	600.8242	2	10.74												
	P.YPPGPLAPPQPF.G	640.8373	2	12.45												
	L.APPQPFGPGFVPPPPPPPY.G	652.6733	3	13.54												
	Y.GPGRIPPPPPAPY.G	658.3615	2	8.13												
	F.GPGFVPPPPPPPY.G	659.8458	2	11.06												
	R.GPYPPGPLAPPQPF.G	717.8744	2	12.78												
	F.GPGFVPPPPPPPYGPG.R	765.3930	2	10.82												
	P.RGPYPPGPLAPPQPF.G	795.9250	2	11.89												
STAT	P.YQPVPEQPL.Y	535.7795	2	8.39												
	P.EQPLYPQPY.Q	567.7769	2	8.34												
	Y.GPYQPVPEQPL.Y	612.8166	2	7.30												
	R.IGRFGYGYGPY.Q	625.3037	2	9.15												
	P.VPEQPLYPQPY.Q	665.8375	2	9.19												
	E.QPLYPQPYQPQ.Y	679.8406	2	7.81												
	P.YQPVPEQPLYPQPYQPQ.Y	691.3423	3	9.89												
	Y.QPVPEQPLYPQPY.Q	778.3932	2	9.69												
	R.FGYGYGPYQPVPEQPL.Y	906.4356	2	11.07												
	Y.QPVPEQPLYPQPYQPQ.Y	954.9782	2	9.22												
	F.GYGYGPYQPVPEQPLYPQPY.Q	1157.0468	2	11.80												
CRNN	L.YSYLRSTKP.-	372.2012	3	4.76												
	E.WVDDHSRET.V	382.1712	3	2.41												
	A.DVIVKPHDPA.T	545.7982	2	5.195												
	R.SQTSQAVTGGHTQIQAGSH.T	632.3053	3	3.625												
	L.DEDHTGTVEFK.E	639.2859	2	4.6												
	M.PQLLQNINGIIE.A	676.3826	2	13.125												
	D.VIVKPHDPATVDE.V	710.3775	2	5.25												
	F.ADVIVKPHDPATVDE.V	803.4096	2	6.295												
IVL	V.ELPVEVPSKQEEKH.M	412.9690	4	5.985												
PPL	L.KTENPGDASDLQGRQL.L	864.9292	2	5.71												
SBSN	R.VVQGLHHGVSQAGR.E	722.8920	2	2.31												
	N.NAAGQVGKEADKLIHHGVHHGAN.Q	787.4037	3	6.295												

Seminal Fluid Substrates					Underwear			Polyester Bed Sheet			PolyBlend Bed Sheet			Denim		
Protein	Sequence	Mass (m/z)	Charge (z)	RT	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3
KLK3	F.LRPGDDSSHDLML.L	485.9014	3	8.80												
	R.FLRPGDDSSHDLML.L	534.9242	3	10.60												
	L.LRLSEPAELTD.A	622.3301	2	8.70												
	L.LRLSEPAELTDA.V	657.8486	2	9.20												
	W.GSIEPEEFLTPK.K	673.8456	2	10.50												
PIP	Y.TIEILKVE.-	472.7868	2	6.60												
	F.YTIEILKVE.-	554.3184	2	11.70												
PPAP	F.AELVGPVIPQDW.S	662.3508	2	13.85												
	F.GIWSKVYDPLY.C	670.8479	2	12.65												
	F.GQLTQLGMEQHYEL.G	823.8958	2	10.55												
	F.GQLTQLGMEQHYELGEY.I	998.4595	2	10.70												
SEMG1	Y.DLNALHKTTKSQRH.L	412.9774	4	1.50												
	Q.HGSHGGLDIVIIE.Q	449.5736	2	11.30												
	Y.GENGVQKDVSQRS.I	468.5673	3	1.60												
	V.VEVREEHS.S	492.7409	2	2.85												
	T.NREQDLLSHEQKGRHQ.H	494.5018	4	2.55												
	I.TIPSQEQEHSQKA.N	494.9110	3	1.30												
	Q.TEKLVAGKSQ.I	530.8035	2	1.70												
	Q.NVVEVREEH.S	555.7805	2	3.05												
	N.TEERLWVHG.L	563.7856	2	6.35												
	R.EQDLLSHEQKGRHQ.H	568.9519	3	2.50												
	Q.NVVEVREEHS.S	599.2966	2	3.05												
	Y.SQTEKLVAGKSQ.I	638.3488	2	2.40												
	Q.NVVEVREEHSSK.V	706.8601	2	2.05												
	Q.STNREQDLLSHEQKGRHQ.H	721.6932	3	2.65												
SEMG2	V.DINDHDWTR.K	391.1757	3	4.75												
	Q.NVVDVREEHS.S	395.1949	3	3.30												
	V.DINDHDWTRK.S	433.8741	3	3.85												
	Q.NVVDVREE.H	480.2433	2	4.30												
	Y.NEDRNPIST.-	523.2491	2	3.50												
	Y.DLNALHKATKSKQH.L	530.9610	3	2.05												
	Q.NVVDVREEHSSKLQ.T	547.2848	3	4.30												
	K.DVSQSSISFQIEKLVEGKSQ.I	553.0391	4	13.35												
	S.SISFQIEKLVEGKSQ.I	564.9771	3	11.40												
	Q.IEKLVEGKSQ.I	565.8244	2	3.15												
	Y.HVDINDHDWTRK.S	768.3711	2	4.60												
	K.DVSQSSISFQIEKLVEGKSQIQ.T	817.4306	3	13.80												
	Y.VLQTEELVVNKQQRETK.N	1021.5657	2	6.05												
CRNN	L.YSYLRSTKP.-	372.2012	3	4.76				NT	NT	NT	NT	NT	NT	NT	NT	NT
	E.WVDDHSRET.V	382.1712	3	2.41				NT	NT	NT	NT	NT	NT	NT	NT	NT
	A.DVIVKPHDPA.T	545.7982	2	5.195				NT	NT	NT	NT	NT	NT	NT	NT	NT
	R.SQTSQAVTGGHTQIQAGSH.T	632.3053	3	3.625				NT	NT	NT	NT	NT	NT	NT	NT	NT
	L.DEDHTGTVEFK.E	639.2859	2	4.6				NT	NT	NT	NT	NT	NT	NT	NT	NT
	M.PQLLQNINGIIE.A	676.3826	2	13.12				NT	NT	NT	NT	NT	NT	NT	NT	NT
	D.VIVKPHDPATVDE.V	710.3775	2	5.25				NT	NT	NT	NT	NT	NT	NT	NT	NT
	F.ADVIVKPHDPATVDE.V	803.4096	2	6.295				NT	NT	NT	NT	NT	NT	NT	NT	NT
IVL	V.ELPVEVPSKQEEKH.M	412.9690	4	5.985				NT	NT	NT	NT	NT	NT	NT	NT	NT
PPL	L.KTENPGDASDLQGRQL.L	864.9292	2	5.71				NT	NT	NT	NT	NT	NT	NT	NT	NT
SBSN	R.VVQGLHHGVSQAGR.E	722.8920	2	2.31				NT	NT	NT	NT	NT	NT	NT	NT	NT
	N.NAAGQVGKEADKLIHHGVHHGAN.Q	787.4037	3	6.295				NT	NT	NT	NT	NT	NT	NT	NT	NT

Saliva Substrates					Polyester Bed Sheet			PolyBlend Bed Sheet			Denim		
Protein	Sequence	Mass (m/z)	Charge (z)	RT	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3
HIS1	H.SHREFPF.Y	460.2247	2	8.25									
	H.REFPFYGDY.G	597.2682	2	10.58									
	K.HHSHREFPF.Y	597.2836	2	6.35									
	S.HREFPFYGDY.G	665.7962	2	9.85									
	F.YGDYGSNYLYDN.-	722.2886	2	7.33									
PRP_1	Q.GGNKPQGPPPPPGKPQ.G	518.2792	3	3.09									
	Q.GPPQQGGNRPQ.G	568.2828	2	0.99									
	A.GNPQGPSPQGGNKPQ.G	731.8553	2	1.42									
PRP_2	Q.GPPSPPGKPQ.G	481.2587	2	2.85									
	Q.GGNKPQGPPSPPGKPQ.G	514.9390	3	3.24									
	A.GNPQGAPPQGGNKPQ.G	723.8578	2	1.52									
PRP_4	Q.RPPPPPGKPQ.G	535.8089	2	1.39									
	Q.GPPPPPQGGRPP.R	577.3093	2	3.77									
	Q.SHRPPPPPGKPE.R	648.3469	2	1.26									
PRPC	Q.GPPQGQSPQ.-	448.2170	2	1.23									
	Q.GPPPPPPGKPQ.G	534.7955	2	2.76									
	Q.QGPPPPQGKPQ.G	565.8013	2	1.50									
	Q.GPPQQGGHPPPPQGRPQ.G	577.9606	3	2.07									
	Q.GPPQQGGHPRPP.R	612.8153	2	2.13									
SMR3B	P.RGPYPPGPL.A	477.2648	2	7.98									
	P.GIFPPPPPQP.-	523.7877	2	10.65									
	G.RIPPPPPAPY.G	552.8137	2	7.69									
	G.FVPPPPPPPY.G	554.2973	2	9.58									
	P.GRIPPPPPAPY.G	581.3244	2	7.55									
	P.GFVPPPPPPPY.G	582.8080	2	10.66									
	Y.GPGIFPPPPPQP.-	600.8242	2	10.74									
	P.YPPGPLAPPQPF.G	640.8373	2	12.45									
	L.APPQPFGPGFVPPPPPPPY.G	652.6733	3	13.54									
	Y.GPGRIPPPPPAPY.G	658.3615	2	8.13									
	F.GPGFVPPPPPPPY.G	659.8458	2	11.06									
	R.GPYPPGPLAPPQPF.G	717.8744	2	12.78									
	F.GPGFVPPPPPPPYGPG.R	765.3930	2	10.82									
	P.RGPYPPGPLAPPQPF.G	795.9250	2	11.89									
STAT	P.YQPVPEQPL.Y	535.7795	2	8.39									
	P.EQPLYPQPY.Q	567.7769	2	8.34									
	Y.GPYQPVPEQPL.Y	612.8166	2	7.30									
	R.IGRFGYGYGPY.Q	625.3037	2	9.15									
	P.VPEQPLYPQPY.Q	665.8375	2	9.19									
	E.QPLYPQPYQPQ.Y	679.8406	2	7.81									
	P.YQPVPEQPLYPQPYQPQ.Y	691.3423	3	9.89									
	Y.QPVPEQPLYPQPY.Q	778.3932	2	9.69									
	R.FGYGYGPYQPVPEQPL.Y	906.4356	2	11.07									
	Y.QPVPEQPLYPQPYQPQ.Y	954.9782	2	9.22									
	F.GYGYGPYQPVPEQPLYPQPY.Q	1157.0468	2	11.80									

Seminal Fluid Contaminants					Water-Based Lubricant			Natural Lubricant			Silicon-Based Lubricant		
Protein	Sequence	Mass (m/z)	Charge (z)	RT	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3
KLK3	F.LRPGDDSSHDLML.L	485.9014	3	8.80									
	R.FLRPGDDSSHDLML.L	534.9242	3	10.60									
	L.LRLSEPAELTD.A	622.3301	2	8.70									
	L.LRLSEPAELTDA.V	657.8486	2	9.20									
	W.GSIEPEEFLTPK.K	673.8456	2	10.50									
PIP	Y.TIEILKVE.-	472.7868	2	6.60									
	F.YTIEILKVE.-	554.3184	2	11.70									
PPAP	F.AELVGPVIPQDW.S	662.3508	2	13.85									
	F.GIWSKVYDPLY.C	670.8479	2	12.65									
	F.GQLTQLGMEQHYEL.G	823.8958	2	10.55									
	F.GQLTQLGMEQHYELGEY.I	998.4595	2	10.70									
SEMG1	Y.DLNALHKTTKSQRH.L	412.9774	4	1.50									
	Q.HGSHGGLDIVIIE.Q	449.5736	2	11.30									
	Y.GENGVQKDVSQRS.I	468.5673	3	1.60									
	V.VEVREEHS.S	492.7409	2	2.85									
	T.NREQDLLSHEQKGRHQ.H	494.5018	4	2.55									
	I.TIPSQEQEHSQKA.N	494.9110	3	1.30									
	Q.TEKLVAGKSQ.I	530.8035	2	1.70									
	Q.NVVEVREEH.S	555.7805	2	3.05									
	N.TEERLWVHG.L	563.7856	2	6.35									
	R.EQDLLSHEQKGRHQ.H	568.9519	3	2.50									
	Q.NVVEVREEHS.S	599.2966	2	3.05									
	Y.SQTEKLVAGKSQ.I	638.3488	2	2.40									
	Q.NVVEVREEHSSK.V	706.8601	2	2.05									
	Q.STNREQDLLSHEQKGRHQ.H	721.6932	3	2.65									
SEMG2	V.DINDHDWTR.K	391.1757	3	4.75									
	Q.NVVDVREEHS.S	395.1949	3	3.30									
	V.DINDHDWTRK.S	433.8741	3	3.85									
	Q.NVVDVREE.H	480.2433	2	4.30									
	Y.NEDRNPIST.-	523.2491	2	3.50									
	Y.DLNALHKATKSKQH.L	530.9610	3	2.05									
	Q.NVVDVREEHSSKLQ.T	547.2848	3	4.30									
	K.DVSQSSISFQIEKLVEGKSQ.I	553.0391	4	13.35									
	S.SISFQIEKLVEGKSQ.I	564.9771	3	11.40									
	Q.IEKLVEGKSQ.I	565.8244	2	3.15									
	Y.HVDINDHDWTRK.S	768.3711	2	4.60									
	K.DVSQSSISFQIEKLVEGKSQIQ.T	817.4306	3	13.80									
	Y.VLQTEELVVNKQQRETK.N	1021.5657	2	6.05									
CRNN	L.YSYLRSTKP.-	372.2012	3	4.76									
	E.WVDDHSRET.V	382.1712	3	2.41									
	A.DVIVKPHDPA.T	545.7982	2	5.19									
	R.SQTSQAVTGGHTQIQAGSH.T	632.3053	3	3.62									
	L.DEDHTGTVEFK.E	639.2859	2	4.6									
	M.PQLLQNINGIIE.A	676.3826	2	13.12									
	D.VIVKPHDPATVDE.V	710.3775	2	5.25									
	F.ADVIVKPHDPATVDE.V	803.4096	2	6.29									
IVL	V.ELPVEVPSKQEEKH.M	412.9690	4	5.98									
PPL	L.KTENPGDASDLQGRQL.L	864.9292	2	5.71									
SBSN	R.VVQGLHHGVSQAGR.E	722.8920	2	2.31									
	N.NAAGQVGKEADKLIHHGVHHGAN.Q	787.4037	3	6.29									

Seminal Fluid Contaminants					10% Bleach			Dish Soap			Menstrual Swab		
Protein	Sequence	Mass (m/z)	Charge (z)	RT	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3
KLK3	F.LRPGDDSSHDLML.L	485.9014	3	8.80									
	R.FLRPGDDSSHDLML.L	534.9242	3	10.60									
	L.LRLSEPAELTD.A	622.3301	2	8.70									
	L.LRLSEPAELTDA.V	657.8486	2	9.20									
	W.GSIEPEEFLTPK.K	673.8456	2	10.50									
PIP	Y.TIEILKVE.-	472.7868	2	6.60									
	F.YTIEILKVE.-	554.3184	2	11.70									
PPAP	F.AELVGPVIPQDW.S	662.3508	2	13.85									
	F.GIWSKVYDPLY.C	670.8479	2	12.65									
	F.GQLTQLGMEQHYEL.G	823.8958	2	10.55									
	F.GQLTQLGMEQHYELGEY.I	998.4595	2	10.70									
SEMG1	Y.DLNALHKTTKSQRH.L	412.9774	4	1.50									
	Q.HGSHGGLDIVIIE.Q	449.5736	2	11.30									
	Y.GENGVQKDVSQRS.I	468.5673	3	1.60									
	V.VEVREEHS.S	492.7409	2	2.85									
	T.NREQDLLSHEQKGRHQ.H	494.5018	4	2.55									
	I.TIPSQEQEHSQKA.N	494.9110	3	1.30									
	Q.TEKLVAGKSQ.I	530.8035	2	1.70									
	Q.NVVEVREEH.S	555.7805	2	3.05									
	N.TEERLWVHG.L	563.7856	2	6.35									
	R.EQDLLSHEQKGRHQ.H	568.9519	3	2.50									
	Q.NVVEVREEHS.S	599.2966	2	3.05									
	Y.SQTEKLVAGKSQ.I	638.3488	2	2.40									
	Q.NVVEVREEHSSK.V	706.8601	2	2.05									
	Q.STNREQDLLSHEQKGRHQ.H	721.6932	3	2.65									
SEMG2	V.DINDHDWTR.K	391.1757	3	4.75									
	Q.NVVDVREEHS.S	395.1949	3	3.30									
	V.DINDHDWTRK.S	433.8741	3	3.85									
	Q.NVVDVREE.H	480.2433	2	4.30									
	Y.NEDRNPIST.-	523.2491	2	3.50									
	Y.DLNALHKATKSKQH.L	530.9610	3	2.05									
	Q.NVVDVREEHSSKLQ.T	547.2848	3	4.30									
	K.DVSQSSISFQIEKLVEGKSQ.I	553.0391	4	13.35									
	S.SISFQIEKLVEGKSQ.I	564.9771	3	11.40									
	Q.IEKLVEGKSQ.I	565.8244	2	3.15									
	Y.HVDINDHDWTRK.S	768.3711	2	4.60									
	K.DVSQSSISFQIEKLVEGKSQIQ.T	817.4306	3	13.80									
	Y.VLQTEELVVNKQQRETK.N	1021.5657	2	6.05									
CRNN	L.YSYLRSTKP.-	372.2012	3	4.76	NT	NT	NT	NT	NT	NT			
	E.WVDDHSRET.V	382.1712	3	2.41	NT	NT	NT	NT	NT	NT			
	A.DVIVKPHDPA.T	545.7982	2	5.19	NT	NT	NT	NT	NT	NT			
	R.SQTSQAVTGGHTQIQAGSH.T	632.3053	3	3.62	NT	NT	NT	NT	NT	NT			
	L.DEDHTGTVEFK.E	639.2859	2	4.6	NT	NT	NT	NT	NT	NT			
	M.PQLLQNINGIIE.A	676.3826	2	13.12	NT	NT	NT	NT	NT	NT			
	D.VIVKPHDPATVDE.V	710.3775	2	5.25	NT	NT	NT	NT	NT	NT			
	F.ADVIVKPHDPATVDE.V	803.4096	2	6.29	NT	NT	NT	NT	NT	NT			
IVL	V.ELPVEVPSKQEEKH.M	412.9690	4	5.98	NT	NT	NT	NT	NT	NT			
PPL	L.KTENPGDASDLQGRQL.L	864.9292	2	5.71	NT	NT	NT	NT	NT	NT			
SBSN	R.VVQGLHHGVSQAGR.E	722.8920	2	2.31	NT	NT	NT	NT	NT	NT			
	N.NAAGQVGKEADKLIHHGVHHGAN.Q	787.4037	3	6.29	NT	NT	NT	NT	NT	NT			

Saliva and Vaginal Fluid Contaminants					10% Bleach			Dish Soap			Menstrual Swab			Menstrual Swab + Saliva		
Protein	Sequence	Mass (m/z)	Charge (z)	RT	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3
HIS1	H.SHREFPF.Y	460.2247	2	8.25							NT	NT	NT			
	H.REFPFYGDY.G	597.2682	2	10.58							NT	NT	NT			
	K.HHSHREFPF.Y	597.2836	2	6.35							NT	NT	NT			
	S.HREFPFYGDY.G	665.7962	2	9.85							NT	NT	NT			
	F.YGDYGSNYLYDN.-	722.2886	2	7.33							NT	NT	NT			
PRP_1	Q.GGNKPQGPPPPPGKPQ.G	518.2792	3	3.09							NT	NT	NT			
	Q.GPPQQGGNRPQ.G	568.2828	2	0.99							NT	NT	NT			
	A.GNPQGPSPQGGNKPQ.G	731.8553	2	1.42							NT	NT	NT			
PRP_2	Q.GPPSPPGKPQ.G	481.2587	2	2.85							NT	NT	NT			
	Q.GGNKPQGPPSPPGKPQ.G	514.9390	3	3.24							NT	NT	NT			
	A.GNPQGAPPQGGNKPQ.G	723.8578	2	1.52							NT	NT	NT			
PRP_4	Q.RPPPPPGKPQ.G	535.8089	2	1.39							NT	NT	NT			
	Q.GPPPPPQGGRPP.R	577.3093	2	3.77							NT	NT	NT			
	Q.SHRPPPPPGKPE.R	648.3469	2	1.26							NT	NT	NT			
PRPC	Q.GPPQGQSPQ.-	448.2170	2	1.23							NT	NT	NT			
	Q.GPPPPPPGKPQ.G	534.7955	2	2.76							NT	NT	NT			
	Q.QGPPPPQGKPQ.G	565.8013	2	1.50							NT	NT	NT			
	Q.GPPQQGGHPPPPQGRPQ.G	577.9606	3	2.07							NT	NT	NT			
	Q.GPPQQGGHPRPP.R	612.8153	2	2.13							NT	NT	NT			
SMR3B	P.RGPYPPGPL.A	477.2648	2	7.98							NT	NT	NT			
	P.GIFPPPPPQP.-	523.7877	2	10.65							NT	NT	NT			
	G.RIPPPPPAPY.G	552.8137	2	7.69							NT	NT	NT			
	G.FVPPPPPPPY.G	554.2973	2	9.58							NT	NT	NT			
	P.GRIPPPPPAPY.G	581.3244	2	7.55							NT	NT	NT			
	P.GFVPPPPPPPY.G	582.8080	2	10.66							NT	NT	NT			
	Y.GPGIFPPPPPQP.-	600.8242	2	10.74							NT	NT	NT			
	P.YPPGPLAPPQPF.G	640.8373	2	12.45							NT	NT	NT			
	L.APPQPFGPGFVPPPPPPPY.G	652.6733	3	13.54							NT	NT	NT			
	Y.GPGRIPPPPPAPY.G	658.3615	2	8.13							NT	NT	NT			
	F.GPGFVPPPPPPPY.G	659.8458	2	11.06							NT	NT	NT			
	R.GPYPPGPLAPPQPF.G	717.8744	2	12.78							NT	NT	NT			
	F.GPGFVPPPPPPPYGPG.R	765.3930	2	10.82							NT	NT	NT			
	P.RGPYPPGPLAPPQPF.G	795.9250	2	11.89							NT	NT	NT			
STAT	P.YQPVPEQPL.Y	535.7795	2	8.39							NT	NT	NT			
	P.EQPLYPQPY.Q	567.7769	2	8.34							NT	NT	NT			
	Y.GPYQPVPEQPL.Y	612.8166	2	7.30							NT	NT	NT			
	R.IGRFGYGYGPY.Q	625.3037	2	9.15							NT	NT	NT			
	P.VPEQPLYPQPY.Q	665.8375	2	9.19							NT	NT	NT			
	E.QPLYPQPYQPQ.Y	679.8406	2	7.81							NT	NT	NT			
	P.YQPVPEQPLYPQPYQPQ.Y	691.3423	3	9.89							NT	NT	NT			
	Y.QPVPEQPLYPQPY.Q	778.3932	2	9.69							NT	NT	NT			
	R.FGYGYGPYQPVPEQPL.Y	906.4356	2	11.07							NT	NT	NT			
	Y.QPVPEQPLYPQPYQPQ.Y	954.9782	2	9.22							NT	NT	NT			
	F.GYGYGPYQPVPEQPLYPQPY.Q	1157.0468	2	11.80							NT	NT	NT			
CRNN	L.YSYLRSTKP.-	372.2012	3	4.76	NT	NT	NT	NT	NT	NT						
	E.WVDDHSRET.V	382.1712	3	2.41	NT	NT	NT	NT	NT	NT						
	A.DVIVKPHDPA.T	545.7982	2	5.19	NT	NT	NT	NT	NT	NT						
	R.SQTSQAVTGGHTQIQAGSH.T	632.3053	3	3.62	NT	NT	NT	NT	NT	NT						
	L.DEDHTGTVEFK.E	639.2859	2	4.6	NT	NT	NT	NT	NT	NT						
	M.PQLLQNINGIIE.A	676.3826	2	13.12	NT	NT	NT	NT	NT	NT						
	D.VIVKPHDPATVDE.V	710.3775	2	5.25	NT	NT	NT	NT	NT	NT						
	F.ADVIVKPHDPATVDE.V	803.4096	2	6.29	NT	NT	NT	NT	NT	NT						
IVL	V.ELPVEVPSKQEEKH.M	412.9690	4	5.98	NT	NT	NT	NT	NT	NT						
PPL	L.KTENPGDASDLQGRQL.L	864.9292	2	5.71	NT	NT	NT	NT	NT	NT						
SBSN	R.VVQGLHHGVSQAGR.E	722.8920	2	2.31	NT	NT	NT	NT	NT	NT						
	N.NAAGQVGKEADKLIHHGVHHGAN.Q	787.4037	3	6.29	NT	NT	NT	NT	NT	NT						

Mixtures					M1 10 µL SA + 2.5 µL SE			M2 50 µL SA + 2.5 µL SE			M3 10 µL SA + 25 µL SE			M4 10 µL SA + 10 µL SE		
Protein	Sequence	Mass (m/z)	Charge (z)	RT	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3
KLK3	F.LRPGDDSSHDLML.L	485.9014	3	8.80												
	R.FLRPGDDSSHDLML.L	534.9242	3	10.60												
	L.LRLSEPAELTD.A	622.3301	2	8.70												
	L.LRLSEPAELTDA.V	657.8486	2	9.20												
	W.GSIEPEEFLTPK.K	673.8456	2	10.50												
PIP	Y.TIEILKVE.-	472.7868	2	6.60												
	F.YTIEILKVE.-	554.3184	2	11.70												
PPAP	F.AELVGPVIPQDW.S	662.3508	2	13.85												
	F.GIWSKVYDPLY.C	670.8479	2	12.65												
	F.GQLTQLGMEQHYEL.G	823.8958	2	10.55												
	F.GQLTQLGMEQHYELGEY.I	998.4595	2	10.70												
SEMG1	Y.DLNALHKTTKSQRH.L	412.9774	4	1.50												
	Q.HGSHGGLDIVIIE.Q	449.5736	2	11.30												
	Y.GENGVQKDVSQRS.I	468.5673	3	1.60												
	V.VEVREEHS.S	492.7409	2	2.85												
	T.NREQDLLSHEQKGRHQ.H	494.5018	4	2.55												
	I.TIPSQEQEHSQKA.N	494.9110	3	1.30												
	Q.TEKLVAGKSQ.I	530.8035	2	1.70												
	Q.NVVEVREEH.S	555.7805	2	3.05												
	N.TEERLWVHG.L	563.7856	2	6.35												
	R.EQDLLSHEQKGRHQ.H	568.9519	3	2.50												
	Q.NVVEVREEHS.S	599.2966	2	3.05												
	Y.SQTEKLVAGKSQ.I	638.3488	2	2.40												
	Q.NVVEVREEHSSK.V	706.8601	2	2.05												
	Q.STNREQDLLSHEQKGRHQ.H	721.6932	3	2.65												
SEMG2	V.DINDHDWTR.K	391.1757	3	4.75												
	Q.NVVDVREEHS.S	395.1949	3	3.30												
	V.DINDHDWTRK.S	433.8741	3	3.85												
	Q.NVVDVREE.H	480.2433	2	4.30												
	Y.NEDRNPIST.-	523.2491	2	3.50												
	Y.DLNALHKATKSKQH.L	530.9610	3	2.05												
	Q.NVVDVREEHSSKLQ.T	547.2848	3	4.30												
	K.DVSQSSISFQIEKLVEGKSQ.I	553.0391	4	13.35												
	S.SISFQIEKLVEGKSQ.I	564.9771	3	11.40												
	Q.IEKLVEGKSQ.I	565.8244	2	3.15												
	Y.HVDINDHDWTRK.S	768.3711	2	4.60												
	K.DVSQSSISFQIEKLVEGKSQIQ.T	817.4306	3	13.80												
	Y.VLQTEELVVNKQQRETK.N	1021.5657	2	6.05												
CRNN	L.YSYLRSTKP.-	372.2012	3	4.76												
	E.WVDDHSRET.V	382.1712	3	2.41												
	A.DVIVKPHDPA.T	545.7982	2	5.19												
	R.SQTSQAVTGGHTQIQAGSH.T	632.3053	3	3.62												
	L.DEDHTGTVEFK.E	639.2859	2	4.6												
	M.PQLLQNINGIIE.A	676.3826	2	13.12												
	D.VIVKPHDPATVDE.V	710.3775	2	5.25												
	F.ADVIVKPHDPATVDE.V	803.4096	2	6.29												
IVL	V.ELPVEVPSKQEEKH.M	412.9690	4	5.98												
PPL	L.KTENPGDASDLQGRQL.L	864.9292	2	5.71												
SBSN	R.VVQGLHHGVSQAGR.E	722.8920	2	2.31												
	N.NAAGQVGKEADKLIHHGVHHGAN.Q	787.4037	3	6.29												

Mixtures					M1 10 µL SA + 2.5 µL SE			M2 50 µL SA + 2.5 µL SE			M3 10 µL SA + 25 µL SE			M4 10 µL SA + 10 µL SE		
Protein	Sequence	Mass (m/z)	Charge (z)	RT	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3
HIS1	H.SHREFPF.Y	460.2247	2	8.25												
	H.REFPFYGDY.G	597.2682	2	10.58												
	K.HHSHREFPF.Y	597.2836	2	6.35												
	S.HREFPFYGDY.G	665.7962	2	9.85												
	F.YGDYGSNYLYDN.-	722.2886	2	7.33												
PRP_1	Q.GGNKPQGPPPPPGKPQ.G	518.2792	3	3.09												
	Q.GPPQQGGNRPQ.G	568.2828	2	0.99												
	A.GNPQGPSPQGGNKPQ.G	731.8553	2	1.42												
PRP_2	Q.GPPSPPGKPQ.G	481.2587	2	2.85												
	Q.GGNKPQGPPSPPGKPQ.G	514.9390	3	3.24												
	A.GNPQGAPPQGGNKPQ.G	723.8578	2	1.52												
PRP_4	Q.RPPPPPGKPQ.G	535.8089	2	1.39												
	Q.GPPPPPQGGRPP.R	577.3093	2	3.77												
	Q.SHRPPPPPGKPE.R	648.3469	2	1.26												
PRPC	Q.GPPQGQSPQ.-	448.2170	2	1.23												
	Q.GPPPPPPGKPQ.G	534.7955	2	2.76												
	Q.QGPPPPQGKPQ.G	565.8013	2	1.50												
	Q.GPPQQGGHPPPPQGRPQ.G	577.9606	3	2.07												
	Q.GPPQQGGHPRPP.R	612.8153	2	2.13												
SMR3B	P.RGPYPPGPL.A	477.2648	2	7.98												
	P.GIFPPPPPQP.-	523.7877	2	10.65												
	G.RIPPPPPAPY.G	552.8137	2	7.69												
	G.FVPPPPPPPY.G	554.2973	2	9.58												
	P.GRIPPPPPAPY.G	581.3244	2	7.55												
	P.GFVPPPPPPPY.G	582.8080	2	10.66												
	Y.GPGIFPPPPPQP.-	600.8242	2	10.74												
	P.YPPGPLAPPQPF.G	640.8373	2	12.45												
	L.APPQPFGPGFVPPPPPPPY.G	652.6733	3	13.54												
	Y.GPGRIPPPPPAPY.G	658.3615	2	8.13												
	F.GPGFVPPPPPPPY.G	659.8458	2	11.06												
	R.GPYPPGPLAPPQPF.G	717.8744	2	12.78												
	F.GPGFVPPPPPPPYGPG.R	765.3930	2	10.82												
	P.RGPYPPGPLAPPQPF.G	795.9250	2	11.89												
STAT	P.YQPVPEQPL.Y	535.7795	2	8.39												
	P.EQPLYPQPY.Q	567.7769	2	8.34												
	Y.GPYQPVPEQPL.Y	612.8166	2	7.30												
	R.IGRFGYGYGPY.Q	625.3037	2	9.15												
	P.VPEQPLYPQPY.Q	665.8375	2	9.19												
	E.QPLYPQPYQPQ.Y	679.8406	2	7.81												
	P.YQPVPEQPLYPQPYQPQ.Y	691.3423	3	9.89												
	Y.QPVPEQPLYPQPY.Q	778.3932	2	9.69												
	R.FGYGYGPYQPVPEQPL.Y	906.4356	2	11.07												
	Y.QPVPEQPLYPQPYQPQ.Y	954.9782	2	9.22												
	F.GYGYGPYQPVPEQPLYPQPY.Q	1157.0468	2	11.80												