Development and Testing of a Rapid Multiplex Assay for the Identification of Biological Stains

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Abstract

While DNA profiling makes it possible to individualize biological stains, the identification of the stain itself can present forensic serologists with a significant challenge. Current antibody- and enzyme activity-based assays used by forensic practitioners for biological stain identification yield only presumptive results. Positive results with non-target body fluids or cross-reactivity with non-human sources has also been well documented. Some tests can consume unacceptable quantities of precious evidence while for some body fluids (vaginal fluid and menstrual blood) there are simply no available tests at all.

This research presented here aims to develop and rigorously test a fast, accurate, and sensitive multiplex assay for the simultaneous identification of saliva, semen, urine, peripheral blood, menstrual blood, and vaginal secretions. This research is based on the following three research phases:

1. Biomarker Identification – Utilizing multidimensional protein separation technologies, bioinformatics tool, and tandem mass spectrometry a database of fluid-specific candidate markers will be developed for each body fluid.
2. Biomarker Verification – Using the most promising candidates from Phase 1 a targeted multiplex assay will be developed on a Quadruple Time-of-Flight mass spectrometer to verify the specificity of these candidate biomarkers with single source laboratory samples as well as single and mixed source casework type samples.

3. Prototype Validation – Development and testing of a case working laboratory prototype assay on a triple quadrupole mass spectrometer using single and mixed source casework-type samples.

This work has the potential to significantly improve the accuracy and sensitivity of forensic serological testing. It will provide practitioners with greatly improved tests for saliva and seminal fluid while also enabling the identification of vaginal secretions for which there are currently no accurate tests. The multiplex design will eliminate the need to perform separate tests on an unknown stain. In short, the successful completion and implementation of this research will provide the forensic community with a powerful tool to aid in criminal investigations.

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Introduction

1 Introduction

The development of DNA typing technologies has transformed the world of forensic science by allowing routine individualization of biological stains. Confirming the source of the stain itself, however, can present a challenge. There is no dependable test for vaginal fluid or menstrual blood and some fluids (*e.g.* saliva) have well established protocols but can provide only presumptive results. Despite the tremendous power of discrimination of DNA testing, it is not difficult to point to situations where the unambiguous identification of a biological fluid can be very informative for investigators.

1.1 History and Importance of Forensic Serology

Forensic serology can be broadly defined as the discipline concerned with the characterization of biological fluids present on evidentiary material. Currently, the identification of unknown stains relies upon the use of microscopic, immunological, chemical, and enzymatic activity tests in order to locate and identify the source of a stain which may be present on a piece of evidentiary material. Once located, stains are processed further in order to generate DNA profiles to identify specific individuals.

In the context described above, forensic serology is being used as a first-pass screen of evidentiary material prior to and independent of DNA analysis. Traditionally, serological techniques attempted to combine both the identification as well as the individualization of a stain in a single method. The most notable example of this being the ABO blood typing system developed by the Austrian physician Karl Landsteiner[1] and implemented for forensic practice by Leon Lattes[2]. While his agglutination method could successfully identify blood, using the technique for individualizing the bloodstain has such a low discriminating power that two

individuals selected at random having the same type is approximately 40%[3]. Furthermore, the agglutination-based test lacked sensitivity and the method to identify an AB type could not be differentiated between a failed test result since the identification relies upon an lack of a reaction[4]. After decades of research and development, the hope to individualize stains using serological markers produced only moderate advances. One notable method combined ABO markers along with polymorphic proteins markers such as PGM (phosphoglucomutase) into a single testing system [5, 6]. While the combination of these two systems was an improvement, there is still a 20% chance that two individuals selected at random will have the exact same type[3].

In recent years modern DNA typing has allowed for the individualization of biological stains such that two individuals selected at random from a population having the same DNA type can be as low as one out of billions to quadrillions [7]. Because the discrimination power of DNA is so high compared to historical serological typing methods, these efforts have been largely abandoned. As such, serological testing in a modern forensic laboratory is considered to be a screening device for locating biological material which is subsequently processed for DNA profiling. While it may appear these techniques have fallen to the wayside, serological screening still holds value as an evidentiary tool in forensic cases. The largest factor being that DNA is unable to provide insight as to the source of a DNA profile. Specifically, DNA alone cannot confirm whether the source of a profile originated from saliva, vaginal fluid, or blood, information which may be able to provide context to a criminal investigation.

For example, consider the case of an alleged sexual assault where DNA consistent with the victim is recovered from the neck of a bottle. The victim states that the suspect penetrated her with the bottle while the suspect claims that no sexual contact occurred – offering instead that the

alleged victim had simulated oral sex on the bottle. Both stories "explain" the presence of the victim's profile on the bottle. The ability to unambiguously detect saliva vs. vaginal secretions could potentially help investigators to refute one of these opposing claims. A number of other scenarios could easily be imagined where the ability to characterize body fluids and to clearly detect and identify even microscopic traces of urine, seminal fluid and saliva or to differentiate between menstrual and peripheral blood, would have important probative value. Such capabilities would have clear benefits for criminal investigations and would enable forensic analysts to make more definitive statements about the potential tissue source of a DNA profile.

1.2 Serological Testing Assays

Serological testing relies upon four general categories of testing methodologies. They include chemical reaction assays, enzyme activity assays, immunoassays, as well as microscopy based assays. Each individual test can be further classified as being presumptive or confirmatory for its designated target fluid. Presumptive tests will disclose the possible presence of a particular body fluid where a confirmatory test will unambiguously identify an unknown stain as having a specific biological origin. The following section will describe the four general testing categories.

1.2.1 Chemical Reaction Assays:

Chemical reaction tests are based off the formation of crystals or a color change from assay reagents interacting with substrates in a target fluid. One example of a colorimetric assay, the Kastle-Meyer test, produces a pink color when exposed to hemoglobin present in blood[8, 9]. In this reaction **(Figure 1),** phenolphathalin is prepared in an alkaline zinc solution and is added to the stain along with hydrogen peroxide. Hemoglobin in the blood has peroxidase-like activity

producing water as well as oxygen-free radicals. The oxygen radicals, in turn, catalyze the oxidation of the phenolphathalin to phenolphathalein, producing a bright pink color.

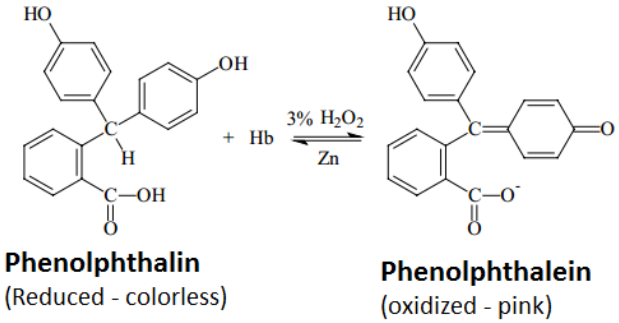


Figure 1 - Kastle-Meyer reaction, phenolphthalein is oxidized in the presence of hydrogen peroxide and hemoglobin giving a chemical indication of blood. Image adapted from Winchester[10].

An example of a crystal producing test is the Teichmann test for blood[11]. Crystals form as a result of the chemical reaction between the iron component of hemoglobin in blood and the Teichman reagent, a solution of potassium bromide, potassium chloride and potassium iodide and glacial acetic acid.

While chemical reaction based assays are sensitive, false positives are common. Peroxides in plants as well as chemical oxidants are common causes of false positive results [12]. In addition, assays requiring the formation of detectible crystals may lack the sensitivity and reliability necessary to be of utility in cases where only trace quantities of evidentiary material are available.

1.2.2 Enzyme Activity Assays

Enzymes are molecules which catalyze a single or series of closely related chemical reactions between substrates to produce a specific product[13]. Enzymes molecules are typically proteins and are present in high concentration in a variety of body fluids. It is the specific catalytic activity of an enzyme which can be used to screen for the possible presence of an

unknown stain. Prostatic acid phosphatase, as the name suggests, will hydrolyze phosphate groups from other molecules. This enzyme is present in high amounts in the prostate gland and its activity is one of the most common methods to screen evidence for the presence of seminal fluid [14]. In this test, the phosphate group on α-naphthyl phosphate is cleaved forming a reactive species. To carry out this test, sodium naphthyl phosphate and the dye Brentamine Fast Blue are applied to a suspected seminal fluid stain. Enzymatically active prostatic acid phosphatase cleaves the phosphate group on naphthyl phosphate which reacts with Brentamine Fast Blue to generate a dark purple color (**Figure 2**).

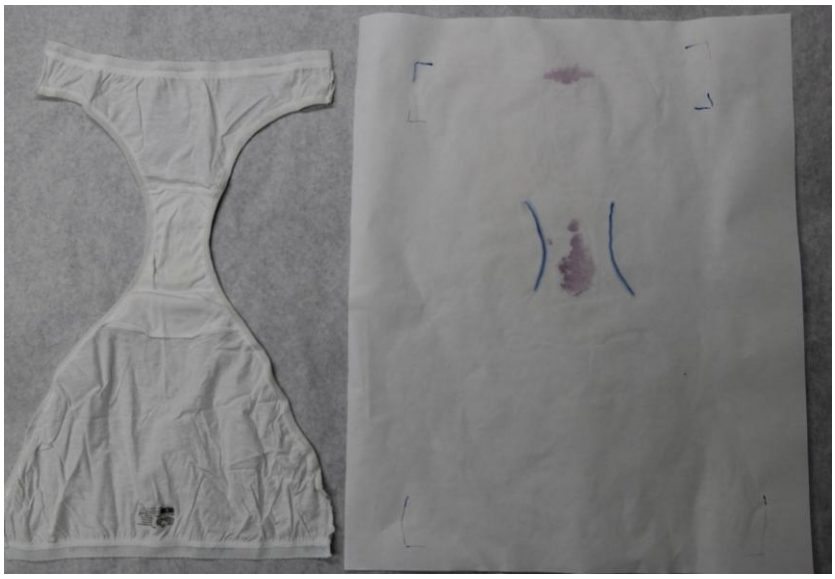


Figure 2 - Acid phosphatase overlay to localize potential seminal fluid stains. Moistened filter paper is pressed on top of the evidentiary material to draw out active enzyme by capillary action. Following the addition of α-naphthyl phosphate and Brentamine Fast Blue, a dark purple color indicates the presence of seminal fluid.

While these tests are typically sensitive, results using are only presumptive for a target body fluid. This is due primarily to expression differences between target fluids vs. absolute specificity of an enzyme. So, while a given enzyme may be an abundant component of the body fluid to which the assay is targeted, the same enzyme may also be present in one or more non-target body fluids or tissues at lower concentrations. Prostatic acid phosphatase, for example, is

used as marker for prostate cancer in males when detected in serum[15]. Additionally, proteins are subject to loss of activity over time due to factors such as microbial degradation, exposure to chemical agents and inhibitors and extremes of temperature or pH [16-18].

1.2.3 Immunoassays

Immunoassays are analytical methods which implement antibody-antigen binding events to generate a response to a specific molecule. When properly and thoroughly characterized, it is usually possible to isolate antibodies that are highly specific and sensitive for nearly any target molecule of interest. In relation to forensic serology the antibody would aim to target molecules which are specific to a particular body fluid. Early assays commonly employed in case working labs were based on the visualization of a precipitation reaction between antigens and their corresponding antibody. Using the ouchterlony double diffusion method, a series of wells are punched into an agarose gel with a central well surrounded by a halo of additional wells. A solution containing antibodies against a specific protein is placed in the central well where controls and unknown stain extracts are applied to the wells in the outer ring. Diffusion of the sample as well as the antibody will occur and a precipitation line will form if an antibody-antigen complex forms, indicating a positive result for the test (**Figure 3**).

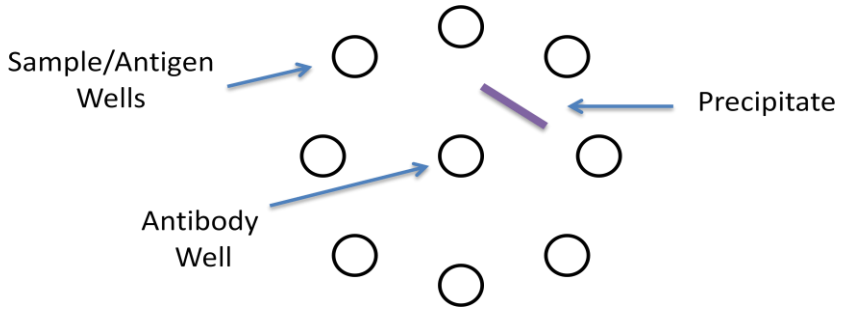


Figure 3 – Antigen-antibody precipitate formation indicating a positive test result.

More sophisticate methods, such as cross-over electrophoresis, use an electric field in as an alternative to diffusion to drive antigens across an agar gel. As with the ouchterlony method, a precipitate will form if an antigen is present.

Immunochromatographic assays have gained popularity in recent years for their simplicity, specificity, cost, and speed [19, 20]. Commercial immunochromatographic assays are sold as single use lateral flow strips with configurations that target specific proteins present in a body fluid. The strips (**Figure 4**) contain a sample loading well, lateral flow membrane, and three antibodies. To run the test, an unknown liquid extract is introduced onto the conjugate pad where it mixes with colloidal gold labeled antibodies. Samples which contain the target protein form an antigen- antibody-colloidal gold complex that migrates by bulk fluid flow across the lateral flow membrane. Immobilized antibodies, specific for the target protein, capture the antigen- antibody-colloidal gold complex as it migrates down the test strip. The aggregation of colloidal gold conjugated antibodies creates a visible band at the Test Line. Free antibodies not captured at the Test Line continue toward the control line where they are captured by immobilized antibodies targeted directly to the antibody portion of the antibody-colloidal gold conjugate. This produces a second visible band at the Control Line indicating that the test performed as designed.

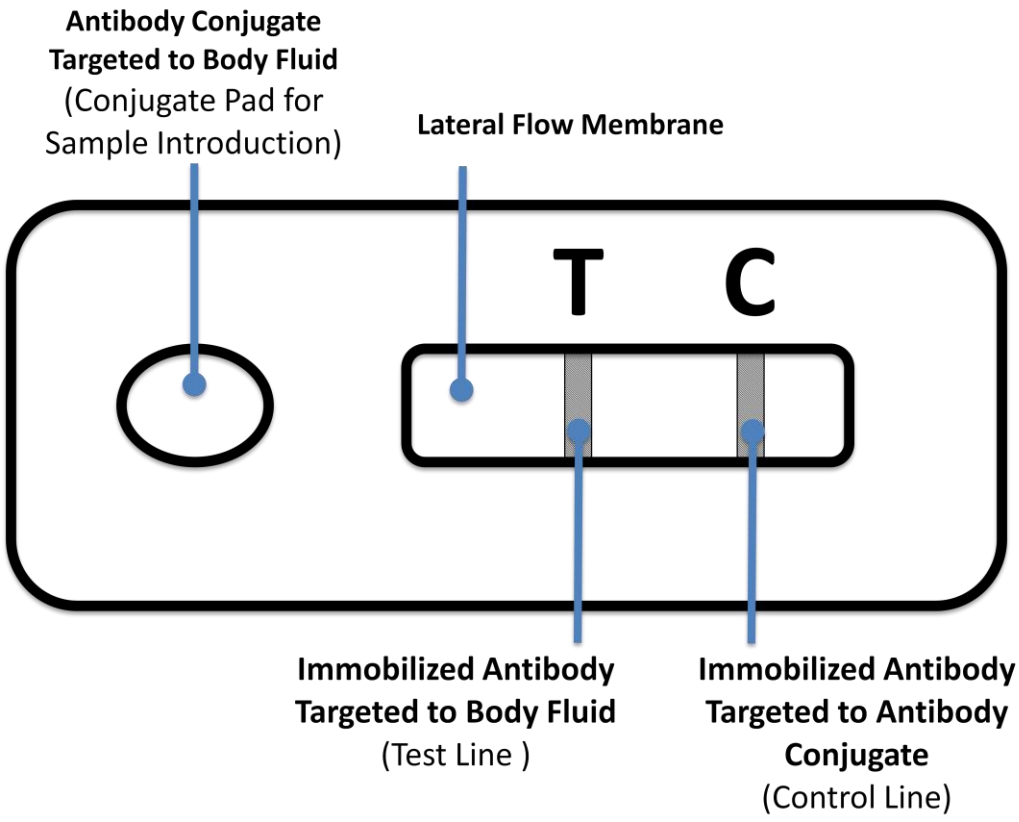


Figure 4 - Lateral Flow Immunochromatographic Strip. A positive result is indicated by visible bands at both the Test Line and the Control Line.

While sensitive and easy to use, antibodies have limitations which limit their probative value. The primary issue being that protein antigen-antibody binding cannot be 100% specific, thus false positive results are possible. This arises from several sources, firstly, proteins are known have variability in structure due to polymorphisms from genetic variability as well as post-translational modifications[21]. Because immunoassays are based on binding a specific epitope of a target protein, any modification to this structure can result in a false negative result. Along with variability of the individual protein, it is possible for non-target molecules with a similar or identical epitope to be recognized by the antibody generating false positive results. Lastly, it is possible for antibodies to over-saturate the target antigen such that the immobilized antibodies at the test line are more likely to bind free antigen than the antigen-complexed mobile

phase antibodies. Referred to as the "hook effect", this can produce false negative or falsely indicate very low amounts of the target body fluid when, in fact, the exact opposite is true [21].

1.2.4 Microscopy Based Assays

Microscopy is simply the use of a series of lenses to view objects which are too small to see with the naked eye. Applied to forensic serology, microscopy has been primarily applied as a means of identifying sperm cells on sexual assault evidence. Sperm cells can be both detected and identified as being of human origin using visible light or fluorescence microscopy by a skilled analyst knowledgeable in the morphological characteristics of human sperm. Because spermatozoa are much smaller than the vaginal or other epithelial cells with which they are often comingled in a forensic context, they can be difficult to localize in the microscopic field. With visible light microscopy, detection is facilitated by the use of histological stains such as the combination of hematoxylin and eosin or nuclear-fast red and picroindigocarmine [22, 23]. The latter more commonly known as "Christmas tree'' stain is particularly useful because it differentially stains spermatozoa and epithelial cells. Using this stain the sperm head will be red with a pink tip, the mid section will be blue and the tail will be yellow-green. Epithelial cells by contrast will appear greenish to blue.

The more recent introduction of fluorescence microscopy has greatly streamlined the process of identifying spermatozoa [24, 25]. The strategy takes advantage of the specificity of fluorochrome-conjugated antibodies targeted to a protein that is unique to the spermatozoa head. The approach, which requires a fluorescence microscope fitted with appropriate excitation and emission filters, makes it possible to selectively and readily visualize human sperm heads even against a background crowded with epithelial cells, microbes and non-cellular debris such as

fecal matter. The spermatozoa heads appear as bright fluorescent ovoid bodies against a dark background.

The major limitations with microscopy are the need for relatively intact spermatozoa and the laborious nature of visually scanning microscopic fields for the presence of relatively few spermatozoa where non-sperm cells and other debris often predominate. Spermatozoa may be difficult to separate from cotton and other clothing fibers and as they degrade, they typically lose their characteristics tails and ovoid shape such that they become more difficult to distinguish in the presence of similarly-sized microbes such as yeast. Finally, fluorescence microscopy involves a greater investment in costly microscopes and, because it is antibody based, may fail to yield optimal results as spermatozoa degrade and antigenic target molecules become unrecognizable by the antibodies.

1.3 Current Testing Methodologies

Seminal fluid, saliva, vaginal fluid and blood are some of the most commonly encountered forms of serological evidence in sexual assault investigations. While not as common, anal secretions, fecal matter, urine, sweat, tears and nasal secretions could also constitute potentially probative evidence depending on the context of the case. Unfortunately, the range of body fluids for which serological assays would be valuable exceeds the number for which assays actually exist. There are no reliable tests for vaginal fluids, anal secretions, sweat, tears or nasal secretions. However, there are numerous assay options available for several body fluids with forensic relevance which offer a wide range of strengths and weaknesses.

1.3.1 Semen and Seminal Fluid

Semen is a mixture of spermatozoa (sperm cells) and seminal plasma. Seminal plasma is mixture of secretions from six different glands/tissues of the male genital system. Semen is ejected as a heterogeneous mixture of these secretions during ejaculation. The initial ejaculate (5% of the total volume) consists of secretions produced by the Cowper (bulbourethral) and Littre glands. The second portion of the ejaculate (15% to 30% of total volume) consists of secretions from the prostate gland. The secretions of the ampulla and epididymis then provide a small contribution while the seminal vesicles account for the balance (approximately 60% of total volume) [26-28]. Spermatozoa (sperm cells) make up a small portion of whole semen (1% to 5% of the total ejaculate volume) [29]. Pre-ejaculation fluid is secreted almost exclusively by the Cowper's gland and can contain traces of acid phosphatase and prostate specific antigen/p30 but not semenogelin. The presence of sperm in pre-ejaculation fluid appears to be rare and may be due to carryover from a prior ejaculatory event. Because of the obvious association with sexual activity, seminal fluid and spermatozoa represent some of the most potentially probative evidence that can be collected as part of a sexual assault investigation.

Chemical reaction-based tests are no longer commonly used for the detection of seminal fluid due to their lack of specificity and reproducibility. Historically, however, the chemical reaction-based tests for seminal fluid were the Barberio and Florence crystal tests.

Enzyme activity based tests such as the Brentamine test for seminal fluid target the activity of seminal acid phosphatase (SAP) which is an enzyme that is an abundant, but not unique, component of seminal fluid. SAP typically retains activity in a vaginal environment for approximately 48 hours post-coitus but in an oral or rectal context this period is typically shorter. A positive SAP result is indicted by a color change in the presence of a coupling agent and an

indicator dye (*e.g.*, α-naphthyl phosphate and Brentamine Fast Blue). The test may be performed as a spot test, commercially prepared test strips or, as an overlay technique to localize possible seminal fluid stains. Although SAP is a sensitive test, positive results cannot confirm the presence of seminal fluid. This is due to the fact that although SAP activity levels are elevated in seminal fluid but positive test results can be obtained with a number of non-seminal fluid associated substances including vaginal fluid, fecal matter, plant material, spermicides and some feminine hygiene products.

Historically, immunoelectrophoresis, Ouchterlony double diffusion and enzyme-linked immunosorbent assays (ELISA) were employed to detect antibody binding. In recent years, these laborious and slow techniques have been steadily supplanted by lateral flow immune-chromatographic strip tests that are faster and commercially available at a relatively low cost. For seminal fluid detection, prostate specific antigen (PSA) and semenogelin are the primary targets using immunochromatographic test strips. The PSA protein is secreted by the prostate and is present in seminal fluid in a concentration range between 70 to 5500 mg/ml with the majority of samples being between 820-1290 mg/ml. Semenogelin (Sg) is produced by the seminal vesicles and serves as the substrate for PSA and is the predominant protein in seminal fluid. In living individuals, the ability to detect PSA and Sg tends to be lost within 24 hours in a vaginal context and even faster in an oral or rectal context. Although antibodies targeted to PSA and Sg are generally sensitive and highly specific for human proteins, positive results must still be interpreted with caution and cannot confirm seminal fluid. There are two reasons, firstly, PSA and Sg are abundant proteins in seminal fluid they can also be found in a variety other body fluids and tissues; although not typically at concentrations that overlap significantly with the

concentration range seen in seminal fluid. In the case of p30/PSA, however, positive results may be obtained with post ejaculate urine; urine from adult males and vaginal fluids (**Figure 5**).

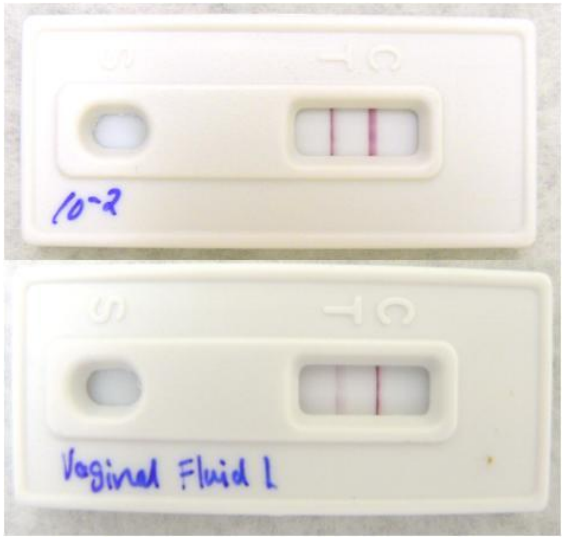


Figure 5 - Abacus Diagnostics ABAcard® p30 immunochromatographic test strips. TOP: Visible bands at the Test Line (T) and the Control Line (C) indicates the detection of prostate specific antigen in a semen sample. BOTTOM: Visible bands at the Test Line (T) and the Control Line (C) indicate the detection of prostate specific antigen in a pre-coital vaginal fluid sample (*i.e.*, in the absence of semen). Image courtesy of Jillian Fesolovich, NMS Labs, Willow Grove, PA.

Similarly, positive results with Sg may be obtained with post ejaculate urine. The second reason is that, by their very nature, all antibodies are subject to cross reactivity with non-target antigens that possess structurally similar epitopes but which may be completely unrelated to the intended target.

Microscopy-based tests provide the most reliable means of indicating the presence of semen. These tests are reliable as they rely upon the direct identification of spermatozoa. On average, male ejaculate contains 3.5 ml of seminal fluid containing 10-50 million spermatozoa per ml. Human spermatozoa have a distinct morphology allowing species differentiation and can be identified for a longer postcoital interval (approximately 72 hours) when compared to chemical or protein components of semen. There are a variety of histological stains used for light

microscopy of spermatozoa. Using methylene blue and acid fuchsin (Baecchi's stain) the heads of the sperm stain red, while the midpieces and tails are blue. The use of hematoxylin and eosin (H&E stain) yields heads characterized by and intense blue colored anterior spike and base-plate and a paler blue nucleus with a pink tail. Most used is the combination of nuclear-fast red and picroindigocarmine (Christmas tree stain) with which the heads are stained red and tails are yellow-green. The major drawback to light microscopy is the specificity of the dyes themselves. Regardless of the stain used, epithelial cells, microbes, bacteria, cotton fibers will stain making the process of identifying the relatively small spermatozoa in a laborious and difficult. The use of fluorescence microscopy circumvents this drawback by allowing the selective visualization of spermatozoa through binding of commercially-available, sperm-specific antibodies conjugated to a fluorochrome. By combining the use of antibodies with microscopy, this approach also circumvents the possibility that antibody cross reactivity might lead to an undetectable false positive result.

When using microscopy in a forensic context, a failure to observe spermatozoa must be interpreted with caution. Some males have abnormally low sperm counts (oligospermia) or may not produce spermatozoa at all (aspermia). Spermatozoa will be absent in the seminal fluid of vasectomized males approximately 2-4 months following surgery. The presence of spermatozoa in seminal fluid can also vary with the age of the male, and can be impacted by a broad range of factors including disease, genetics, radiation exposure, environmental toxins, undescended testis, varicocele, trauma, diet, tobacco use and/or illicit drug consumption.

1.3.2 Vaginal Fluid

Similarly to semen and the connection with sexual activity, the ability to detect vaginal fluid on evidentiary material has enormous potential to provide evidence as part of a sexual assault investigation. However, in contrast to current testing methods for semen, development of reliable tests for evidence of vaginal fluids has been largely unsuccessful.

Over the years, there have been attempts to identify vaginal epithelial cells in evidentiary samples. While the use of histochemical stains to detect sperm cells[23] is routine, staining to differentiate epithelial cells types (*e.g.* skin, buccal and vaginal cells) has not been as successful. In the 1960s, the iodine-based Lugol's test held promise for identifying vaginal cells[30, 31]. This was based on studies suggesting that vaginal cells contained more glycogen than other epithelial cells. Unfortunately Lugol's positive cells are also present in the male urethra[32], male urine deposits [33] on >50% of penile swabs from males who had abstained from sex for several days and the oral mucosa [34]. More recently, a methanol fixation protocol and Dane's staining technique (stains targeting pre-keratin, keratin and mucin) was able to differentiate pure samples of vaginal, buccal and skin cells[35] but was unable to distinguish between a pure buccal cell sample and a mixed preparation of vaginal and skin cells. Given that forensic samples often contain cell mixtures, this essentially forcloses the forensic utility of this approach as even a moderately reliable means of identifying vaginal epithelial cells.

1.3.3 Saliva

Saliva is a fluid produced by the major (90%) and minor (10%) salivary glands. The fluid is made up of 99% water but also consists of a complex mixture of low-abundance proteins. Saliva provides lubrication, aids in digestion, maintains pH, assists with tooth enamel re-mineralisation and plays a role in innate immune defense. The protein component of saliva is

rich in the enzyme α-amylase which breaks down complex carbohydrates into smaller sugar molecules. As such, α-amylase activity or the ability to detect it through antibody binding serves as the basis of most serological assays.

Enzyme activity tests for α-amylase most commonly use the Starch-Iodine radial diffusion test. Starch in the gel incubates along with suspected saliva samples and are then stained with iodine which stains starch a dark bluish-black. The passive radial diffusion of α-amylase into the gel and the subsequent breakdown of starch molecules produces an unstained "halo effect" around the test well that is characteristic of a positive result. There is a semi logarithmic correlation between size of the halo and the amount of α-amylase loaded into the well[36]. Another approach is based on the use of degradable starch which contains a chemically-bound blue dye. Degradation of the starch due to α-amylase releases the blue dye to produce a color change that is visible to naked eye. The test may be performed as a spot test; using commercially prepared test strips or; as an overlay technique to which may help to localize possible saliva stains on an article of clothing or other evidentiary material.

As with other fluids Immunochromatographic tests have improved the presumptive detection of saliva via α-amylase specific antibodies. Similar to semen, lateral flow immunochromatographic strip tests are employed and a positive test result is indicated by the appearance of visible lines at the "test" and "control" positions on the assay membrane.

Regardless of whether the detection of α-amylase is based on enzyme activity or antibody binding, a positive result can only be considered presumptive. The α-amylase protein has been well conserved in evolution and as a result enzyme activity assays will yield positive results regardless of whether the amylase is from bacteria, fungi, or some domestic pets. Similarly it is

not possible on the basis of enzyme activity to distinguish between four variants (salivary and pancreatic) of α-amylase. Amylase is also found in many body fluids other than saliva at levels that yield positive results with both enzyme activity and antibody-binding tests. These include breast milk, vaginal fluid, fecal matter urine and blood and semen. Although rare, cross reactivity between antibodies and non-target antigens that possess structurally similar epitopes is also a possibility.

1.3.4 Blood

One of the most commonly encountered body fluids in criminal investigations is blood. Blood includes a cellular fraction consisting of erythrocytes, leukocytes and platelets (45%) suspended in liquid fraction consisting of blood plasma (55%). Plasma contains 92% water along with a complex mixture of proteins, glucose, mineral ions, hormones as well as lipids. The metalloprotein hemoglobin is responsible for oxygen transport and is the major protein contained within erythrocytes. Because of the high abundance of this protein the majority of blood detection strategies target this specific protein.

Among the many chemical reaction-based presumptive tests for blood are two crystal tests, the Teichmann and Takayama tests, which rely on the heme group ferrous iron catalyzed formation of crystals of heme derivatives. A positive result with the Teichmann test is indicated by the detection of brownish-yellow rhombic crystals of chlorohemin formed through combination of a halogen with ferriprotoporphyrin. The Takayama test is based on the formation of salmon-pink feathery crystals of pyridine hemochromogen resulting from the reaction of the ferriprotoporphyrin with pyridine. While crystal tests have not been found to yield positive results with body fluids other than blood, they are only presumptive for human blood for two reasons. The first is that a positive result will be obtained with both human and non-human

blood. The second reason is that crystal formation only reflects a chemical reaction with the ferriprotoporphyrin group of hemoglobin. Thus false positives are technically possible with non-blood substances since iron porphyrin-containing hemoproteins such as cytochrome P450, cytochrome c oxidase, peroxidases and a number of other cytochromes occur frequently in biological systems[37]. As a practical consideration, however, these proteins are typically found in natural systems at concentrations well below the detection sensitivity of the crystal tests.

A number of other chemical reaction-based assays widely employed are based on reactions involving the iron-containing heme group of hemoglobin. This protoporphyrin IX ring structure with its associated iron ion displays peroxidase-like activity that can be harnessed to produce chemoluminescence or colorimetric changes in any of several indicator dyes. The Luminol test is most sensitive of these assays. Luminol and its contemporary derivatives are chemoluminescent substrates, the oxidation of which is catalyzed by heme in the presence of hydrogen peroxide to form an unstable intermediate. Decay of this intermediate, liberates light[38]. Similarly the Kastle-Meyer (KM) and the Leucomalachite Green (LMG) tests rely on the peroxidase-like activity of heme to catalyze the reduction of hydrogen peroxide. This reaction liberates oxygen which then oxidizes either phenolphthalein to produce a pink color in the case of the KM test or the triphenylmethane dye leucomalachite green to produce a blue/green color which indicates a positive LMG result. Some commercially available test strips employ filter paper that has been pre-treated with diisopropylbenzene dihydroperoxide and the indicator dye tetramethylbenzidine (*i.e.* Hemastix). The peroxidase-like activity of the heme group catalyzes a change from yellow to green as a presumptive positive for the detection of blood. Positive results with these peroxidase reaction-based tests should be viewed as indicating peroxidase-like activity and by extension the presumed identification of blood. False positive

results have been obtained with non-blood hemoprotein containing substances such as potato and horseradish as well as chemical oxidizing agents such as bleach, rust, ferric sulphate and copper-salts (*e.g.*, cupric sulphate which is an aquatic algaecide commonly used in aquaculture) [39, 40].

Immunochromatographic tests have greatly improved presumptive detection human blood by making it possible to more confidently identify blood. Hemoglobin has historically been targeted as the protein of choice for antibody-based tests with glycophorin-A being implemented more recently via the RSID™ Blood kit[41]. Glycophorin-A is the primary membrane protein located in red blood cells and is involved with cell-cell binding interactions[42]. The specificity of the assay is such that it is even capable of distinguishing human from primate glycophorin A. As with the commercially available antibody-based tests for seminal fluid and α-amylase, lateral flow immunochromatographic strip tests are employed, a positive test result is indicated by the appearance of visible lines at the "test" and "control" positions on the assay membrane. Although antibodies targeted specifically to human hemoglobin or glycophorin A are generally sensitive and specific for human and often primate proteins, positive results should still be interpreted with caution. Antibody cross reactivity to non-human blood are possible. For example, one antibody binding based-assay which specifically targets human hemoglobin has also been found to yield a positive result with ferret blood[43]. In addition, the potential for binding to unrelated proteins with structurally similar epitopes is difficult or impossible to predict and cannot be eliminated as a possibility.

1.3.5 Menstrual Blood

Menstrual blood is present between 3 to 7 days during an average 28 day female menstrual cycle. Menstrual blood has not been considered a critical fluid with regards to forensic serology, particularly in comparison to the identification of semen and/or vaginal fluid. The

reality, however, is that the ability to identify of menstrual blood components has the potential to provide critical evidence as part of a sexual assault investigation.

Initial work on the identification of this fluid attempted to utilize the polymorphic isoenzyme Lactate Dehydrogenase (LDH)[44]. As with Hemoglobin, LDH quatnernary structure consists of a tetramer of two subuints, M and H. Unlike Hemoglobin, LDH can exist as five separate isoforms; LDH-1 with 4H subunits, LDH-2 or 3H1M, LDH-3 or 2H2M, LDH-4 or 1H3M, and LDH-5 or 5M. Serological differentiation relies upon specific localization of each enzyme. Peripheral blood contains LDH-1, LDH-2, and LDH-3 where menstrual blood will also contains LDH-4 and LDH-5[3]. Similarly to other protein-based serological markers, LDH can be found in other tissues limiting the value as a specific test for menstrual blood[45].

1.3.6 Urine

Urine contains a variety of inorganic ions including chloride, phosphate, and sulfate which are not specific to urine but which are found at concentrations that are significantly higher in urine than other body fluids[37]. In addition, urine also contains a variety of peptides and amines which can serve as presumptive indicators for the presence of urine including urea, uric acid, and creatinine. A number of chemical reaction- and enzyme activity-based tests have been developed to identify possible urine stains. Two that are in more common use are tests for urea and creatinine. Both tests provide a presumptive indication of the presence of urine since both urea and creatinine are found at detectable concentrations in other body fluids including sweat and seminal fluid[46].

Tests for urea are based on the detection of urease enzyme activity which breaks down urea to liberate ammonia and carbon dioxide. The production of ammonia is then detected by Nessler's reagent (mercuric iodide in potassium iodide) which turns yellow or p-

dimethylaminocinnamaldehyde (DMAC) which produces a strong red color. One of the first commercially available test strips based on this reaction employ paper impregnated with urease and bromothymol blue as a pH indicator to measure the formation of ammonia hydroxide. This same chemical system can be used to locate suspected urine stains by spraying a combination of urease and bromothymol blue on an item of evidence. The presence of urine is indicated by the appearance of a blue spot[47].

Tests for creatinine include the Jaffe and Salkowski tests. The former mixes a solution of picric acid in toluene or benzene with an extract of a suspected urine stain. A positive reaction is indicated by the formation of red creatinine picrate. The latter uses sodium nitroprusside so that a positive indication of creatinine is indicated by formation of a blue product upon heating. Commercially available test strips based on the detection of creatinine employ a proprietary indicator system which produces a purplish-brown color in the presence of creatinine.

Antibody-binding based-assays for urine currently provide the only specific means of identifying urine. A commercially available lateral flow immunochromatographic strip test is based on the use of antibodies that are targeted to the Tamm-Horsfall (THP) glycoprotein (*i.e.*, uromodulin) which is the most abundant protein present in urine. It is secreted into urine at a rate of 80-200 mg/day by the thick ascending limb of the loop of Henle[48]. As with other immunochromatographic strip tests, a positive test result is indicated by the appearance of visible lines at the "test" and "control" positions on the assay membrane. A positive test result, however, should be considered presumptive for the identification of human urine. As currently formulated, the tests do not display cross-reactivity with human saliva, semen, whole blood, vaginal fluid, or menstrual blood but do show cross reactivity with urine from non-human samples.

1.3.7 Fecal Matter

Fecal material is most often identified by testing for the presence of urobilin which is formed as the terminal degradation product of heme in the enterohepatic urobilinogen cycle. Urobilin is responsible for the characteristic color of urine and fecal matter. In the large intestine, urobilinogen (a precursor of urobilin) is present at high levels and can be readily detected using the Edelman test which oxidizes the urobilinogen to urobilin by treatment with alcoholic mercuric chloride. This is followed by the addition of alcoholic zinc chloride to yield an urobilin-zinc salt compound which fluoresces a bright apple green under UV light. A positive result is not human specific and is presumptive for the fecal matter since urobilin is also present in urine, albeit at lower concentrations.

Light microscopy can also be used for the identification of fecal matter. This approach requires a skilled analyst familiar with the appearance of various enteric microbes as well as undigested fibrous particles of food which may include both plant and animal products. Although many microbial species will be present in both human and non-humans, a careful analysis of partially decomposed food debris may provide a more reliable indication of whether the material is consistent with a human diet.

1.4 Emerging Techniques

While serological tests for blood, semen, saliva and urine have long existed[19, 49-51], Many of these are laborious, some pose health and safety risks, others consume significant amounts of a valuable sample, others fail to provide adequate sensitivity or specificity. Additionally, there are some fluids (*i.e.* vaginal fluid and menstrual blood) which lack presumptive or confirmatory tests

entirely. A number of promising methodologies have been in development to resolve the pitfalls of today's current techniques. Emerging proteomic, transcripomic, epigenomic and other technologies are being explored as a way to rapidly identify human body fluids with greater specificity and sensitivity than that of assays currently in use.

1.4.1 mRNA/miRNA Biomarkers

Messenger ribonucleic acid (mRNA) acts an intermediary molecule for the transfer of genetic information stored in DNA to the cells functional units proteins [52]. Significant research interest has been devoted to these molecules as a means of identifying body fluids on the basis of differential expression profiles[53].

Several research groups have been investigating the use of mRNA-based techniques for body fluid identification. In 2003, pioneering work identified markers for saliva by searching the Cancer Genome Anatomy Project for proteins expressed only in the salivary glands. Combining reverse transcription PCR, visualization through gel electrophoresis, and quantiation using RiboGreen®, several of these candidate markers, including histatin 3, statherin, and PRB1/2/3, were shown to be expressed in saliva but not blood or semen[54].

More recently, a comprehensive method for identifying blood, saliva, seminal fluid, and menstrual blood was successfully developed using reverse transcriptase PCR. This multiplex method paired mRNA genetic markers along with a housekeeping gene to normalize for differences in mRNA expression between different body fluids which allowed for a more confident expression patterns to be developed for each bio fluid[55]. An in-depth study of RNA recovery under a variety of conditions also demonstrated that mRNA remained stable and detectable in some casework-type samples after 547 days[56].

The use of mRNA biomarkers for body fluid identification also has the advantage of enabling forensic practitioners to employ familiar sample preparation methods that are fundamental to work involving nucleic acids. Methods for the co-extraction of DNA and RNA from the same stain have been developed[57] so that both mRNA and STR profiles can be developed from the same extract thus facilitating both individualization and body fluid identification in a single method [58].

Micro RNAs (miRNA) are small, 21-25 base pair long, non-coding nucleotide RNA molecules which are involved in the regulation of gene expression[59]. Along with mRNA, these molecules have been explored as promising markers for the characterization of body fluids. The first study, published in 2009, used a combination of database evaluation and empirical testing to identify miRNA's candidates which showed differential expression for blood, semen, saliva, vaginal fluid, and menstrual blood. The results of this work found that each body fluid (with the exception of semen) could be reliably differentiated on the basis of a unique pattern of miRNA expression. Further work to evaluate the reliability of miRNA biomarkers, examined twenty one different tissues (*e.g.*, cervix, esophagus *etc.*) and found no expression patterns matching those of the forensically relevant body fluids [60]. In a more recent study, a microarray strategy was used to identify candidate miRNA markers for saliva, semen, vaginal fluid, peripheral and menstrual blood. miRNA biomarkers that differentiated peripheral blood and semen were identified but the approach was not able to identify reliable markers for saliva, vaginal fluid or menstrual blood[61].

While this technology is in its infancy, the approach has the same key advantages as mRNA based assays with respect to integration with the current forensic workflow for DNA. As an added advantage, the small size of miRNA molecules relative to mRNA makes them potentially more robust for the analysis of aged or degraded samples[62].

1.4.2 Epigenetic Markers

Epigenetics is the study of transmissible changes in DNA expression that are not related to modifications in the DNA sequence [63] and is generally associated with DNA methylation related gene silencing. DNA methylation targets cytosine residues and localizes at CpG islands in promoter regions upstream of protein coding genes[64]. What makes these sites significant is their capability of gene silencing [65]. Put simply, a gene with an unmethylated promoter region will freely transcribe genetic information whereas a methylated promoter region will have its genetic transmission quenched[66].

The use of epigenetic biomarkers for body fluid identification, therefore, relies on detecting tissue-associated differences in DNA methylation patterns[63, 66]. As with RNA markers, this approach employs pattern analysis but has the advantage of making it possible to directly "query" the DNA in a sample to determine the tissue from which it originated. Initial studies of epigenetic markers have demonstrated the potential utility of the approach using semen, saliva and skin tissue[67, 68]. A commercially available assay uses methylation specific PCR to target genetic loci which are differentially methylated between semen and non-semen samples. In stains containing semen, three semen specific loci will preferentially amplify creating a strong signal which can be visualized on an electropherogram whereas non-semen samples will not amplify strongly at this locust thus generating a weaker signal [69, 70].

A more extensive study aimed at identifying methylation response ratios that could be used to differentiate between semen, blood, saliva and skin cells identified 38 different loci which showed differential metlyation patterns[68]. Of those, 15 were selected for inclusion in a multiplex assay which successfully differentiated target stains that were assayed as either pure or mixed samples. Tests with aged casework-type samples demonstrated that semen and blood

could be clearly distinguished from each other in 20-month old samples. As with other nucleic acid based assays, this methodology can also be readily incorporated into the standard DNA workflow.

1.4.3 Raman Spectroscopy

Non-destructive strategies to body fluid identification are particularly attractive to forensic practitioners for whom conventional methods of serological testing can be unacceptably consumptive of what are often minimal samples. Raman spectroscopy is based on a spectroscopic technique which determines the identity of an unknown substance[71] by measuring the inelastic scattering of light produced from the chemical constituents within the sample. Every unique "substance", therefore should produce a unique signature of spectroscopic peaks which upon which substances can be differentiated from each other[72]. The technology is robust and sensitive such that little if any sample preparation is needed, and pictogram quantities of a sample can be detected [72].

This technique has been applied to body fluids in order to identify unique "multidimensional spectroscopic signatures" for specific body fluids of interest. In an effort to accommodate sample heterogeneity, advanced statistical algorithms also employed to search for the best match between an expected "spectroscopic signature" and that of a questioned sample. Encouraging results have been reported for the identification of single-source vaginal fluid, sweat, blood, and semen stains using this approach[43] [73]. Additional studies have evaluated raman spectroscopy as capable of differentiating menstrual from peripheral blood[48] as well as being capable of differentiating between human, canine, and feline blood samples[64].

Because of its reliance on statistical pattern fitting, however, additional studies may be needed to ensure that the pattern matching algorithms can accommodate more challenging mixed

stains as well as those containing contaminants or that are degraded, *i.e.*, any forensic type sample that deviates substantially from the reference "spectroscopic signature".

Because of its ability to analyze samples in situ, an added potential benefit of this technique would be the ability to screen evidentiary material at the crime scene effectively eliminating the need in some cases to transport cumbersome items of evidence to the lab to screen for potentially probative material.

1.4.4 Proteomic Assays

Among the most promising of the emerging strategies for body fluid identification are those made possible by advances in proteomics. The proteome is the final product of genome expression and comprises all the proteins present in a cell at a particular time[74]. In contrast to traditional experimentation in molecular biology where a single or small subset of proteins would be analyzed, proteomic techniques make it possible to identify and monitor hundreds to thousands of proteins in a single experiment[75]. It is now possible, for example, to map entire proteomes with high reproducibility using comparative multidimensional HPLC and mass spectrometry to identify potentially useful biomarkers. Once identified, targeted mass spectrometry can be used to facilitate the detection and quantitation of low abundance protein biomarkers of interest[76]. This has resulted in a wealth of new opportunities to develop protein-based assays for medical and forensic purposes.

The application of proteomics to challenges in forensic serology has been the focus of research and development activities of multiple research groups[77]. By comparing the proteomes of different body fluids, researchers seek to discover protein biomarkers that are unique to a given body fluid and thus could serve as the basis for the development of confirmatory tests for

the identification of saliva, seminal fluid, peripheral blood, menstrual blood, vaginal fluid, and urine.

One group has reported the successful development of a multiplex assay for blood, saliva and semen on a MALDI-TOF/TOF tandem mass spectrometer[77, 78]. Using protein biomarkers identified through database mining as well as empirical research, several front-end sample preparation protocols were evaluated. These include 2-dimensional fractionation by combining isoelectric focusing and reverse phase liquid chromatography (IEF-LC-MALDI); 1-dimensional fractionation by reverse phase liquid chromatography (LC-MALDI) and; direct spotting of unfractionated samples followed by MALDI-TOF/TOF analysis. These approaches made it possible to successfully detect several body fluid protein biomarkers including cystatin-SA and α-amylase 1 as markers of saliva; semenogelin I/II and kallikrein 4 as markers of semen and; hemoglobin subunit α and β as markers of blood.

A major strength of the MALDI-TOF/TOF platform is the potential speed of analysis. Using the direct spotting method, a 96 well plate of samples can be processed in a matter of minutes. As with all mass spectrometry strategies, however, there is a relationship between the amount of pre-fractionation and sensitivity of the assay. The trade off is that fractionation steps are consumptive in terms of time and cost but achieve superior sensitivity. For example, using 0.1nl of semen as starting material, the direct spotting approach was only able to detect semenogelin-1 and 2 whereas the LC-MALDI approach detected several additional biomarkers in the seminal fluid panel. This relationship also carried over to the analysis of mixtures by MALDI-TOF/TOF. The more rigorous sample pre-fractionation achieved by the IEF-LC-MALDI strategy made it possible to detect salivary α-amylase in a 10:1 mixture of semen and saliva while the less rigorous LC-MALDI and direct spotting approaches were not able to detect the minor saliva

component. Overall, however, these approaches made it possible to characterize single and mixed body fluid stains in the nanoliter range and worked well with forensic type samples aged up to 20 months[79].

1.5 Proteins as Serological Candidates

One of the significant advantages of a protein biomarker approach is the tremendous diversity of potential targets that are made possible due to post-translational modification in different tissues. As a result, a single protein may be differentially modified by one's metabolism in two different body fluids, making it possible to develop highly specific assays in cases where epigenomic patterns or mRNA expression profiles might not differ. Another key advantage is the stability of many proteins under conditions that lead to degradation of other molecules. Proteins are among the most long-lasting of all biological molecules having been routinely isolated from even ancient biological material[80, 81]. In a more forensically applicable study, a 99.5% decrease in mRNA levels was observed in post-mortem brain tissue while protein levels remained relatively constant[82]. Still, as is the case with all biological molecules, proteins do fragment and degrade over time. The use of protein biomarkers, however, can be readily adapted to detect protein fragments. Thus even partially degraded target biomarkers may be detected[83].

1.6 Proteomics

Proteomics is a global or large scale study of expressed proteins. Because of recent advancements in instrumentation and techniques, the field of proteomics has expanded such that research scientists have the ability to profile entire proteomes of nearly any tissue or fluid. The

primary objective of most proteomic-based research projects has been to develop novel diagnostic tests for the clinical setting by identifying protein biomarkers that could detect disease states earlier to generating tools that may be able to personalize treatments based on disease stage[84]. While the identification of disease biomarkers is not the goal of a proteomics project focused on forensic serology, the same principals, methodologies, and instrumentation can be utilized in order to identify proteins which can act as biomarkers which can identify a specific biological fluid. These methodologies employed follow what is now a well established proteomics pipeline that begins with a discovery phase to experimentally derive a panel of candidate biomarkers followed by a validation phase to confirm their specificity across a larger sample population.

1.6.1 Proteomics Front End Technologies

One of the primary challenges of Proteomics discovery research is the enormous number of different proteins that are present within the biological fluids. Human plasma, for example, is estimated to contain several thousand different proteins. Additionally, the difference in concentration between the most and lease abundant proteins can vary across ten orders of magnitude [85]. As a consequence, high abundance proteins (*e.g.*, serum albumin or immunoglobulins in blood) which are not highly specific for a body fluid of interest can easily mask the detection of less abundant proteins that are more "diagnostically relevant" for forensic applications. To circumvent this masking effect proteomes are typically fractionated. As stated above, the primary goal of proteomics discovery research has been to identify protein biomarkers. In a medical diagnostics setting biomarker should have a differential expression profile between two samples – one from a diseased patient and another from a healthy individual. Adapted to forensic serology, biomarkers will ideally not be differentially expressed between

different body fluids but should be 100% unique. Both of these objectives rely on the combination of qualitative and quantitative measurements of a proteome and include traditional gel based methods, metabolic and chemical labeling methods, label free methodologies, as well as targeted quantitative methods in order to identify biomarkers.

1.6.1.1 Gel-Based Methods

Traditionally, proteome analysis has relied on two-dimensional polyacrylamide gel electrophoresis coupled with mass spectrometry. These methods have been successful in separating highly complex mixtures to give a global view of the proteome where proteins gels can be compared, spots can be picked, and subsequently identified. However, these techniques have been shown to be highly variable from gel to gel making it difficult to distinguish between actual protein variability versus gel-to-gel experimental variation [86]. More recent improvements have utilized two-dimensional difference gel electrophoresis (2D-DIGE). In this technique several samples are fluorescently labeled, pooled, and run on a single gel. Each spot pattern is then detectable by its unique wavelength and the runs can be normalized and analyzed to detect differentially expressed protein spots for identification though mass spectrometry [87]. While these techniques have improved, there still are limitations that impede the accurate analysis of proteins with very high or low pI values, proteins with large Trans membrane domains as well as limitations to proteins with extreme molecular masses [88, 89].

1.6.1.2 Labeling Based Methods

Metabolic and chemical strategies offer a different approach than in-gel methods. Stable isotope labeling with amino acids in cell culture (SILAC) is one option. In this method cells are grown in cultures with differentially labeled amino acids. One culture media will contain normal amino acids and the other will have heavy isotope labels, *e.g.* C-13 vs. C-12 or N-15 vs. N-14.

The amino acids in culture will be incorporated into the protein. When run on the mass spectrometer, peptides will behave identically in the LC and mass spectrometer but have a measurable mass shift associated with them, allowing quantiation and detection of differential expression between two states [90]. This method has advantages due to a very far upstream incorporation of heavy/light amino acids. The strategy has been successful in many cell culture based systems. Nirmalan *et al.* successfully measured proteomic changes in the parasite *Plasmodium falciparum* with regard to its protein expression during development as well as responses to anti-malaria drugs [91]. A large drawback in this approach was the requirement for the incorporation of labeled amino acids (which occurred in cell culture) thus leaving many human/clinical samples (*e.g.* serum and tissues) incompatible with the assay.

Chemical labeling strategies exist as well. Two well known methods of chemical labeling are isotope-coded affinity tag or ICAT system and isotope tagging for relative and absolute quantitation or the ITRAQ system. Both methods use a chemical label that is incorporated into the protein. In the ICAT system a heavy or light label plus an affinity tag is incorporated into cysteine –SH groups. The biotin affinity tag is used to isolate the ICAT peptides through affinity chromatography and quantitative differences can be measured [92]. ITRAQ uses a different system with the tag attaching to the N terminal of digested peptides. The tagging molecule consists of three groups, a reporter group, an isobaric tag, and a reactive group that will link to peptides. Differentially labeled peptides co-elute but have different reporter ions that dissociate during CID. Quantitative information is achieved by examining reporter ion abundances [93]. Numerous issues are present in these workflows. ICAT, for instance, relies on cysteines to be present. Without the presence of the amino acid the affinity isolation process fails resulting in missed tryptic peptides for quantification. Another issue relevant to both methods is the addition

of labels far downstream. This can be compromised, however, by potential errors in the efficiency of any process before addition of tags such as fractionation, depletion, or digestion[94]. In addition, these methods are typically expensive, rely on specific software, and aside from ITRAQ, are limited to two or three comparisons at a time [95].

1.6.1.3 Label-Free Methods

Two major label free methodologies exist. Compared to labeling techniques where samples are pooled and run simultaneously label free methods have each sample prepared independently. Both methods follow the same processes of sample prep, digestion, sample separation, chromatographic separation, and analysis on a tandem mass spectrometer. Data are quantified either through spectral peak intensities or spectral counting after identification [95]. Using spectral peak intensity method extracted ion chromatograms are found for each peptide and peak areas are found in relation to the total chromatogram. The spectral counting method compares the amount of protein in a sample to the MS/MS spectra relating to that. This method has the advantage of removing laborious and potentially expensive labeling strategies; the capability of running on any sample type, and with no limitation to how many samples can be compared. Conversely, these techniques rely heavily on reproducible chromatography and numerous sample replicates for accurate and precise quantitative measurements [96].

1.6.1.4 Targeted Proteomics

The final methodology is targeted proteomics. The more popular methods utilize the multiple reaction monitoring technique and single reaction monitoring on a triple quadrupole mass spectrometer [97]. This method employs two quadrupoles in the mass spectrometer to isolate specific precursor and product ions. Quantiation is achieved through a stable isotope dilution strategy where labeled standards with known concentration are added to the sample. Both the

synthetic peptides and the native digested peptide will behave identically in the chromatographic separation and mass spectrometer therefore co-eluting and fragmenting in the same way, but, with a known mass shift. Calculating the ratio between standard and native peptide allows quantitation of the peptides [98] . This method has become popular because of its high sensitivity when targeting known compounds. The very high duty cycle, multiplexing capability, and enhanced sensitivity increase due to the reduction in the background noise [99].

1.6.1.5 High Performance Liquid Chromatography

Protein mass spectrometry quality is directly linked to separation technologies to simplify samples before protein identification through tandem mass spectrometry [100] . Such technologies and techniques have been evolving in an attempt to dive even deeper into the proteome while increasing the dynamic range capabilities of the separation techniques. Two such methods, both utilizing different approaches to proteomics, are two-dimensional liquid chromatography (2D-HPLC) on the ProteoSep™system and Multidimensional Protein Identification Technology (mudPIT). Both have left gel-based methods behind in favor of precision HPLC based separations. Along with the different separation methods, these two strategies differ in how the protein/peptides are analyzed. Using the ProteoSep™system proteins are separated in tact; this is commonly referred to as "top down" proteomics. Alternatively, mudPIT performs the separation techniques on proteins that have undergone proteolytic digestion. The latter strategy is commonly referred to as "bottom up" analysis [100]. Both approaches utilize two methods of separation, the ProteoSep™ employing chromatofocusing and reverse phase chromatography while mudPIT uses strong cation exchange coupled with reverse phase chromatography.

With the ProteoSep™2D-HPLC, the first step is chromatofocusing which separates proteins based off their isoelectric point. Proteins are loaded into a positively charged column at a pH of 10 and begin a gradient down to a pH of 4. As the gradient decreases, proteins will begin to elute off the column into a 96 well fraction collector when they reach the point where their net charge is zero. Reverse phase chromatography is performed in sequence on the pH fractions. In this step, a stationary phase with octadecyl carbon chains, or C18, and mobile phase gradient (acetonitrile) elute proteins based off hydrophobic characteristics. Proteins are initially bound to the hydrophobic chains until a sufficient percent of acetonitrile gradient disrupts the interaction from the solid phase [101]. As proteins elute from the reverse phase column they are collected in eight 96 well plates. During the reverse phase runs, a 214 nm UV detector monitors eluents as they are released from the column. Reverse phase runs are then combined to generate a proteome map (**Figure 6**) representing all the proteins successfully fractionated by the system. The multidimensional maps can then be compared using software programs that can detect up/down expression across multiple samples. Based on the users scientific question, individual reverse phase fractions can be selected and tandem mass spectrometry performed to identify proteins in a particular fraction.

Figure 6 - Example of the 2D pI/hydrophobicity maps (*i.e.*, proteome maps) obtained body fluid samples. Proteins which are represented as colored bands across the image are separated based on pH on the x-axis followed by hydrophbicity on the y-axis.

MudPIT utilizes a 2D LC separation technology based off of strong cation exchange coupled to reverse phase chromatography. What makes this methodology unique is that both the RP and SXC resins are packaged together in a single micro capillary column with a direct interface to the mass spectrometer [102]. During a mudPIT run, proteins digested using trypsin are loaded into the SXC portion of the column. A salt "bump" washes a small portion of peptides from the SCX onto the reverse phase resin. Following the salt bump, an acetonitrile gradient separates the peptides by hydrophobicity with elution directly into the mass spectrometer [102]. Following the reverse phase separation and subsequent mass injection into the mass spectrometer, another salt "bump" washes another small amount of peptides into the reverse phase column. The mass spec data can then be examined and quantitative differences can be found where proteins are differentially expressed.

1.6.2 Liquid Chromatography Tandem Mass Spectrometry for Proteomics

The most powerful tool in proteomics research is the mass spectrometry systems which allow scientists to identify, characterize, as well as quantitate proteins in complex biological matrices. These systems incorporate high performance liquid chromatography systems to fractionate protein digests, an ion source to generate charged gas phase molecules, and a mass spectrometer to analyze the ions. Once data have been acquired, bioinformatic software tools are employed to identify proteins by comparing acquired data to a protein database.

1.6.2.1 Reverse-Phase High Performance Liquid Chromatography

Chromatography is a general term for a technique which is used to separate mixtures. All chromatographic techniques utilize a mobile phase as well as a stationary phase. A mixture is dissolved in the mobile phase and is then passed through a stationary phase. Every compound in a mixture will have a higher or lower affinity for the mobile and stationary phases, thus allowing complex mixtures to be separated.

The most popular chromatographic technique for protein analysis is reverse phase high performance liquid chromatography (HPLC). This technique uses a pumping system to deliver a gradient of polar mobile phase to a non-polar stationary phase. The mobile phase consists of a blended aqueous and organic buffer commonly termed buffers A and B. As the name implies, Buffer A consists of water where Buffer B will be made up of an organic solvent (typically acetonitrile or methanol) which is less polar than the aqueous buffer.

During separation, a protein mixture is loaded onto the stationary phase under high aqueous conditions causing hydrophobic moieties on the protein/peptides to bind the non-polar solid phase particles. A linear gradient with an increasing percentage of organic solvent is applied to the stationary phase causing the mobile phase to become increasingly non-polar. As a

result, hydrophilic proteins adsorb to the stationary phase poorly whereas hydrophobic proteins adhere strongly, thus a hydrophilic proteins elute quickly where a higher percentage of organic solvent will be required to elute hydrophilic proteins from the column (**Figure 7**). Once eluted from the stationary phase, individual proteins/peptides are measured via a detector, in the case of proteomics work a mass spectrometer.

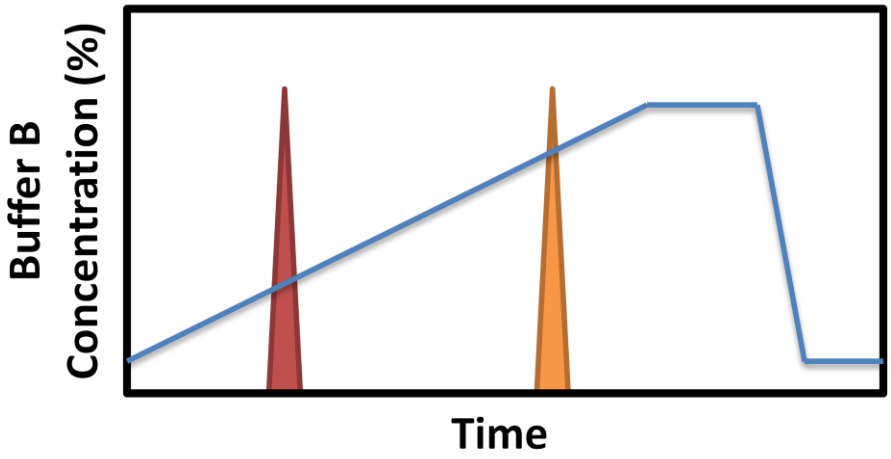


Figure 7 – Acetonitrile gradient used to elute peptides (orange and red peaks) using reverse-phase HPLC.

1.6.2.2 Electrospray ionization

Many modes of ionization exist for generating charged particles for analysis via mass spectrometry. Currently, peptide and protein analysis is restricted to 'soft' ionization techniques via Electrospray (ESI) or Matrix Associated Laser Desorption Ionization (MALDI). This restriction to ESI and MALDI for protein analysis is due to the generation of fragments in ionization from harsh sources such as chemical or electron ionization.

When coupling an HPLC system to a mass spectrometer an electrospray ionization source is commonly used as it allows molecules to become ionized directly from the liquid phase (**Figure 8**).

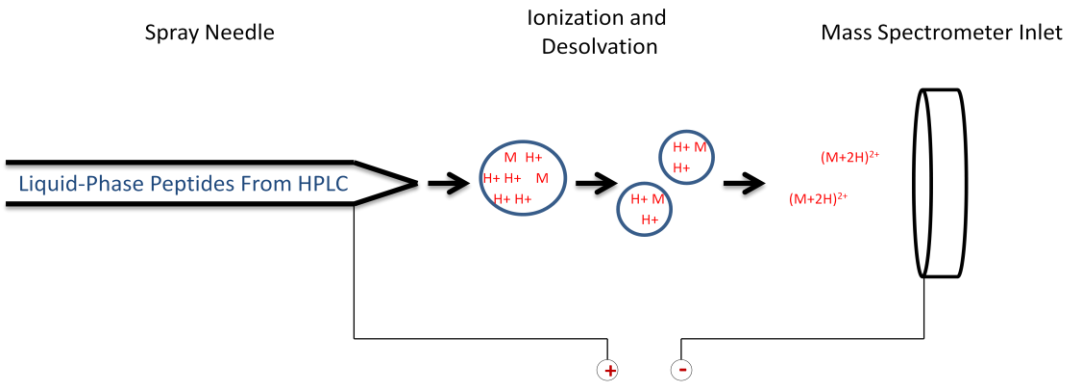


Figure 8 - Electrospray ionization source. Peptides eluting into the source are charged, dried, and travel into the mass spectrometer inlet.

In this process, the volatile organic solvent and acidic eluent (*i.e.* acetonitrile with acid modifier such as formic acid) from the HPLC is forced through a capillary under a high voltage potential (~2000 Volts) between the spray needle and entrance to the mass spectrometer. This process generates positively charged droplets containing solvent, acid, as well as analyte which will travel together towards the negatively charged MS inlet. Heated nitrogen drying gas (~300°C) aids in evaporation of the solvent. As the solvent evaporates, the repulsive forces increase due to the excess charge contained in the droplets [103]. The repulsive force eventually becomes strong enough that a coulombic fission occurs splitting the initial droplet into a number of smaller droplets. Repeated evaporation and fission events eventually lead to protonated gas-phase peptides which enter the mass spectrometer[104].

Proteome analysis by LC-MS/MS represents one of the most difficult analytical challenges when trying to biomarkers in complex biological matrices. Sample quantities are typically limited and individual analytes/proteins in a preparation are generally low abundant. To circumvent these problems, nanoscale liquid chromatography is typically employed to increase

the sensitivity of the LC-MS platform as well as decrease the amount of material necessary to generate quality data.

As the method implies, nanoscale liquid chromatography is simply a scaled down version of standard electrospray. As a comparison, a standard HPLC column with a 4.6 mm inner diameter and a 150 mm length would have a column volume of approximately 2.5 mL. On the other hand, a nanoscale column with a .075 µm inner diameter and a 150 mm length would have a column volume of only 700 nL. Translating this to elution volumes, a peptide eluting from the column on a standard HPLC column would enter the ESI source in 500 µL where the same peptide in a nanoscale system would have a peak volume of only 135 nL[105]. In addition to the concentration advantage in nanoflow systems, nanoscale electrospray typically generates smaller charged droplets leading to increased ionization efficiency.

1.6.2.3 Mass Spectrometry

Mass spectrometers consist of a mass analyzer to separate ions on the basis of mass as well as a detector to record the amount of each mass value [106].More specifically, mass spectrometers measure compounds using mass-to-charge (m/z) ratios. m/z ratios are simply the mass number divided by its charge. For example, a peptide sequence Serine-Glycine-Alanine-Valine-Methionine-Lysine (**Figure 9**) will protonate and enter the mass spectrometer with a +2 charge. The m/z ratio for this compound would computed by determining the mass of the compound 591 Da, adding two protons, and dividing by a charge state of 2 to give the following formula (591+2) / 2 = 296.5 m/z.

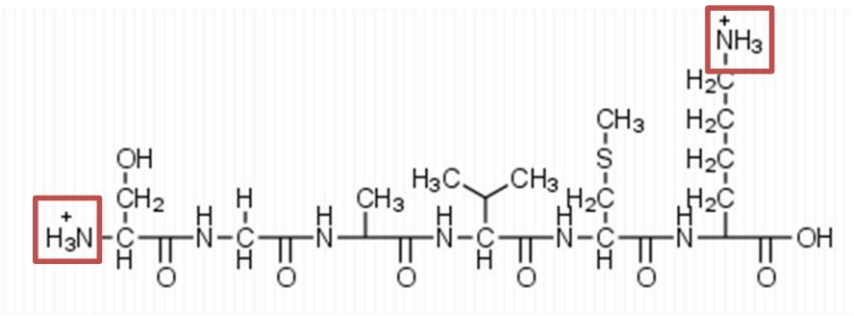


Figure 9 – Peptide with the sequence Serine-Glycine-Alanine-Valine-Methionine-Lysine. The m/z ratio for this doubly charged compound would be 296.5 m/z.

There are various mass spectrometers capable of performing proteomics experiments. Each instrument can be differentiated by the mass analyzers integrated into the instrument with the quadrupole, ion trap, and time-of-flight being some of the most common[106, 107]. Modern proteomics instruments are capable of multiple steps of mass measurements. This process, also called tandem mass spectrometry, will generate a mass spectrum of both the intact ionized peptide (MS) as well as its fragmentation spectra (MS/MS). Some instruments such as an ion trap can perform MS and MS/MS using the same mass analyzer where other instruments, call hybrid mass spectrometers, require multiple analyzers working together.

Ion trap mass spectrometers (**Figure 10**) utilize alternating electric fields to trap and eject ions from the trap. Using this system, peptides are trapped and subsequently ejected from an oscillating electric field produced from static DC and alternating radio frequency AC currents[108]. More specifically, a multi-stage tandem-in-time process is carried out where ions are first stored or "trapped" in orbitals specific to their m/z ratios. The RF amplitude is then ramped up which ejects peptides from the trap to a detector. Acquisition software will examine the MS spectra and select specific precursor ions for fragmentation. Ions not selected for fragmentation are ejected from a new batch of ions in the trap. An increased RF signal is then applied at a specific resonance frequency to induce fragmentation of the isolated compound.

Product ions are then ejected in the same manner as the initial MS scan, with the spectra representing product ions generated during fragmentation[103].

One notable feature of ion traps is the ability to perform additional MS scans on product ions (MS^n). MS^n allows for a compound to be fragmented and the resulting fragments further isolated in the ion trap. This feature allows ion traps to provide greater explanation of the chemical structure of compounds compared to other systems[109].

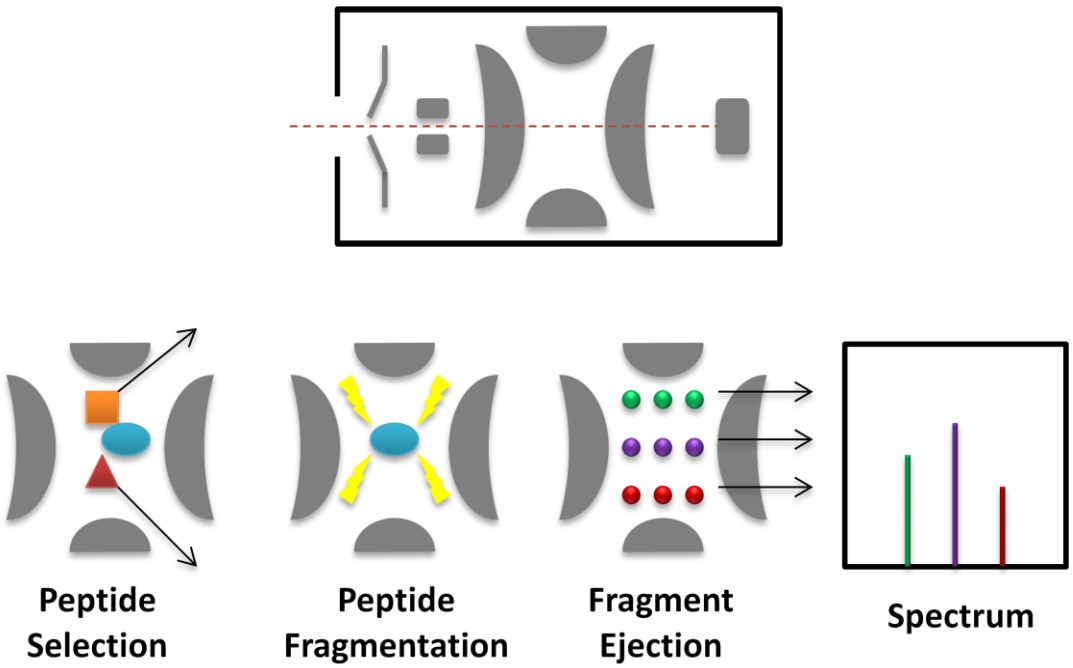


Figure 10 Top – Schematic of an ion trap mass spectrometer. Bottom – MS/MS spectra generation through peptide isolation, fragmentation, and ejection from the ion trap.

Quadrupole Time-of-Flight (QTOF) is a hybrid mass spectrometer which combines a quadrupole and time of flight mass analyzers (**Figure 11**). Like the ion trap, these instruments are capable of MS and MS/MS scans as well as ion targeting using inclusion lists. In the standard

acquisition mode, the quadrupole mass filter applies an oscillating electric field to stabilize only a narrow window of m/z ratios. The quadrupole will perform a series of scans over small mass window allowing low to high m/z ratios to pass through the quadrupole. Ions are then accelerated through the Time-of-flight (TOF) analyzer and detected to produce an MS scan of the peptides entering the instrument. Acquisition software then selects specific precursor ions from the ms scan, the quadrupole filters for only that specific m/z, the precursor ion is fragmented in the collision cell and the product ions are accelerated and resolved in the TOF before detection. The latter precursor isolation, fragmentation, separation, and detection produce the MS/MS fragmentation spectra.

The advantages to the Q-TOF compared to the ion trap are twofold. First, the quadruple mass analyzer allows the system to operate using an inclusion list. This acquisition directs the instrument to only acquire data over a specific m/z ratio and time period during the reverse phase separation, leading to increased sensitivity and reproducibility for selected analytes. Additionally, the system can achieve very good mass accuracy (<10 ppm) coupled with high resolution. These factors combine to give the QTOF the advantage of being able to accurately determine precursor and product ions when compared to a triple quadrupole or ion traps, leading to more confident qualitative information [110, 111]. However, the TOF analyzer, which allows for highly accurate mass measurements, also imposes a low duty cycle on the instrument, which is proportional to the time the instrument in useful operation [112].

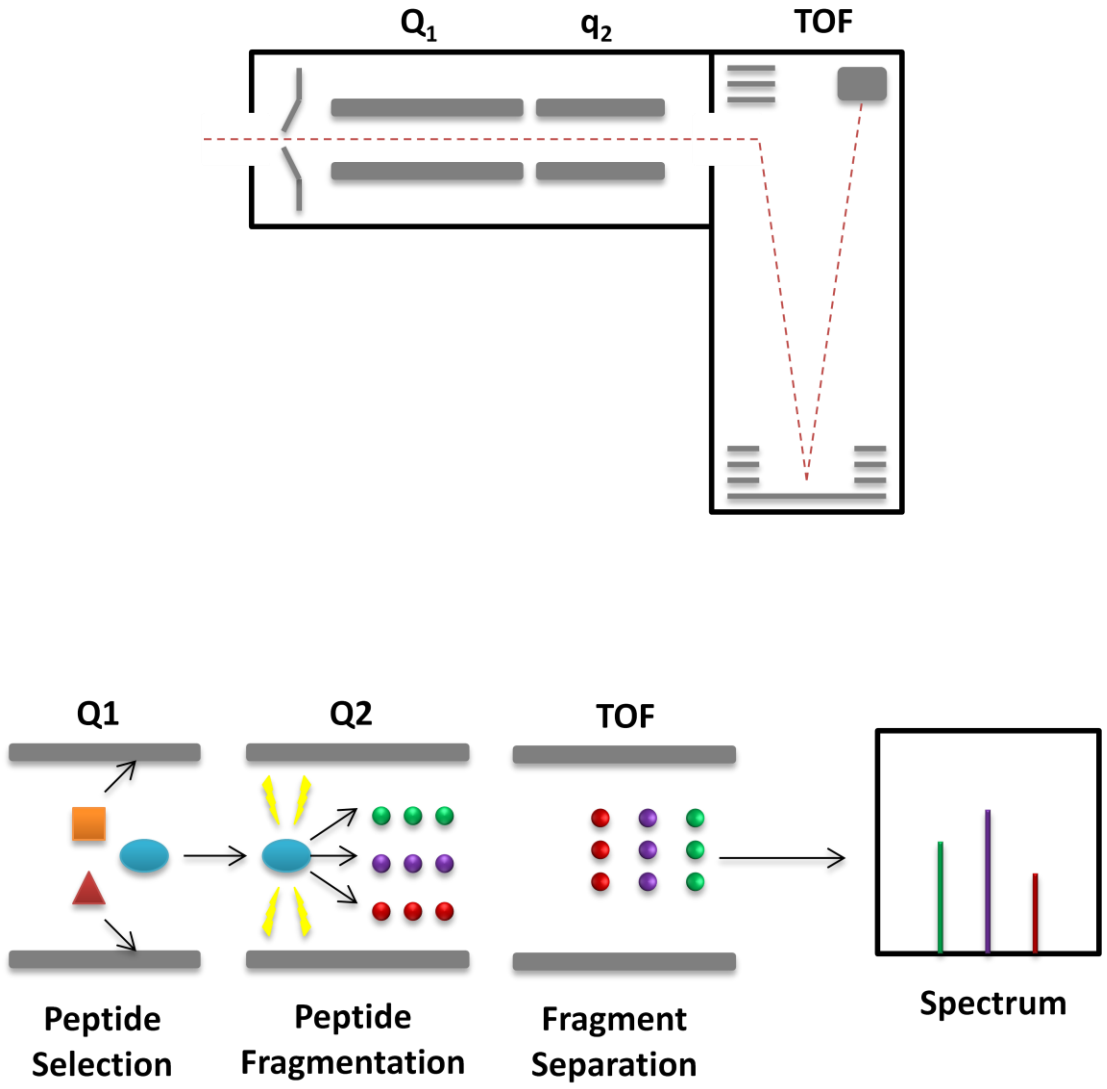


Figure 11 - Schematic of a Quadrupole Time-of-Flight mass spectrometer. Bottom – MS/MS spectra generation through peptide isolation in quadrupole one (Q1), fragmentation in the collision cell (Q2), followed by separation by the TOF mass analyzer.

Triple quadrupole mass spectrometers (QQQ) have been described as the gold standard for modern quantitative analysis [113]. This instrument (**Figure 12**) is made up of two quadrupole mass filters with a collision cell between them. The gold standard classification has been awarded due to the sensitivity, selectivity, and ease of operation of these instruments due to the ability to perform selective ion monitoring (SRM) and multiple reaction monitoring (MRM). In this mode of operation two separate selection steps are performed. First, quadrupole one will

isolate a select precursor ion, precursor ions are selected fragmented in the collision cell q2, and then specific fragmentation or product ions are selected for in Q3 and subsequently detected. While this instrument has much lower mass accuracy/resolution compared to the QTOF, the QQQ mass spectrometers in MRM mode achieve a greatly enhanced signal-to-noise ratio which ensures that target ions are detected with improved sensitivity and confidence even with low amounts of starting material [114].

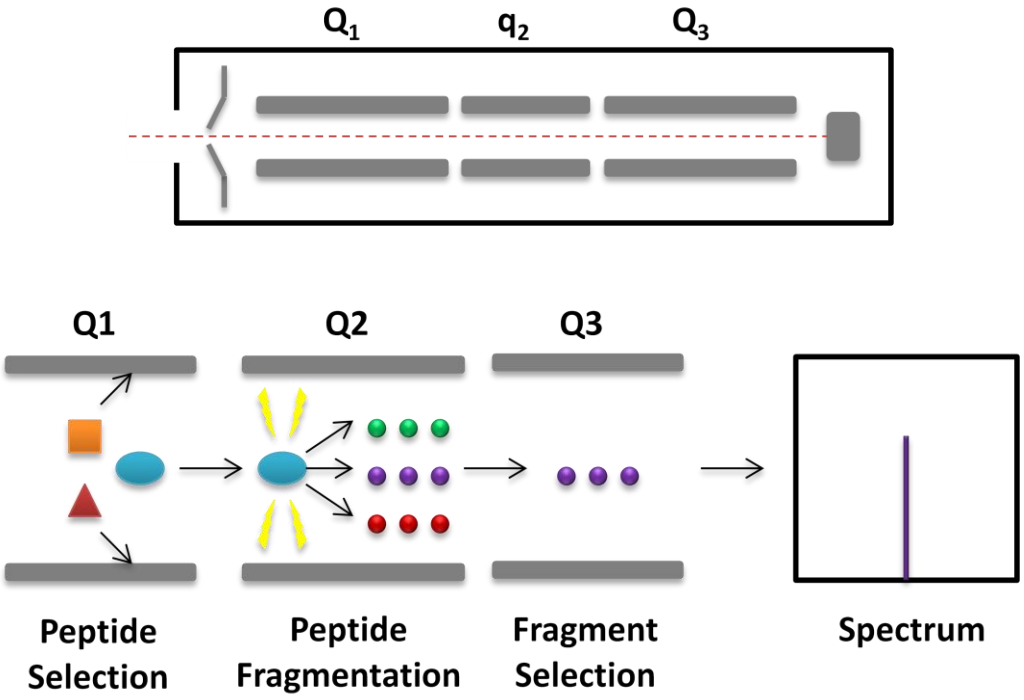


Figure 12 - Schematic of a triple quadrupole mass spectrometer. Bottom – MS/MS spectra generation through MRM mode with peptide isolation in quadrupole 1 (Q1), fragmentation in the collision cell (Q2), product ion isolation in quadrupole 2 (Q33.

1.7 Research Objectives

In the current research study, proteomics techniques will be applied to six forensically significant body fluids including semen, saliva, urine, peripheral blood, menstrual blood and vaginal fluid in order to develop a multiplex body fluid assay on a mass spectrometer. To do this, a standard proteomic workflow will be employed encompassing a biomarker discovery phase and a verification phase followed by the development of a prototype assay for validation/casework.

The discovery phase of the research aims to generate a panel of candidate protein biomarkers which are unique to six body fluids including saliva, seminal fluid, vaginal fluid, menstrual blood, peripheral blood, and urine. Front end fractionation will be performed with the ProteoSep™ PF2D 2D-HPLC in order to generate and compare PF2D proteome maps for protein identification via tandem mass spectrometry. This will generate a database of candidate high-specificity protein biomarkers.

The biomarker verification phase aims to evaluate the reliability and specificity of the candidate biomarkers. A multiplex, targeted, Q-TOF assay will be designed to analyze samples of each body fluid from fifty individual donors to evaluate whether the biomarker candidates are consistently expressed across every individual while determining if the candidates are strictly specific to the intended target body fluid.

The prototype validation phase will examine the viability a mass spectrometry based serological assay for use in a case working lab. The most promising targets from the verification phase will be used to develop a multiple reaction monitoring assay on a triple quadrupole mass spectrometer. Additionally, a series of casework-type samples will be prepared which will include the evaluation of a DNA/Proteomic co-extraction procedure.

1.8 Hypothesis

The central hypothesis being tested is that a mass spectrometry based proteomics assay will provide an accurate and robust multiplex assay for the simultaneous identification of semen, saliva, urine, peripheral blood, menstrual blood and vaginal fluid for use in processing serological samples in a crime lab. The assay will improve upon current methodologies by providing true confirmatory results for all the target fluids in a single multiplex assay. The core hypothesis of this dissertation research is therefore:

(1) Proteomic fractionation and tandem mass spectrometry is capable of generating a database of body fluid specific proteins for the differentiation of biological stains.

(2) A mass spectrometry based assay can successfully be designed to screen for six forensically relevant body fluids in a single assay.

(3) Mass spectrometry is sufficiently robust for processing process casework samples in a production lab.

(4) Mass spectrometry is compatible with existing DNA workflows as well as to the demands of a testing laboratory.

1.9 Dissertation Structure

Each chapter contains a brief introduction, description of the experimental methods employed, results as well as a discussion of the research findings. Chapter 2 will focus on the discovery of a list of candidate biomarkers through three identification approaches. The focus of chapter 3 is the development and testing of a multiplex assay on a Q-TOF which can screen for six biological fluids simultaneously. Chapter 4 will assess the feasibility of a mass spectrometry

based body fluid assay in a casework lab, this will include experiments which examine the compatibility of sample preparation for this approach with existing DNA methodologies.

Chapter 2:

Biomarker Discovery

2 Introduction

The objective for this phase of the dissertation research was to develop a database of candidate biomarkers candidates that are specific to individual biological fluids using proteomic techniques. These fluids include saliva, semen, urine, vaginal fluid, peripheral blood, and menstrual blood. Ideally, this section of the dissertation research will identify five to ten total candidates for each fluid as it is possible/likely that initially promising but ultimately non-specific proteins will exist in the dataset.

Traditionally, fractionating and recovering proteins from complex mixtures for downstream analysis has been handled by 2D gel electrophoresis (2DGE) and manual excision/purification of proteins of potential interest [115]. While these methods have a long history, it is difficult to resolve proteins that are lipophilic, very large (>150 kDa), very small (<5 kDa) or less abundant. Also, poor reproducibility requires that numerous gels be run to obtain data sets that can be reliably compared with each other [116].

More recently, multidimensional liquid chromatography platforms have been introduced to alleviate to limitations to traditional gel-based approaches [117]. The use of liquid phase separation avoids the solubility problems associated with gels. Second, a more complete profile of each body fluid can be generated because more proteins are fractionated and recovered. Third, less abundant proteins in complex mixtures can be screened as potential markers of specific body fluids because there was a higher efficiency of recovery (>95%) and more total protein could be injected (50µg-30mg) without the band distortion that occurs with 2DGE.

With the known limitations of 2DGE in mind, a single method to characterize a proteome may require multiple approaches including 1D fractionation, 2D fractionation, as well as a "whole" proteome analysis. Thus, a series of experiments were performed in order to populate a

database of body fluid-specific proteins. The current study investigated the use of the ProteoSep™ PF2D 2D-HPLC and tandem mass spectrometry to fulfill these goals. First, pH and reverse-phase fractionation (2D separation) was used in combination with a custom in-house software package. Next, pH fractionation alone (1D separation) will be performed. Finally, a whole proteome approach using unfractionated samples was analyzed using tandem mass spectrometry.

2.1 Materials and Methods

All research conducted under this project was IRB reviewed, approved and conducted in full compliance with U.S. Federal Policy for the Protection of Human Subjects. **Figure 13** is an overview of the workflow performed from sample collection through biomarker identification.

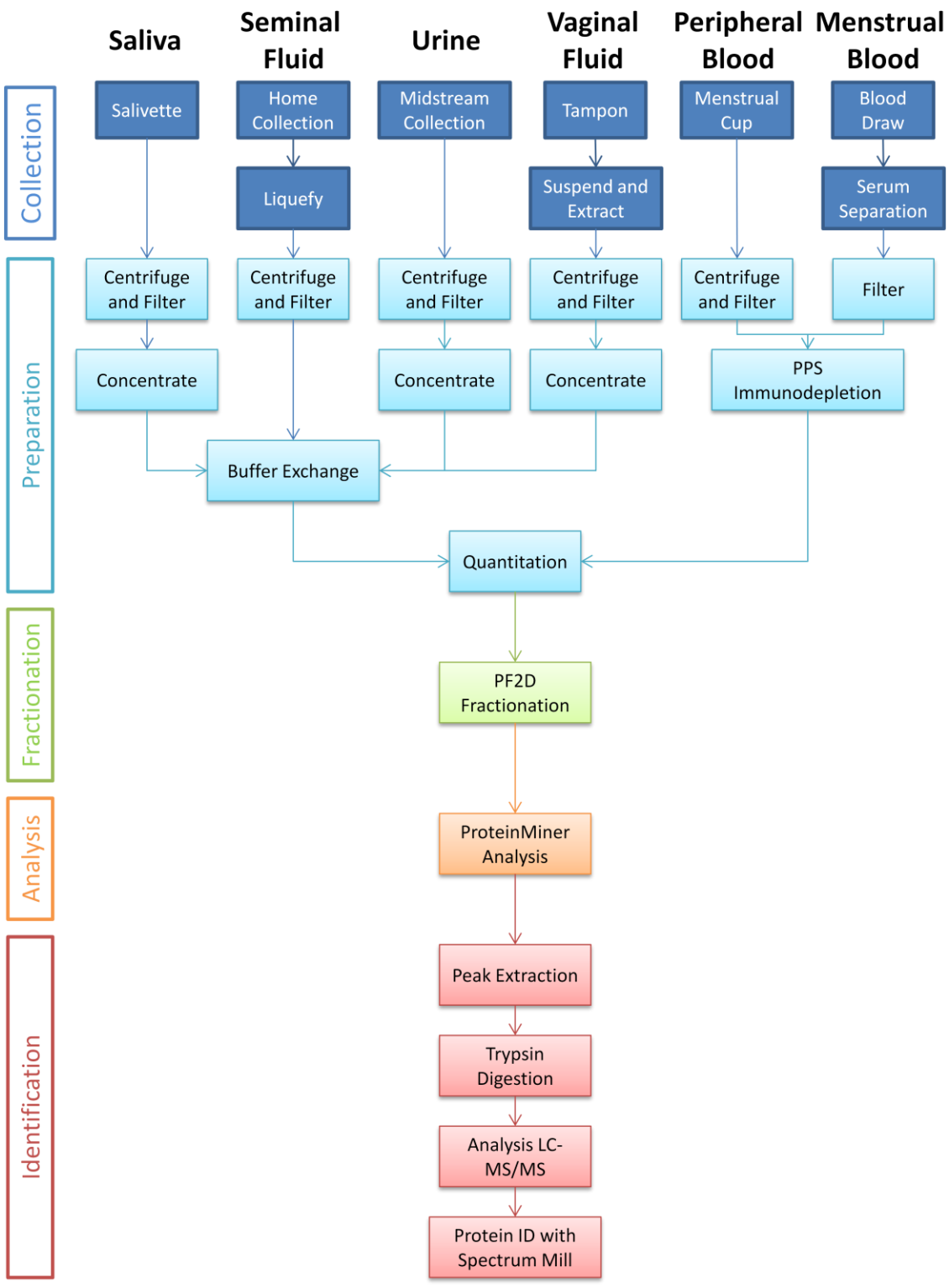


Figure 13 - Overview of Biomarker discovery phase from sample collection to protein identification.

2.1.1 Sample Collection and Preparation

2.1.1.1 Saliva

The donor was directed to thoroughly rinse their mouth with sterile water to remove residual food particles. After 5 minutes to allow secretion of saliva, the donor was instructed to place a Sarstedt Salivette™ saliva collection sponge into their mouth and to gently chew and roll the sponge around in their mouth for 3-4 minutes. The sponge was then placed into a sterile plastic conical tube and repeated 5 additional times.

Salivette™ sponges were centrifuged for 2 min at 1500 x g at 4°C to recover saliva. Individual Salivette™ sponge fractions were then pooled for each donor for a total volume between 5 and 10 mL. Pooled samples were centrifuged again at 10,000 x g for 20 minutes at 4°C to pellet cells and insoluble material. Supernatant was filtered through a .45 µm filter to remove remaining debris prior to concentration. Each sample was then concentrated using an Amicon Ultra-15 with a 5K NMWCO centrifugal filter units at 4000 x g for 30 minutes to concentrate sample to 2.5 mL.

2.1.1.2 Seminal Fluid

Donors were directed to refrain from sexual activity for a minimum of 24 hours and then to obtain a 3-6ml sample of seminal fluid by masturbation in the privacy of their home. The subject was requested to directly deposit the fluid into a sterile plastic collection cup provided by the laboratory and then to refrigerate the sample until it could be transported to the lab within 1 hour.

Freshly collected semen was then incubated at room temperature for 30 minutes to allow it to liquefy. Liquefied samples were transferred to a 15 mL conical vial and diluted with a 1/3 volume of phosphate buffered saline. Sample was then centrifuged at 10,000 x g for 20 minutes

at 4°C to pellet spermatozoa. The protein-rich supernatant was then passed through .45 µm filter to ensure cellular removal.

2.1.1.3 Urine

Donors were directed to deposit a morning urine sample (>50ml) into a sterile collection cup provided by the laboratory. Protein concentration varied substantially between individuals thus > 20 ml was typically used to ensure a sufficient quantity of protein for proteome mapping.

Freshly collected Urine was centrifuged at 10,000 x g for 20 minutes at 4°C and passed through a .45 µm filter to ensure cellular removal. Two 15 mL volumes of urine were then concentrated using an Amicon Ultra-15 with a 5K NMWCO centrifugal filter units at 4000 x g for 30 minutes to concentrate sample to 2.5 mL total volume.

2.1.1.4 Vaginal Fluid

Donors were directed to insert a commercially available 100% cotton tampon and were encouraged to use lubricant to minimize the risk of tissue abrasion and/or microbial infections. The tampon was left in place for the period of approximately 10 minutes, gently removed and placed in a 15mL conical tube. Donors were directed to refrigerate the sample until it could be transported to the lab within 1 hour.

Tampons were saturated with PBS and allowed to sit at room temperature for 30 min with occasional vortexing to elute proteins. The tampon was then placed in a 50cc syringe to force out the fluids and eluted proteins. The liquid was transferred to 50 ml conical vials and centrifuge at 10,000 x g for 20 minutes at 4°C. The resulting supernatant was passed through a .45 µm filter to ensure cellular removal prior to concentration. Sample was then concentrated using an Amicon Ultra-15 with a 5K NMWCO centrifugal filter units at 4000 x g for 30 minutes to concentrate total sample to 2.5 mL.

2.1.1.5 Peripheral Blood

Donors were escorted to the Student Health Center where a 15ml sample of whole blood was obtained by a certified nurse using venipuncture. The blood was drawn into a sterile vacuum tube containing an anticoagulant (EDTA) and gel barrier to separate cellular material.

EDTA gel barrier tubes were spun for 10 minutes at 1500 x g to separate serum. Blood serum was then passed through a .45 um filter unit to ensure cellular removal from sample.

2.1.1.6 Menstrual Blood

Menstrual blood was self collected by study participants in the privacy of their home. The collection protocol employed an FDA-approved over-the-counter latex-free, hypoallergenic cup (DivaCup™) for the collection of menstrual flow. The donor was directed to insert the cup into the vagina during menses for a period of approximately 1 hour. The cup was then gently removed; the contents were poured into a sterile 50ml conical tube and refrigerated until delivered to lab.

Fresh menstrual blood was transferred to a 15 mL conical vial and diluted with 1/3 total volume with phosphate buffered saline. Sample was homoginized by vortexing and added to EDTA gel barrier blood collection tube and allowed to incubate for 15 minutes at room temperature. Tubes were then spun for 10 minutes at 1500 x g to separate serum. Menstrual serum was then passed through a .45 μm filter unit to ensure cellular removal from sample.

2.1.1.7 Hemoglobin Removal from Menstural Blood

HemogloBind™ from Biotech Support Group was used to remove hemoglobin from lysed erythrocytes commonly encountered in menstrual blood samples. To 250 μL menstrual serum 500 μL of HemogloBind™ was added. Solution was vortexed and gently mixed for 15

minutes. Sample saw then centrifuged at 9000 rpm for 2 minutes. The supernatant containing hemoglobin free menstrual serum was then stored until needed.

2.1.1.8 Protein Partitioning System: Immunodepletion

Both menstrual blood and peripheral blood were depleted of twelve non-specific high-abundance proteins which had the potential to mask the detection of greater specificity lower-abundance species. These included albumin, IgG, IgA, IgM, fibrinogen, transferrin, HDL, haptoglobin, α1-antitrypsin, α1-acid glycoprotein and α2-macroglobin. For Protein partitioning, 250 µL of sample was diluted with 375 µL of dilution buffer (10 mM Tris-HCL, 150 mM NaCl, pH 7.4). The partitioning column was first equilibrated for 30 minutes at 2 mL/min with dilution buffer. Proteins were loaded onto column in dilution buffer at 2 mL/min. Unbound flow through was collected in 50 mL conical tubes using a Beckman FC module. Bound high abundant proteins were eluted with stripping buffer (100 mM glycine, pH 2.5) at 2 mL/min. Lastly, the column was equilibrated with dilution buffer at of 2 mL/min This process was repeated with 7 total runs for each sample. Flow through from each individual sample was pooled and concentrated with an Amicon Ultra-15 with a 5K NMWCO centrifugal filter units at 4000 x g for 30 minutes to concentrate sample to 1 mL total volume. PF2D start buffer was added for a total volume of 4 mL and concentrated again to exchange buffer contents. PF2D start buffer was added again to a final volume of 2 mL.

2.1.1.9 Buffer exchange

Non-blood samples which had not been treated by PPS immunodepletion column required buffer exchange prior to PF2D injection. PD-10 Desalting Columns from GE Healthcare were equilibrated with 25 mL of PD2D start buffer. 2.5 mL sample was added to PD-

10 column and the flow through was discarded. 3.5 mL of PF2D start buffer was added to column to elute proteins in clean 3.5 mL conical tube.

2.1.1.10 Protein Quantitation with Pierce Micro BCA kit

Dilution standards were prepared as follows using Bovine Serum albumin (BSA) as a calibrator. A total of eight serial dilutions were prepared with concentrations ranging from .5 µg/mL to 200 µg/mL.

Working reagent was prepared with 25 parts BCA Reagent A and 25 parts BCA Reagent B with 1 part BCA Reagent C. In a 1.5 mL microcentrifuge tube 150 µl of working reagent was added to 150 µL of sample or standard. Tubes were vortexed, spun briefly, and allowed to incubate in an Eppendorf Thermomixer® at 37ºC for 2 hours. Samples were allowed to cool to room temperature for 10 minutes and read on a visible spectrophotometer at 562 nm. BSA curve was used to generate a linear equation which was used to determine protein concentration of unknowns.

2.1.2 PF2D 2D Fractionation

Fractionation using the Beckman Coulter ProteomeLab™ PF2D was performed in two steps, high-performance chromatofocusing followed by high-performance reverse-phase chromatography.

High-performance chromatofocusing column was used to fractionate protein mixtures based on the isoelectric points. An in-line pH meter controlled the output of the eluent to a 96-well plate in 0.1 pH increments from pH 8.5 to pH 4.0. The first dimension chromatofocusing (HPCF) was equilibrated with 30 column volumes of start buffer at pH 8.5 at a flow rate of 0.2 ml/min for 130 minutes. Following equilibration, 5 mg of protein was injected into the HPCF module followed by 20 min of start buffer at 0.2 ml/min. At 20 minutes, eluent buffer (pH 4) was run at 0.2 ml/min for 115 min to create a pH gradient with fractions collected at 0.1 pH intervals

and stored in chilled autosampler. At 115 min 1 M NaCl was run as a high ionic strength salt wash.

Following completion of HPCF fractionation each fraction collected from the first dimension were automatically injected into a high-performance reverse-phase (HPRP) column where proteins are separated by hydrophobicity. This second dimension HPRP column was initially flushed with 5 column volumes of 0.08% TFA in acetonitrile followed by 10 column volumes of 0.1% TFA in H2O running at 0.75 ml/min. Sample proteins were bound with 2 min of 0.1% TFA in water at a flow rate of 0.75 ml/min. At 2 minutes, a 0-100% of 0.08% TFA in acetonitrile gradient was performed over 30 minutes creating a 3.33% change in solvent/minute. At 0.5 min intervals, fractions were collected with a Gilson FC-204 fraction collector in a series of twenty 96-well plates. The collected fractions (approximately 400), containing intact proteins, were stored frozen in a locked -70°C freezer until required for further characterization.

2.1.3 PF2D 2D Data Analysis

ProteinMiner™, which is based on a combination of C++, Perl, and Matlab, was used to address three aspects of data analysis. These were: 1) porting datasets from the ProteomeLab™ PF2D System to consistent and useful formats capable of making tab/comma delimited files; 2) data visualization and graphical manipulation to facilitate detailed visual analysis aimed at detecting possible forensic protein targets and; 3) "number crunching" to combine and average data from individual samples to create a single "consensus map" for a given body fluid and then to compare consensus maps across body fluid proteomes. The specific functionalities of ProteinMiner™ software application are addressed in greater detail in the "Results and Discussion" section of this report.

2.1.4 PF2D 2D Biomarker Identification

2.1.4.1 Protein Recovery

Proteins that were of interest as potential biomarkers needed to be recovered from 96 well plates. To do this a novel fraction analysis software package from the University of Michigan was used to rapidly locate a specific protein fraction that has been eluted from the PF2D second dimension. By simply placing the cursor over a desired peak, the software outputs the specific deep-well plate location of the protein fraction of interest.

2.1.4.2 Trypsin digestion

Proteins located by the fraction analysis software were lyophilized in a vacuum evaporator. Dried protein samples were reconstituted in 40ul of 100 mM Tris-HCl pH 8.5, 1.2ul of 100 mM TCEP reducing agent and then shaken for 20 minutes at room temperature. Then, 0.88ul of 500 mM IAA was added and the sample was shaken in the dark for an additional 15 minutes to alkylate the proteins. The proteins were digested with 1.25 ug of trypsin for 8 hours at 37°C. Samples were sonicated and digested with a second equal volume of trypsin for an additional 8-10 hours at 37°C. Digested samples were dried and re-suspended suspend in 3% acetonitrile and 0.1% formic acid for mass spectrometry analysis.

2.1.4.3 MS/MS Acquisition

Mass spectrometry was performed on an Agilent 6300 series ion trap mass spectrometer coupled to a 1200 series HPLC-Chip/MS system (Protein ID “short chip” specifications 43mm 300 A C18 chip) using 0.1 to 1.5ul of digested sample per injection. Columns were equilibrated in 0.1% Formic acid in water. A 0-45% of 0.1% formic acid in acetonitrile gradient was performed over 11 minutes followed by a 4 minute column re-equilibration.

2.1.4.4 Database Searching

Data analysis was performed using Spectrum Mill software suite by Agilent Technologies. The Swiss-Prot database was used to match MS/MS spectrum generated on mass spectrometer. Typically proteins identified with 2+ peptides and scores >16 were considered confident matches.

2.1.5 PF2D 1D pH Fraction Biomarker Identification

2.1.5.1 pH Fraction Desalting

Corning Spin-X UF 500 concentrators (5000 MWCO) were used to concentrate and desalt PF2D pH fractions. 500 µL of sample was added to the filter unit and spun at 10,000 x g until filtrate had passed through column. Three 500ul volumes of 50 mM ammonium bicarbonate was then passed through the column to remove salts. Filtrate was then quantified for total protein using the Pierce Micro BCA Assay previously described.

2.1.5.2 Trypsin Digestion

30 micrograms of total protein were lyophilized in a vacuum evaporator. Dried protein samples were reconstituted in 40ul of 100 mM Tris-HCl pH 8.5, 1.2ul of 100 mM TCEP reducing agent and then shaken for 20 minutes at room temperature. Then, 0.88ul of 500 mM IAA was added and the sample was shaken in the dark for an additional 15 minutes to alkylate the proteins. The proteins were digested with 1.25 ug of trypsin for 8 hours at 37°C. Samples were sonicated and digested with a second equal volume of trypsin for an additional 8-10 hours at 37°C. Digested samples were dried and re-suspended suspend in 3% acetonitrile and 0.1% formic acid for mass spectrometry analysis.

2.1.5.3 MS/MS Acquisition and Data Analysis

Mass spectrometry was performed on an Agilent 6300 series ion trap mass spectrometer coupled to a 1200 series HPLC-Chip/MS system (Protein ID chip specifications 150mm 300 A C18 chip) using 1ul = 1 ug of digested sample per injection. Columns were equilibrated in 0.1% Formic acid in water. A 0-45% of 0.1% formic acid in acetonitrile gradient was performed over 40 minutes followed by a 6 minute column re-equilibration. Data analysis was performed as previously described.

2.1.6 Unfractionated Biomarker Identification

2.1.6.1 Sample Preparation and Trypsin Digestion

All samples were prepared as described in section 2.1.1. Trypsin digestion was performed as previously described using 30 ug of total non-fractionated protein.

2.1.6.2 MS/MS Acquisition and Data Analysis

Mass spectrometry was performed on an Agilent 6510 series Q-TOF mass spectrometer coupled to a 1200 series HPLC-Chip/MS system (Protein ID chip specifications 150mm 300 A C18 chip) using 1ul = 1 ug of digested sample per injection. Columns were equilibrated in 0.1% Formic acid in water. A 0-45% of 0.1% formic acid in acetonitrile gradient was performed over 44 minutes followed by a 6 minute column re-equilibration. Data analysis was performed as previously described.

2.2 Results and Discussion

In total, over 1000 proteins were identified in the course of the biomarker discovery experiments. This included candidate proteins identified by: (1) Analysis of peaks from the PF2D which were identified as unique using an in-house software package; (2) Analysis of protein rich pH fractions and; (3) Analysis of unfractionated body fluid samples. Table 2.1 summarizes all candidate biomarkers identified under these three approaches. The details of each experiment will be described separately.

Table 1 - Summary of identified protein biomarkers across all three separation approaches.

Fluid	Protein	Accession Number	PF2D Fraction	pH Fraction	Non Fraction
Semen	Epididymal secretory protein E1	P61916		√	√
	Glycodelin	P09466			√
	Semenogelin-1	P04279	√	√	√
	Semenogelin-2	Q02383	√	√	√
	Prolactin-inducible protein	P12273	√	√	
	Prostate-specific antigen	P07288	√	√	√
	Prostatic Acid Phosphatase	P15309	√	√	√
Saliva	Cystatin_D	P28325			√
	Cystatin_SA	P09228		√	√
	Histatin-1	P15515	√		√
	Mucin-5B	Q9HC84			√
	Salivary acidic proline-rich phosphoprotein 1/2	P02810	√		
	Statherin	P02808	√		√
	Submaxillary gland androgen-regulated protein 3B	P02814			√

Vaginal Secretions	Cornulin	Q9UBG3	√	√	√
	IgGFc-binding protein	Q9Y6R7			√
	Involucrin	P07476	√	√	√
	Ly6/PLAUR domain-containing protein 3	O95274		√	√
	Matrix metalloproteinase-9	P14780			√
	Mucin-5B	Q9HC84			√
	Neutrophil gelatinase-associated lipocalin	P80188		√	√
	Periplakin	O60437	√	√	
	Suprabasin	Q6UWP8			√
	Vimentin	P08670	√		
Urine	Osteopontin	P10451	√	√	√
	Uromodulin	P07911		√	√
Menstrual Blood	Calpastatin	P20810		√	
Peripheral Blood	α-1-antitrypsin	P01009		√	√
	Complement C3	P01024	√	√	√
	Hemoglobin subunit beta	P68871		√	√
	Hemopexin	P02790	√	√	√

2.2.1 Biomarker Identification: 2D PF2D Fractionation

Five individuals were recruited to donate samples of each body fluid being analyzed. This redundancy was intended to help to discriminate between proteins that are consistently expressed in a specific body fluid versus those that might reflect inter-individual variability in protein expression - and thus not be suitable as biomarkers. Five individuals reflected an additional effort to obtain the maximum amount of proteomic information within a reasonable budget and timeframe. Additionally, while mapping proteomes from a larger number of study participants

would provide a stronger assessment of protein expression among different humans, the primary objective of this research stage was to identify candidate biomarkers of body fluids that could be verified for use across a larger population.

Samples were prepared as summarized in **Figure 13.** Saliva, Seminal fluid, Urine, and Vaginal Fluid were prepared similarly; samples were pelleted at high speed to remove cellular material followed by concentration and buffer exchange into PF2D loading buffer.

Menstrual and peripheral blood needed to be treated independently primarily due to the complexity of the blood proteome. Peripheral blood was collected in EDTA gel barrier tube in order to generate blood serum devoid of cells as well as clotting factors. Menstrual blood, however, were typically contaminated with hematosed red blood cells. As hemoglobin comprises 32-36% of all the proteins found in red blood cells the serum from menstrual blood samples contained large quantities of hemoglobin which served to mask the detection of less abundant menstrual blood specific proteins. For this reason, hemoglobin was removed from collected serum prior to proteome fractionation through use of HemogloBind™.

Because the dynamic range of protein concentration in serum spans more than ten orders of magnitude, the presence of these high-abundance proteins (*e.g.* albumin, IgG, transferrin) make it difficult to identify proteins that are abundant but which are more likely to be specific markers of each body fluid [118]. To circumvent this problem, commercially available IgY-12 Proteome Partitioning columns were employed. These antibody-based columns made it possible to remove twelve highly abundant proteins (albumin, IgG, transferrin, fibrinogen, IgA, α-2-Macroglobin, IgM, α-1-acid antitrypsin, haptoglobin, α-1-acid glycoprotein, apolipoprotein A-I

and A-II) from human blood serum. This yielded an enriched pool of the less abundant but more body fluid specific blood proteins in the flow-through fraction.

Each body fluid sample was quantified using the Pierce Micro BCA kit as described and a standardized 3 mg of total protein was loaded onto the PF2D system. In total, thirty proteome maps were generated. In each of these maps, data from the chromatofocusing column (X axis) is combined with data from the reverse phase column (Y axis) to yield a .dat file containing a X, Y, and Z (height) values which can be processed and visualized with the ProteoVue™ software suite. Examples of the 2D pI/hydrophobicity maps for peripheral blood, urine, semen and saliva are shown in **Figure 14**. The intensity and color of the bands represent the abundance of the protein detected. Red, orange and yellow bands represent more abundant proteins and while green and blue represent less abundant proteins.

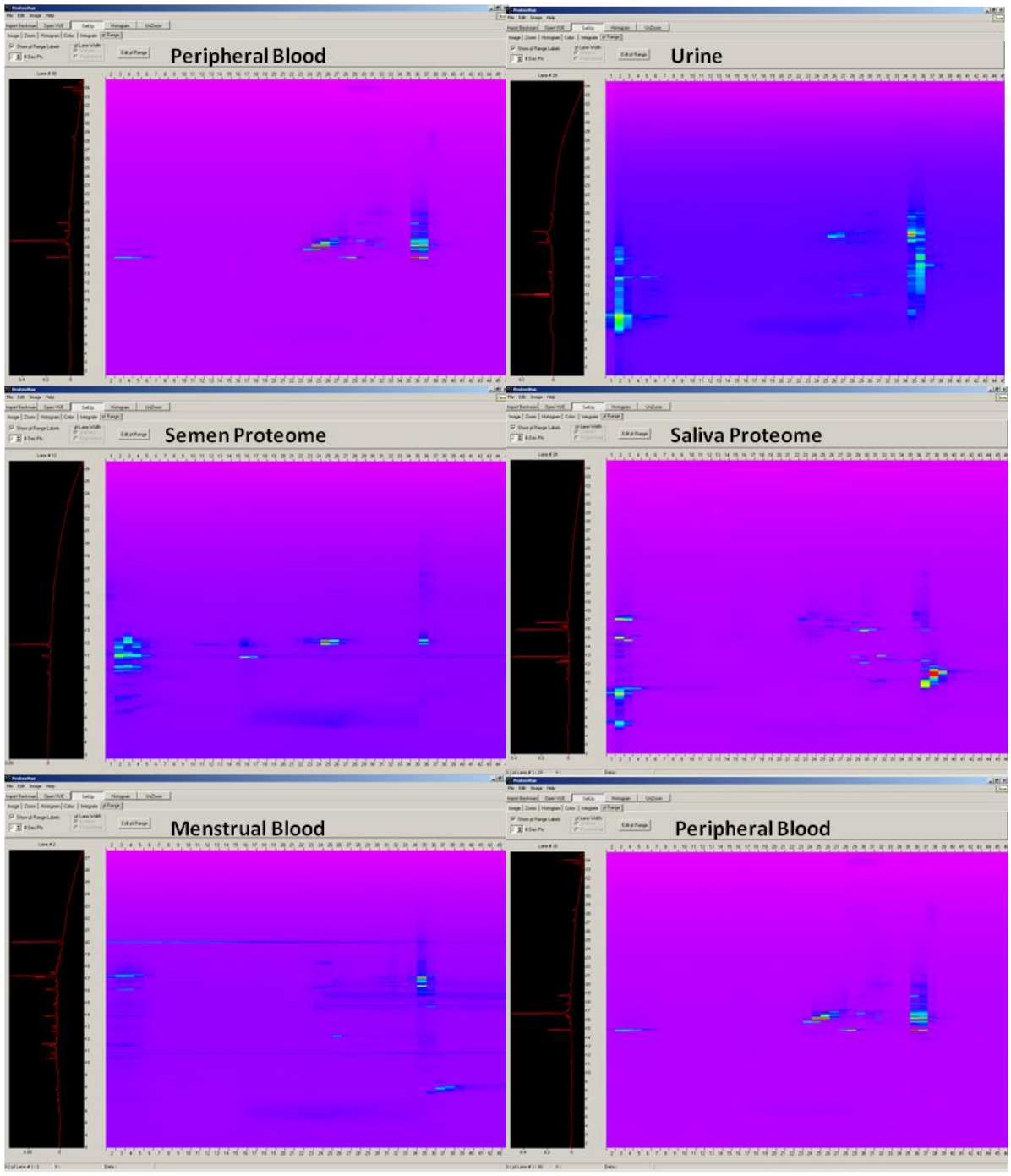


Figure 14 - Examples of the 2D pI/hydrophobicity maps (i.e., proteome maps) obtained from 4 different body fluid samples (peripheral blood, urine, semen and saliva). Differences in bands are indicative of potential protein markers of interest.

2.2.1.1 Proteome Map Comparison

Pair-wise comparisons of the 2D pI/hydrophobicity proteome maps were the next step in the identification of body fluid specific candidate biomarkers. Initially, the commercial DeltaVue™ software suite which was integrated into the ProteomeLab™ PF2D System was used for comparing proteome maps. The software requires a .dat file input to generate color-coded "differential display" maps that highlighted differences between the proteomic profiles of any two samples (**Figure 15**).

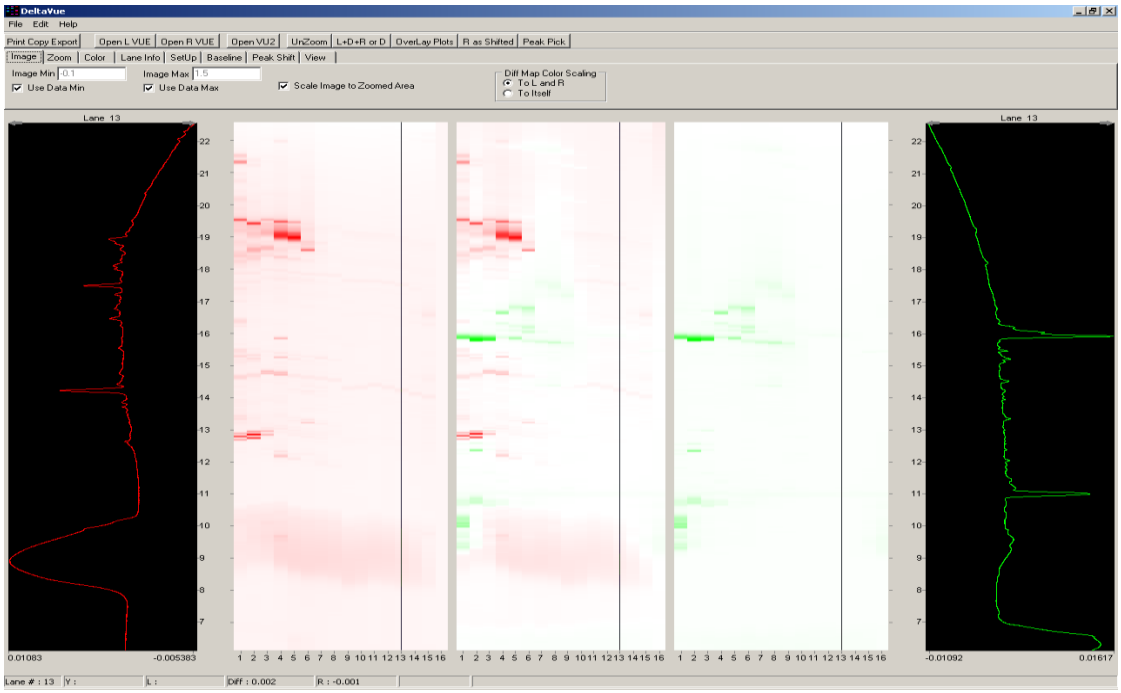


Figure 15 - DeltaVue™ difference profile comparing urine (left) with saliva (right). Loss of resolution of lower abundance proteins and the inability to normalize for subtle retention time differences and pH variances between proteome maps imported into DeltaVue™ make it difficult to reliably identify true protein differences between body fluids.

Following several pair-wise comparisons, however, the data analysis limitations of the software became apparent. The software was designed to be used in traditional biomedical based proteomics projects where diseased samples are compared to healthy samples. Similar to our

workflow, five individuals from each state (healthy and diseased) would be run on the PF2D. In that case, two nearly identical proteomes would be compared for quantitative differences encompassing only ten total samples. Contrasted with adapting the PF2D for six body fluids, six highly dissimilar proteomes with five samples each must be compared in order to identify proteins with absolute specificity to a target fluid. Doing the comparisons using the DeltaVue™ software would easily require hundreds of one to one comparisons between different proteome maps.

With the limitations in the current software, a custom software suite was developed to compare multiple proteome maps at once. To do this, a series of data optimization procedures were developed combined with a data mining algorithm to identify body fluid specific peak clusters within the PF2D maps. Those peaks flagged by the software can then be identified using tandem mass spectrometry.

2.2.1.2 Dataset Optimization

As stated above, each proteome map is stored in a .dat file produced by the PF2D System. The file consisted of a 45 x 10,501 data point matrix. A large portion of the >450,000 data points, however, represent uninformative background noise created when no proteins are eluting from the system or between peaks during the reverse phase separation (**Figure 16**).

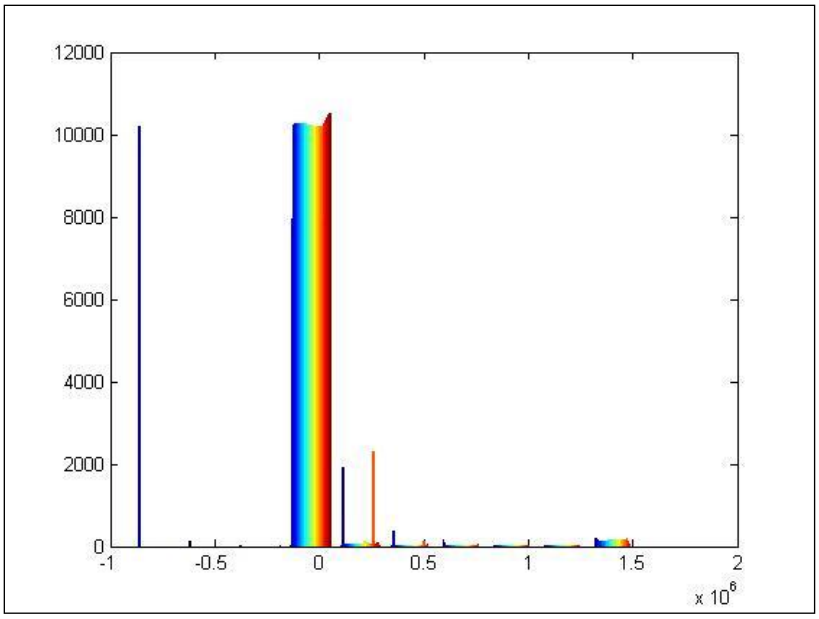


Figure 16 – Histogram of data point distribution stored by the PF2D system. The majority of the stored data exists as background/non-protein regions.

To circumvent this problem, a protein peak identification algorithm was implemented to create a simplified dataset for each proteome containing only peak locations (**Figure 17**). The algorithm works on a simple principle, that the point at which a slope changes from positive to negative (or the point where a derivative changes signs) represents the coordinates on the proteome map where a protein peak is likely to exist. Using this technique virtually all of the underlying noise can be eliminated while increasing the resolution of the signal itself.

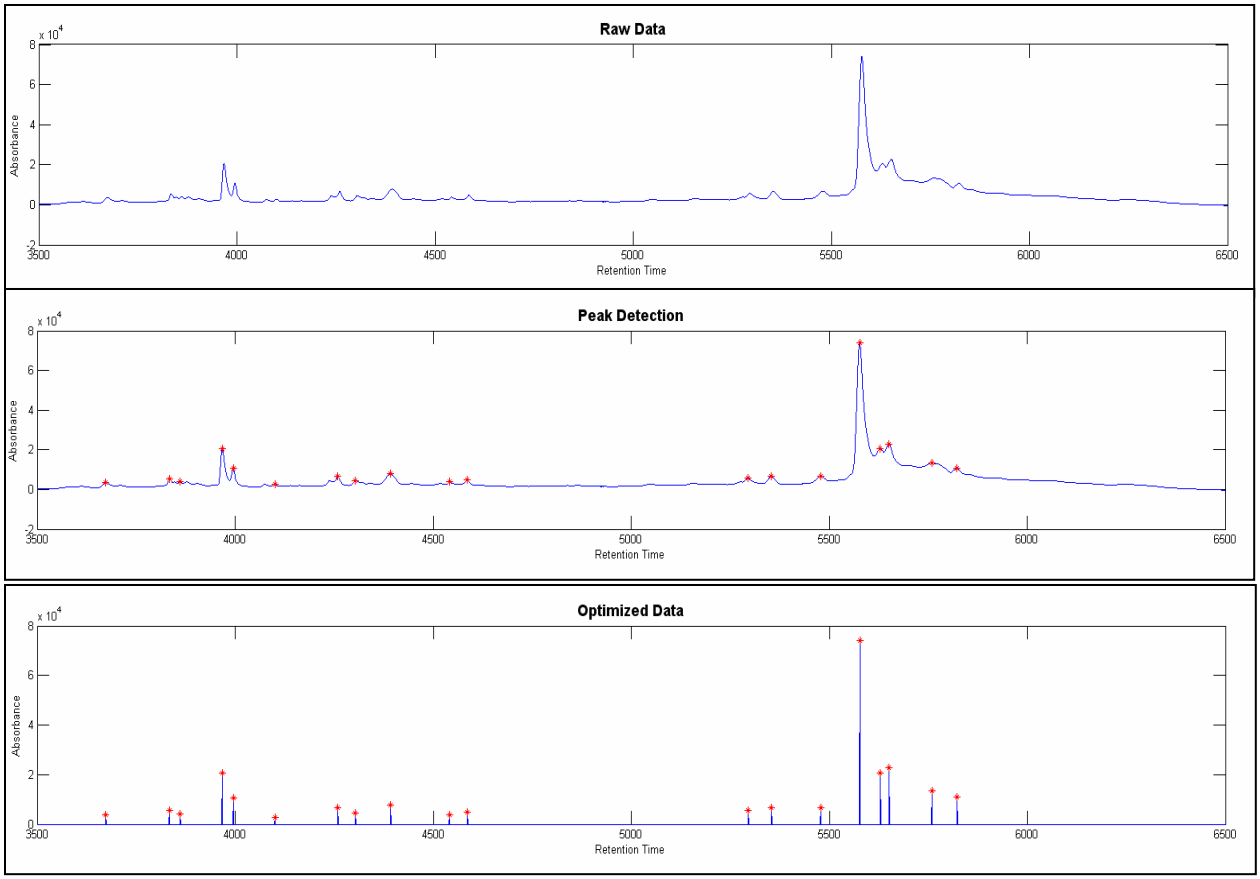


Figure 17 - Application of a protein peak identification algorithm to raw hydrophobicity data (top) generated from a single first-dimension ProteomeLab™ PF2D fraction. Protein peaks are called based on the point at which the slope changes from positive to negative or the point where a derivative changes signs (middle). Once identified, called protein peaks are used to create a normalized representative dataset from which > 99.9% of the underlying noise has been eliminated (bottom).

2.2.1.3 Data Mining of Optimized Proteome Maps

It was also necessary to creating a reliable means of comparing different proteomes while taking into consideration the subtle differences between sample runs and individuals. For this task, a data mining algorithm was implemented to combine individual proteome maps for the same body fluid into a single consensus proteome map. Data mining for this purpose was defined as grouping like objects together. This "clustered/consensus map" was then used to easily compare one body fluid to another. The specific algorithm that was implemented is known as the k-means clustering - an algorithm that organizes a data set into k subsets. The algorithm involves

a four step procedure. First, a location is assigned for each of the subset centers k (centroids); second, each data point is assigned to its nearest center; third, the optimal position of each center is calculated based off a distance measure to each point assigned to it and; fourth, steps 2 and 3 are repeated until the centers are "stable" with each center representing the set of individual protein points from multiple proteome maps from the same fluid. The overall product of this procedure was a set of points split into k partitions, meaning that all of the proteins from each fluid were grouped together in three dimensional space with a single central point representing the closest peaks. The clustered "Consensus" proteome maps were overlaid and cluster centers were between each fluid. Any cluster which appeared in a single fluid and did not overlap with any other fluid was flagged for identification through tandem mass spectrometry.

2.2.1.4 Biomarker Identification from PF2D Fractions

The translation of identified proteome map coordinates output by Protein Miner™, into actual candidate protein biomarkers was a relatively straight forward process. The first step of this workflow begins with the retrieval of ProteomeLab™ PF2D second dimension fractions identified as containing potentially unique proteins. A novel fractional analysis software package from the University of Michigan was used to rapidly locate a specific protein fraction that has been eluted from the PF2D second dimension. By placing the cursor over a desired peak, the software outputs the specific deep-well plate location of the protein fraction of interest (**Figure 18**).

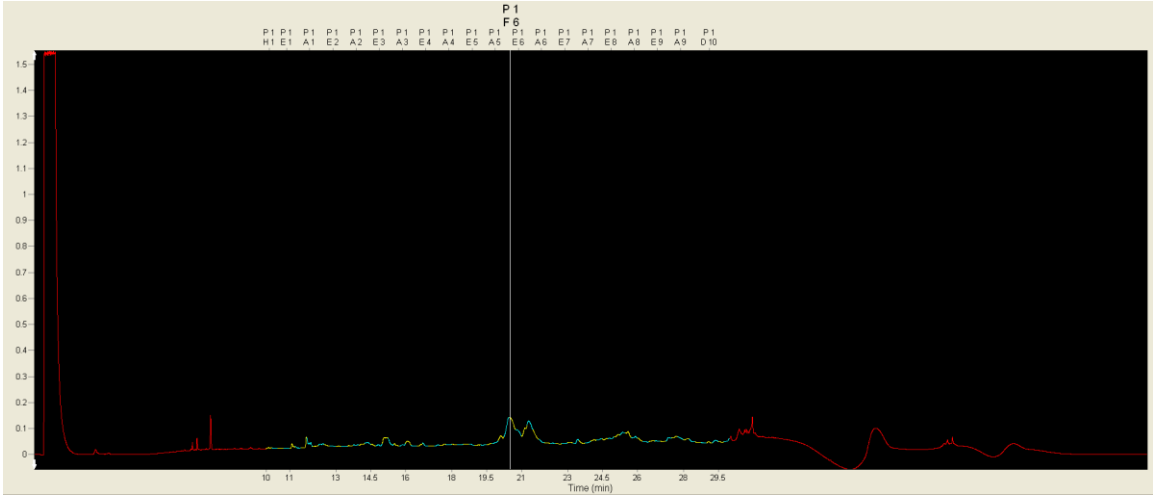
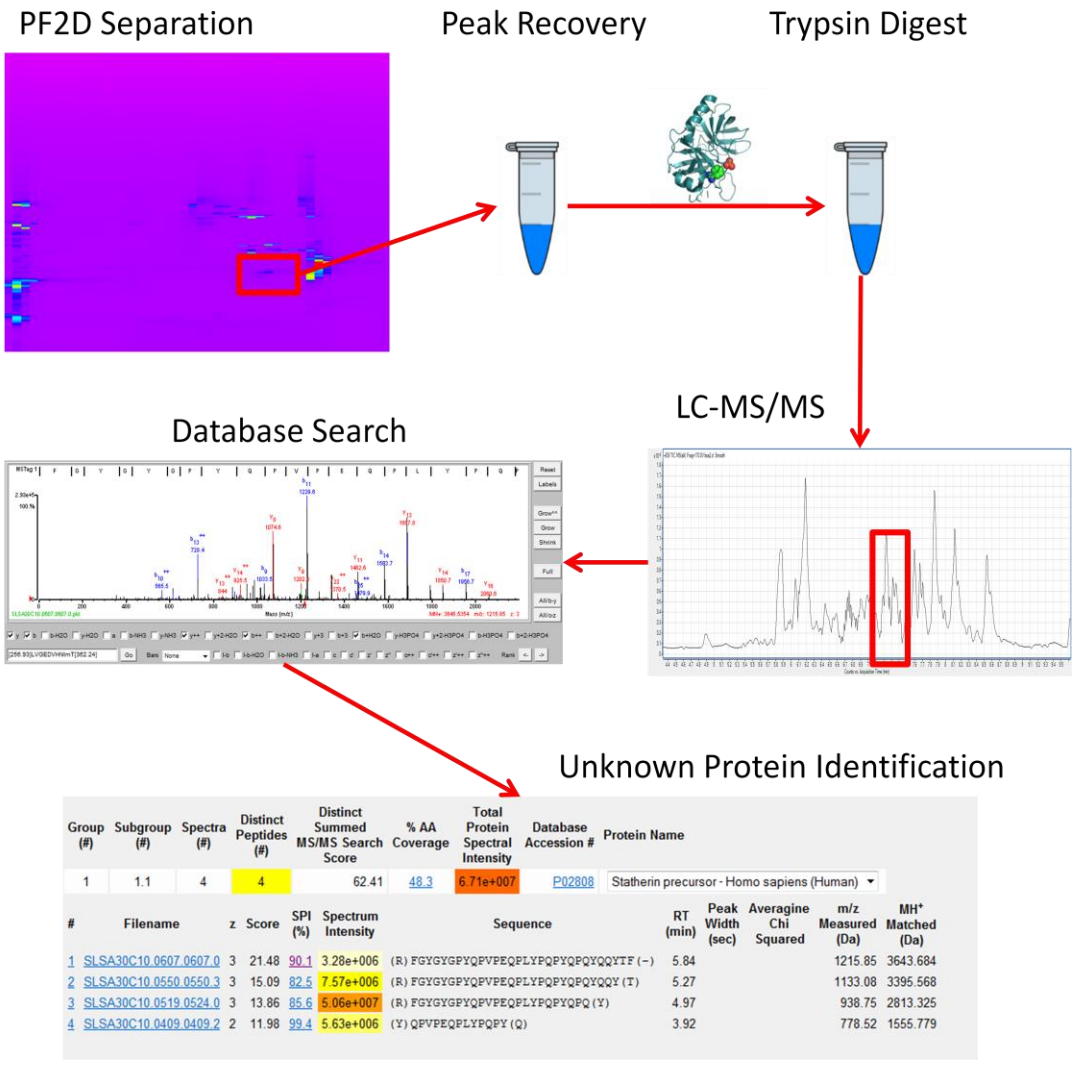


Figure 18 – Fraction analysis software acquired from the University of Michigan. Cursor placement reveals exact plate and well where unidentified peak can be found in 96 well plate.

The proteins contained in these fractions were prepared as stated in section 2.1.4.2. Briefly, protein was denatured and digested into peptide fragments using the enzyme trypsin. Mass spectrometry (see section 2.1.4.3) was performed on an Agilent 6300 series ion trap mass spectrometer coupled with a nanoflow-LC 1200 series HPLC-Chip/MS system. Because of the simplicity of a PF2D fraction (1-3 proteins expected per fraction) a 43mm analytical column with an 11 minute gradient was used to produce quality protein identifications.

Database identification was performed using Agilent's Spectrum Mill search engine as described in section 2.1.4.4. **Figure 19** is the workflow and search result for a cluster of peaks from saliva sample SLSA, using the peak selection software pH fraction 30 and reverse phase fraction C10 correlated to plate P1F6. After recovery, digestion, MS/MS analysis and database search results the saliva biomarker statherin was identified. This result was very encouraging as statherin has been independently identified as a possible saliva marker by gene expression database searches and by forensic researchers working on the development of mRNA markers for saliva [54, 119]. Similarly, the identification of semenogelin-1 and -2 as markers of seminal

fluid [120] and periplakin as marker of vaginal secretions are consistent with what has been reported by biomedical researchers [121]. The accuracy with which the proteomic approach was able to identify these markers was seen as boding well for the likely specificity of numerous other candidate biomarkers that were identified - but for which information on tissue specificity in the literature was lacking.



Group (#)	Subgroup (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Total Protein Spectral Intensity	Database Accession #	Protein Name
1	1.1	4	4	62.41	48.3	6.71e+007	P02808	Statherin precursor - Homo sapiens (Human)

#	Filename	z	Score	SPI (%)	Spectrum Intensity	Sequence	RT (min)	Peak Width (sec)	Averagine Chi Squared	m/z Measured (Da)	MH+ Matched (Da)
1	SLSA30C10.0607.0607.0	3	21.48	90.1	3.28e+006	(R)FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF(-)	5.84			1215.85	3643.684
2	SLSA30C10.0550.0550.3	3	15.09	82.5	7.57e+006	(R)FGYGYGPYQPVPEQPLYPQPYQPQYQQY(T)	5.27			1133.08	3395.568
3	SLSA30C10.0519.0524.0	3	13.86	85.6	5.06e+007	(R)FGYGYGPYQPVPEQPLYPQPYQPQ(Y)	4.97			938.75	2813.325
4	SLSA30C10.0409.0409.2	2	11.98	99.4	5.63e+006	(Y)QPVPEQPLYPQPY(Q)	3.92			778.52	1555.779

Figure 19 – Protein identification workflow. Briefly, proteins flagged as unique are recovered, digested and analyzed on an ion trap mass spectrometer. Acquired data is then processed using Agilent's spectrum mill search engine to identify unknown proteins in the fraction.

This methodology of cluster analysis, peak recovery, and identification through tandem mass spectrometry was repeated for each biofluid to populate a database of candidate high-specificity biomarkers based on the identification results, database searches and independent reports in the professional proteomic literature. These include seminal fluid markers semenogelin-I/II, prolactin inducible protein, prostate specific antigen, and prostatic acid phosphatase. Salivary markers include histatin-1, salivary acidic proline rich phosphoprotein 1/2, as well as statherin. Markers for vaginal fluid included cornulin, involucrin, periplakin, as well as vimentin. osteopontin was found as a urinary candidate biomarker. Peripheral blood markers selected included α-1-antitrypsin, complement C3, hemoglobin subunit β, as well as hemopexin. Unfortunately, no Menstrual Blood markers were identified using this approach. Analysis of each candidate based on information in Swiss-Prot /uniprot/ncbi as well as published biomedical literature made it possible to arrive at a reasonably accurate listing of candidate biomarkers. Biomarker candidates will be described further in relation to their biological function as well as specificity in a later section.

While this particular approach provided promising result, the original intention of generating 5-10 candidate biomarkers for each fluid was not met. Additionally, no viable menstrual blood proteins were identified using the PF2D mapping strategy. The basic assumption employed in this approach is that any peak located consistently in only one fluid must be unique – *i.e.* a peak cluster only found in saliva will contain a saliva specific protein. The reality, however, is a unique peak cluster may not contain any viable markers for that fluid. **Figure 20** illustrates a search result from a Vaginal Fluid sample well that was flagged as unique. Search results identified several ubiquitously expressed serpin isoforms which act as serine protease inhibitors as well as S100 proteins which act as calcium binding proteins [122, 123].

Group (#)	Subgroup (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Total Protein Spectral Intensity	Database Accession #	Protein Name
1	1.1	3	3	39.46	31.1	5.14e+007	P05109	Protein S100-A8 OS=Homo sapiens GN=S100A8 PE=1 SV=1
2	2.1	3	3	34.30	9.4	1.57e+007	P29508	Serpin B3 OS=Homo sapiens GN=SERPINB3 PE=1 SV=2
2	2.1	2	2	24.14	5.8	1.10e+007	P48594	Serpin B4 OS=Homo sapiens GN=SERPINB4 PE=1 SV=2
2	2.1	1	1	13.19	3	6.23e+006	Q9UIV8	Serpin B13 OS=Homo sapiens GN=SERPINB13 PE=2 SV=2
3	3.1	2	2	27.11	24.5	2.64e+007	P06702	Protein S100-A9 OS=Homo sapiens GN=S100A9 PE=1 SV=1
Totals:	11	11						

Figure 20 – Peak cluster flagged as unique in vaginal fluid but contains no uniquely expressed proteins.

In fact, upon a more thorough analysis of the database results, these proteins were all identified via identical sequence conserved by all three serpins (**Figure 21**). Thus, from these results, it is impossible to tell exactly which of the serpin proteins are present in the sample.

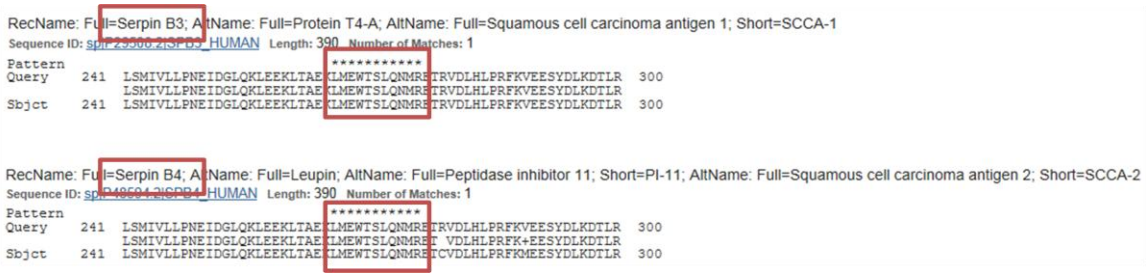


Figure 21 - Conserved peptide sequences are shown in red which identifies multiple Serpin proteins via the same peptide.

Results like these establish that flagged clusters of peaks do not always contain unique proteins. Therefore, the inverse assumption that clusters with similar coordinates must have contain the same proteins is likely to be untrue as well. Therefore, a more thorough analysis of the proteome should lead to the identification of more biomarkers which would not be flagged as unique by the software.

2.2.2 Biomarker Identification: 1D PF2D Fractionation

As stated above, peaks from two separate fluids co-fractionating does not necessarily indicate the peaks represent the same protein. To test this, protein rich pH fractions for each body

fluid were desalted, quantified, and run on a longer gradient in order to identify additional biomarkers which may have been missed by the initial cluster analysis. **Figure 22** is a vaginal proteome map indicating pH fractions 20 through 27 which were selected for MS/MS analysis using the methods described.

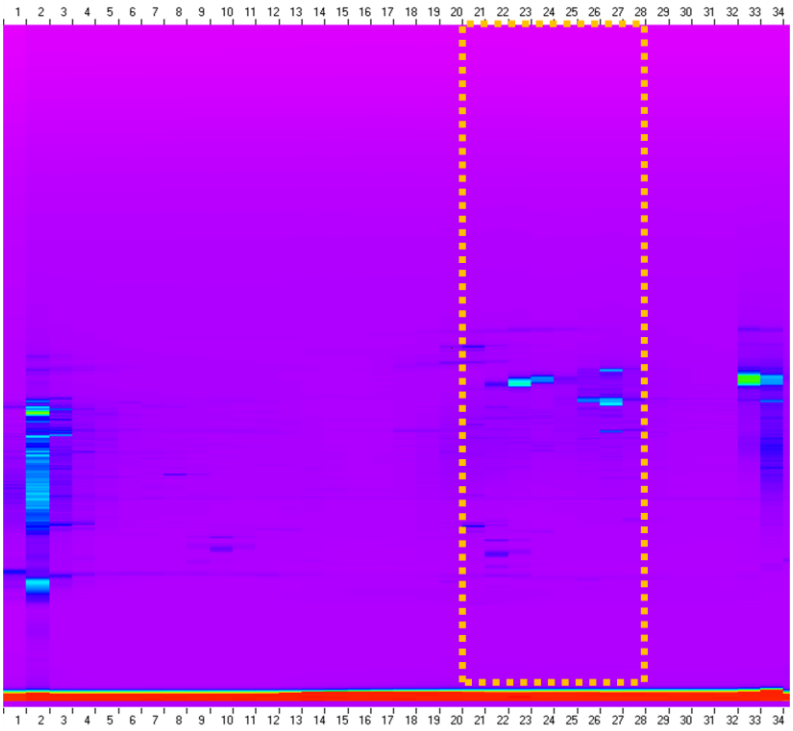


Figure 22 – Vaginal fluid sample with protein rich pH fractions 20-27 which were extracted for analysis.

pH fraction analysis added several candidate biomarkers for each fluid and successfully identified the first candidate for menstrual blood. Analysis of seminal fluid detected all previously identified biomarkers with one additional protein, epididymal secretory protein E1, being added as a candidate marker. Cystatin-SA was added as an additional biomarker for saliva. Analysis was successful as well with several new biomarkers were added to vaginal fluid including Ly6/PLAUR domain-containing protein 3 and neutrophil gelatinase-associated lipocalin. Uromodulin was added o the candidate list for urine. Menstrual blood pH fraction analysis identified calpastatin as well.

These results reinforce the concept that a single pass approach to biomarker discovery may not be the best option for a thorough analysis of the proteome. This additional approach added six new candidate biomarkers while providing redundancy in the identification of many other biomarkers (*e.g.* semenogelin-I/II) increasing the likelihood that the initial protein identification results are reliable and that these proteins can be reliably detected.

2.2.3 Biomarker Identification: Unfractionated Body Fluids

With the success of the pH fraction analysis an additional series of experiments were performed analyzing unfractionated body fluids. It is known that some proteins are not compatible with all separation approaches. For example, highly hydrophobic proteins may not be able to bind a non-polar stationary phase under which reverse-phase chromatography is performed. Additionally, these data would offer a "real world" assessment of the biomarkers performance as no fractionation or enrichment was performed.

To alleviate variability, three samples of each fluid were prepared in a similar fashion to those as listed in section to section 2.1.1. However, neither menstrual nor peripheral blood samples were passed through an immunodepletion column and hemoglobin was not removed during these experiments.

The results further supplemented the candidate database for saliva as well as vaginal fluid while reconfirming several candidate markers as being detectable in non-fractionated samples. In saliva cystatin-D, histatin-1, mucin5B, as well as submaxillary gland androgen-regulated protein 3B were added to the candidate list. For Vaginal fluid IgGFc-binding protein, matrix metalloprotinase-9, mucing5B, as well as suprabasin were identified as high-specificity biomarker candidates.

2.2.4 Biomarker Candidate Review

A reasonably accurate list of candidate biomarkers (Table 1) was reached after the completion of the biomarker discovery phase of this dissertation research. This list was propagated through database descriptions as well as a brief literature review. Moving forward, a more thorough investigation as to function and specificity of these markers must also be performed. Biomarker candidates for each fluid are described in detail in the following section.

2.2.4.1 Seminal Fluid

Seminal fluid showed highly consistent results between all three experimental methods, only epididymal secretory protein E1 and prolactin-inducible protein were not consistently expressed across all experiments. Epididymal secretory protein E1 was first identified as a major secretary protein in the epididymis [124]. The protein is involved in the transportation of cholesterol from lysosomes and is proposed to have a role in sperm formation [125, 126]. This protein was not identified in the PF2D 2D fractions but found in the pH fractions as well as unfractionated samples.

Semenogelin-I/II is the most predominant proteins in seminal plasma, and is responsible for the gel-like matrix of human semen. Both isoforms act as substrates for prostate specific antigen, where upon lysis, sperm are able to move freely through the seminal matrix [127, 128]. Both protein variants were identified in all three discovery methods.

Prolactin-inducible protein is a small 17 kDa glycoprotein which appears to be located in numerous biological fluids including seminal fluid, salivary, and in the sweat glands. Because of the co-expression in seminal fluid and saliva this protein would not be able to confirm the presence of seminal fluid [129]. The protein has numerous biological functions including protection of spermatozoa from damage by endogenous IgG antibodies [130]. Prolactin-inducible

protein was detected in the PF2D fractions as well as the pH fractions but was absent from unfractionated experiments.

Prostate-specific antigen is a serine protease produced by epithelial cells located in the prostate[131]. The primary function of prostate-specific antigen is to cleave semenogelin-I/II thus creating a soluble, liquid medium, for spermatozoa movement [132]. Prostate-specific antigen has well studied utility as an indicator for prostate cancer when PSA serum levels reach approximately 4-10 ng/ml [133, 134]. While this proves PSA is not seminal fluid specific, the detection limits using even the most sensitive mass spectrometric or immunological platforms will be unable to detect the protein [135]. This is compounded by the fact that these assays are designed for laboratory serum samples, not whole blood, which is encountered in forensic casework. As with semenogelin, PSA was detected in all three experimental strategies.

Prostatic acid phosphatase is a glycoprotein secreted by the epithelial cells of the prostate gland which is capable of to hydrolyzing phosphate groups from substrate molecules [136]. Prostatic acid phosphatase is another seminal fluid protein which has seen utility as a clinical marker for prostate cancer [137, 138]. While largely replaced by PSA for screening purposes, the same argument of detection limits makes this protein essentially specific to seminal fluid.

All the proposed protein biomarkers have been well characterized in proteomic experiments and have been seen in numerous studies demonstrating the potential reliability of these candidate markers [139, 140].

2.2.4.2 Saliva

Salivary biomarkers were much more variable in their detection across the three experiments. Cystatin D and cystatin SA originate from the submandibular and sublingual glands and act as protease inhibitors in saliva[141]. Both of these proteins are salivary specific with no

expression in seminal vesicle, prostate, ovaries, as well as other tissues [142]. Cystatin D was only detecting in the unfractionated assay where cystatin SA was detectable in the pH fractionation experiments as well as the unfractionated assay.

Histatin-1 - is secreted into saliva and originates from the submandibular and sublingual glands and is associated with the regulation of salivary calcium [143, 144]. Histatin-1 was identified in the Pf2D fractions as well as the unfractionated experiments.

Salivary acidic proline-rich phosphoprotein is secreted into saliva where it acts to regulates calcium phosphate salts providing a protective / reparative environment for dental enamel [145, 146]. This protein as was identified in the PF2D fractions.

Statherin, also secreted into saliva, has a similar function as salivary acidic proline-rich phosphoprotein 1/2. Statherin inhibits potentially harmful calcium phosphate precipitation in saliva and was detected in both PF2D fractionation and unfractionated experiments [147, 148].

All the proposed biomarkers have been well characterized in numerous proteomics experiments increasing the likelihood that these are reliable biomarkers for saliva [149, 150].

2.2.4.3 Vaginal Secretions

Vaginal fluid was the most difficult body fluid to analyze as the proteins associated with the fluid appear to be less consistent across multiple experiments and are poorly documented in the literature as to their tissue localization as well as biological function. Cornulin, for example, is involved in squamous cell differentiation in epithelial cells and may play a role in mucosal-epithelial immune response. The protein has been characterized in the esophagus as well as the cervix [151, 152]. This protein as one of only two identified via all three approaches.

IgGFc-binding protein, on the other hand, is involved with mucosal layers of epithelial cells and has been found in colon and placenta and aid in immunity [153]. IgGFc was only detected in the unfractionated samples.

Involucrin is a structural protein contained within squamous epithelial cells [154]. Similar to cornulin, involucrin was detected in all three experiments.

Ly6/PLAUR domain-containing protein 3 is involved with regulation between extracellular structural support scaffolding and the epithelial cell layers [155]. Ly6/PLAUR domain-containing protein 3 was identified in the pH experiments as well as the unfractionated experiments.

Matrix metalloproteinase are a family of proteins which break down and remodel components of the extracellular matrix[156]. Matrix metalloproteinase-9 was found only in the unfractionated experiments. This protein has been detected in serum as well as blood plasma. However, this protein may be under the detection limit of current testing methodologies and thus may remain a viable candidate biomarker [157, 158].

Mucin-5B – Mucins are large highly glycosylated proteins which form elastic matrices on mucosal surfaces [159]. Mucin 5B is reportedly expressed in the mucosal glands of the airway epithelium as well as the uterine endocervix [160-162]. It is expected that this protein will be present in all saliva and vaginal samples. Nonetheless, Mucin5B is a very abundant protein and may be able to act as a presumptive marker for both fluids. Mucin 5B was detected in the unfractionated experiments.

Neutrophil gelatinase-associated lipocalin belongs to the lipocalin family of transport proteins and has been associated with innate immunity though iron sequestration [163]. As such, this protein can be found in found in tissues prone to bacterial and other microorganism exposure

including respiratory tract, salivary glands, uterus, and prostate [163, 164]. Neutrophil gelatinase-associated lipocalin was detected in the pH experiments as well as the unfractionated experiments.

Periplakin is a protein component of the cornified envelope in human skin and was identified in the pH and unfractionated experiments [165].

Suprabasin is expressed in keratinocytes and plays a role in epidermal differentiation, it can be found in the uterus as well as esophagus [166]. Suprabasin was identified in the unfractionated experiments.

Vimentin acts as the major intermediate filament in mesenchymal stem cells [167]. Vimentin was identified in the PF2D fractions.

Vaginal fluid was the most difficult body fluid to analyze with respect to the specificity and thus the potential utility of the candidate biomarkers. The presence of these markers being consistently identified in a series of vaginal proteomics projects was reviewed in the professional literature. Table 2 presents a compiled list of eight vaginal proteomics research reports and lists the study in which the candidate biomarker was identified.

Table 2 – Meta-analysis of vaginal fluid candidates identified in other vaginal fluid proteomics studies. Table adapted from Study 7; In numerical order; Dasari [168], DiQuinzio [169], Klein [170], Pereira [171], Shaw [172], Tang [173], Venkataraman [174], Ziegels [175]

Protein	Accession	Study 1	Study 2	Study 3	Study 4	Study 5	Study 6	Study 7	Study 8
Cornulin	Q9UBG3					√			√
IgGFc-binding protein	Q9Y6R7					√			
Involucrin	P07476	√		√	√	√			√
Ly6/PLAUR domain-containing protein 3	O95274					√			√
Matrix metalloproteinase-9	P14780	√			√	√			
Mucin-5B	Q9HC84	√			√	√			√
Neutrophil gelatinase-associated lipocalin	P80188	√		√	√	√	√	√	√
Periplakin	O60437	√			√	√			√
Suprabasin	Q6UWP8					√			√
Vimentin	P08670	√			√	√			√

Each of the candidate protein biomarkers was identified in at least two projects with several being identified in three to six of the eight published studies. Because these results are consistent with what has been reported by biomedical researchers it is likely that the identifications have been accurately identified in this study as well. However, due to the limited literature available on some of these biomarkers it is likely some of the proposed candidates may not be unique to vaginal fluids.

2.2.4.4 Urine

Through the course of the experiments two urine biomarkers were identified. The protein osteopontin plays a primary role in the urinary tract in protection from kidney stone formation through the inhibition of calcium crystal formation [176]. Uromodulin is also involved in bone

matrix formation as a extracellular protein [177]. Osteopontin can also be found in plasma with connections varying from autoimmune disorders to tumor progression [178, 179]. Osteopontin was detectable in all three experiments.

Uromodulin, also known as Tamm-Horsfall urinary glycoprotein, is the most abundant protein found in human urine [180]. Uromodulin function is proposed to be involved in preservation of water and electrolyte levels as well as linked to infection prevention [180, 181]. Uromodulin was detectable in the pH fraction experiments as well as the unfractionated experiments. Both of these proteins are repeatable found in urine and thus would make for good targets for urine identification [182, 183].

2.2.4.5 Menstrual Blood

Only a single menstrual blood-specific biomarker was identified under the pH fraction experiments. The marker, calpastatin, is another cysteine protease inhibitor. More specifically, this protein is thought to be involved in the regulation of some cellular apoptosis events[184] and plays a role in tissue preservation and muscle degradation through proteolysis inhibition [185]. To date, no menstrual blood proteomics projects have been performed. The absence of this marker from whole unfractionated analysis does raise some concern. It is possible that the protein may not be universally expressed which would explain the absence in the three samples analyzed. It is also a possibility that removing the immunodepletion step puts the protein below the detection limits of the instrument.

However, there were vaginal-specific candidate biomarkers detected in menstrual blood. Table 3 summarizes the prevalence of these proteins in menstrual blood. Because vaginal fluid will be a component of any vaginal sourced sample, these data suggest that it may be possible to differentiate a sample as being vaginal-specific. However, there would be no way to completely

classify a sample as menstrual blood versus a mixture of peripheral blood and vaginal fluid with 100% specific menstrual blood proteins.

Table 3 – Vaginal fluid proteins identified in Menstrual Blood.

Fluid	Protein	Accession Number	PF2D Fraction	pH Fraction	Non Fraction
Vaginal Biomarkers in Menstrual Blood	Cornulin	Q9UBG3			√
	IgGFc-binding protein	Q9Y6R7			
	Involucrin	P07476			
	Ly6/PLAUR domain-containing protein 3	O95274			
	Matrix metalloproteinase-9	P14780			
	Mucin-5B	Q9HC84			
	Neutrophil gelatinase-associated lipocalin	P80188	√	√	
	Periplakin	O60437			
	Suprabasin	Q6UWP8			
	Vimentin	P08670			√

2.2.4.6 Peripheral Blood

Four peripheral blood biomarkers were selected during the course of the discovery-based experiments. α-1-antitrypsin is a non-specific serine protease inhibitor found in human plasma. This proteins primary role is to act as an inhibitor of neutrophil elastase thus protecting tissues from proteolysis attack [186, 187]. α-1-antitrypsin was identified in the pH experiments as well as the unfractionated experiments.

Complement C3 is a serum protein implicated in the complement system of the immune system. More specifically, complement C3 is involved with innate immunity and aids antibodies

as well as phagocytes in response to pathogen infection [188, 189]. Complement C3 was identified in all experiments performed.

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[190]. Hemoglobin subunit beta was not found in the PF2D fractionation experiments but was present in the other two experiments performed.

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 [191, 192]. As with complement C3 this protein was identified in each experiment performed.

The selected blood proteins have been well studied and characterized and all represent quality blood biomarkers [193].

2.3 Conclusion

Proteomics-based biomarker discovery experiments can routinely identify hundreds of proteins in a biological system [194]. The overall objective of these experiments was to identify candidate high-specificity protein biomarkers for use as a diagnostic tool; be it for a clinical diagnosis, or in the case of the current study, for use as a diagnostic marker for a specific body fluid for use in forensic casework.

In the study performed, over one thousand proteins were identified utilizing three approaches; 2D-PF2D fractionation and cluster analysis of individual protein peak analysis by MS/MS, 1D-PF2D pH fractionation and analysis by MS/MS, as well as analysis of unfractionated body fluid by MS/MS. After review of the data, results of these experiments populated a database containing thirty body fluid specific protein biomarkers with candidates

linked to each target body fluid. These include biomarker candidates to identify semen, saliva, urine, peripheral blood, menstrual blood and vaginal fluid.

While these results are highly encouraging, the discovery process was performed on the samples from only five individuals per bodily fluid. It is important to stress that these protein biomarkers were identified by mapping the protein profiles of just five individuals per bodily fluid and thus these proteins can only be considered candidate biomarkers. For example, the possibility cannot be ignored that some candidate biomarkers might be secreted into non-target fluids, particularly with the less documented proteins associated with vaginal fluid. Because of this, a larger-scale study must be completed in order to more rigorously confirm these candidates as being body fluid specific.

Chapter 3:

Biomarker Verification

3 Introduction

The proteomics biomarker pipeline includes a methodical discovery phase to identify putative biomarker candidates followed by high throughput verification to assess the diagnostic potential of the initial biomarker candidates. Historically, verification in the clinical setting has been performed using immunoassays in order to generate accurate and reproducible quantitative measurements for specific protein biomarkers [195, 196]. While immunoassays are well established techniques, a disconnect exists between translation of biomarker candidates to clinical utility. This exists primarily due to the lack of commercially available antibodies/reagents as well as the prohibitive cost of *de novo* immunoassay development. This problem is compounded by the fact that typical proteomics discovery projects may identify as few as ten or as many as fifty candidate proteins with potential clinical utility [76, 197]. Recent advances in targeted mass spectrometry offer an alternative approach to traditional antibody-based testing. Using ion scanning techniques on quadrupole mass spectrometers it is possible to identify as well as quantify hundreds of protein biomarkers in a single injection, without the added cost of immunoassay design and characterization [198, 199].

In developing a verification platform for a body fluid specific serological assay it must be reinforced that with any antibody-based assay results are "presumptive by definition". This is because the potential for antibody cross-reactivity with non-target molecules can never be eliminated [200]. Thus, for a serological biomarkers specify characterization assay, a targeted mass spectrometry based platform offers a lower cost, a shorter development time, as well as the reliability of detection by structural identification by mass spectrometry instead of indirect identification based on a binding event.

The core objective for this phase of the dissertation research was to verify the specificity of six panels of candidate protein biomarkers with potential utility for the reliable detection and identification biological stains of forensic utility (*i.e.*, saliva, semen, peripheral blood, menstrual blood, vaginal secretions, and urine). This was performed utilizing a Quadrupole Time-of-Flight instrument.

3.1 Materials and Methods

All research conducted under this project was IRB reviewed, approved and conducted in full compliance with U.S. Federal Policy for the Protection of Human Subjects. **Figure 23** is an overview of the workflow performed from sample collection through biomarker identification.

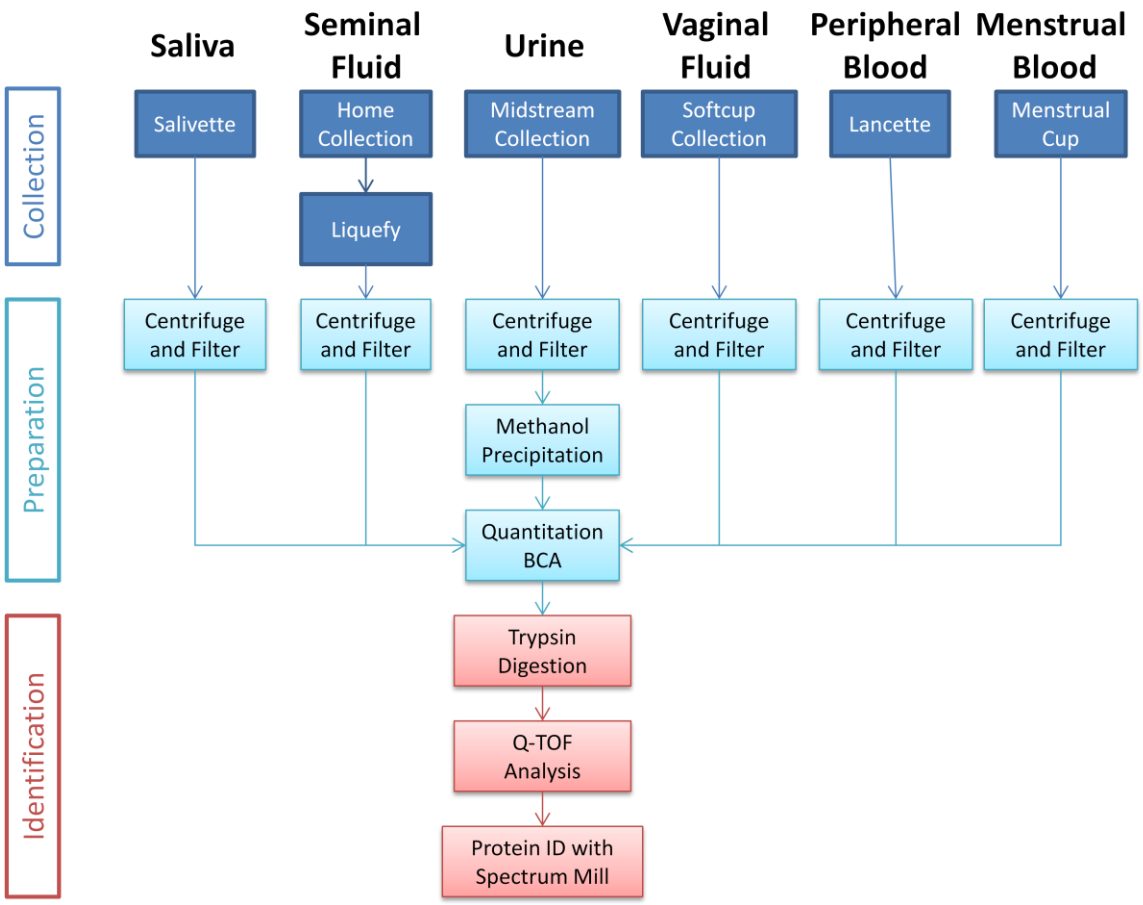


Figure 23 – Biomarker verification workflow from sample collection to biomarker identification.

3.1.1 Sample Collection and Preparation

3.1.1.1 Saliva

The donor was directed to thoroughly rinse their mouth with sterile water to remove residual food particles. After 5 minutes to allow secretion of saliva, the donor was instructed to place a Sarstedt Salivette™ saliva collection sponge into their mouth and to gently chew and roll the sponge around in their mouth for 3-4 minutes. The sponge was then placed into a sterile plastic conical tube and repeated with one more cotton sponge. This allowed for the collection of

large quantities of relatively pure saliva while reducing protein contamination from food items. Salivette™ sponges were centrifuged for 2 min at 1500 x g at 4°C to recover saliva.

Saliva was recovered from Salivette™ and pooled. Pooled samples were centrifuged at 10,000 x g to pellet cells and insoluble material. Supernatant was then passed through a .45 um spin filter unit.

3.1.1.2 Seminal Fluid

Donors were directed to refrain from sexual activity for a minimum of 24 hours and then to obtain a 3-6ml sample of seminal fluid by masturbation in the privacy of their home. The subject was requested to directly deposit the fluid into a sterile plastic collection cup provided by the laboratory and then to refrigerate the sample until it could be transported to the lab within 1 hour.

Freshly collected semen was allowed to liquefy at room temperature for 30 minutes. Sample was added to 2 1.5 mL microcentrifuge tubes and centrifuged at 10,000 x g for 10 minutes at 4°C and passed through a .45 µm filter to ensure cellular removal.

3.1.1.3 Urine

Donors were directed to deposit a morning urine sample (>50ml) into a sterile collection cup provided by the laboratory. Protein concentration varied substantially between individuals thus > 20 ml was typically used to ensure a sufficient quantity of protein.

Freshly collected Urine was centrifuged at 10,000 x g for 10 minutes at 4°C and passed through a .45 µm filter to ensure cellular removal. Protein was then precipitated with 3 parts ice cold -20ºC Methanol (300 µL urine:900 µL Methanol) and incubated for 10 min at -20ºC. Solution was spun at 12.000 x g for 10 min at 4ºC and supernatant discarded. one volume (300

µL) ice cold -20ºC acetone was added and incubated for 10 minutes at min at 4ºC. Sample was spun again at 12.000 x g for 10 min at 4ºC and supernatant discarded. 100 µL of 25 mM Ammonium Bicarbonate was added and shaken at 37 ºC for 10 minutes to re-solublize protein pellet.

3.1.1.4 Vaginal Fluid

Vaginal fluid collection employed an FDA-approved over-the-counter latex-free, hypoallergenic cup (SoftCup™). For the collection of vaginal secretions, donors were instructed to insert the Softcup™ for one hour and then deposit the cup into a 50 mL sterile collection container. Donors were directed to refrigerate the sample until it could be transported to the lab within 1 hour.

Softcup™ with vaginal fluid was delivered in a 50 mL sterile collection container. 2-3 mL of phosphate buffered saline was added to dissolve/collect fluid from sides of softcup and then transferred to 2-3 1.5 mL microcentrifuge tubes, these were spun at 10,000 x g for 10 minutes at 4°C and passed through a .45 µm filter to ensure cellular removal.

3.1.1.5 Peripheral Blood

Small volumes of peripheral blood were collected using the Unistik® 3 lancet and the StatSampler EDTA collection vial.. Volunteers were instructed to wash their hands with disinfectant soap for two minute under warm water. The volunteer's finger was then cleaned with an alcohol swab and punctured using the lancet on the side of the volunteer's ring finger. Blood was expressed and collected from volunteer using the using the capillary tube from the StatSampler collection kits. Once the capillary tube was full (200 µL) the volunteer was given a Band-Aid® to cover the puncture site. StatSampler capillary tube was then expelled into a gel

barrier microcentrifuge tube and spun for 2 minutes at 1500 x g to recover serum. Blood sera from StatSampler collection kits were spun at 10,000 x g for 20 minutes at 4°C and passed through a .45 µm filter to ensure cellular removal.

3.1.1.6 Menstrual Blood

Menstrual blood was self collected by study participants in the privacy of their home. The collection protocol employed an FDA-approved over-the-counter latex-free, hypoallergenic cup (DivaCup™) for the collection of menstrual flow. The donor was directed to insert the cup into the vagina during menses for a period of approximately 1 hour. The cup was then gently removed; the contents were poured into a sterile 50ml conical tube and refrigerated until delivered to lab.

Menstrual blood was delivered in a sterile 50ml sterile collection container. The blood was then transferred to a pink-cap 5 mL EDTA blood collection tube. The blood was rinsed with 2-3 mL PBS and returned to a pink-cap EDTA blood tube. If necessary these were divided between additional pink-cap EDTA blood tubes. Sample was allowed to stand for 5 minutes on and then spun for 10 minutes at 1500 x g to separate serum. Menstrual serum was spun at 10,000 x g for 10 minutes at 4°C and passed through a .45 µm filter to ensure cellular removal.

3.1.1.7 Protein Quantitation with Pierce Micro BCA kit

Dilution standards were prepared as follows using Bovine Serum albumin (BSA) as a calibrator. A total of eight serial dilutions were prepared with concentrations ranging from .5 µg/mL to 200 µg/mL.

Working reagent was prepared with 25 parts BCA Reagent A and 25 parts BCA Reagent B with 1 part BCA Reagent C. In a 1.5 mL microcentrifuge tube 150 µl of working reagent was added to 150 µL of sample or standard. Tubes were vortexed, spun briefly, and allowed to incubate in an Eppendorf Thermomixer® at 37ºC for 2 hours. Samples were allowed to cool to

room temperature for 10 minutes and read on a visible spectrophotometer at 562 nm. BSA curve was used to generate linear equation and used to determine protein concentration of unknowns.

3.1.2 Body Fluid Identification

3.1.2.1 Trypsin Digestion

30 ug of total protein was lyophilized in a vacuum evaporator. Dried protein samples were reconstituted in 40ul of 8M urea in 100 mM Tris-HCl pH 8.5, 1.2ul of 100 mM TCEP reducing agent and then shaken for 20 minutes at room temperature. Then, 0.88ul of 500 mM IAA was added and the sample was shaken in the dark for an additional 15 minutes to alkylate the proteins. 120 ul of 100 mM Tris-HCl was added to sample to dilute urea to 2M. The proteins were digested with 1 ug of trypsin for 14-16 hours at 37°C.

3.1.2.2 Peptide Cleanup and Desalting

Pierce C-18 spin columns were used to cleanup and concentrate digested peptides. Spin column was placed in 1.5 mL microcentrifuge tube and activated by adding 200 ul of 50% acetonitrile followed by centrifugation at 1500 x g for 1 minute at room temperature. This was repeated one additional time. Resin was equilibrated with 200 ul of 5% acetonitrile and .5% triflouroacetic acid and spun at 1500 x g for 1 minute at room temperature. This was repeated one additional time. Digested samples were loaded onto column and spun at 1500 x g for 1 minute at room temperature. Flow through was collected and passed through the column again at 1500 x g for 1 minute at room temperature. The column was washed with 200 ul of 5% acetronitrile and .5% triflouroacetic acid and spun at 1500 x g for 1 minute at room temperature. This was repeated three additional times. Peptides were eluted by adding 20 ul of 70% acetonitrile and centrifugation at 1500 x g for 1 minute at room temperature. This was repeated

one additional time. Peptides were lyophilized in a vacuum evaporator and resuspended in 3% acetonitrile and .1% formic acid for analysis by LC-MS/MS.

3.1.2.3 Q-TOF Analysis

Mass spectrometry was performed on an Agilent Technologies HPLC-chip/MS system coupled to an Agilent 6510 Quadrupole Mass Spectrometer. The HPLC chip column used was a 150mm 300 A C18 Analytical with a 160 nL enrichment column. Columns were equilibrated in 0.1% Formic acid in water. A total of 500 ng of protein were injected onto the column with the following run conditions; buffer A (.1% formic acid in water) and B (90% Acetonitile, 10% water, .1% formic acid). An initial 44 minute run employed a gradient of 3% B to 36% B over 38 minutes. This was followed by 80% B from 40 min to 44 min and then 5 minute post-run equilibration at 3% A.

Data Analysis - Data analysis was performed using Spectrum Mill software suite by Agilent Technologies. The Swiss-Prot database was used to match MS/MS spectrum generated on mass spectrometer. Typically proteins identified with 2+ peptides and scores >16 were considered confident matches.

3.1.3 Preparation and Analysis of Casework Samples

3.1.3.1 Stains on Swabs

50 ul of body fluid, collected and prepared as described previously was spotted on standard cotton swabs. Swabs were allowed to air dry in a biosafety hood for 40 minutes to 1 hour. Once dried, the cotton was removed from swab with a scalpel, placed into 1.5 mL microcentrifuge tube. Proteins were extracted by adding 300 ul phosphate buffered saline to swab and shaken at 37°C for 10 minutes. After incubation, the swab was placed into spin basket

and centrifuged for 30 seconds at 13,000 x g to recover all fluid. Samples were then quantified using Pierce Micro BCA protocol. Sample identification was performed as discussed in section 3.2.3.

3.1.3.2 Stains on Substrates

50 ul of body fluid was collected and prepared as described previously and was spotted on various 5x5 cm substrate cuttings such as cotton, denim, and leather. Cuttings were allowed to air dry in a biosafety hood for 40 minutes to 1 hour. Once dried, cuttings were placed into 1.5 mL microcentrifuge tube. Proteins were extracted by adding 300 ul phosphate buffered saline to cuttings and shaken at 37°C for 10 minutes. After incubation cuttings was placed into spin basket and centrifuged for 30 seconds at 13,000 x g to recover all fluid. Samples were then quantified using Pierce Micro BCA protocol. Sample identification was performed as discussed previously.

3.1.3.3 Stains on Swabs with Contaminants

50 µl of body fluid was collected and prepared as described previously and was spotted on standard cotton swabs which had been dipped in various environmental contaminants such as bleach, Bluestar®, and spermicidal lubricant. Swabs were allowed to air dry under the hood for 40 minutes to 1 hour. Once dried, cotton was removed from swab with a scalpel, placed into 1.5 mL microcentrifuge tube. Proteins were extracted by adding 300 µl phosphate buffered saline to swab and shaken at 37°C for 10 minutes. After incubation swab was placed into spin basket and centrifuged for 30 seconds at 13,000 x g to recover all fluid. Samples were then quantified using Pierce Micro BCA protocol. Sample identification was performed as discussed previously.

3.1.3.4 Mixed Stains on Swabs

25 µL of each body fluid in mixture was prepared as described previously was spotted on standard cotton swabs. Swabs were allowed to air dry under the hood for 40 minutes to 1 hour. Once dried, cotton was removed from swab with a scalpel, placed into 1.5 mL microcentrifuge tube. Proteins were extracted by adding 300 µL phosphate buffered saline to swab and shaken at 37°C for 10 minutes. After incubation swab was placed into spin basket and centrifuged for 30 seconds at 13,000 x g to recover all fluid. Samples were then quantified using Pierce Micro BCA protocol. Sample identification was performed as discussed previously.

50 µL of body fluid was collected and prepared as prepared as described previously was spotted on standard cotton swabs. Swabs were allowed to air dry under the hood for 40 minutes to 1 hour. Once dried, cotton was removed from swab with a scalpel, placed into 1.5 mL microcentrifuge tube. Proteins were extracted by adding 300 µL phosphate buffered saline to swab and shaken at 37°C for 10 minutes. After incubation swab was placed into spin basket and centrifuged for 30 seconds at 13,000 x g to recover all fluid. Samples were then quantified using Pierce Micro BCA. Sample identification was performed as discussed previously using 15 ug of each of each fluid to be included in the mixture.

3.1.3.5 Body fluid Dilutions

To test lower limit of detection of detection 50 µL of body fluid was collected and prepared as described in sections 3.2.1 and 3.2.2 and was spotted on standard cotton swabs. Swabs were allowed to air dry under the hood for 40 minutes to 1 hour. Once dried, cotton was removed from swab with a scalpel, placed into 1.5 mL microcentrifuge tube. Proteins were extracted by adding 300 µL phosphate buffered saline to swab and shaken at 37°C for 10 minutes. After incubation swab was placed into spin basket and centrifuged for 30 seconds at 13,000 x g to recover all

fluid. Samples were then quantified using Pierce Micro BCA protocol. Sample identification was performed as discussed previously using varying amounts of protein injection amounts from 500 ng to 12.5 ng.

3.2 Results and Discussion

Under this objective of the dissertation research, thirty-five single source samples for Saliva, Vaginal fluid, Seminal Fluid, Urine, Peripheral Blood, and Menstrual Blood were collected and run on a Quadrupole Time-of-Flight mass spectrometer operating in targeted MS/MS mode. Additionally, a series of casework-type samples were prepared which included dried sample extraction from swabs, substrates, chemical contaminants, mixture analysis, as well as sensitivity studies.

3.2.1 Sample Collection

Fifty single source samples were collected for analysis by targeted mass spectrometry. These were collected and quantified as described. In order to appropriately represent samples to be analyzed by the method, no prefractionation or pre-treatment was applied this stage. For example, menstrual blood was not treated with HemogloBind™ to remove hemoglobin. Additionally, menstrual and peripheral blood was not passed through an immunodepletion column in order to remove interfering proteins. In order to properly assess the capability of the method under real world conditions, casework-type samples will be assessed below.

3.2.2 Method Development

Due to the complexity of designing a method which can process six biological fluids simultaneously, the overall method development was divided into a series of smaller objectives.

These include: Protein selection, peptide selection, single-fluid multiplex assay followed by the six fluid multiplex assay (**Figure 24**).

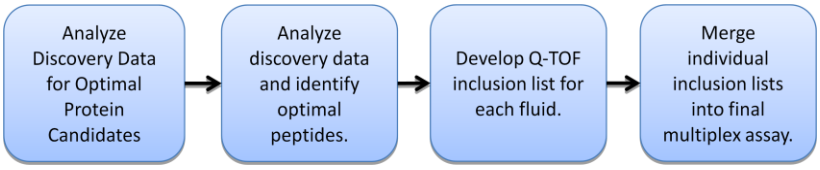


Figure 24 – Method development workflow from peptide selection through multiplex assay design.

More specifically, using data generated from samples during the initial biomarker discovery project, three to five of the most promising candidate protein biomarkers were selected for the multiplex body fluid assay. Once selected, optimal precursor ions (peptides) for each protein will be selected for use in an inclusion list which is read by the mass spectrometer in order to scan for specific peptides during the analytical run. This selective scanning, which is made possible by the quadrupole mass filter, allows the instrument to selectively scan for only peptides of interest even when present in trace amounts. The isolated precursor ions are then fragmented to produce product ions whose fragmentation spectra confirm the presence of the original biomarker. Also during this phase an analytical gradient was developed. Once a single fluid multiplex assays are designed the individual inclusion lists were merged into the final multiplex assay.

3.2.2.1 Protein Selection

Results from the discovery phase of the dissertation research identified numerous candidate biomarkers for body fluid differentiation. From these candidates, ideal targets to move forward with and integrate into the targeted Q-TOF assay should ideally be consistently identified consistently across multiple experiments and, most importantly, are stain specific.

In general, seminal fluid candidate markers were identified consistently across all three separation approaches. Only two proteins were missed, epididymal secretory protein E1 was

missed in the PF2D fractions and prolactin inducible protein was not identified in the unfractionated experiments. All the candidate biomarkers aside from prolactin inducible protein were included in the development of the targeted assay. Prolactin inducible protein, however, has been identified in several salivary proteomics studies and thus is not seminal fluid specific [201, 202].

Saliva biomarkers selection was slightly more difficult as no biomarker was detected across all three experiments. In this case more weight is given to proteins identified in the unfractionated experiments to ensure that proteins are abundant enough to be reliably detected in the unfractionated analysis. Included candidates were statherin, histatin-1, as well as cystatin-SA which were identified in two experiments and cystatin D, mucin 5B, and submaxillary gland androgen regulated protein were identified in the unfractionated experiments. Salivary acidic proline-rich phosphoprotein 1/2 was omitted due to its only being detectable in the PF2D fractions.

Vaginal fluid markers were challenging as only one candidate biomarker, cornulin, was identified across multiple experiments. Additionally, according to a literature review, the vaginal candidates appear to have the least specificity. For example, cornulin, involucrin, Ly6/PLAUR domain-containing protein 3, periplakin and suprabasin were all reported to be associated with the epithelium. Thus it can be assumed that similar tissues may contain these proteins as well. Therefore, it is very possible that other non-keratinized tissues such as the mouth, esophagus, male reproductive tract, as well as the intestines may contain many of the same proteins. As an example, cornulin has been linked to disorders of non cervico-vaginal tissue including esophageal cancer [203]. While of the vaginal fluid candidates that were detected in the reviewed seminal fluid or urine proteomics studies [139, 140, 182], several of the markers were also identified

in salivary proteome projects. Table 4 reviews the four studies where vaginal candidates were identified.

Table 4 – Salivary proteomic studies surveyed where vaginal fluid candidate markers were identified. In numerical order; Hu [204], Wilmarth [205], Xie [206], Hu [207]

			Study 1	Study 2	Study 3	Study 4
Vaginal Secretions	Cornulin	Q9UBG3				
	IgGFc-binding protein	Q9Y6R7				
	Involucrin	P07476			√	
	Ly6/PLAUR domain-containing protein 3	O95274				
	Matrix metalloproteinase-9	P14780			√	
	Mucin-5B	Q9HC84	√	√	√	
	Neutrophil gelatinase-associated lipocalin	P80188			√	
	Periplakin	O60437			√	
	Suprabasin	Q6UWP8				
	Vimentin	P08670			√	

Several markers have been identified in saliva proteome projects and these results must be taken into consideration when designing the final target ion list for the targeted QTOF assay. However, it should also be noted that none of these markers were identified in any of the three separation strategies employed during this dissertation research. Based on these findings, cornulin, IgGFc-binding protein, Ly6/PLAUR domain-containing protein 3, as well as suprabasin were added to the targeted assay. Additionally, matrix metalloproteinase-9, mucin-5B, as well as neutrophil gelatinase-associated lipocalin were added to the assay. While these markers have been identified in saliva, all four studies which were reviewed utilized extensive two dimensional fractionation strategies. While the proteins may be expressed in both fluids, the quantity of protein that can be found may hold value in discerning saliva from vaginal fluid.

Vimentin, involucrin, as well as periplaklin were not detected in the unfractionated assay and were thusly removed for the targeted assay method development.

Urine biomarkers osteopontin as well as uromodulin were both selected for integration into the targeted assay. Both markers appear highly specific to urine and both proteins were identified in nearly all the separation strategies employed.

Calpastatin was the only menstrual blood marker identified in the three separation strategies. This protein was unfortunately not detected in any experiment aside from the pH fraction experiments. Because of this an attempt was made to locate the protein manually using the results from the pH fractions. Two peptides GTVPDDAVEALADSLGK and LAAAISEVVSQTPASTTQAGAPP, which were identified previously, were used in an attempt to manually locate the protein in the raw data from the unfractionated experiments. Using the exact masses for the peptides **Figure 25** shows the extracted ion chromatograms (EIC) for the calpastatin peptides (top) as well as an EIC for the Hemopexin peptide NFPSPVDAAFR (bottom).

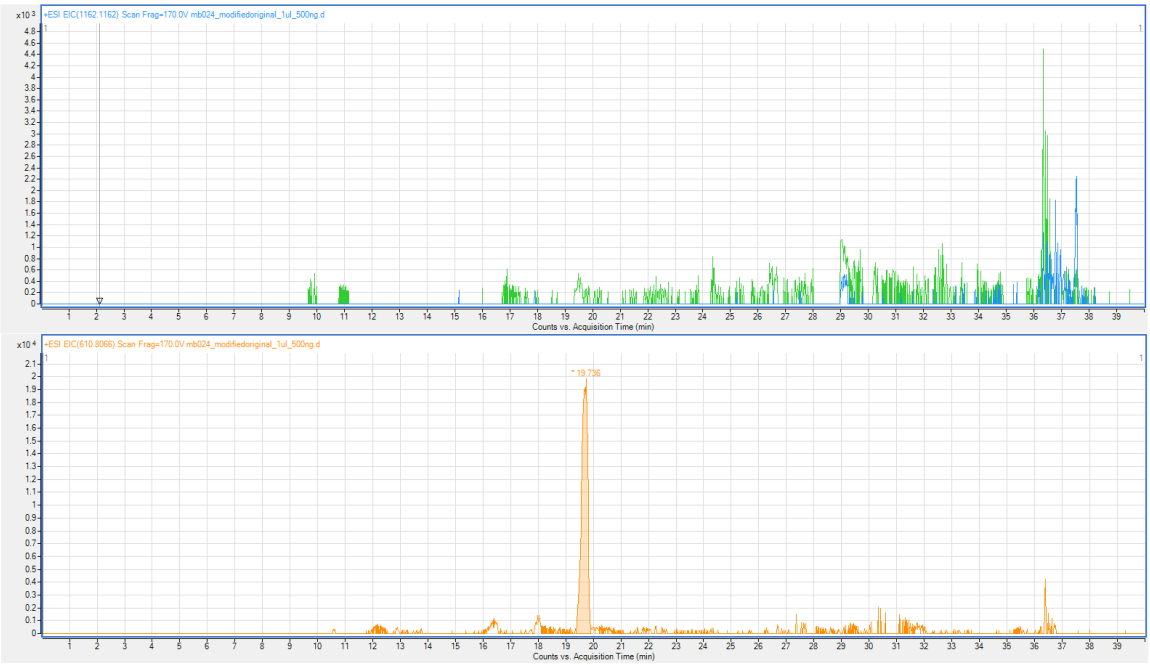


Figure 25 –Extracted ion chromatogram for Calpastatin and Hemopexin. Hemopexin (Bottom) was reliably detectable in the menstrual blood sample where the Calpastatin (Top) peptide could not be manually located.

At 19.5 minutes a clear peak exists for Hemopexin where the calpastatin peptides only reveal noise. This result was observed across all sample replicates for the other unfractionated samples. These results imply that the protein is either not present in the three samples or that omitting the preparation steps (*i.e.*, HemogloBind, immunodepletion, and fractionation) made the protein undetectable in the assay. Regardless, this marker is most likely unreliable as a marker for menstrual blood. However, some of the vaginal-specific biomarkers had also been identified in menstrual blood. Therefore, the best option available is that some of these will be consistently expressed across the menstrual blood sample population.

Similar to seminal fluid, Peripheral blood was a relatively straightforward as all the candidate biomarkers were expressed across nearly all the experiments performed. Complement C3, hemoglobin subunit beta, as well as Hemopexin were included in the targeted assay.

3.2.2.2 Peptide Selection

The next step in assay development was selecting optimal peptides to represent the candidate biomarkers. Ideal peptides were those which: (1) were detected consistently across multiple experiments, with additional weight given to peptides identified in the unfractionated experiments (2) had no post-translational modifications that would alter the mass-charge ratio (3) were of high abundance so as to facilitate detection (4) are human specific.

Following the above criteria as closely as possible, each protein was examined in order to select two to three optimal peptides. In some cases this was a relatively straightforward process. statherin, for instance, is based on the identification of only one peptide. While two to three peptides per protein was the goal, this single peptide represents approximately 50% of the entire sequence of the protein (see below in red) and has been consistently identified across multiple individuals as well as experiments.

MKFLVFAFILALMVSMIGADSSEEKFLRRIGRFGYGYGPYQPVPEQPLYPQPYQPQYQQYTF

Other proteins, such as cystatin-SA, were relatively straightforward as a select few peptide sequences were consistently detected in the unfractionated experiments. Table 5 shows the results from the unfractionated experiments where SQPNLDTCAFHEQPELQKK, QLCSFQIYEVPWEDR, as well as IIEGGIYDADLNDER were consistently detected and thus selected as targets for cystatin SA.

Table 5 – Cystatin SA identification results across three unfractionated QTOF injections.

Sample	Peptide	Modifications	Spectrum Intensity	Score	SPI	Charge	m/z	m+	RT
Unfractionated Run 1	IIEGGIYDADLNDER		2.30E+05	18.91	79.6	2	846.906	1692.81	11.9
	QLCSFQIYEVPWEDR	C:Carbamidomethylation	1.74E+05	16.73	92.5	2	985.458	1969.91	19.8
	QLCSFQIYEVPWEDR	C:Carbamidomethylation	1.57E+05	16.47	85.2	3	657.309	1969.91	19.8
	SQPNLDTCAFHEQPELQKK	C:Carbamidomethylation	1.07E+07	15.69	74.7	4	568.282	2270.09	10
	SQPNLDTCAFHEQPELQKK	C:Carbamidomethylation	2.11E+06	9.02	75.6	3	757.369	2270.09	10
Unfractionated Run 2	IIEGGIYDADLNDER		2.61E+05	23.06	91	2	846.91	1692.81	11.7
	QLCSFQIYEVPWEDR	C:Carbamidomethylation	1.28E+05	13.88	74.6	2	985.456	1969.91	19.7
	QLCSFQIYEVPWEDR	C:Carbamidomethylation	1.28E+05	14.56	74.6	2	985.456	1969.91	19.8
	SQPNLDTCAFHEQPELQKK	C:Carbamidomethylation	3.62E+05	10.77	78.5	3	757.369	2270.09	9.9
Unfractionated Run 3	ALHFVISEYNK		1.09E+06	9.14	65.2	3	440.901	1320.7	18.1
	IIEGGIYDADLNDER		5.17E+05	12.78	70.7	2	846.905	1692.81	11.8
	QLCSFQIYEVPWEDR	C:Carbamidomethylation	3.65E+05	16.45	86.5	3	657.307	1969.91	19.8
	SQPNLDTCAFHEQPELQKK	C:Carbamidomethylation	5.12E+06	8.82	50.8	3	757.364	2270.09	10
	SQPNLDTCAFHEQPELQKK	C:Carbamidomethylation	5.51E+06	10.96	55.3	3	757.364	2270.09	10

In other cases peptide selection was made difficult due to the sheer number of potential targets that were identified. High abundant proteins such as semenogelin, and hemoglobin resulted in more than 20 viable protein targets. In this case the most abundant/non-modified peptides were selected for the targeted assay.

3.2.2.3 Q-TOF Method Development

With proteins and peptides selected, an analytical gradient needed to be designed. Once a gradient was chosen, replicates for each body fluid were then run in order to identify the exact retention time at which a target peptide eluted into the mass spectrometer. Using the mass-to-charge ratio as well as the retention time an inclusion list is generated for the Q-TOF for each fluid to selectively target each peptide for each biomarker.

The unfractionated discovery experiments employed a 44 minute analytical run. While this method was successful in identifying numerous biomarker candidates for each fluid, chromatographic separation could be improved to spread out the elution time of the peptides into the mass spectrometer. Using the original method as a starting point four gradients were tested (**Table 6**).

Table 6 – Four analytical gradients tested.

Gradient 1		Gradient 2		Gradient 3		Gradient 4	
Original 40 minute		Short 22 minute		Modified 40 minute		Modified 40 minute	
Time	% Organic	Time	% Organic	Time	% Organic	Time	% Organic
0	3	0	3	0	3	0	3
1	10	1	10	1	6		
33	45	20	45	33	38	33	38
35	80	20.1	80	35	80	35	80
40	80	23	80	40	80	40	80
40.1	3	23.1	3	40.1	3	40.1	3

In an attempt to speed sample throughput, a 22 minute gradient (gradient 2) was built and tested against all target fluids. For most fluids, this offered a substantial increase in speed with no chromatographic issues. However, problems arose with saliva using the faster throughput methods. The saliva proteome consists of a considerable (~50% of the total protein content) number of small, hydrophilic, low molecular weight proteins [208]. Using reverse-phase chromatography hydrophilic peptides are retained weakly and elute first whereas hydrophobic peptides are retained strongly and elute later in the run. This translated into column overloading issues (**Figure 26**) where the majority of the salivary peptides were entering the mass spectrometer at the front end of the run.

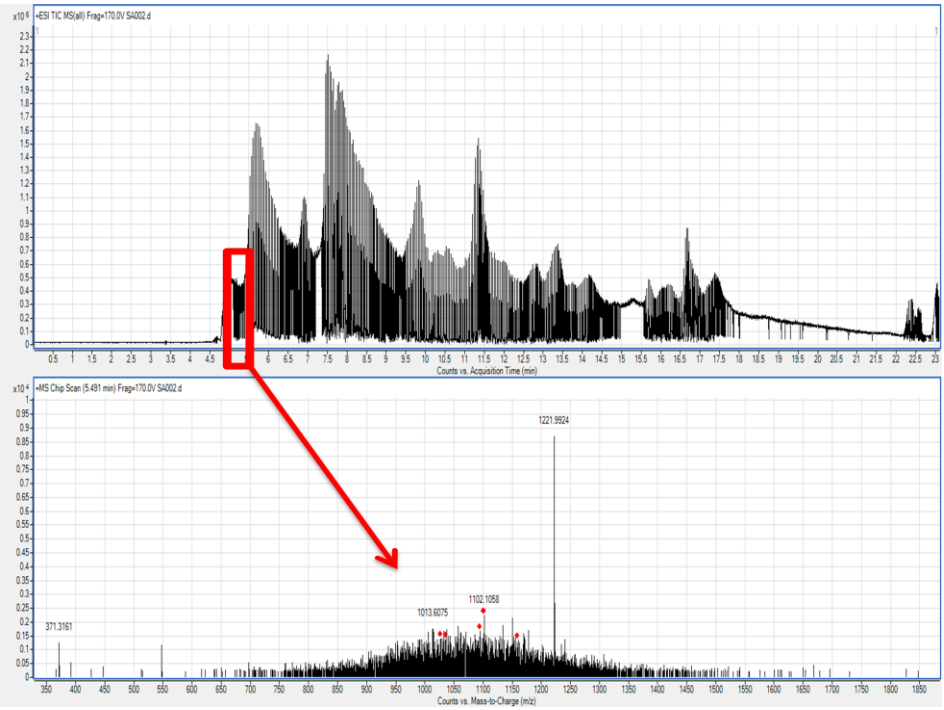


Figure 26 – Column overloading with saliva on a fast 22 minute gradient generating irresolvable MS scan.

Poor chromatograph and column overloading results in poor peak, reproducibility issues, as well as severe ion suppression for any peptide that elutes during that time frame. Additionally, if a peptide from another fluid co-elutes during that time point in the analytical run a significant signal suppression may make it impossible to detect that ion in a mixture with saliva. Because of this overload problem, the original 40 minute gradient was revisited and optimized. **Figure 27** represents the three gradients tested for the same sample of saliva, Gradient 1 is the original one used for the unfractionated experiments. It is clear that from approximately 25 minutes to 40 minutes very few peptides are eluting out of the column essentially "wasting" instrument run time. To address this, two additional methods were created and tested to improve the overall chromatography. From the results, gradient 3 (**Center Figure 27**) was selected as the overloading issues were alleviated with the greatest peptide distribution of the methods tested.

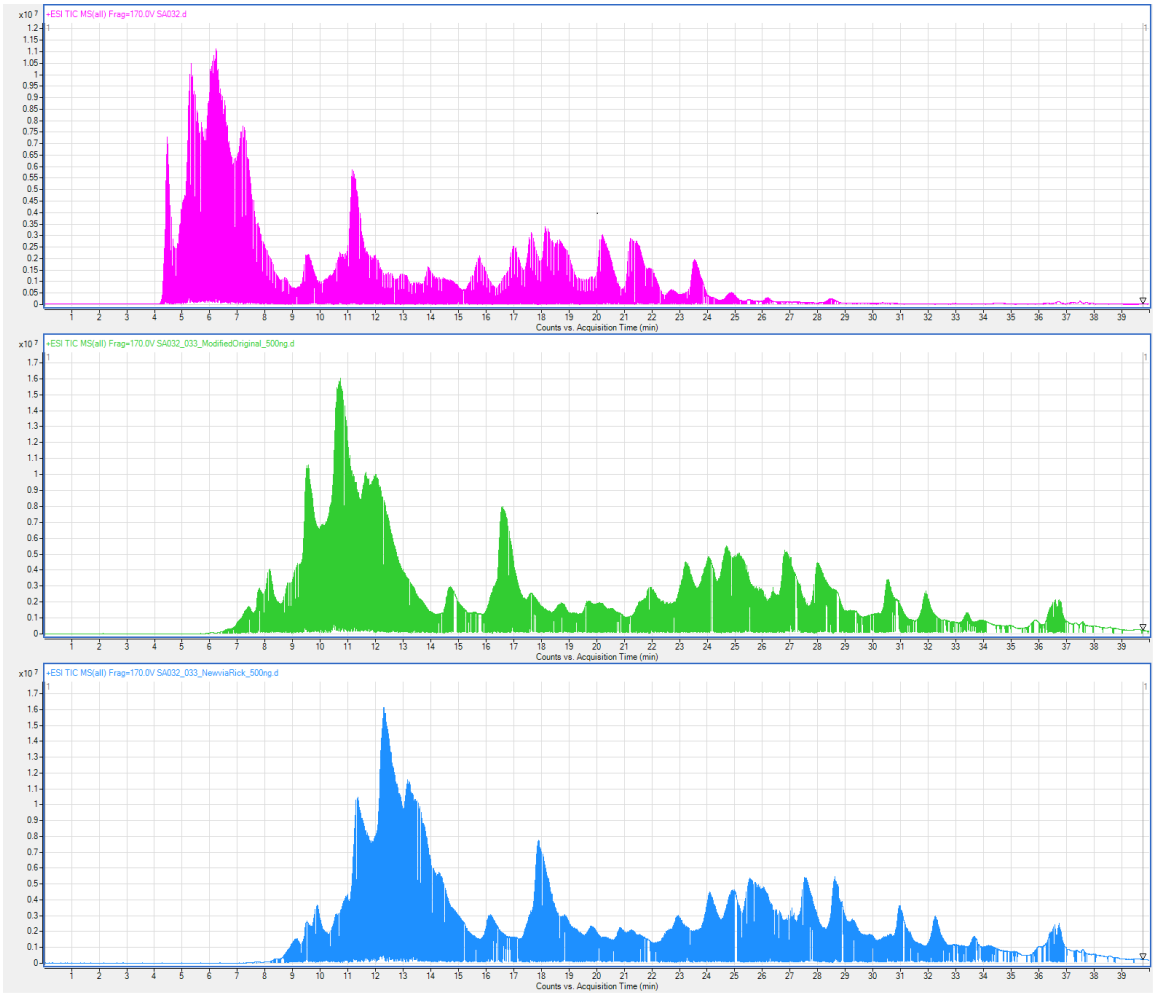


Figure 27 – Three 40 minute gradients tested for chromatography optimization. Gradient 1 (top) is the original used for the unfractionated experiments where Gradient 3 (center) and Gradient 4 (bottom) are modifications.

With a gradient selected, two samples from each fluid were run in order to identify the exact retention time in which each peptide elutes into the system. Once located, peptide m/z, retention time, as well as charge was compiled into an inclusion list for each body fluid, (Tables 7A-E). For each body fluid, the inclusion list describes the candidate proteins being assayed, their corresponding target peptides, retention times, and exact mass-to-charge ratios. The target ion inclusion lists for each "singleplex" body fluid assay were then tested before being combined

into the final multiplex assay. This process resulted in the development of the final multiplex assay illustrated in **Figure 28**.

Table 7A Peripheral Blood Peptide Inclusion List

Protein Biomarker	Target Biomarker Peptide	Prec m/z	Charge	Retention Time (min)
Hemoglobin subunit beta	LLVVYPWTQR	637.8732	2	20.8
	VVAGVANALAHKYH	483.9403	3	12.4
	GTFATLSELHCDK	739.8534	2	14.4
Complement C3	TMQALPYSTVGNSNNYLHLSVLR	860.1049	3	23.43
	VYAYYNLEESCTR	834.3748	2	14.77
	VFLDCCNYITELR	851.9012	2	21.4
Hemopexin	NFPSPVDAAFR	610.8107	2	18.3
	YYCFQGNQFLR	748.474	2	18.3

Table 7B Saliva Peptide Inclusion List

Protein Biomarker	Target Biomarker Peptide	Prec m/z	Charge	Retention Time (min)
Cystatin SA	IIEGGIYDADLNDER	846.9146	2	17.5
	SQPNLDTCAFHEQPELQKK	757.3652	3	14.5
	QLCSFQIYEVPWEDR	985.4583	2	26.3
Mucin 5B	GYQVCPVLADIECR	840.3965	2	22.2

Protein Biomarker	Target Biomarker Peptide	Prec m/z	Charge	Retention Time (min)
	AQAQPGVPLGELGQVVECSLDFGLVCR	967.1439	3	36.2
	AAGGAVCEQPLGLECR	844.3968	2	15.2
Cystatin D	SQPNLDNCPFNDQPK	887.3956	2	13.8
	TLAGGIHATDLNDK	475.9143	3	12.2
Submaxillary gland androgen regulated protein	GPYPPGPLAPPQPFGPGFVPPPPPPPYGPGR	776.1541	4	28.8
	IPPPPPAPYGPGIFPPPPPQP	710.7205	3	25.25
Statherin	FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF	1215.8982	3	28.4
Histatin-1	EFPFYGDYGSNYLYDN	982.4056	2	26.27

Table 7C Seminal Fluid Peptide Inclusion List

Protein Biomarker	Target Biomarker Peptide	Prec m/z	Charge	Retention Time (min)
Semenogelin 1	KQGGSQSSYVLQTEELVANK	722.7071	3	18.5
	DIFTTQDELLVYNK	842.9264	2	23.45
Semenogelin 2	DVSQSSISFQIEK	734.3714	2	18.31
	DIFTTQDELLVYN	842.9264	2	24.1
PSA	VMDLPTQEPALGTTCYASGWGSIEPEEFLTPK	1175.5551	3	30.5

	AVCGGVLVHPQWVLTAAHCIR	586.8051	4	24.2
	LSEPAELTDAVK	636.8399	2	16.75
Prostatic Acid Phosphatase	ELSELSLLSLYGIHK	567.9866	3	28.5
	FQELESETLKSEEFQK			16.3
	SPIDTFPTDPIK	665.8475	2	19.35
Glycodelin	VHITSLLPTPEDNLEIVLHR	574.8187	4	27.3
	VLVEDDEIMQGFIR	555.2807	3	25.6
Epididymal secretory protein E1	AVVHGILMGVPVPFPIPEPDGCK	810.7632	3	32.2
	EVNVSPCPTQPCQLSK	922.4355	2	12.8

Table 7D Urine Peptide Inclusion List

Protein Biomarker	**Target Biomarker Peptide**	**Prec m/z**	**Charge**	**Retention Time (min)**
Osteopontin	GDSVVYGLR	927.9524	2	13
	QLYNKYPDAVATWLNPDPSQK	816.7445	3	23.8
	AIPVAQDLNAPSDWDSR	927.9533	2	20.5
Uromodulin	VLNLGPITR	491.8078	2	18.2
	STEYGEGYACDTDLR	868.8572	2	13.1
	DGPCGTVLTR	538.2658	2	10.21

Table 7E Vaginal Fluid and Menstrual Blood Peptide Inclusion List

Protein Biomarker	Target Biomarker Peptide	Prec m/z	Charge	Retention Time (min)
Mucin 5B/Cervical	GYQVCPVLADIECR	840.3965	2	22.4
	AQAQPGVPLGELGQVVECSLDFGLVCR	967.1501	3	36.51
	AAGGAVCEQPLGLECR	844.3968	2	14.8
Cornulin	ISPQIQLSGQTEQTQK	893.4706	2	13.4
	TLSESAEGACGSQESGSLHSGASQELGEGQR	1036.125	3	21.35
IgGFc-binding protein	APGWDPLCWDECR	831.3529	2	25.3
	SLAAYTAACQAAGVAVKPWR	697.6999	3	22.2
	AGCVAESTAVCR	640.7912	2	640.79
Ly6/PLAUR containing protein 3	DGVTGPGFTLSGSCCQGSR	971.925	2	16.02
	GLDLHGLLAFIQLQQCAQDR	766.0686	3	32.2
	GCVQDEFCTR	636.2653	2	11.24
Matrix metallo-proteinase-9	GSRPQGPFLIADKWPALPR	527.2981	4	25.22
Neutrophil gelatinase-associated lipocalin	SYPGLTSYLVR	628.3402	2	21.2
	TFVPGCQPGEFTLGNIK	622.3172	3	22.6

Protein Biomarker	Target Biomarker Peptide	Prec m/z	Charge	Retention Time (min)
Suprabasin	ALDGINSGITHAGR	461.2476	3	12.4
	LGQGVNHAADQAGKEVEK	617.652	3	9.45

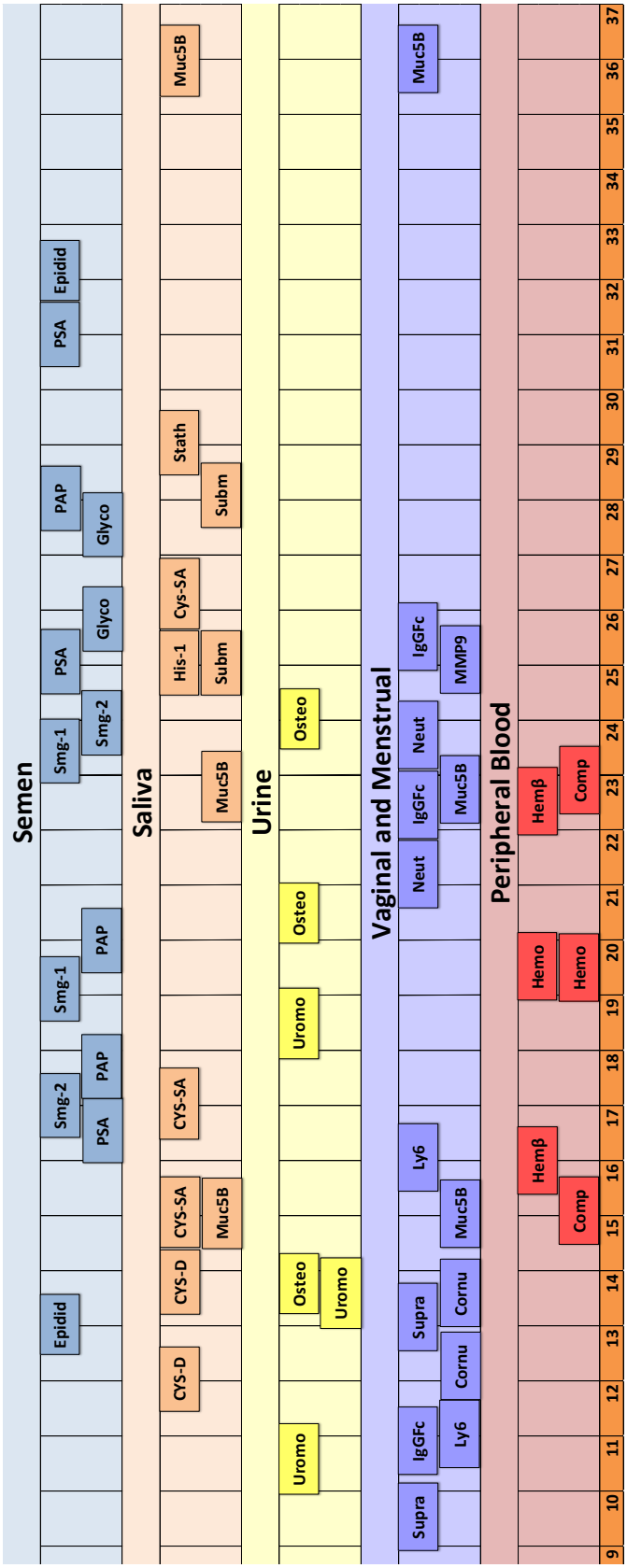


Figure 28 - Precursor ions employed in the Q-TOF body fluid multiplex assay. Ions are presented in order of elution (*i.e.*, retention time) and are segregated by body fluid specificity.

Analysis of a representative urine sample is shown in **Figure 29**. Chromatography and database search results revealed the identification of the two targeted biomarkers for urine (osteopontin and uromodulin) with no non-urine associated proteins detected.

Results obtained with samples of the other five body fluids also proved to be of high quality and specificity (**Figures 30-34**). Specifically, the analysis of a representative seminal fluid sample resulted in the detection of semenogelin I/II, prostate specific antigen, epididymal secretory protein E1, and prostatic acid phosphatase. No non-seminal fluid associated proteins were detected (**Figure 30**).

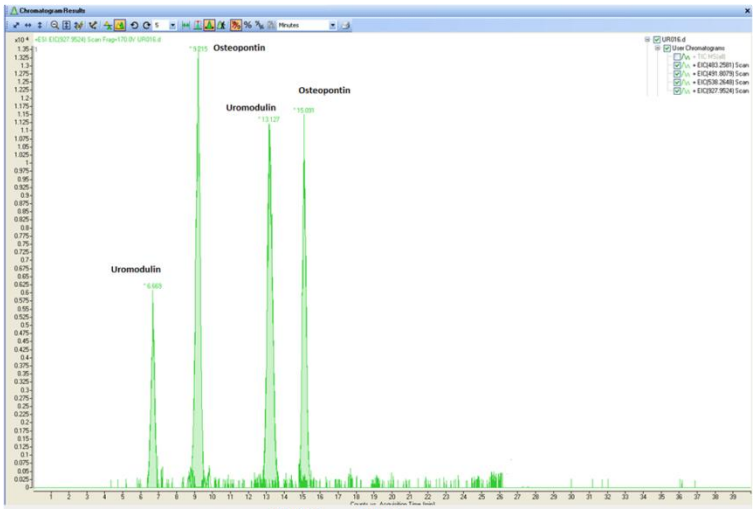
The multiplex assay of a representative saliva sample produced results that revealed the presence of mucin 5B, cystatin SA, cystatin D, submaxillary gland androgen regulated protein, statherin and histatin-1. Here again, no unexpected non-saliva associated protein was detected (**Figure 31**). It should be noted that while mucin 5B is a saliva-associated protein, it is also expressed and expected to be found in vaginal secretions.

Analysis of a vaginal fluid sample revealed the presence of the targeted markers cornulin, IgGFc-binding protein, Ly6/PLAUR containing protein 3, neutrophil gelatinase-associated lipocalin, suprabasin, and matrix metallo-proteinase-9. No unanticipated non-vaginal associated proteins were detected (**Figure 32**). As noted above, mucin 5B is a vaginal secretion- associated protein, it is co-expressed in saliva. Similarly, cornulin, also appears in menstrual blood.

The multiplex assay of a representative menstrual blood sample resulted in the detection of the target ions for complement C3, hemoglobin subunit beta, hemopexin, and cornulin (**Figure 33**). Because menstrual blood contains peripheral blood as a major component, it was anticipated that all of the peripheral blood markers would also appear in this sample. In addition to the peripheral blood proteins, cornulin was also detected in this sample. This biomarker was

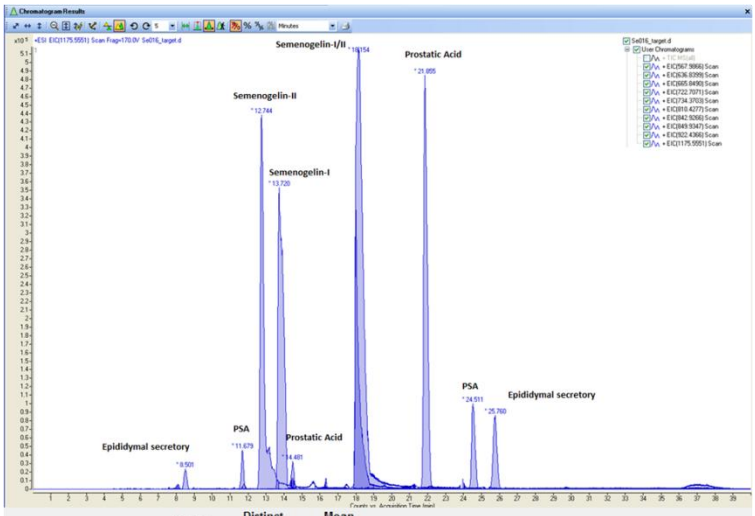
initially employed as a prospective vaginal fluid-specific marker. It is not clear whether its detection in menstrual blood is the result of mixing between the menstrual blood and vaginal fluid during collection or if cornulin is also a component of menstrual blood. In either event, the ability to detect cornulin in these cases may have potential utility for differentiating between peripheral and menstrual blood.

Finally, the analysis of a representative peripheral blood sample resulted in the unambiguous detection of complement C3, hemoglobin subunit beta and hemopexin (**Figure 34**). Aside from the expected occurrence of these proteins in menstrual blood, these proteins were not detected in any other body fluid.



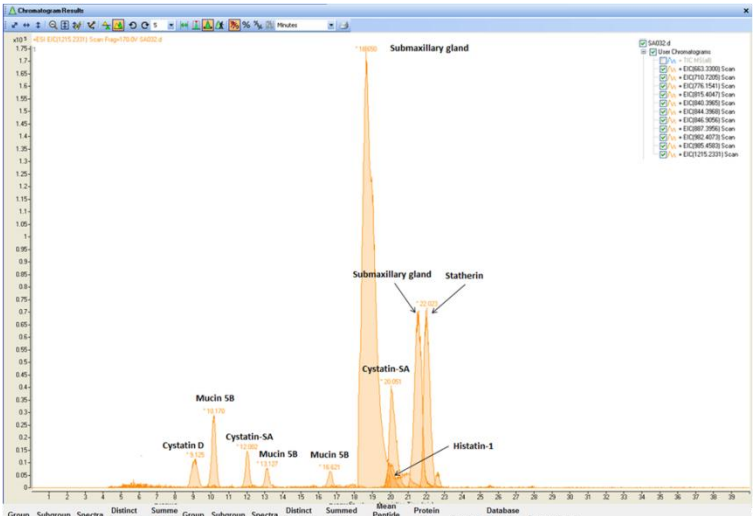
Group (#)	Subgroup (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
1	1.1	2	2	33.30	1.33e+005	35422.9	HUMAN	2	Osteopontin
2	2.1	3	2	26.52	8.64e+004	69761.4	HUMAN	1	Uromodulin
Totals:		5	4						

Figure 29 - TOP - Raw Q-TOF multiplex data indication a urine hit. BOTTOM – Database search results indicate high-confidence confirmatory identification Human urine. All targeted urine biomarkers were detected. No biomarkers indicative of other body fluids were detected



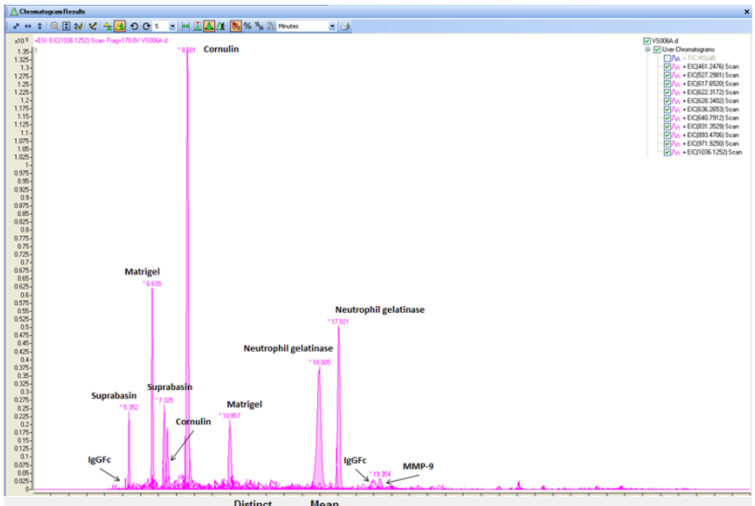
Group (#)	Subgroup (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
1	1.1	5	2	48.50	1.09e+007	65444.7	HUMAN	7	Semenogelin-2
2	2.1	6	2	42.59	1.04e+007	52131.1	HUMAN	6	Semenogelin-1
3	3.1	2	2	39.91	3.66e+005	28741.5	HUMAN	3	Prostate-specific antigen
4	4.1	3	2	39.41	1.29e+006	16570.3	HUMAN	5	Epididymal secretory protein E1
5	5.1	4	2	32.63	1.32e+006	44566.4	HUMAN	4	Prostatic acid phosphatase
Totals:		20	10						

Figure 30 TOP – Raw Q-TOF multiplex data indication a seminal fluid hit. BOTTOM – Database search results indicate high-confidence confirmatory identification Human seminal fluid. All targeted seminal fluid biomarkers were detected. No biomarkers indicative of other body fluids were detected.



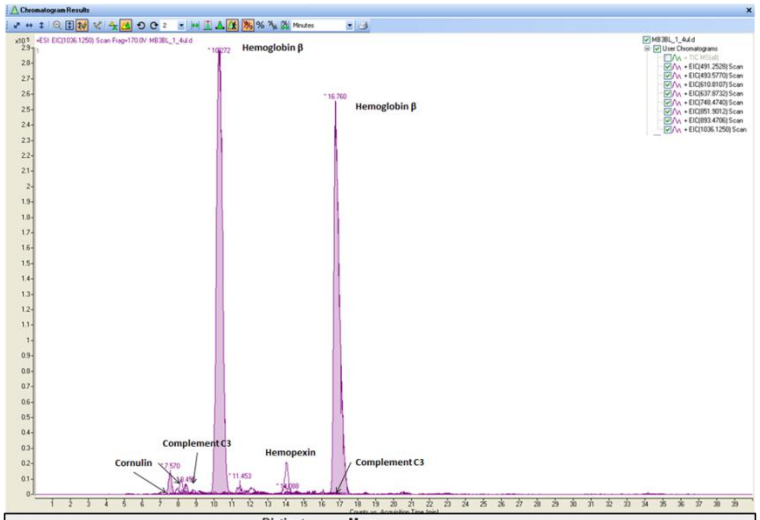
Group (#)	Subgroup (#)	Spectra (#)	Distinct Peptides (#)	Summed MS/MS Search Score	Group (#)	Subgroup (#)	Spectra (#)	Distinct Peptides (#)	Summed MS/MS Search Score	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
1	1.1	8	3	4[illegible]	1	1.1	8	3	45.14	6.63e+004	596343.8	HUMAN	Q9HC84	Mucin-5B OS=Homo sapiens GN=MUC5B PE=1 SV=3
2	2.1	7	2	4[illegible]	2	2.1	7	2	43.40	5.00e+005	8187.7	HUMAN	P02814	Submaxillary gland androgen-regulated protein 3B OS=Homo sapiens
3	3.1	3	2	3[illegible]	3	3.1	3	2	35.72	8.62e+004	16080.6	HUMAN	P28325	Cystatin-D OS=Homo sapiens GN=CST5 PE=1 SV=1
4	4.1	6	2	2[illegible]	4	4.1	6	2	28.38	7.57e+004	16444.8	HUMAN	P09228	Cystatin-SA OS=Homo sapiens GN=CST2 PE=1 SV=1
5	5.1	3	1	14	5	5.1	3	1	14.39	3.30e+005	7304.5	HUMAN	P02808	Statherin OS=Homo sapiens GN=STATH PE=1 SV=2
6	6.1	2	1	[illegible]	6	6.1	2	1	9.33	7.81e+004	6962.9	HUMAN	P15515	Histatin-1 OS=Homo sapiens GN=HTN1 PE=1 SV=2
Totals:		29	11		Totals:		29	11						

Figure 31 - TOP - Raw Q-TOF multiplex data indication a saliva hit. BOTTOM – Database search results indicate high-confidence confirmatory identification Human saliva. All targeted saliva biomarkers were detected. No biomarkers indicative of other body fluids were detected.



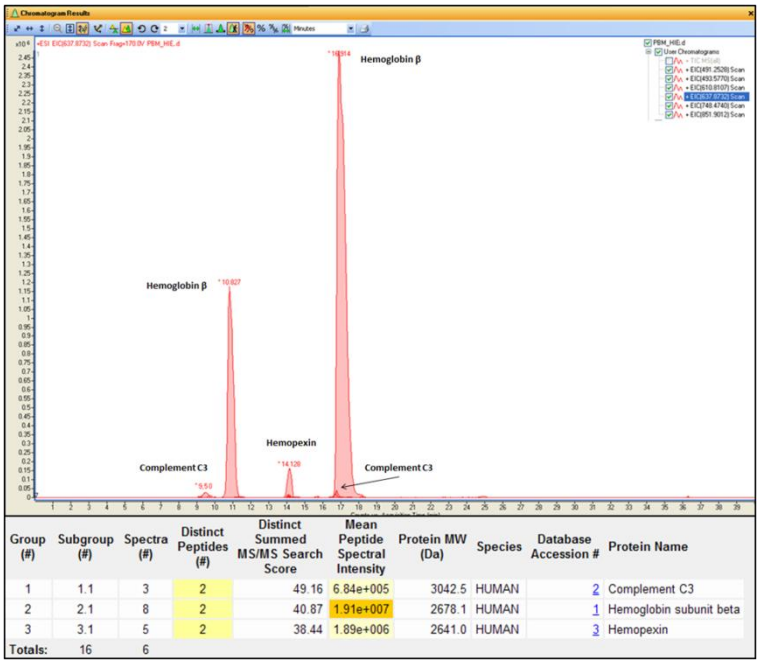
Group (#)	Subgroup (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
1	1.1	8	2	41.29	2.12e+006	22588.2	HUMAN	18	Neutrophil gelatinase-associated lipocalin
2	2.1	8	2	38.34	3.12e+006	53533.5	HUMAN	14	Cornulin
3	3.1	8	2	32.92	1.28e+006	35971.0	HUMAN	16	Ly6/PLAUR domain-containing protein 3
4	4.1	6	2	26.08	6.31e+005	25335.2	HUMAN	19	Suprabasin
5	5.1	2	1	16.52	6.54e+005	78458.7	HUMAN	17	Matrix metalloproteinase-9
Totals:	32	9							

Figure 32 - TOP - Raw Q-TOF multiplex data indication a vaginal fluid hit. BOTTOM – Database search results indicate high-confidence confirmatory identification Human vaginal fluid. Targeted vaginal fluid biomarkers were detected. No biomarkers indicative of other body fluids were detected.



Group (#)	Subgroup (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
1	1.1	2	2	46.85	2.37e+005	3042.5	HUMAN	2	Complement C3
2	2.1	28	2	42.21	3.32e+007	2678.1	HUMAN	1	Hemoglobin subunit beta
3	3.1	7	2	39.10	3.41e+005	4818.1	HUMAN	4	Cornulin
4	4.1	3	2	30.62	7.47e+005	2641.0	HUMAN	3	Hemopexin
Totals:	40	8							

Figure 33 - TOP - Raw Q-TOF multiplex data indicating a possible menstrual blood hit. BOTTOM – Database search results indicate high-confidence confirmatory identification Human blood biomarkers and a moderate-specificity vaginal fluid biomarker. No biomarkers indicative of other body fluids were detected. Taken together, these results may indicate the presence of menstrual blood or a mixture of peripheral blood and vaginal fluid.



Group (#)	Subgroup (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
1	1.1	3	2	49.16	6.84e+005	3042.5	HUMAN	2	Complement C3
2	2.1	8	2	40.87	1.91e+007	2678.1	HUMAN	1	Hemoglobin subunit beta
3	3.1	5	2	38.44	1.89e+006	2641.0	HUMAN	3	Hemopexin
Totals:		16	6						

Figure 34 - TOP - Raw Q-TOF multiplex data indication a blood hit. BOTTOM – Database search results indicate high-confidence confirmatory identification Human blood. All targeted blood biomarkers were detected. No biomarkers indicative of other body fluids were detected.

3.2.3 Single Source Population Study

The applicability of the candidate biomarkers requires thorough verification for stain specificity across a larger population sample. To assess this, multiplexed Q-TOF analyses of single-source body fluid samples from a sample population of fifty human research participants were used. This made it possible to assess the frequency at which target biomarkers may be detected in both target and non-target body fluids. The results obtained are summarized in table 8 and are described for each fluid.

Table 8 - Results of the targeted ion Q-TOF 6-body fluid multiplex assay. Targeted biomarkers are listed across the top of the figure and the body fluids tested are listed along the left side of the figure. Green cells indicate protein biomarkers that display high-specificity for their target body fluid and which were detected in all samples tested. Yellow cells indicate protein biomarkers that were detected in less than 100% of their target body fluid samples or. Red cells indicate cases where a specific biomarker was not detected in any of the samples tested.

	Seminal Fluid					Urine		Saliva						Vaginal Fluid and Menstrual Blood						Peripheral Blood		
	Semenogelin-1	Semenogelin-2	Epididymal protein E1	PSA	PAP	Uromodulin	Osteopontin	Submaxillary gland	Cystatin_D	Cystatin_SA	Statherin	Histatin 1	mucin 5b	Cornulin	Neutrophil gelatinase-associated lipocalin	Ly6/PLAUR domain- containing protein 3	IgGFc-binding protein	Matrix mmp-9	Suprabasin	Hemoglobin subunit beta	Complement C3	Hemopexin
Seminal Fluid	100.0%	100.0%	100.0%	100.0%	100.0%	0.00%	0.0%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Saliva	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%	76.0%	94.0%	90.0%	30.0%	96.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	4.0%	0.0%	0.0%
Urine - Male	24.0%	20.0%	40.0%	60.0%	80.0%	100.0%	100.0%	0.0%	0.0%	0.0%	0.0%	0.0%	4.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Urine - Female	0.0%	0.0%	30.0%	0.0%	0.0%	100.0%	100.0%	0.0%	0.0%	0.0%	0.0%	0.0%	4.0%	4.0%	0.0%	4.0%	0.0%	0.0%	0.0%	12.0%	0.0%	6.0%
Vaginal Fluid	0.0%	0.0%	4.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	38.0%	100.0%	100.0%	100.0%	68.0%	20.0%	22.0%	6.0%	0.0%	4.0%
Menstrual Blood	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	20.0%	20.0%	6.0%	4.0%	0.0%	0.0%	0.0%	100.0%	54.0%	76.0%
Peripheral Blood	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%	100.0%	96.0%

3.2.3.1 Seminal Fluid

Seminal fluid candidate biomarkers semenogelin I/II, epididymal secretory protein E1, prostatic acid phosphatase and prostate specific antigen were consistently detected in all semen samples. These markers were generally undetectable in non-target body fluids markers with a few exceptions. Semenogelin I/II, epididymal secretory protein E1, prostatic acid phosphatase and prostate specific antigen were observed in 20-80% of male urine samples and epididymal secretory protein E1 was observed in 4% of female Urine samples. While seminal fluid proteins were detected in other fluids, no urine specific biomarkers were detectable in any seminal fluid sample.

Seminal fluid donors were instructed to refrain from ejaculation for 5 days prior to urine collection. Although it is impossible to confirm compliance with this other than by self-reporting, it is possible that some seminal fluid proteins may persist in the male urinary/reproductive tract. This may be due to leakage from the reproductive system or residual ejaculate from many days prior. While unfortunate, seminal fluid proteins from this study have also been observed in other urinary proteomics publications involving male subjects [209]. Even with these cross-identifications, semenogelin I/II, prostatic acid phosphatase and prostate specific antigen were only identified in male samples. Also, no urine specific markers were detected in any seminal fluid sample. This leads to the conclusion that these markers may have utility in tracing source of the stain to a male donor.

However, epididymal secretory protein E1 was detected in female urine at nearly the same rate as in male urine samples. It is not possible to determine if this is an endogenous component of vaginal fluid or urine or if the detected protein represented residual protein from a prior sexual encounter. As with male participants, females were instructed to refrain from sexual

intercourse for 5 days prior to the collection of urine, vaginal fluids and menstrual blood although it was impossible to confirm compliance with this other than by self-reporting. As a result of these findings, epididymal secretory protein E1 was eliminated as a marker of seminal fluid.

3.2.3.2 Saliva

Saliva candidate biomarkers consisted of cystatin SA, cystatin D, submaxillary gland androgen-regulated protein, histatin-1, statherin as well as mucin 5B. Of these markers several showed a high degree of specificity and consistency across the population. Submaxillary gland androgen-regulated protein was detected in 100% saliva samples where cystatin SA, statherin and mucin 5B were identified in 90% or greater of the samples processed on the Q-TOF. cystatin D was identified in 76% of samples while histatin 1 was only detectable in 30% of samples assayed. Of these markers only one protein, mucin5B, was detected in any of the other non-targets body fluids analyzed.

It was initially hypothesized that mucin 5B would be specific to vaginal fluid as well as saliva. However, it was also detected in 20% and 38% of menstrual blood and vaginal fluid samples as well as 4% of male/female urine samples. From these results it is unlikely that mucin 5B will offer any discriminatory power between these fluids and thus it was removed as a biomarker candidate.

Improvements in assay sensitivity (*e.g.* use of a QQQ platform) may allow for more consistent detection of cystatin SA, cystatin D, as well as statherin. Alternatively, histatin 1 could be removed from further experiments due to the low identification rate in the samples.

3.2.3.3 Urine

Urine candidate biomarkers uromodulin and osteopontin were detected in all male and female urine samples. Additionally, these two proteins were not detected in any non-target body fluids. Thus, uromodulin and osteopontin appeared to be suitable biomarkers for urine.

3.2.3.4 Vaginal fluid

Vaginal fluid biomarker candidates included cornulin, IgGFc-binding protein, neutrophil gelatinase-associated lipocalin, Ly6/PLAUR containing protein 3, suprabasin, and matrix metallo-proteinase-9. Of these, cornulin, neutrophil gelatinase-associated lipocalin and Ly6/PLAUR containing protein 3 were detected in 100% of samples assayed. IgGFc-binding protein was detected in 68% of samples, matrix metallo-proteinase-9 in 20% of samples, and suprabasin was detected in 22% of samples analyzed on the Q-TOF. Other than the anticipated cross identification (including cornulin, neutrophil gelatinase-associated lipocalin and Ly6/PLAUR containing protein 3) with menstrual blood, no vaginal markers were detected in any other fluid.

3.2.3.5 Menstrual Blood

As discussed in the method development section, there were no reliable markers which could definitively differentiate menstrual blood from vaginal fluid. It was hypothesized that vaginal fluid markers may allow the differentiation of menstrual blood from other fluids with peripheral blood being particularly important. However, cornulin, neutrophil gelatinase-associated lipocalin and Ly6/PLAUR containing protein 3, were only found in 30%, 6%, and 4% of menstrual blood samples assayed. This may be due to the complexity of the blood proteome and sensitivity limits of the Q-TOF method. Accordingly, these markers should be evaluated should be evaluated for their potential utility since a more sensitive assay may allow reliable

detection of these proteins. However, it must be noted that without a 100% specific menstrual blood protein it is impossible to differentiate between pure menstrual blood *vs.* a mixture of menstrual blood and vaginal fluid.

3.2.3.6 Peripheral Blood

Biomarker candidates for peripheral blood included hemoglobin subunit beta, complement C3, and hemopexin. Hemoglobin and complement C3 were detectable in all peripheral blood samples while hemopexin was identified in 96% of analyzed samples.

These markers were detectable in several other fluids including menstrual blood, vaginal fluid, female urine as well as saliva. It was expected that these candidates would be identified in menstrual blood as peripheral blood is a major component of this fluid. Hemoglobin subunit beta, complement C3, and hemopexin were detected in 100%, 54% and 76% of these samples. While peripheral blood is a main component of menstrual blood, the lower percent detection of these proteins in this fluid is likely due to matrix suppression from cellular/endometrial tissue. Blood detection in vaginal fluid, female urine, and saliva may be due to trace amounts of these proteins in these fluids. This could be due to small abrasions from normal activity. Additionally, hemoglobin, complement C3, and hemopexin have been well documented in salivary, vaginal, as well as urinary proteomics projects aside from the findings presented here [149, 210].

3.2.4 Casework-Type Samples

Laboratory samples from the completed population study were used to further aid in the identification of candidates which demonstrate a high degree of consistency and specificity. While these laboratory samples assisted in the selection of the most promising biomarkers for further validation, they did not assess the use of a mass spectrometry platform for the processing of samples typically encountered in a forensic lab. In order to evaluate this, a series of studies

were performed to evaluate the reliability of biomarker detection in a forensic context. More specifically, the biomarkers must be recovered and identified from a variety of substrates such as cotton, leather or denim. The markers must detectable when exposed to a variety of environmental and chemical contaminants as well as recoverable from items typically associated with sexual assaults. Additionally, sample mixtures containing two to three body fluid components must reliably detected. For these studies 37 casework-type samples were analyzed in order to review the applicability of the method for use by forensic practitioners (**Tables 9 and 10**). These 37 samples were divided into five specific categories which included single source body fluids extracted from swabs; substrate extractions; volumetric mixtures; contaminant extractions, as well as weight mixtures.

3.2.4.1 Single Source Swabs

50 µL of sample was added to cotton swabs, allowed to dry, and re-suspended with phosphate buffered saline. Saliva, semen, vaginal fluid, peripheral blood and urine spotted onto cotton swabs were accurately identified by one or more body fluid specific proteins. Unfortunately, no vaginal specific proteins were identified in the menstrual blood swab assayed. Inclusion of vaginal proteins in the menstrual blood sample could support an interpretation that the source of the sample was from the vaginal canal, but without an identification of any of these proteins it is impossible to differentiate menstrual blood from peripheral blood. Additionally, aside from the anticipated epididymal secretory protein E1 in urine, no unexpected proteins were identified.

3.2.4.2 Single Source Substrate Studies

Similar to above, 50 µL of sample was added to a variety of substrates, allowed to dry, and re-suspended with phosphate buffered saline. Similar to the results from the single-source

swab studies saliva, semen, vaginal fluid, peripheral blood and urine spotted were accurately identified whereas menstrual blood could not be differentiated from peripheral blood.

As expected, epididymal secretory protein E1 was detected in the urine sample tested. In addition to epididymal secretory protein E1, Ly6/PLAUR containing protein 3 was detectable in all three saliva samples (cigarette, bottle, and digital swabBased on prior work this protein has been considered a vaginal fluid specific biomarker.Upon further research, it was found that Ly6/PLAUR containing protein 3 has been identified in the squamous esophageal epithelium, specifically in relation to esophageal cancer [211]. The detection of this protein in a non-target body fluid at this stage of the research is likely due to the modified collection protocols for the casework samples. In the population study saliva was collected using cotton material directly next to the salivary ducts producing a pure and cell-free saliva solution. To more realistically simulate what would be seen in real world samples saliva was directly deposited from the mouth into a 50 mL collection tube before application onto the cotton swab. This modified method deposited a greater amount of cellular material and protein from the oral cavity. This unexpected result highlights the importance of conducting studies on casework-type samples.

3.2.4.3 Volume Mixtures

Six two-component mixtures were run on the multiplex assay to evaluate potential matrix interference between two different body fluids. In all but one case (equivalent volumes of saliva and peripheral blood), at least one biomarker for each body fluid present in the mixture was detected. This loss of detection is likely due to the quantitative difference in protein content between saliva and peripheral blood when mixed in equal volumes. The total protein content of saliva is approximately 1.3 mg/mL while the protein concentration in peripheral blood is approximately 120 mg/mL. Applying 25 µl of saliva to the swab results in ~32 µg of total saliva

on the swab compared to ~3,000 µg of total blood protein onto the swab. Based on seeing these values it is likely that there is not enough saliva protein to detect against the background of more abundant blood proteins.

3.2.4.4 Contaminant Studies

A series of single-source body fluid samples were also assayed for the influence of potential endogenous inhibitors on biomarker detection. These inhibitors included spermicidal lubricant, BlueStar®, soil, chewing tobacco, coffee as well as chewing gum. Of the inhibitors assayed only chewing tobacco juice appeared to prevent the identification of saliva. This was not entirely unexpected given that tobacco juice is also known to act as a potent inhibitor of DNA typing chemistries. Aside from saliva, there were no interferences from any contaminant tested.

3.2.4.5 Weight Mixtures

It is hypothesized that the failure to detect the saliva component of a saliva and peripheral blood mixture is due to matrix effects from the peripheral blood proteins which are in significantly greater abundance than the salivary proteins. Support for this hypothesis is found in the results of a second set of 2-component mixtures that were normalized to provide for equivalent total protein input. Aside from menstrual blood, these samples were successful in identifying all two component mixtures.

Table 9 - Results of the Q-TOF multiplex assays using casework-type samples. Targeted biomarkers are listed across the top of the figure and the description of the type of body fluid and substrate are provided along the left side of the figure. Green cells indicate protein biomarkers that were detected. Red cells indicate protein biomarkers that were not detected. (VF: vaginal fluid; MB: menstrual blood; PB: peripheral blood)

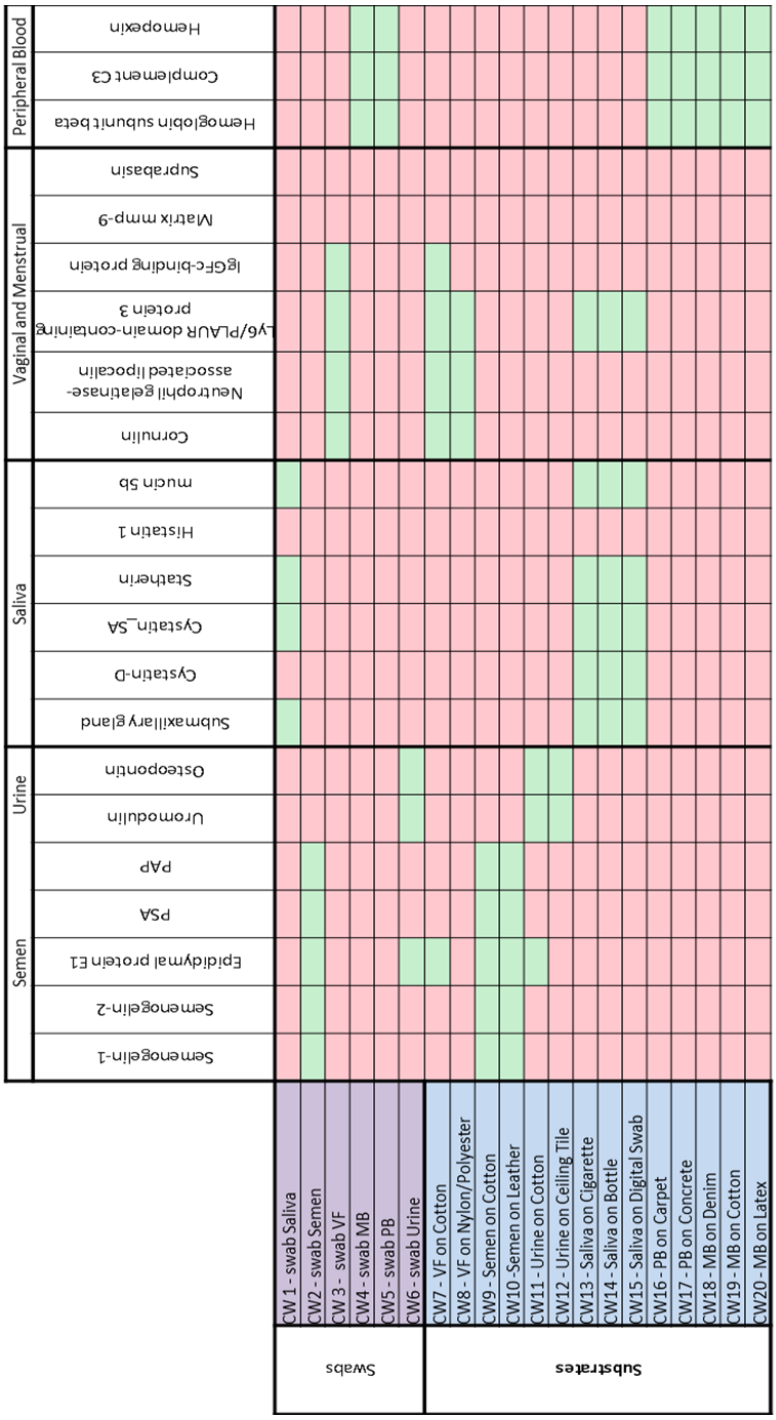
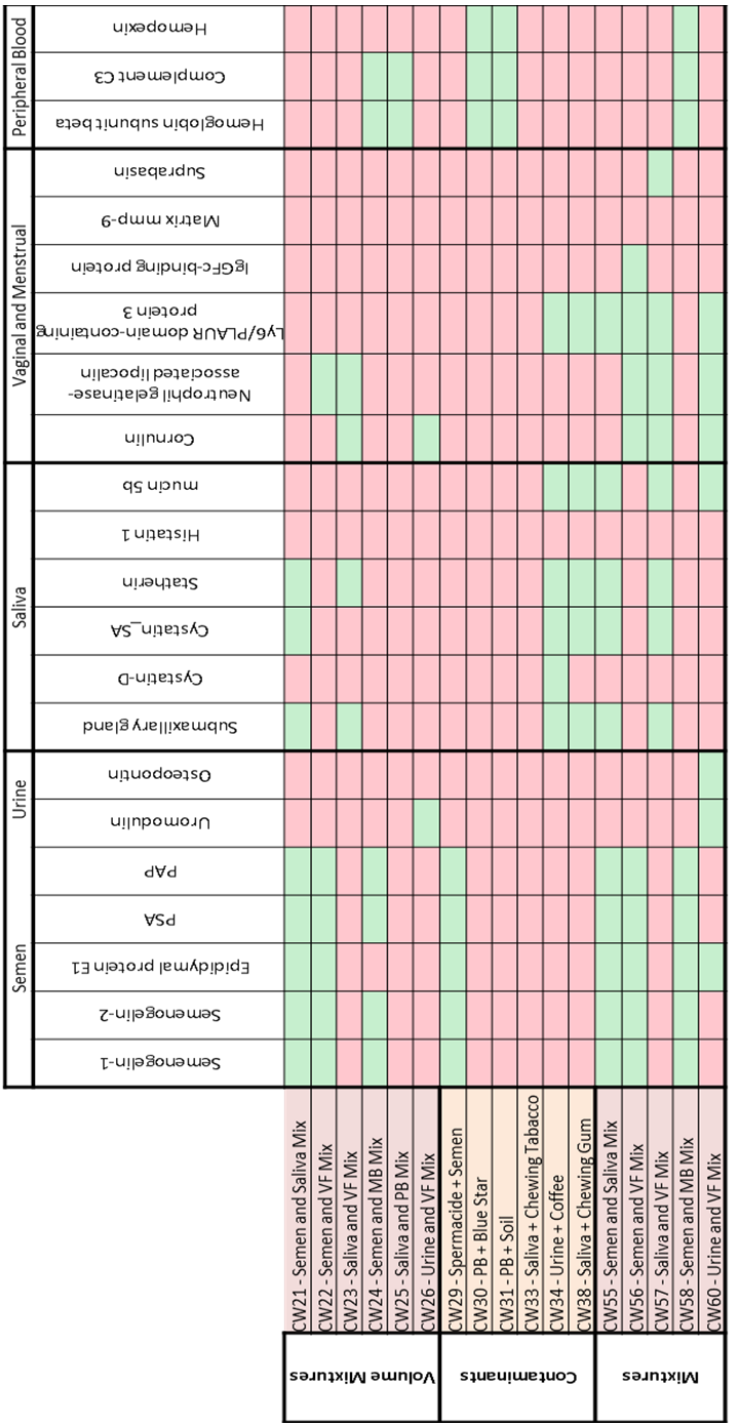


Table 10 - Results of the Q-TOF multiplex assays using casework-type mixed-source samples and samples containing mixtures. Green cells indicate protein biomarkers that were detected. Red cells indicate protein biomarkers that were not detected. (VF: vaginal fluid; MB: menstrual blood; PB: peripheral blood)



3.3 Conclusion

The second phase in the proteomics pipeline is to assess the biological applicability of candidate biomarkers to the specific biological question. In the case of the current research, the two major objectives were to assess whether the candidates were specific to as well as constantly present in their target fluid. In addition, because the overall goal of this project was to replace traditional/non-specific forensic serological tests with a confirmatory multiplex assay, it was also necessary to assess the viability of a mass spec based assay for processing forensic-type samples.

To assess the specificity and consistency of the initial biomarker candidates, twenty one of the most promising protein markers identified during the discovery phase were selected for investigation. These markers consisted of six seminal fluid candidates, two urine candidates, six saliva candidate biomarkers, seven vaginal/menstrual candidates as well as three peripheral blood candidate biomarkers. Using these proteins, a 44 minute targeted assay was designed and tested with fifty single-source laboratory samples. Results from this study identified several biomarkers which were fluid specific and which were consistently detected. In these studies, several candidate markers which were non-specific or inconsistently expressed across several body fluids were also identified. In addition to the population study, a series of casework-type samples were processed in order to evaluate the performance of the method for use in a caseworking laboratory. Of these casework-type samples, detection of the target biomarkers made it possible to correctly identify the body fluid components in all but a few samples.

Overall, this series of experiments was able to successfully assess the specificity, consistency, and applicability of a mass spectrometry-based assay for body fluid identification. However, even with these successes, a number of serious limitations to the instruments performance were recognized that may act as barriers to adoption by practitioners in forensic

testing laboratories. Most significantly, the instrument is not optimally engineered for multiplex assays[212] and at a top speed of 44 minutes/sample and one sample/run it is not suited to the much higher throughput needs of most forensic labs. With this limitation of speed in mind a different mass spectrometry-based platform may be a superior choice for the throughput requirements of a typical caseworking lab. Triple quadrupole mass spectrometers performing multiple reaction monitoring (QQQ-MRM) have become the "workhorse" instrument for high-throughput clinical and toxicological screening. This is due to advantages in sensitivity, selectivity, easier operation and lower cost of these instruments compared to Q-TOFs. In addition, QQQ instruments offer faster analytical gradients which can match the needs of a caseworking/production lab. Therefore, the next step in the dissertation research was to develop a QQQ multiplex assay incorporating the optimal biomarkers identified in the current phase of the research.

Chapter 4:

Prototype Validation

4 Introduction

This dissertation research has followed the biomarker pipeline from the discovery of candidate biomarkers for body fluid identification through candidate verification. The biomarker discovery phase resulted in the generation and characterization of a panel of over thirty candidate biomarkers for identification of biological stains. Under the biomarker verification phase a novel Q-TOF assay was designed to verify the specificity of the most promising biomarker candidates in a larger population. The Q-TOF verification phase was able to successfully confirm the specificity of numerous protein markers as well as flag several proteins which were cross-reactive with other biological fluids, thus eliminating them as specific biomarkers. Additionally, the viability of a mass spectrometry based serological assay for adoption was successfully assessed using casework-type samples. Results of these two studies produced a panel of fluid-specific biomarkers which can be reliably detected using mass spectrometry. The next step in the process, with an emphasis on "real-world" application, is a full developmental validation to assess the reliability, reproducibility, accuracy, sensitivity and limitations of a mass-spectrometry based assay for human stain identification with the implementation of a MS-based serological assay for the forensic biology laboratory as an overreaching goal.

While the Q-TOF assay was successfully able to confidently identify a biological stain, the instrument is not well suited for multiplex assays or the throughput required by a caseworking labratory. Due to these limitations, a developmental validation using this instrument would be undesirable. However, in recent years triple quadrupole mass spectrometers performing multiple reaction monitoring (QQQ-MRM) have been coined as a "workhorse" instrument for proteomics validation assays, clinical laboratory testing, as well as for confirmatory testing of illicit

substances in toxicology laboratories . This is due to the greater sensitivity, greater selectivity, easier operation, and lower cost compared to the Q-TOF.

The use of the QQQ-MRM platform provides analysts with much greater confidence in the accuracy of the results obtained for a given stain. This is because each individual body fluid body is identified based on the presence of multiple biomarker proteins (*e.g.* statherin and cystatin-SA for saliva). The presence of each biomarker protein itself will be based on the detection of multiple pieces (*i.e.* precursor ions) derived from the original biomarker. The presence/identity of each precursor ion will then be independently reconfirmed by detection of its breakdown products (*i.e.* product ions). The two fold filtration mechanism in these interments lead to a much more sensitive assay when targeting specific analytes. Additionally, this internal confirmation and reconfirmation of targets stands in sharp contrast to existing commercial forensic assays where identification is typically based on a single binding event between an antibody and its presumed target protein.

The following chapter will focus on the development and testing of a QQQ-MRM prototype assay for the identification of seminal fluid, saliva, and vaginal fluid in laboratory and casework type samples (*i.e.*, a "sexual assault" targeted assay). Additionally, a workflow was developed in order to simultaneously process evidentiary material for STR-based DNA analysis (for stain individualization) coupled with an MS-based serological identification of the of the stain body fluid source.

4.1 Materials and Methods

All research conducted under this project was IRB reviewed, approved and conducted in full compliance with U.S. Federal Policy for the Protection of Human Subjects. **Figure 35** presents

an overview of the workflow performed from sample collection through biomarker identification.

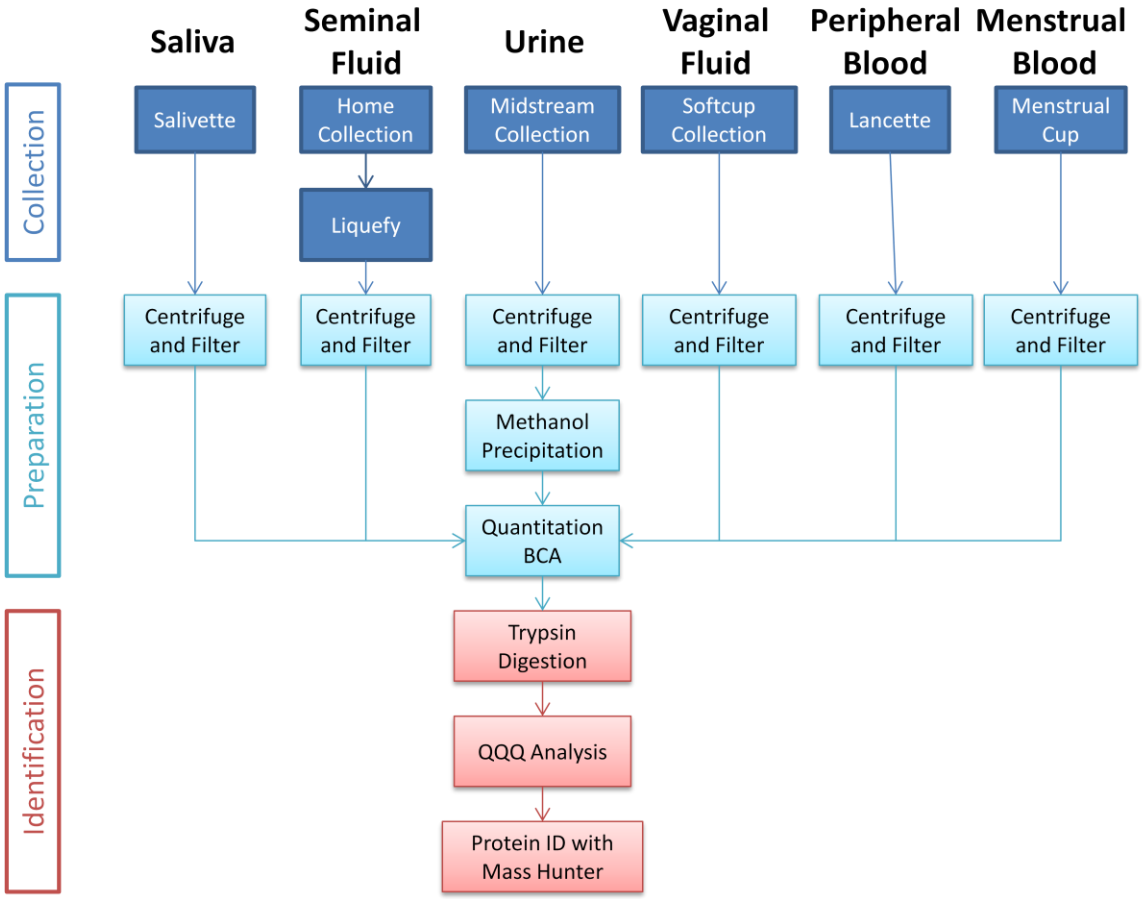


Figure 35 - Overview of QQQ Prototype phase from sample collection to protein identification.

4.1.1 Sample Collection and Preparation

4.1.1.1 Saliva

The donor was directed to thoroughly rinse their mouth with sterile water to remove residual food particles. After 5 minutes to allow secretion of saliva, the donor was instructed to place a Sarstedt Salivette™ saliva collection sponge into their mouth and to gently chew and roll the sponge around in their mouth for 3-4 minutes. The sponge was then placed into a sterile

plastic conical tube and repeated with one more cotton sponge. This allowed for the collection of large quantities of relatively pure saliva while reducing protein contamination from food items. Salivette™ sponges were centrifuged for 2 min at 1500 x g at 4°C to recover saliva.

Saliva was recovered from Salivette™ and pooled. Pooled samples were centrifuged at 10,000 x g to pellet cells and insoluble material. The supernatant was then passed through a .45 um spin filter unit.

4.1.1.2 Semen

Donors were directed to refrain from sexual activity for a minimum of 24 hours and then to obtain a 3-6ml sample of seminal fluid by masturbation in the privacy of their home. The subject was requested to directly deposit the fluid into a sterile plastic collection cup provided by the laboratory and then to refrigerate the sample until it could be transported to the lab within 1 hour.

Freshly collected semen was allowed to liquefy at room temperature for 30 minutes. Sample was added to two 1.5 mL microcentrifuge tubes and centrifuged at 10,000 x g for 10 minutes at 4°C and passed through a .45 µm filter to ensure cellular removal.

4.1.1.3 Urine

Donors were directed to deposit a morning urine sample (>50ml) into a sterile collection cup provided by the laboratory. Protein concentration varied substantially between individuals thus > 20 ml was typically used to ensure a sufficient quantity of protein.

Freshly collected Urine was centrifuged at 10,000 x g for 10 minutes at 4°C and passed through a .45 µm filter to ensure cellular removal. Protein was then precipitated with three parts ice cold -20ºC Methanol (300 µL urine:900 µL Methanol) and incubated for 10 min at -20ºC.

Solution was spun at 12.000 x g for 10 min at 4ºC and supernatant discarded. One volume (300 µL) ice cold -20ºC acetone was added and incubated for 10 minutes at min at 4ºC. Sample was spun again at 12.000 x g for 10 min at 4ºC and supernatant discarded. 100 µL of 25 mM Ammonium Bicarbonate was added and shaken at 37 ºC for 10 minutes to resolublize protein pellet.

4.1.1.4 Vaginal Fluid

Vaginal fluid collection employed an FDA-approved over-the-counter latex-free, hypoallergenic cup (SoftCup™). For the collection of vaginal secretions, donors were instructed to insert the Softcup™ for one hour and then deposit the cup into a 50 mL sterile collection container. Donors were directed to refrigerate the sample until it could be transported to the lab within 1 hour.

Softcup™ with vaginal fluid was delivered in a 50 mL sterile collection container. 2-3 mL of phosphate buffered saline was added to dissolve/collect fluid from sides of softcup and then transferred to 2-3 1.5 mL microcentrifuge tubes, these were spun at 10,000 x g for 10 minutes at 4°C and passed through a .45 µm filter to ensure cellular removal.

4.1.1.5 Peripheral Blood

Small volumes of peripheral blood were collected using the Unistik® 3 lancet and the StatSampler EDTA collection vial. Volunteers were instructed to wash their hands with disinfectant soap for two minute under warm water. Volunteer's finger was then cleaned with an alcohol swab and punctured using the lancet on the side of the volunteer's ring finger. Blood was collected from volunteer using the using the capillary tube from the StatSampler collection kits. Once the capillary tube was full (200 µL) the volunteer was given a Band-Aid® to cover the

puncture site. The StatSampler capillary tube was then expelled into a gel barrier microcentrifuge tube and spun for 2 minutes at 1500 x g to recover serum.

Blood serum from StatSampler collection kits were spun at 10,000 x g for 20 minutes at 4°C and passed through a .45 µm filter to ensure cellular removal.

4.1.1.6 Menstrual Blood

Menstrual blood was self collected by study participants in the privacy of their home. The collection protocol employed an FDA-approved over-the-counter latex-free, hypoallergenic cup (DivaCup™) for the collection of menstrual flow. The donor was directed to insert the cup into the vagina during menses for a period of approximately 1 hour. The cup was then gently removed; the contents were poured into a sterile 50ml conical tube and refrigerated until delivered to lab.

Menstrual blood was delivered in a sterile 50ml sterile collection container then placed into a 5 mL EDTA blood collection tube. Rinse with 2-3 mL PBS and add to Pink EDTA blood tube. If necessary these were divided between additional EDTA blood tubes. Sample was allowed to stand for 5 minutes on and then spun for 10 minutes at 1500 x g to separate serum. Menstrual serum was spun at 10,000 x g for 10 minutes at 4°C and passed through a .45 µm filter to ensure cellular removal.

4.1.1.7 Protein Quantitation with Pierce Micro BCA kit

Dilution standards were prepared as follows using Bovine Serum albumin (BSA) as a calibrator. A total of eight serial dilutions were prepared with concentrations ranging from .5 µg/mL to 200 µg/mL.

Working reagent was prepared with 25 parts BCA Reagent A and 25 parts BCA Reagent B with 1 part BCA Reagent C. In a 1.5 mL microcentrifuge tube 150 µl of working reagent was added to 150 µL of sample or standard. Tubes were vortexed, spun briefly, and allowed to

incubate in an Eppendorf Thermomixer® at 37ºC for 2 hours. Samples were allowed to cool to room temperature for 10 minutes and read on a visible spectrophotometer at 562 nm. BSA curve was used to generate a linear equation which was used to determine protein concentration of unknowns.

4.1.2 Body Fluid Identification

4.1.2.1 Trypsin Digestion

30 µg of total protein was lyophilized in a vacuum evaporator. Dried protein samples were reconstituted in 40ul of 8M urea in 100 mM Tris-HCl pH 8.5, 1.2 µL of 100 mM TCEP reducing agent and then shaken for 20 minutes at room temperature. Then, 0.88 µL of 500 mM IAA was added and the sample was shaken in the dark for an additional 15 minutes to alkylate the proteins. 120 µL of 100 mM Tris-HCl was added to sample to dilute urea to 2M. The proteins were digested with 1 µg of trypsin for 14-16 hours at 37°C.

4.1.2.2 Peptide Cleanup and Desalting

Pierce C-18 spin columns were used to cleanup and concentrate digested peptides. The spin column was placed in 1.5 mL microcentrifuge tube and activated by adding 200 µL of 50% acetonitrile followed by centrifugation at 1500 x g for 1 minute at room temperature. This was repeated one additional time. The resin was equilibrated with 200 µL of 5% acetronitrile and .5% triflouroacetic acid and spun at 1500 x g for 1 minute at room temperature. This was repeated one additional time. Digested sample was loaded onto column and spun at at 1500 x g for 1 minute at room temperature. The flow through was collected and passed through the column again at 1500 x g for 1 minute at room temperature. The column was washed with 200 µL of 5% acetronitrile and 0.5% triflouroacetic acid and spun at 1500 x g for 1 minute at room temperature. This was repeated three additional time. Peptides were eluted by adding 20 µL of

70% acetonitrile and spun at 1500 x g for 1 minute at room temperature. This was repeated one additional time. Peptides were lyophilized in a vacuum evaporator and resuspended in 3% acetonitrile and 0.1% formic acid for analysis by LC-MS/MS.

4.1.2.3 QQQ Analysis

Mass spectrometry was performed on an Agilent Technologies 6410 triple quadrupole mass spectrometer. The method employed a 150x1.0mm 3.5um 300SB-C18 analytical column with 12.5x2.1mm 5µM SB-C8 enrichment column. Solvent A contained H_20 with .1% formic acid and Solvent B contained a 90:10 mixture of Acetonitrile: H_20 with .1% formic acid. Samples were initially loaded on enrichment column at .2 mL/min with flow through going to waste. At 1 minute, the column was switched to analysis mode with buffer A and B passing through both enrichment and analytical columns with a gradient of 5% B to 35% B over 15 minutes. This was followed by 80% B from 15.01 to 16 minutes and 100% B from 16.01 to 17.01. A 5 minute post run followed gradient to re-equilibrate column.

4.1.2.4 Data Analysis

Data analysis was performed using Agilent MassHunter QQQ Quantitative software.

4.1.3 Preparation and Analysis of Casework-Type Samples

4.1.3.1 Body Fluids on Swabs

50 µL of body fluid was collected and prepared as described in sections 4.2.1 and 4.2.2 and was spotted on standard cotton swabs. Swabs were allowed to air dry in a biosafety hood for 40 minutes to 1 hour. Once dried, cotton was removed from the swab with a scalpel and placed into a 1.5 mL microcentrifuge tube. Proteins were extracted by adding 300 µL phosphate buffered saline to the swab which was then shaken at 37°C for 10 minutes. After incubation, the

swab was placed into spin basket and centrifuged for 30 seconds at 13,000 x g to recover all fluid. Samples were then quantified using Pierce Micro BCA protocol. Sample identification was performed as discussed.

4.1.3.2 Body Fluids on Substrates

50 ul of body fluid was collected and prepared as described in sections 3.2.1 and 3.2.2 and was spotted on various 5x5 cm substrate cuttings such as cotton, denim, and leather. Cuttings were allowed to air dry in a biosafety hood for 40 minutes to 1 hour. Once dried, cuttings were placed into 1.5 mL microcentrifuge tube. Proteins were extracted by adding 300 µL phosphate buffered saline to cuttings which was then shaken at 37°C for 10 minutes. After incubation cuttings was placed into spin basket and centrifuged for 30 seconds at 13,000 x g to recover all fluid. Samples were then quantified using the Pierce Micro BCA protocol previously described.

4.1.3.3 Body Fluids on Swabs with contaminants

50 µL of body fluid was collected and prepared as described in sections 3.2.1 and 3.2.2 and was spotted on standard cotton swabs which had been dipped in various environmental contaminants such as bleach, Bluestar®, and spermicidal lubricant. Swabs were allowed to air dry under the hood for 40 minutes to 1 hour. Once dried, cotton was removed from the swab with a scalpel, placed into 1.5 mL microcentrifuge tube. Proteins were extracted by adding 300 µL phosphate buffered saline to the swab which was then shaken at 37°C for 10 minutes. After incubation, the swab was placed into spin basket and centrifuged for 30 seconds at 13,000 x g to recover all fluid. Samples were then quantified using the Pierce Micro BCA protocol previously described.

4.1.3.4 Body Fluid Mixtures

25 µL of each body fluid in mixture was prepared as described in sections 4.2.1 and 4.2.2 was spotted on standard cotton swabs. Swabs were allowed to air dry in a biosafety hood for 40 minutes to 1 hour. Once dried, the cotton was removed from swab with a scalpel and placed into 1.5 mL microcentrifuge tube. Proteins were extracted by adding 300 µL phosphate buffered saline to swab which was then shaken at 37°C for 10 minutes. After incubation, the swab was placed into spin basket and centrifuged for 30 seconds at 13,000 x g to recover all fluid. Samples were then quantified using the Pierce Micro BCA kit.

50 µL of body fluid was collected and prepared as described in sections 3.2.1 and 3.2.2 was spotted on standard cotton swabs. Swabs were allowed to air dry under the hood for 40 minutes to 1 hour. Once dried, cotton was removed from swab with a scalpel, placed into 1.5 mL microcentrifuge tube. Proteins were extracted by adding 300 µL phosphate buffered saline to the swab which was then shaken at 37°C for 10 minutes. After incubation, the swab was placed into spin basket and centrifuged for 30 seconds at 13,000 x g to recover all fluid. Samples were then quantified using Pierce Micro BCA protocol and identified as discussed previously.

4.1.3.5 Body Fluid Dilutions

To test lower limit of detection of detection 50 µL of body fluid was collected and prepared as described in sections 4.2.1 and 4.2.2 and was spotted on standard cotton swabs. Swabs were allowed to air dry under the hood for 40 minutes to 1 hour. Once dried, the cotton was removed from swab with a scalpel, placed into a 1.5 mL microcentrifuge tube. Proteins were extracted by adding 300 µL phosphate buffered saline to the swab which was then shaken at 37°C for 10 minutes. After incubation, the swab was placed into spin basket and centrifuged for

30 seconds at 13,000 x g to recover all fluid. Samples were then quantified using Pierce Micro BCA protocol and identified as discussed previously.

4.1.4 Combined DNA Typing and Serological Analysis

Mixtures and swabs were prepared as described previously with slight modification. After cellular centrifugation step the supernatant was removed and processed for protein analysis. The cellular pellet (*i.e.*, DNA-rich material) was processed further to isolate DNA suitable for STR-based analyses.

4.1.4.1 DNA extraction with BioRobot EZ1

190 µL of Buffer G2 and 10 µL of Protinase K were added to the pelleted material and incubated for 15 minutes at 56°C. Tube was spun and supernatant processed using the EZ1 DNA Tissue kit (Qiagen).

4.1.4.2 DNA Quantitation with Quantifiler™ Duo

Quantifiler™ Duo primer mix and PCR reaction mix were used to make a master mix of 4.62ul/sample and 5.5 µL/sample. In addition, eight standard mixes were prepared at a concentration of 50, 16.7, 5.56, 1.85, 0.62, 0.21, 0.068 and 0.023 ng/µL. To 9.2 µL of master mix .8 µL of sample or standard was mixed in each reaction well on a standard optical 96 well plate and analyzed on the real time PCR instrument.

4.1.4.3 STR Amplification with Identifiler™

For DNA profiling, 4.6µL/ sample of Identifiler™ master mix and2.3 µL/sample Identifiler primer set were combined for the master mix. To a 0.2 mL tube 6 µL of master mix and 4 µL of DNA was added at a concentration of 0.1 ng/µL. Thermal cycling conditions (**Table 11**) consisted of the following:

Table 11 – Thermocycler conditions for STR amplification with Identifiler™.

Initial Incubation Step	**Denature**	**Anneal**	**Extend**	**Final Extension**	**Final Step**
HOLD	CYCLE (standard: 28 cycles, LCN: 34 cycles)			HOLD	HOLD
95°C 11 min	94 °C 1 min	59°C 1 min	72°C 1 min	60°C 60 min	4 – 25°C forever

4.2 Results and Discussion

4.2.1 Method Development

The first objective of this phase of the research was to identify ions for use in an MRM biomarker assay for saliva, seminal fluid, and vaginal secretions as well as to develop a working QQQ assay for the detection of these markers. While this approach yields very dependable results, method design and development involves a substantial amount of effort to identify biologically relevant protein targets, optimal tryptic peptides as well as the specific product ions which the QQQ-MRM assay will measure. The first two requirements were completed as part of the biomarker discovery and verification stages of this project

From the Q-TOF verification study, several biologically relevant proteins in saliva, seminal fluid, and vaginal fluid were identified as consistently detectable across multiple individuals with little to no cross detection in other fluids. Using these data (**Table 8**) three markers were selected for the MRM assay for each body fluid including: cystatin_SA, statherin, and submaxillary gland androgen-regulated protein 3B as markers for saliva, prosatic acid

phosphatase and semenogelin-I/II for seminal fluid, and cornulin, Ly6/PLAUR domain-containing protein 3, and neutrophil gelatinase-associated lipocalin as markers for vaginal fluid. Since these markers had been examined previously the optimal peptides for each protein were already known (**Figures 7A-7E**) and will be used again on the QQQ assay. Briefly, the peptides selected for the Q-TOF were consistently identified between individuals, displayed strong signal intensity, had high database match statistics, were unique to a given protein, were devoid of missed cleavages, and did not include peptides with potential amino acid modifications including those with methionine, cystine, and N-Terminus glutamic acid residues.

The final assay design aspect involves selection of product ions from the peptide fragmentation. Peptide exact mass as well as a unique fragmentation pattern is what the Q-TOF uses to confirm the detection of a specific marker. The MRM assay uses the same fragmentation mechanism but only detects specific product ions rather than the entire product ion spectra, thus selection of the most abundant ions are needed to ensure that the greatest level of detection and sensitivity are achieved. Figure 36 presents the sequence specific fragmentation pattern generated on the Q-TOF for the peptide FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF which is diagnostic for the salivary protein marker statherin. The m/z for this peptide is 1215.2332 with a charge of +3, for product ion selection high abundant ions are selected which are larger than the precursor m/z. This is to ensure specificity such that a multiply charged fragment will not be inadvertently monitored twice. From the fragmentation in Figure 36 the product ions 1462.6 and 1229.5 m/z were targeted for this Statherin peptide. Figure 37 correspond to the QQQ raw data showing the much cleaner signal when compared to the Q-TOF. Using this same approach, the optimal precursor-product ion pairs were determined for each protein biomarker which can be seen in Table 12.

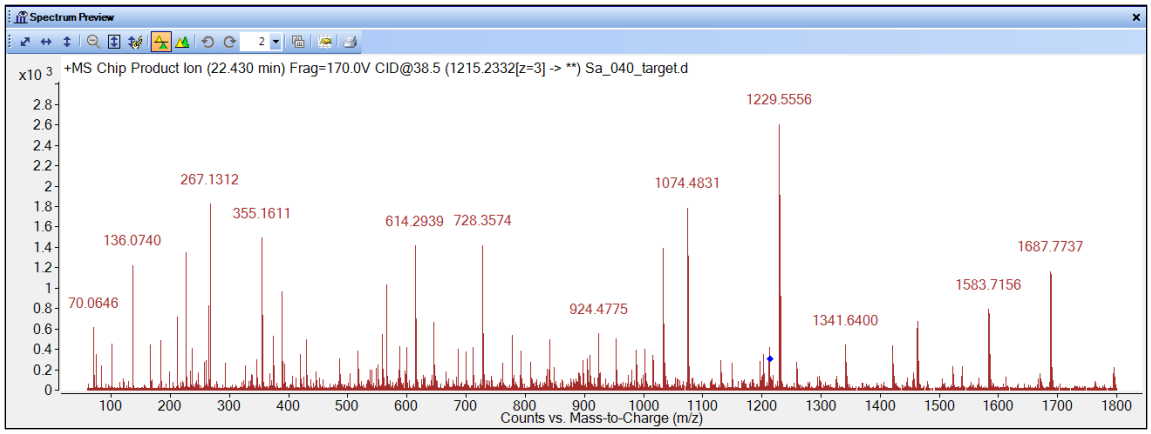


Figure 36 - In a Q-TOF assay, inclusion list precursor ions are only isolated in the first quadrupole (Q1). After the isolated precursor ions are fragmented in the collision cell all product ions reach the detector. These fragments generate a complex fragmentation spectrum which can be analyzed to confirm the presence of a particular protein biomarker in a database.

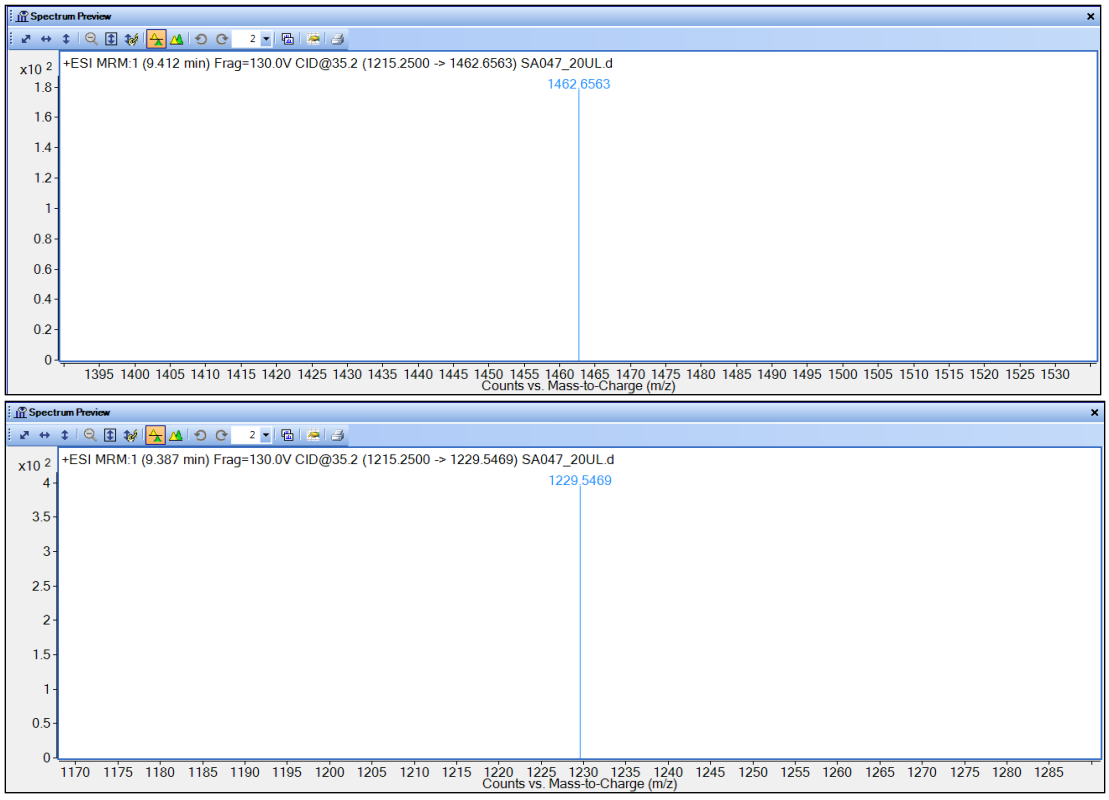


Figure 37 - When using a QQQ in MRM mode, target-ion list precursor ions are isolated in the first quadrupole (Q1); fragmented and then sent to the second quadrupole (Q2) which acts as a second filter for the isolation of specific product ions. This two-fold filtration process greatly improves the signal-to-noise ratio for target ion detection and increases the sensitivity of the assay by 10-100 fold. Shown are two product ions (1462.6 and 1229.5 m/z) from salivary biomarker statherin peptide FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF.

Table 12 - QQQ-MRM transition list which targets seminal fluid, saliva, and vaginal fluid.

Compound Group	Compound Name	ISTD?	Precursor Ion	Product Ion	Dwell	Fragmentor	Collision Energy	Ion Name
sp|Q9UBG3|CRNN_HUMAN	TLSESAEGACGSQESGSLHSGASQFLGEGQR	FALSE	1036.125319	1131.53889	20	130	28.6	y11
sp|Q9UBG3|CRNN_HUMAN	TLSESAEGACGSQESGSLHSGASQFLGEGQR	FALSE	1036.125319	1259.54659	20	130	28.6	y25
sp|Q9UBG3|CRNN_HUMAN	TLSESAEGACGSQESGSLHSGASQFLGEGQR	FALSE	1036.125319	1195.02529	20	130	28.6	y24
sp|Q9UBG3|CRNN_HUMAN	ISPQIQLSGQTEQTQK	FALSE	893.470697	1247.62262	20	130	30	y11
sp|Q9UBG3|CRNN_HUMAN	ISPQIQLSGQTEQTQK	FALSE	893.470697	1006.47998	20	130	30	y9
sp|Q9UBG3|CRNN_HUMAN	ISPQIQLSGQTEQTQK	FALSE	893.470697	919.44795	20	130	30	y8
sp|P80188|NGAL_HUMAN	TFVPGCQPGEFTLGNIK	FALSE	622.313583	1075.57824	20	130	13.2	y10
sp|P80188|NGAL_HUMAN	TFVPGCQPGEFTLGNIK	FALSE	622.313583	978.525472	20	130	13.2	y9
sp|P80188|NGAL_HUMAN	TFVPGCQPGEFTLGNIK	FALSE	622.313583	759.374483	20	130	13.2	y14
sp|P80188|NGAL_HUMAN	SYPGLTSYLVR	FALSE	628.337695	1005.57276	20	130	16.5	y9
sp|P80188|NGAL_HUMAN	SYPGLTSYLVR	FALSE	628.337695	908.519993	20	130	16.5	y8
sp|P80188|NGAL_HUMAN	SYPGLTSYLVR	FALSE	628.337695	738.414465	20	130	16.5	y6
sp|O95274|LYPD3_HUMAN	GCVQDEFCTR	FALSE	636.260804	1054.46222	20	130	16.9	y8
sp|O95274|LYPD3_HUMAN	GCVQDEFCTR	FALSE	636.260804	955.393806	20	130	16.9	y7
sp|O95274|LYPD3_HUMAN	GCVQDEFCTR	FALSE	636.260804	827.335229	20	130	16.9	y6
sp|O95274|LYPD3_HUMAN	DGVTGPGFTLSGSCCQGSR	FALSE	971.922731	1212.50958	20	130	34	y11
sp|O95274|LYPD3_HUMAN	DGVTGPGFTLSGSCCQGSR	FALSE	971.922731	1111.4619	20	130	34	y10
sp|O95274|LYPD3_HUMAN	DGVTGPGFTLSGSCCQGSR	FALSE	971.922731	998.377839	20	130	34	y9
sp|P04279|SEMG1_HUMAN	KQGGSQSSYVLQTEELVANK	FALSE	722.706085	1031.53677	20	130	17	y9
sp|P04279|SEMG1_HUMAN	KQGGSQSSYVLQTEELVANK	FALSE	722.706085	903.478188	20	130	17	y8
sp|P04279|SEMG1_HUMAN	KQGGSQSSYVLQTEELVANK	FALSE	722.706085	1022.49015	20	130	17	b10
sp|P04279|SEMG1_HUMAN	DIFSTQDELLVYNK	FALSE	842.92506	1309.66342	20	130	27.4	y11
sp|P04279|SEMG1_HUMAN	DIFSTQDELLVYNK	FALSE	842.92506	1121.58372	20	130	27.4	y9
sp|P04279|SEMG1_HUMAN	DIFSTQDELLVYNK	FALSE	842.92506	993.525138	20	130	27.4	y8
sp|Q02383|SEMG2_HUMAN	DIFTTQDELLVYNK	FALSE	849.932885	1222.63139	20	130	27.8	y10
sp|Q02383|SEMG2_HUMAN	DIFTTQDELLVYNK	FALSE	849.932885	1121.58372	20	130	27.8	y9
sp|Q02383|SEMG2_HUMAN	DIFTTQDELLVYNK	FALSE	849.932885	993.525138	20	130	27.8	y8
sp|Q02383|SEMG2_HUMAN	DVSQSSISFQIEK	FALSE	734.36992	1038.5466	20	130	21.9	y9
sp|Q02383|SEMG2_HUMAN	DVSQSSISFQIEK	FALSE	734.36992	951.514573	20	130	21.9	y8
sp|Q02383|SEMG2_HUMAN	DVSQSSISFQIEK	FALSE	734.36992	751.398481	20	130	21.9	y6
sp|P15309|PPAP_HUMAN	SPIDTFPTDPIK	FALSE	665.848092	1033.52005	20	130	18.4	y9
sp|P15309|PPAP_HUMAN	SPIDTFPTDPIK	FALSE	665.848092	918.49311	20	130	18.4	y8
sp|P15309|PPAP_HUMAN	SPIDTFPTDPIK	FALSE	665.848092	817.445431	20	130	18.4	y7
sp|P15309|PPAP_HUMAN	FQELESETLKSEEFQK	FALSE	657.98971	895.451973	20	130	14.6	y7
sp|P15309|PPAP_HUMAN	FQELESETLKSEEFQK	FALSE	657.98971	848.917432	20	130	14.6	y14
sp|P15309|PPAP_HUMAN	FQELESETLKSEEFQK	FALSE	657.98971	784.396135	20	130	14.6	y13
sp|P15309|PPAP_HUMAN	ELSELSLLSLYGIHK	FALSE	567.985572	930.540728	20	130	11.2	y8
sp|P15309|PPAP_HUMAN	ELSELSLLSLYGIHK	FALSE	567.985572	817.456664	20	130	11.2	y7
sp|P15309|PPAP_HUMAN	ELSELSLLSLYGIHK	FALSE	567.985572	730.424636	20	130	11.2	y6
sp|P02808|STAT_HUMAN	FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF	FALSE	1215.233017	1687.7751	20	130	35.2	y13
sp|P02808|STAT_HUMAN	FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF	FALSE	1215.233017	1462.66376	20	130	35.2	y11
sp|P02808|STAT_HUMAN	FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF	FALSE	1215.233017	1229.56259	20	130	35.2	b11
sp|P02808|STAT_HUMAN	FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF	FALSE	1215.233017	1583.71652	20	130	35.2	b14
sp|P09228|CYTT_HUMAN	IIEGGIYDADLNDER	FALSE	846.907398	1337.5968	20	130	27.6	y12
sp|P09228|CYTT_HUMAN	IIEGGIYDADLNDER	FALSE	846.907398	1110.46981	20	130	27.6	y9
sp|P09228|CYTT_HUMAN	IIEGGIYDADLNDER	FALSE	846.907398	947.406479	20	130	27.6	y8
sp|P09228|CYTT_HUMAN	QLCSFQIYEVPWEDR	FALSE	985.459275	1093.4949	20	130	34.7	y8
sp|P09228|CYTT_HUMAN	QLCSFQIYEVPWEDR	FALSE	985.459275	801.388979	20	130	34.7	y6
sp|P09228|CYTT_HUMAN	QLCSFQIYEVPWEDR	FALSE	985.459275	702.320565	20	130	34.7	y5
sp|P02814|SMR3B_HUMAN	GPYPPGPLAPPQPFGPGFVPPPPPPPYGPGR	FALSE	776.15636	1228.64732	20	130	18.9	y12
sp|P02814|SMR3B_HUMAN	GPYPPGPLAPPQPFGPGFVPPPPPPPYGPGR	FALSE	776.15636	850.445765	20	130	18.9	b9
sp|P02814|SMR3B_HUMAN	GPYPPGPLAPPQPFGPGFVPPPPPPPYGPGR	FALSE	776.15636	1172.60987	20	130	18.9	b12
sp|P02814|SMR3B_HUMAN	IPPPPPAPYGPGIFPPPPPQP	FALSE	710.718937	729.393001	20	130	16.5	y7
sp|P02814|SMR3B_HUMAN	IPPPPPAPYGPGIFPPPPPQP	FALSE	710.718937	987.529829	20	130	16.5	b10
sp|P02814|SMR3B_HUMAN	IPPPPPAPYGPGIFPPPPPQP	FALSE	710.718937	1141.60406	20	130	16.5	b12

From the transition list in Table 12, a 16-minute method was developed. The assay was first run using a pooled sample of each fluid (saliva, seminal fluid, and vaginal fluid) to alleviate variation between individuals. The pooled data were acquired during a single time segment where the instrument cycles between all precursor-product ion pairs during the chromatographic run. Using this un-segmented method the instrument scans for all potential biomarkers across the

entire chromatographic separation without taking into account the exact elution times for each pair. Monitoring many compounds simultaneously will lower the dwell time (accumulation of ions) for each individual precursor-product ion pair causing a loss in sensitivity and an increase in the signal-to-noise ratio. While this method was not optimal, it did allow for the discovery of the retention times for each of the peptides. Using the known retention times a method was developed to divide the targets into three time segments which allowed the instrument to only target ions which elute during that specific time segment – thereby increasing the allowable dwell time. Table 13 represents all targeted peptides and their location in three time segments.

Table 13 – Segmented method dividing the MRM acquisition into three time segments.

Segment	Protein	Accession	Peptide	RT (min)
2	Ly6/PLAUR domain-containing protein 3	O95274	GCVQDEFCTR	5.71
	Ly6/PLAUR domain-containing protein 3	O95274	TLSESAEGACGSQESGSLHSGASQELGEGQR	6.61
	Cornulin	Q9UBG3	ISPQIQLSGQTEQTQK	7.6
3	Ly6/PLAUR domain-containing protein 3	O95274	DGVTGPGFTLSGSCCQGSR	8.5
	Prostatic acid phosphatase	P15309	FQELESETLKSEEFQK	8.57
	Cystatin_SA	P09228	IIEGGIYDADLNDER	8.957
	Semenogelin-2	Q02383	DVSQSSISFQIEK	9.16
	Semenogelin-1	P04279	KQGGSQSSYVLQTEELVANK	9.65
	Prostatic acid phosphatase	P15309	SPIDTFPTDPIK	10.28
	Neutrophil gelatinase-associated lipocalin	P80188	SYPGLTSYLVR	10.9
4	Neutrophil gelatinase-associated lipocalin	P80188	TFVPGCQPGEFTLGNIK	11.85
	Semenogelin-1	P04279	DIFSTQDELLVYNK	11.93
	Semenogelin-2	Q02383	DIFTTQDELLVYNK	11.93
	Cystatin_SA	P09228	QLCSFQIYEVPWEDR	13.26
	Submaxillary gland	P02814	IPPPPPAPYGPGIFPPPPPQP	13.47
	Prostatic acid phosphatase	P15309	ELSELSLLSLYGIHK	14
	Statherin	P02808	FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF	14.41
	Submaxillary gland	P02814	GPYPPGPLAPPQPFGPGFVPPPPPPPYGPGR	14.91

4.2.2 Method Evaluation

Using the segmented method developed a series of single source samples were run to evaluate the consistency of the assay and markers on the QQQ platform. Additionally, theses samples were used to develop preliminary interpretation guidelines using the Agilent

Quantitative Analysis software in order to evaluate the results from the mixture and casework-type samples.

A representative example of results obtained using a QQQ-MRM multiplex assay to identify human seminal fluid can be seen in **Figure 38**. Examples of assay results obtained for a known saliva sample (**Figure 39**) and a known vaginal fluid sample (**Figure 40**) illustrate the specificity and accuracy the MRM-QQQ multiplex assay.

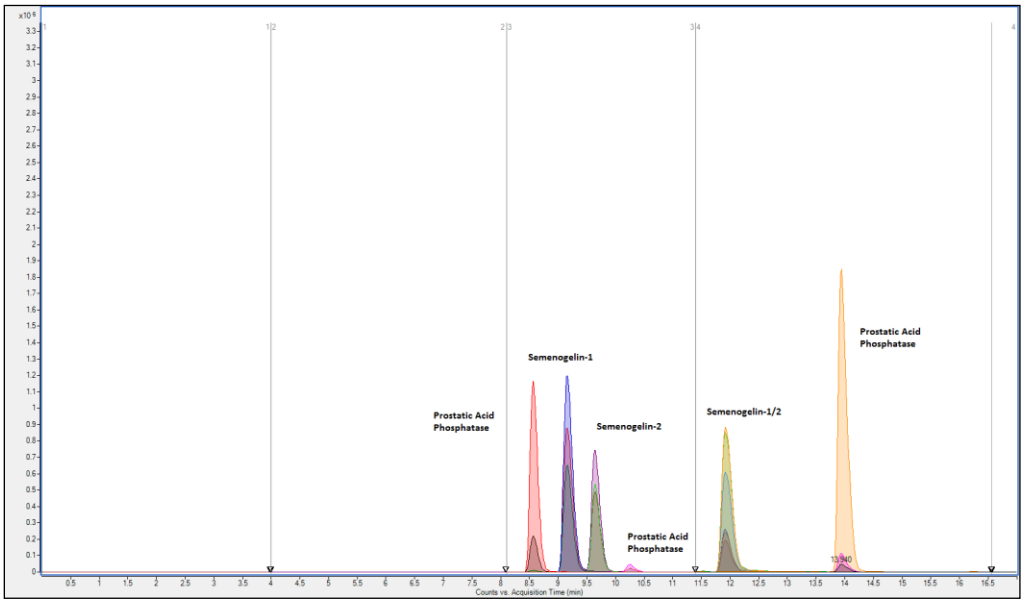


Figure 38 - QQQ-MRM multiplex results showing seminal fluid. The assay achieved clear detection of all target ions indicating the high-confidence confirmatory identification of Human seminal fluid. No biomarkers indicative of other body fluids were detected.

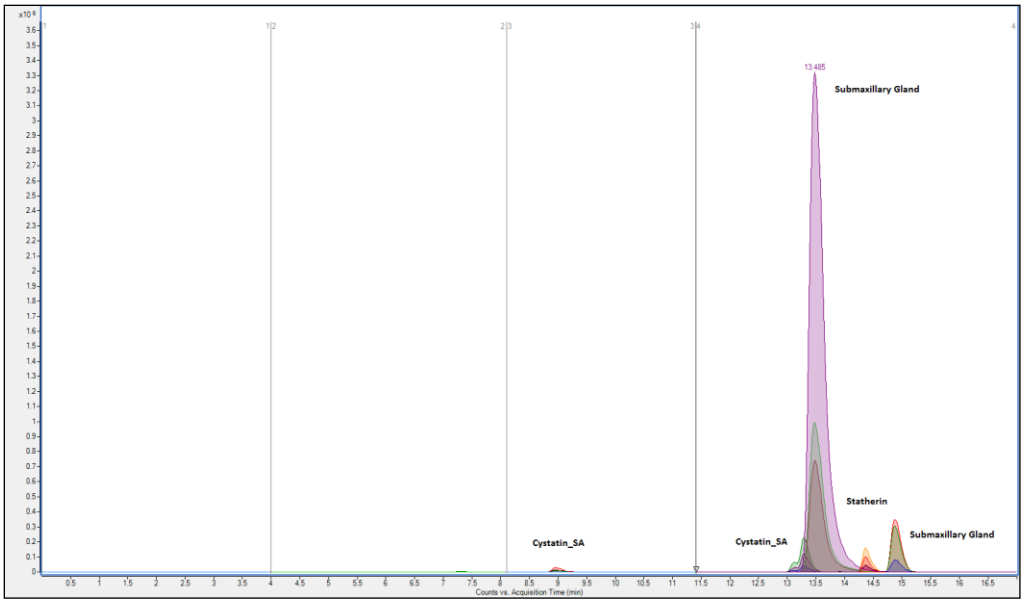


Figure 39 - QQQ-MRM multiplex results showing a saliva hit. The assay achieved clear detection of all target ions indicating the high-confidence confirmatory identification of Human saliva. No biomarkers indicative of other body fluids were detected.

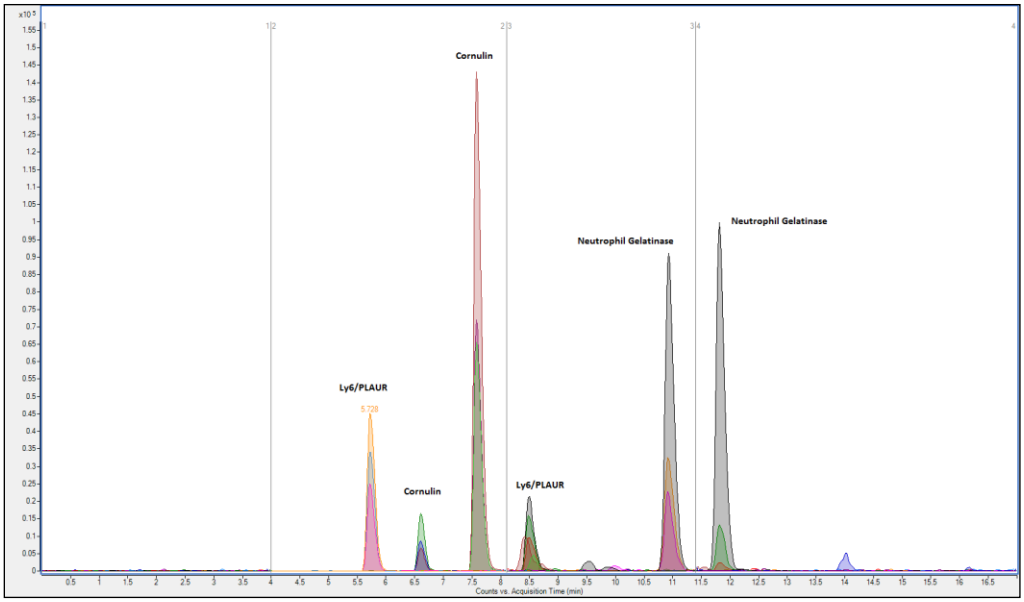


Figure 40 - QQQ-MRM multiplex results of a three component mixture. The assay achieved clear detection of all target ions indicating the high-confidence confirmatory identification of Human vaginal fluid. No biomarkers indicative of other body fluids were detected.

While the Q-TOF identification was confirmed through matching the entire product ion spectra to a protein database, the QQQ identification criteria is based on the response of only the selected transitions for the protein. Therefore, before analysis of the results, an interpretation method was designed using Agilent's Quantitative Analysis software. To do this, a mixture of all three samples was run and loaded into the analysis software. From the mixed reference sample, the retention time as well as relative response ratios were determined and used to analyze the rest of the samples in the batch.

The outcomes of these experiments were consistent with results from previous studies conducted using a Q-TOF mass spectrometer-based assay platform. However, because of the increased sensitivity associated with the QQQ platform, it became possible to detect protein biomarkers which might have fallen below the level of detection for the Q-TOF platform. As a result, the QQQ-MRM assay will provide a means of further confirming the specificity of the selected protein biomarkers.

Tables 14 and 15 provide summaries of the results from the ten single source assays using two different identification parameters. **Table 14** shows a low-stringency assay in which biomarker detection was based on the presence of at least one peptide per protein. In contrast to this, **Table 15** shows the assay results where detection was based on the confident identification of all peptides associated with that marker. Since each biomarker (aside from statherin) can be identified using two peptides and their associated fragments, the detection of both peptides represents a more confident identification of a targeted protein biomarker.

Table 14 - Frequency of protein identification in reference samples of saliva, seminal fluid and vaginal fluid (ten samples each). Protein identification in this table is based on at least one targeted peptide per biomarker.

Biomarker Identification - One or Two Peptides Detected										
		Saliva			Seminal Fluid			Vaginal fluid		
		Cystatin_SA	Statherin	Submax Gland	Semenogelin-1	Semenogelin-2	Prostatic Acid	Cornulin	Ly6	Neutrophil Gel
Samples	Saliva	100.00%	90.00%	100.00%	0.00%	10.00%	0.00%	30.00%	100.00%	0.00%
	Seminal Fluid	0.00%	0.00%	0.00%	100.00%	100.00%	100.00%	0.00%	0.00%	0.00%
	Vaginal fluid	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	100.00%	100.00%	100.00%

Table 15 - Frequency of protein identification in reference samples of saliva, seminal fluid and vaginal fluid (ten samples each). Protein identification in this table is based on the detection of two targeted peptides per biomarker.

Biomarker Identificaion - Two Peptides Detected										
		Saliva			Seminal Fluid			Vaginal fluid		
		Cystatin_SA	Statherin	Submax Gland	Semenogelin-1	Semenogelin-2	Prostatic Acid	Cornulin	Ly6	Neutrophil Gel
Samples	Saliva	100.00%	90.00%	100.00%	0.00%	0.00%	0.00%	0.00%	80.00%	0.00%
	Seminal Fluid	0.00%	0.00%	0.00%	100.00%	100.00%	100.00%	0.00%	0.00%	0.00%
	Vaginal fluid	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	100.00%	100.00%	100.00%

Detection of both peptides for a protein identification (**Table 15**) results in an identification pattern similar to what was standardized using the Q-TOF assay although the QQQ-MRM assay still achieved greater sensitivity. For example, Ly6 which had initially appeared promising as a vaginal fluid biomarker had been previously identified in a small number of casework-type samples which contained saliva. Its unambiguous identification using the more sensitive QQQ-MRM platform, therefore, was not unexpected.

Biomarker identification based on the detection of only a single peptide results in a more complicated interpretation. Cornulin, which is involved with mucosal/epithelial immune response, was also detected with one peptide in three of the ten saliva reverence samples. While this protein has not been detected in previous experiments, it has been previously characterized in the esophagus where it is thought to have an association with oral cancer [213, 214]. Its presence in a salivary sample, therefore, is not unreasonable.

Detection of a semenogelin-2 peptide in a single saliva sample was unanticipated in an assumed “saliva only" sample and has never been seen in previous assays involving either the Q-

TOF or QQQ. **Figure 41** shows the raw data comparing the semenogelin-2 peptide detection in saliva sample SA044 as well as a representative hit from seminal fluid sample SE022. By appearance alone it is clear that three fragment ion peaks are present in both samples, but the response (peak area) of the peptide in the seminal fluid sample is approximately 1,700 times larger. While the peak height and response are significantly higher in the seminal fluid sample, other qualitative information leads to the conclusion that the peak detected in this one saliva sample is real regardless of whether it is an endogenous salivary protein.

Using quantitative analysis software (**Table 16**), the signal-to-noise ratio of the peptide was calculated to be 18.81 in SA044 compared to 255.57 in SE022. Typically, a lower limit of detection requires a signal-to-noise ratio greater than 5 to be valid. Additionally, the ratio between the responses of all three product ions must be within an accepted error limit. In this case an uncertainly of 20% is acceptable compared to the reference samples. Lastly, the retention times must be the same. Using these qualifying parameters, there is every indication that this peptide is a genuine in both the saliva and seminal fluid samples. While this result was not expected, there is some potential corroborating support in the biomedical literature. Using an RT-PCR approach, Semenogelin transcripts were detected in the trachea as well as the salivary gland [215]. Whether this represents a peptide that can be reproducibly detected in a broad range of humans at very low levels versus an unrecognized artifact in a single study remains to be determined. In addition, it is possible that this result is merely a artifact from sample carryover. Moving forward, this must be assessed in a full developmental validation study.

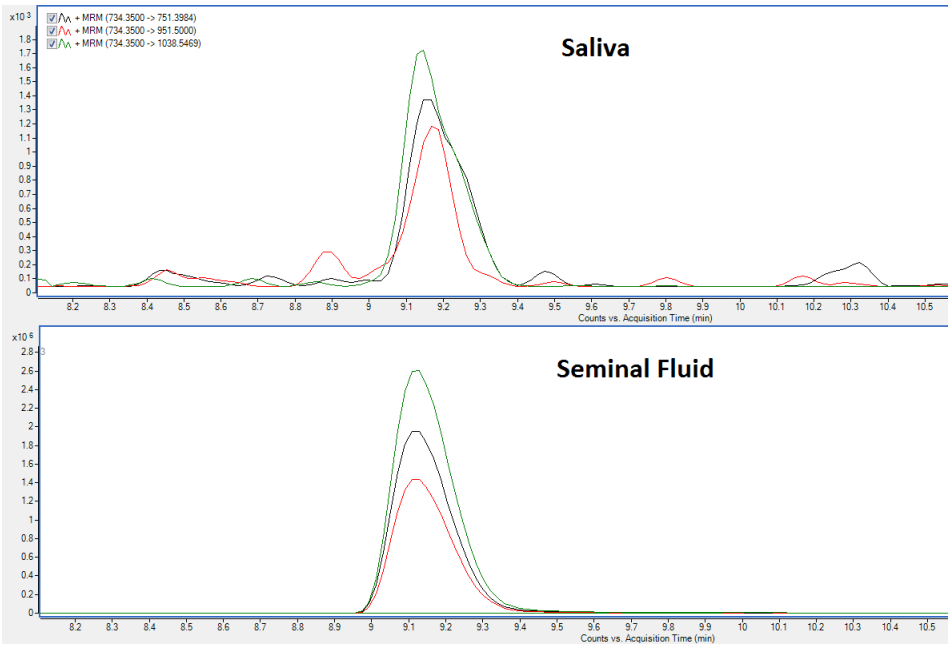


Figure 41 - Detection of Semenogelin-2 peptide DVSQSSISFQIEK in saliva (top) and Seminal fluid (bottom)

Table 16 - Quantitative software generated values from corresponding Semenogelin-2 peptide DVSQSSISFQIEK hit. This information is used to determine the accuracy of a peptide identification.

Sample					SMG_2 - DVSQSSISFQIEK Results					Qualifier (734.4 -> 951.5) Results			Qualifier (734.4 -> 751.4) Results		
Name	Data File	Type	Level	Acq. Date-Time	RT	Resp.	MI	Final Conc.	S/N	Ratio	MI	S/N	Ratio	MI	S/N
SA044_10ul	SA044_10ul.d	Sample		11/27/2012 4:58 PM	9.147	16554		0.0208	18.81	58.4		19.44	83.3		7.35
SE022_10ul	SE022_10ul.d	Sample		11/27/2012 6:18 PM	9.128	29382814		1.7016	255.57	54.8		278.81	74.5		392.57

In order for a protein biomarker to be confidently identified, therefore, it is the opinion of the author that that good practice dictates that both peptides must be detected in a sample. It is also suggested that more samples be run in the future and that external standards be used as a quantitative means of assessing analyte concentrations. Finally, it should be noted that these samples were run on an older microflow LC-MS/MS system and not on a nanoflow LC-MS/MS. If a nano LC-QQQ were to be employed the same samples, sufficient sensitivity may be achieved to confidently ID the missing peptides and thus confidently demonstrate the presence of a given biomarker.

4.2.3 Casework Type Samples

The multiplex body fluid identification assay has been successfully tested on a series of samples representing all targeted body fluids and a variety of casework-type biological stains. These included both single-source and mixed-source body fluid samples stained onto a variety of substrates including denim, leather and nylon. To assess the potential impact of environmental/chemical insult on assay performance, biological stains were subjected to commonly encountered agents including gasoline, soil, laundry detergent and personal lubricants. The complete list of casework-type samples that were tested can be found in **Table 17**.

Table 17 - Results of casework-type samples run on the QQQ-MRM assay. Positive protein identification requires both target peptides to be valid.

		Biomarkers								
		Saliva			Seminal Fluid			Vaginal fluid		
		Cystatin_SA	Statherin	Submaxi Gland	Semenogelin-1	Semenogelin-2	Prostatic Acid	Cornulin	Ly6	Neutrophil Gel
Substrates	CW01 - Saliva Swab									
	CW02 - Semen Swab									
	CW03 - Vaginal Swab									
	CW04 - Vaginal Fluid on Cotton									
	CW05 - Vaginal fluid on Nylon									
	CW06 - Seminal Fluid on Denim									
	CW07 - Seminal fluid on Leather									
	CW08 - Saliva on Cigraette									
	CW09 - Saliva on Bottle									
Contaminants	CW10 - Semain Fluid with Spermicide									
	CW11 - Saliva with Chewing Tobacco									
Mixtures	CW12 - Mixture Swab 1SA:3VS									
	CW13 - Mixture Swab 1SA:1VS									
	CW14 - Mixture Swab 3SA:1VS									
	CW15 - Mixture Swab 1SE:3SA									
	CW16 - Mixture Swab 1SE:1SA									
	CW17 - Mixture Swab 3SE:1SA									
	CW18 - Mixture Swab 1SE:3VS									
	CW19 - Mixture Swab 1SE:1VS									
	CW20 - Mixture Swab 3SE:1VS									
	CW21 - 3 Component Mixture Swab									
Dilution	CW22 - 3 Component 2 Fold Dilution									
	CW23 - 3 Component 4 Fold Dilution									
	CW24 - 3 Component 8 Fold Dilution									

The data obtained using casework-type samples were consistent with expected results as well as the results of previous Q-TOF studies. Irrespective of substrate and/or chemical contaminant, all of the targeted protein biomarkers appeared in the appropriate sample. Mixture studies were performed using cotton swabs onto which 2-component body fluid mixtures were prepared at three different ratios (1:3, 1:1, 3:1). At every assayed mixture ratio, all expected biomarkers were

detected. The only identification of a non-specific protein occurred with the 1:1 and 1:3 seminal fluid:saliva mixture swabs where the vaginal fluid marker Ly6 was detected. This has been previously observed on the Q-TOF as well with the QQQ reference samples. This marker is clearly non-specific for vaginal fluids and thus should be removed in future studies. Finally additional dilution studies to assess matrix effects on the lower limit of detection for each body fluid are clearly indicated as a focus for additional study.

4.2.4 Combined DNA and Serological Analysis

This research has shown that stain identification from a variety of casework-type samples is precise and reliable. However, in order for a mass spectrometry assay for serological analysis to be implemented into routine forensic casework it must be possible to recover a DNA profile from the same sample. To test this, a modified protocol similar to a differential extraction was developed (**Figure 42**). Similar to previous casework, samples were added to swabs before re-hydration in PBS, spin baskets were then used to remove liquid in the swabs followed by a high speed centrifugation step to pellet cellular and insoluble material. The protein rich supernatant fraction was processed for QQQ analysis while the cellular (DNA) fraction was extracted using the EZ1 BioRobot, quantified using Quantifiler® Duo, followed by STR profile generation with the Identifiler® Kit.

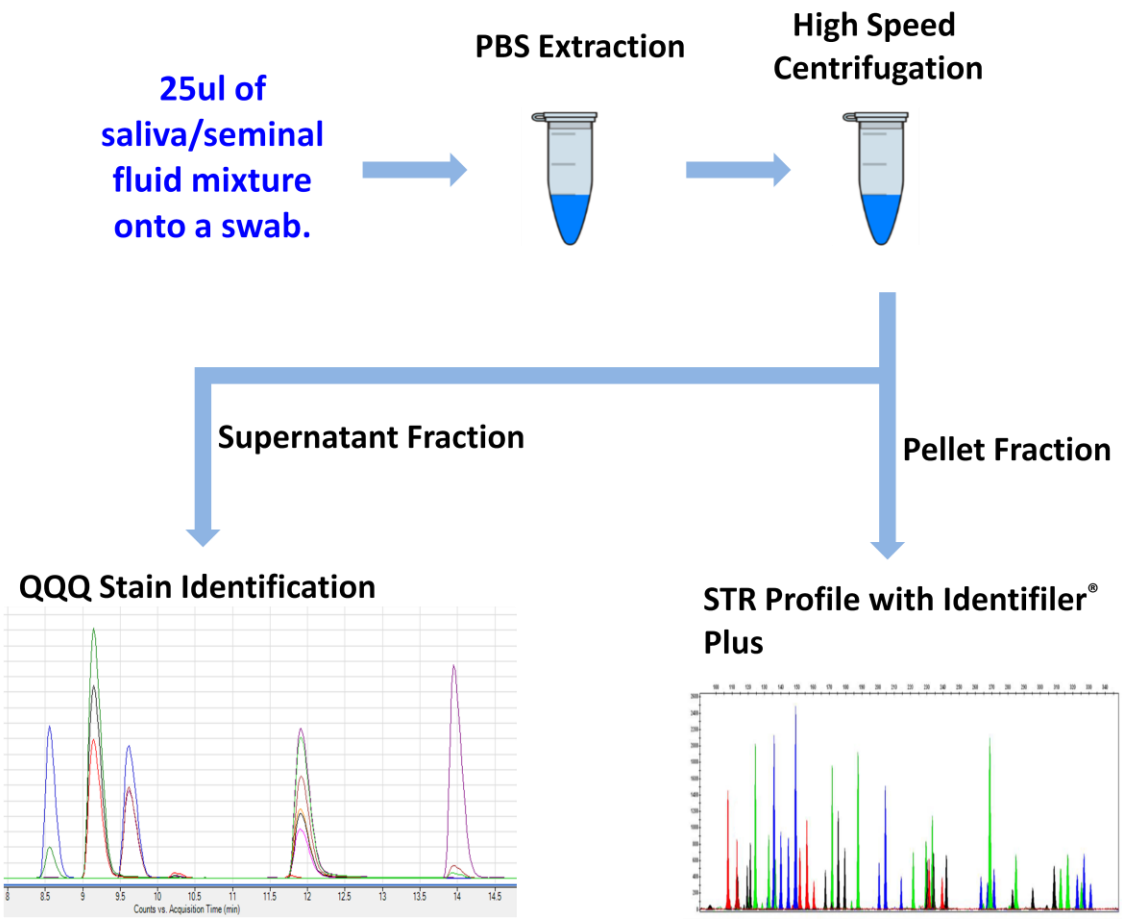


Figure 42 – Generation of a full STR profile and stain identification for a mixed stain containing seminal fluid and saliva mixture. Swab was rehydrated in phosphate buffer saline and placed in a filter basket to remove retained fluid. Sample was then spun at high speed to pellet the cellular material for STR analysis The DNA-depleted supernatant was processed for proteomic analysis. DNA was quantified using ABI Quantifiler® duo and a full DNA profile was generated using ABI Identifiler® Plus kit. Protein concentration was determined using Pierce micro BCA assay kit, and confirmatory stain identification was achieved using the Q-TOF multiplex assay.

In total, six swabs were processed using the modified protocol – two reference samples from semen and vaginal fluid as well as four male/female mixed samples. For the mixed samples a semen/saliva swab, semen/vaginal fluid swab, saliva/vaginal fluid swab, as well as a semen/peripheral blood swab were prepared. QQQ analysis of these swabs, as expected, successfully identified all anticipated proteins from single and mixed source body fluids (**Table 18**).

Table 18 – Results of DNA/Protein samples processed for joint genetic and serological analysis. For each swab the fluid was confidently identified.

	Biomarkers								
	Saliva			Seminal Fluid			Vaginal fluid		
	Cystatin_SA	Statherin	Submaxi Gland	Semenogelin-1	Semenogelin-2	Prostatic Acid	Cornulin	Ly6	Neutrophil Gel
Semen (M) Saliva (F)									
Semen (M) Vaginal (F)									
Saliva (M) Vaginal (F)									
Semen (M) Peripheral Blood (F)									
Semen Reference									
Vaginal Reference									

Genetic analyses (**Table 19**) were able to successfully produce single and mixed source profiles. In all reference and mixed samples the major contributor resulted in a complete STR profile. The saliva/vaginal fluid sample resulted in a full profile for the minor contributor. In the semen/saliva as well as the semen/vaginal fluid samples 50% of the distinct alleles were present not counting shared alleles. In the semen/peripheral blood sample 79% of distinct alleles were present not counting shared alleles. The end result of this experiment provides ample support for the proposition that a mass spectrometry-based serological assay is compatible with existing STR profiling methodologies.

Table 19 – Summary Table of STR DNA Results using the Identifiler kit. "NR"= No results, "NT"= Not tested, "(M)"=Major contributor, "(m)"= Minor contributor.

EXHIBIT NUMBER	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	TH01	D13S317	D16S539	D2S1338	D19S433	vWA	TPOX	D18S51	AMEL	D5S818	FGA
Male Donor (Semen)	10,13	31.2, 32.2	10,12	10,11	15,17	6,7	10,12	11,12	17,24	13,14	17,18	8,11	11,15	X,Y	11,12	20,24
Female Donor (Vaginal)	14,15	29,30	10,11	10,11	15	6,9.3	11,12	9,12	19,21	13,15	17,18	8	16	X	11	25,27
Semen/Saliva (M)	10,13	31.2, 32.2	10,12	10,11	15,17	6,7	10,12	11,12	17,24	13,14	17,18	8,11	11,15	X,Y	11,12	20,24
Semen/Saliva (m)	14,15	30	11			9.3	11		19							
Semen/Vaginal (M)	10,13	31.2, 32.2	10,12	10,11	15,17	6,7	10,12	11,12	17,24	13,14	17,18	8,11	11,15	X,Y	11,12	20,24
Semen/Vaginal (m)	14,15		11				11		21				16			25
Saliva/Vaginal (M)	10,13	31.2, 32.2	10,12	10,11	15,17	6,7	10,12	11,12	17,24	13,14	17,18	8,11	11,15	X,Y	11,12	20,24
Saliva/Vaginal (m)	14,15	29,30	11			9.3	11	9	19,21	15			16			25,27
Semen/PBlood (M)	14,15	29,30	10,11	10,11	15	6,9.3	11,12	9,12	19,21	13,15	17,18	8	16	X	11	25,27
Semen/PBlood (m)	10,13	31.2, 32.2	12		17		10	11	17	14		11	11,15	Y	12	20,24

4.3 Conclusion

The central objective of this phase of the dissertation research was to improve upon the previous Q-TOF assay by increasing the speed of analysis and to test the feasibility of a mass-spectrometry based assay for stain identification for routine casework testing. A prototype QQQ-MRM "sexual assault" assay was designed to identify semen, saliva, and vaginal fluid.

Starting with the data from the Q-TOF studies, the four most promising biomarkers for the three fluids were selected for use in the prototype QQQ-MRM assay. Because these proteins (and subsequent peptides) had already been evaluated previously, the only task that remained was to identify optimal product ions for inclusion in the QQQ-MRM assay. Once selected, the transitions were integrated into a QQQ-MRM triplex biological stain assay. The assay accurately identified seminal fluid, saliva, and vaginal secretions on the basis of 12 high-specificity protein biomarkers with an instrument run time of 18 minutes. Compared to the previous Q-TOF assay, this achieves more than a 75% reduction in total analysis time.

The QQQ-MRM three-stain sexual assault assay was tested using single-source samples (7 samples each) as well as a series of casework-type samples. Data from these experiments confirmed the accuracy and consistency of the biomarkers on this novel, high speed platform. In addition to the single source laboratory samples a novel protocol was developed to co-extract the DNA and protein fractions from a single swab. This new protocol was able to identify the specific body fluid stains present as well as to generate accurate STR profiles. In summary, a high speed QQQ assay for stain identification can act as a launching point for the subsequent full developmental validation of a six-body fluid multiplex for forensic casework on a QQQ-MRM platform.

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Appendices

Appendix A: PF2D 2D Protein Identification Results

Seminal Fluid PF2D 2D Protein Identification Results

Distinct Peptides	MS/MS Score	% AA Coverage	Spectral Intensity	SwissProt Accession	Protein Name
18	303.34	37.2	2.83E+10	P04279	Semenogelin-1 precursor
15	230.45	27.3	8.80E+09	Q02383	Semenogelin-2 precursor
6	109.76	10.5	2.41E+08	P04264	Keratin, type II cytoskeletal 1
4	57.15	6.2	1.18E+08	P35908	Keratin, type II cytoskeletal 2 epidermal
6	95.35	10.2	1.60E+08	P13645	Keratin, type I cytoskeletal 10
1	14.42	1.9	1.14E+07	P02533	Keratin, type I cytoskeletal 14
1	14.42	1.9	1.14E+07	P08779	Keratin, type I cytoskeletal 16
6	90.52	9.2	7.12E+07	P02788	Lactotransferrin
6	88.65	12.9	7.10E+07	P10909	Clusterin
3	46.79	7.2	4.23E+07	P15309	Prostatic acid phosphatase
3	45.76	5.2	3.86E+07	P02768	Serum albumin
3	45.36	49.1	3.96E+07	P06702	Protein S100-A9
2	34.78	10.7	2.08E+07	P07288	Prostate-specific antigen

Urine PF2D 2D Protein Identification Results

Distinct Peptides	MS/MS Score	% AA Coverage	Spectral Intensity	SwissProt Accession #	Protein Name
32	561.94	63.7	2.79E+09	P02768	Serum albumin
10	155.15	39.6	1.21E+08	P25311	Zinc-alpha-2-glycoprotein
8	153.58	2.4	6.00E+08	P98160	Basement membrane-specific heparan sulfate proteoglycan protein
6	111.72	16.7	1.23E+09	P02760	Protein AMBP
6	104.17	9.8	1.83E+08	Q14624	Inter-alpha-trypsin inhibitor heavy chain H4
5	77.59	8.3	1.53E+08	P01042	Kininogen-1
4	59.89	10.6	1.46E+08	P07602	Proactivator polypeptide
4	58.86	35.5	4.55E+07	P05451	Lithostathine-1-alpha
2	32.74	13.8	3.40E+07	P48304	Lithostathine-1-beta
3	57.26	11.7	1.23E+08	P10451	Osteopontin
4	54.88	25.1	1.21E+10	P00761	Trypsin
3	53.21	5.3	5.09E+07	P55290	Cadherin-13
3	49.91	6.2	2.55E+07	P13645	Keratin, type I cytoskeletal 10
3	48.83	5.4	4.96E+07	P04264	Keratin, type II cytoskeletal 1
1	14.86	2	3.31E+07	P19013	Keratin, type II cytoskeletal 4
1	14.86	2.2	3.31E+07	P05787	Keratin, type II cytoskeletal 8
1	14.86	2.3	3.31E+07	P08729	Keratin, type II cytoskeletal 7
1	14.86	2.4	3.31E+07	Q6KB66	Keratin, type II cytoskeletal 80

1	14.86	2.5	3.31E+07	P14136	Glial fibrillary acidic protein
3	48.68	14.1	7.39E+07	P06870	Kallikrein-1
4	47.36	66	3.05E+07	P01834	Ig kappa chain C region
2	45.33	25.8	3.31E+07	Q14508	WAP four-disulfide core domain protein 2
3	44.7	10.9	5.89E+07	P28799	Granulins
2	36.06	5.1	2.14E+07	P01833	Polymeric immunoglobulin receptor
2	34.04	3.3	7.05E+07	P01133	Pro-epidermal growth factor
2	31.1	17.3	1.28E+07	P41222	Prostaglandin-H2 D-isomerase
2	29.7	16.4	4.09E+07	P02753	Retinol-binding protein 4
2	28.93	23.8	3.39E+06	P02766	Transthyretin
2	28.52	18.7	9.68E+07	P13987	CD59 glycoprotein
2	25.8	7	2.95E+07	P01876	Ig alpha-1 chain C region
1	13.92	2.9	1.91E+07	P01877	Ig alpha-2 chain C region
1	22.74	1	4.22E+07	Q14767	Latent-transforming growth factor beta-binding protein 2
1	21.07	4.7	1.81E+07	P04156	Major prion protein
1	21.01	2.3	2.26E+07	P05060	Secretogranin-1 O
1	19.38	5.9	1.73E+06	P05452	Tetranectin
1	17.8	2.9	1.06E+07	Q9ULV1	Frizzled-4
1	15.91	1.6	9.02E+06	O75339	Cartilage intermediate layer protein 1
1	15.23	6.2	4.84E+06	Q7Z3B1	Neuronal growth regulator 1
1	14.51	10.8	1.71E+07	P31151	Protein S100-A7
1	14.51	10.8	1.71E+07	Q86SG5	Protein S100-A7A
1	14.4	9.5	8.04E+06	P09564	T-cell antigen CD7

1	14.34	5.4	4.05E+07	Q9UNN8	Endothelial protein C receptor
1	14.03	5.1	1.94E+07	Q9BT78	COP9 signalosome complex subunit
1	13.31	2.1	7.41E+06	Q14574	Desmocollin-3
1	13.26	3.3	1.34E+07	Q6NXE6	Armadillo repeat-containing protein 6

Saliva PF2D 2D Protein Identification Results

Distinct Peptides	MS/MS Score	% AA Coverage	Spectral Intensity	SwissProt Accession	Protein Name
4	62.41	48.3	6.71E+07	P02808	Statherin precursor - Homo sapiens (Human)
2	35.56	3.7	8.29E+06	P04264	Keratin, type II cytoskeletal 1
1	17.04	1.8	8.25E+06	P35908	Keratin, type II cytoskeletal 2 epiderma
2	34.81	5.2	6.57E+06	P13645	Keratin, type I cytoskeletal 10
1	21.17	8.1	1.40E+06	P61626	Lysozyme C precursor
1	17.68	10.2	3.13E+05	P02810	Salivary acidic proline-rich phosphoprotein 1/2
1	17.16	3.5	4.80E+07	P14314	Glucosidase 2 subunit beta precursor
1	16.53	6	9.94E+06	Q96DU7	Inositol-trisphosphate 3-kinase C
1	15.98	5.8	4.24E+07	Q9UM11	Fizzy-related protein homolog -
1	15.44	17.5	1.98E+05	P15515	Histatin-1 OS=Homo sapiens
1	14.79	4.2	2.94E+05	P24158	Myeloblastin OS=Homo sapiens
1	14.4	9.7	5.68E+06	Q9UK76	Hematological and neurological expressed 1 protein
1	13.77	0.2	2.72E+06	P49792	E3 SUMO-protein ligase RanBP2
1	13.77	0.5	2.72E+06	Q53T03	RANBP2-like and GRIP domain-containing protein 6
1	13.77	0.5	2.72E+06	Q99666	RANBP2-like and GRIP domain-containing protein 5

1	13.77	0.5	2.72E+06	A6NKT7	RANBP2-like and GRIP domain-containing protein 3
1	13.77	0.5	2.72E+06	Q7Z3J3	RANBP2-like and GRIP domain-containing protein 4
1	13.77	0.5	2.72E+06	Q68DN6	RANBP2-like and GRIP domain-containing protein 1
1	13.77	0.9	2.72E+06	Q9H0B2	RANBP2-like and GRIP domain-containing protein 7

Vaginal Fluid PF2D 2D Protein Identification Results

Distinct Peptides	MS/MS Score	% AA Coverage	Spectral Intensity	SwissProt Accession	Protein Name
18	301.33	37.1	1.87E+09	P02768	Serum albumin precursor
15	250.88	38.7	5.00E+09	P30740	Leukocyte elastase inhibitor
1	17.92	2.4	3.82E+07	O75830	Serpin I2
1	17.92	2.6	3.82E+07	P50452	Serpin B8
1	17.92	2.6	3.82E+07	P50453	Serpin B9
13	205.85	32.1	1.05E+09	P07476	Involucrin
10	172.07	29.4	6.32E+08	P07355	Annexin A2
8	130.66	24.1	4.01E+08	A6NMY6	Putative annexin A2-like protein
11	167.88	20.7	1.20E+09	P13646	Keratin, type I cytoskeletal 13
5	83.51	9.2	4.66E+08	P19012	Keratin, type I cytoskeletal 15
3	50.89	4.8	1.55E+08	P02533	Keratin, type I cytoskeletal 14
3	50.89	4.8	1.55E+08	P08779	Keratin, type I cytoskeletal
2	30.68	2.5	5.90E+07	P35900	Keratin, type I cytoskeletal 20
2	30.68	2.5	5.90E+07	Q04695	Keratin, type I cytoskeletal 17
2	25.96	3.8	8.75E+07	Q99456	Keratin, type I cytoskeletal 12
2	25.77	2	1.56E+08	P13645	Keratin, type I cytoskeletal 10
1	15.23	2.2	8.94E+06	P08727	Keratin, type I cytoskeletal 19
1	15.23	3	8.94E+06	Q8N1A0	Keratin-like protein KRT222
1	14.96	2	1.14E+08	Q2M2I5	Keratin, type I cytoskeletal 24
10	153.97	16.9	4.57E+08	P05164	Myeloperoxidase
9	140.8	31.7	1.20E+09	P29508	Serpin B3
1	13.29	3	2.30E+07	Q9UIV8	Serpin B13

8	116.33	27.1	9.32E+08	P48594	Serpin B4
8	131.35	31.2	3.82E+08	P04083	Annexin A1
7	114.87	12.8	2.84E+08	P04264	Keratin, type II cytoskeletal 1
1	13.22	2	2.26E+06	P05787	Keratin, type II cytoskeletal 8
6	104.98	13.1	1.06E+09	P02538	Keratin, type II cytoskeletal 6A -
6	101.44	13.1	5.51E+08	P04259	Keratin, type II cytoskeletal 6B -
5	85.83	10.4	5.40E+08	P48668	Keratin, type II cytoskeletal 6E
4	70.18	7.9	4.14E+08	P13647	Keratin, type II cytoskeletal 5
7	111.65	53.5	2.69E+10	P06702	Protein S100-A9
5	89.22	11.4	1.08E+09	P19013	Keratin, type II cytoskeletal 4
6	81.1	5.4	1.47E+08	A8K2U0	Alpha-2-macroglobulin-like protein 1
5	61.68	23.3	1.23E+08	P01857	Ig gamma-1 chain C region
2	22.99	6.3	3.99E+07	P01860	Ig gamma-3 chain C region
2	22.42	9.7	3.00E+07	P01861	Ig gamma-4 chain C region
3	52.48	32.6	1.47E+08	P02766	Transthyretin
3	50.41	5.7	1.21E+08	P02787	Serotransferrin
3	49.55	28.4	6.16E+08	Q9UBC9	Small proline-rich protein 3
3	48.85	4.4	7.43E+07	P11021	78 kDa glucose-regulated protein precursor
3	43.94	31.1	1.72E+08	P05109	Protein
3	39.41	7	9.41E+07	P13796	Plastin-2
1	14.47	1.4	3.31E+07	P13797	Plastin-3
1	14.47	1.4	3.31E+07	Q14651	Plastin-1
2	36.42	16.2	3.50E+07	P61626	Lysozyme C

2	36.19	11.1	8.94E+07	Q9UBG3	Cornulin
2	31	24	4.12E+07	Q9HD89	Resistin
2	28.87	6.6	1.26E+08	P07951	Tropomyosin beta chain
2	28.87	7.6	1.26E+08	P67936	Tropomyosin alpha-4 chain
1	14.82	3.5	7.18E+07	P06753	Tropomyosin alpha-3 chain
1	14.82	3.5	7.18E+07	P09493	Tropomyosin alpha-1 chain
2	28.49	2.9	5.99E+07	P02788	Lactotransferrin OS
2	27.99	17.7	5.90E+07	Q14508	WAP four-disulfide core domain protein
2	27.48	34.8	9.57E+07	P35321	Cornifin-A
2	27.3	34.8	7.56E+07	P22528	Cornifin-B
2	25.23	1.4	2.83E+07	O60437	Periplakin OS
2	25.06	7.5	9.35E+07	P08670	Vimentin
1	18.22	3.5	6.32E+07	Q96LW4	Coiled-coil domain-containing
1	18.12	1.7	1.14E+07	O00391	Sulfhydryl oxidase 1
1	17.15	1.7	1.82E+07	P34931	Heat shock 70 kDa protein 1L
1	17.15	1.7	1.82E+07	P08107	Heat shock 70 kDa protein 1
1	17.03	12.3	8.59E+07	P10599	Thioredoxin OS=Homo sapiens
1	16.53	2	4.11E+07	Q2M2Z5	Uncharacterized protein C20orf19
1	16.21	18	3.95E+07	P35326	Small proline-rich protein 2A
1	16.2	9	2.77E+07	P00441	Superoxide dismutase [Cu-Zn]
1	16.19	4.5	2.41E+07	O15519	CASP8 and FADD-like apoptosis regulator
1	15.69	1.2	9.89E+06	Q9NQ38	Serine protease inhibitor Kazal-type 5

1	15.62	8.5	4.81E+06	P04632	Calpain small subunit 1
1	14.98	4.1	1.82E+07	O95948	One cut domain family member 2
1	14.57	3.6	6.85E+05	P35527	Keratin, type I cytoskeletal 9
1	13.56	2.2	3.06E+06	Q8IV53	DENN domain-containing protein
1	13.54	1.2	1.24E+07	P06731	Carcinoembryonic antigen-related cell adhesion molecule 5
1	13.54	1.7	1.24E+07	P13688	Carcinoembryonic antigen-related cell adhesion molecule 1
1	13.54	2.5	1.24E+07	P31997	Carcinoembryonic antigen-related cell adhesion molecule 8
1	13.33	2.2	5.19E+07	P42261	Glutamate receptor 1
1	13.33	2.2	5.19E+07	P42263	Glutamate receptor 3
1	13.33	2.2	5.19E+07	P48058	Glutamate receptor 4
1	13.33	2.2	5.19E+07	P42262	Glutamate receptor 2

Menstrual Blood PF2D 2D Protein Identification Results

Distinct Peptides	MS/MS Score	% AA Coverage	Spectral Intensity	SwissProt Accession	Protein Name
38	647.31	35.2	5.91E+09	P01024	Complement C3
33	523.43	35.6	4.88E+09	P01023	Alpha-2-macroglobulin
4	59.92	3.5	6.40E+08	P20742	Pregnancy zone protein
22	373.98	41.2	4.60E+09	P02787	Serotransferrin
16	271.67	54.6	1.16E+10	P02647	Apolipoprotein A-I
16	263.75	37.6	2.14E+09	P02675	Fibrinogen beta chain
17	261.74	29.4	1.75E+09	P00450	Ceruloplasmin
15	240.5	46.8	4.26E+09	P01009	Alpha-1-antitrypsin
15	234.68	15.1	6.75E+08	P0C0L5	Complement C4-B
15	234.68	15.1	6.75E+08	P0C0L4	Complement C4-A
11	178.42	37.8	3.40E+09	P02790	Hemopexin
1	20.35	3.4	1.70E+07	P20058	Hemopexin
10	167.17	77.5	1.68E+10	P68871	Hemoglobin subunit beta
8	133.25	62.5	8.07E+09	P02042	Hemoglobin subunit delta
9	144.91	31.2	1.31E+09	P02774	Vitamin D-binding protein
9	139.82	28.9	2.16E+09	P02679	Fibrinogen gamma chain
8	137.61	30.7	8.06E+08	P00738	Haptoglobin
4	73.02	14.6	4.86E+08	P00739	Haptoglobin-related protein
9	130.79	11.3	3.68E+08	P08603	Complement factor H
7	121.94	64	6.42E+09	P69905	Hemoglobin subunit alpha
8	121.8	3.1	3.00E+08	P04114	Apolipoprotein B-100
7	120.93	24.2	3.08E+07	P30740	Leukocyte elastase inhibitor

6	101.04	32.4	4.84E+08	P02749	Beta-2-glycoprotein 1
5	96.27	17.1	4.60E+08	P01019	Angiotensinogen
6	88.06	13.2	1.74E+08	P00751	Complement factor B
5	80.28	17.1	5.16E+08	P04217	Alpha-1B-glycoprotein
5	80.08	7.8	3.46E+08	P02671	Fibrinogen alpha chain
5	77.37	27.7	2.89E+07	P32119	Peroxiredoxin-2
1	15.62	5.5	1.07E+07	Q06830	Peroxiredoxin-1
4	74.91	21	3.39E+07	P00915	Carbonic anhydrase 1 OS=Homo sapiens GN=CA1 PE=1 SV=2
4	67.01	21.5	1.01E+09	P02765	Alpha-2-HS-glycoprotein
4	64.11	48	1.16E+09	P02652	Apolipoprotein A-II
4	63.4	10.5	3.09E+08	P01042	Kininogen-1
4	60.11	6.5	1.88E+08	P19823	Inter-alpha-trypsin inhibitor heavy chain H2
4	55.39	13.4	9.81E+07	P01871	Ig mu chain C region
3	38.64	11.5	8.85E+07	P04220	Ig mu heavy chain disease protein
3	50.98	18.9	7.13E+08	P02763	Alpha-1-acid glycoprotein 1
2	32.08	12.9	3.84E+08	P19652	Alpha-1-acid glycoprotein 2
4	49.62	66	2.05E+08	P01834	Ig kappa chain C region
3	47.89	7.2	1.35E+08	P00734	Prothrombin OS=Homo sapiens
3	47.6	9.6	1.06E+08	P04004	Vitronectin OS=Homo sapiens
3	47.03	5.1	2.99E+08	P19827	Inter-alpha-trypsin inhibitor heavy chain H1
3	41.72	11.5	1.17E+08	P10909	Clusterin
2	39.68	4.8	8.11E+07	P01833	Polymeric immunoglobulin receptor

2	37.96	23.8	5.87E+07	P02766	Transthyretin
2	37.47	5	6.49E+06	Q13228	Selenium-binding protein 1
2	37.33	16.8	4.56E+07	P00441	Superoxide dismutase [Cu-Zn]
2	35.13	7.8	2.03E+08	P01011	Alpha-1-antichymotrypsin
2	30.6	27.2	4.04E+08	P02656	Apolipoprotein C-III
2	29.64	7.9	5.77E+07	P01008	Antithrombin-III
2	29.2	20.5	4.50E+06	Q9NZT1	Calmodulin-like protein 5
2	28.98	17.7	7.92E+07	Q14508	WAP four-disulfide core domain protein 2
2	28.39	6.5	1.05E+08	P02760	Protein AMBP
2	24.16	7.7	1.19E+07	P00761	Trypsin
1	21.5	1.8	1.37E+06	P35908	Keratin, type II cytoskeletal 2 epidermal
1	21.5	2	1.37E+06	P13647	Keratin, type II cytoskeletal 5
1	21.5	2.1	1.37E+06	P02538	Keratin, type II cytoskeletal 6A
1	21.5	2.1	1.37E+06	P04259	Keratin, type II cytoskeletal 6B
1	21.5	2.1	1.37E+06	P48668	Keratin, type II cytoskeletal 6C
1	21.5	2.1	1.37E+06	O95678	Keratin, type II cytoskeletal 75
1	21.5	2.2	1.37E+06	Q5XKE5	Keratin, type II cytoskeletal 79
1	20.65	2.3	6.86E+07	Q14624	Inter-alpha-trypsin inhibitor heavy chain
1	20.02	4.5	3.62E+06	P13645	Keratin, type I cytoskeletal 10
1	17.58	1.8	1.47E+06	P04264	Keratin, type II cytoskeletal 1
1	17.19	2.7	1.44E+07	Q8BMT4	Leucine-rich repeat-containing protein 33
1	16.65	9	6.14E+07	P03973	Antileukoproteinase

1	16.06	11.8	1.81E+06	Q01469	Fatty acid-binding protein, epidermal
1	15.88	1	9.21E+07	O00160	Myosin-If
1	15.75	3.2	1.56E+07	P06727	Apolipoprotein A-IV
1	15.69	8.5	4.06E+07	P80188	Neutrophil gelatinase-associated
1	15.44	2.7	1.20E+06	P07476	Involucrin
1	15.37	4.1	2.53E+06	Q61468	Mesothelin
1	15.21	6.8	3.89E+07	Q6PII5	Hydroxyacylglutathione hydrolase-like protein
1	15	15.7	2.98E+07	P22528	Cornifin-B
1	15	15.7	2.98E+07	P35321	Cornifin-A
1	14.83	4.2	3.25E+07	P68133	Actin, alpha skeletal muscle
1	14.83	4.2	3.25E+07	P68032	Actin, alpha cardiac muscle 1
1	14.83	4.2	3.25E+07	P62736	Actin, aortic smooth muscle
1	14.83	4.2	3.25E+07	P63267	Actin, gamma-enteric smooth muscle
1	14.56	5.3	3.65E+07	P01860	Ig gamma-3 chain C region
1	14.47	18.4	1.49E+07	P61769	Beta-2-microglobulin
1	14.38	1.7	2.12E+07	P04196	Histidine-rich glycoprotein
1	14.33	1.1	9.85E+07	Q9P1Z9	Uncharacterized protein KIAA1529
1	14.26	0.6	1.02E+06	Q9BTC0	Death-inducer obliterator 1
1	14.18	3.2	3.02E+06	Q8R4E9	DNA replication factor Cdt1
1	14.14	3.4	5.55E+06	Q9NZN3	EH domain-containing protein 3
1	14.09	0.8	4.00E+07	Q9P212	1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase
1	13.94	5.8	2.74E+06	P28289	Tropomodulin-1

1	13.64	7.1	9.06E+07	P25311	Zinc-alpha-2-glycoprotein
1	13.56	0.6	1.80E+06	Q13535	Serine/threonine-protein kinase ATR
1	13.4	4.3	2.34E+06	Q9H0R3	Transmembrane protein 222
1	13.36	3.9	5.82E+06	Q8N807	Protein disulfide-isomerase-like protein of the testis
1	13.26	1.4	6.21E+06	P01031	Complement C5
1	13.17	3.5	4.82E+06	Q86UB2	Basic immunoglobulin-like variable motif-containing protein
1	13.13	1	1.38E+08	Q702N8	Xin actin-binding repeat-containing protein 1
1	13.11	3.3	1.07E+07	O14492	SH2B adapter protein 2

Peripheral Blood PF2D 2D Protein Identification Results

Distinct Peptides	MS/MS Score	% AA Coverage	Spectral Intensity	SwissProt Accession	Protein Name
32	584.65	61.5	2.51E+10	P02768	Serum albumin
32	557.01	40.4	4.46E+09	P08603	Complement factor H
3	50.51	13.3	1.48E+08	Q03591	Complement factor H-related protein 1
19	344.32	55	3.66E+09	P02774	Vitamin D-binding protein
13	244.61	33.1	9.66E+08	P04196	Histidine-rich glycoprotein
15	231.06	42.8	2.37E+09	P02790	Hemopexin
12	214.92	31.6	1.32E+09	P00751	Complement factor B
12	205.55	5	4.47E+08	P04114	Apolipoprotein B-100
12	202.1	24.9	4.57E+08	P00747	Plasminogen
12	200.29	12.3	7.95E+08	P01024	Complement C3
8	143.68	69.3	2.35E+09	P02766	Transthyretin
7	111.8	10.7	2.07E+08	P00450	Ceruloplasmin
7	106.06	26.7	7.40E+08	P01008	Antithrombin-III
6	87.77	12	2.50E+08	Q14624	Inter-alpha-trypsin inhibitor heavy chain H4
4	64.15	32.3	4.36E+08	P02753	Retinol-binding protein 4
3	54.93	10.1	7.16E+07	P43652	Afamin OS=Homo sapiens
2	39.75	2.9	1.11E+08	P0C0L5	Complement C4-B
2	39.75	2.9	1.11E+08	P0C0L4	Complement C4-A
1	18.85	3.5	1.82E+07	P01019	Angiotensinogen
1	15.85	1.4	3.84E+07	P01023	Alpha-2-macroglobulin

1	15.64	7.2	8.72E+06	P02749	Beta-2-glycoprotein 1
1	15.64	3.4	2.80E+07	P08697	Alpha-2-antiplasmin
1	14.62	10.3	3.76E+07	P05452	Tetranectin
1	14.18	4.2	1.45E+08	Q3MIP1	Inositol 1,4,5-triphosphate receptor-interacting protein-like 2
1	14.01	3.9	1.88E+08	P40818	Ubiquitin carboxyl-terminal hydrolase 8
1	13.86	0.9	3.15E+07	P35498	Sodium channel protein type 1 subunit alpha
1	13.06	9.7	6.99E+06	O14626	Probable G-protein coupled receptor 171

Appendix B: PF2D 1D Protein Identification Results

Seminal Fluid PF2D 1D Protein Identification Results

Distinct Peptides	MS/MS Score	% AA Coverage	Spectral Intensity	SwissProt Accession	Protein Name
16	281.63	35.6	6.65E+06	P02788	Lactotransferrin
11	196.27	28.5	1.26E+08	P04279	Semenogelin-1
10	190.82	22.6	1.35E+08	Q02383	Semenogelin-2
10	161.67	31.3	8.09E+06	P15309	Prostatic acid phosphatase
8	138.07	6.9	1.11E+06	P02751	Fibronectin
8	132.47	20.6	5.82E+06	P02768	Serum albumin
7	111.84	51.3	4.58E+06	P12273	Prolactin-inducible protein
6	108.85	16.4	3.98E+06	P10909	Clusterin
4	87.07	36.7	6.20E+06	P07288	Prostate-specific antigen
2	38.88	6.7	8.03E+04	P49221	Protein-glutamine gamma-glutamyltransferase 4
2	35.24	25.8	1.25E+06	P61916	Epididymal secretory protein E1
1	22.49	4.6	9.85E+05	P16870	Carboxypeptidase E
1	21.67	5.4	1.06E+06	P25311	Zinc-alpha-2-glycoprotein
1	15.48	5	1.34E+05	P80303	Nucleobindin-2
1	15.33	7.1	1.06E+05	P04406	Glyceraldehyde-3-phosphate dehydrogenase
1	14.87	18.4	4.47E+05	P61769	Beta-2-microglobulin
1	13.96	8	7.35E+04	P54107	Cysteine-rich secretory protein 1

Urine PF2D 1D Protein Identification Results

Distinct Peptides	MS/MS Score	% AA Coverage	Spectral Intensity	SwissProt Accession	Protein Name
17	322.36	38	2.78E+07	P02768	Serum albumin
7	140.98	27.2	1.29E+08	P02760	Protein AMBP
5	93.39	28.7	4.71E+05	P01857	Ig gamma-1 chain C region
2	40.77	11.6	2.73E+05	P01861	Ig gamma-4 chain C region
1	21.03	4.2	1.61E+05	P01860	Ig gamma-3 chain C region
4	74.5	11.6	3.35E+05	P01833	Polymeric immunoglobulin receptor
4	73.21	60.3	1.57E+07	P01834	Ig kappa chain C region
4	72.3	19.1	4.00E+05	P19961	Alpha-amylase 2B
3	53.5	13.3	1.03E+05	P04745	Alpha-amylase 1
3	52.98	14	3.60E+05	P04746	Pancreatic alpha-amylase
3	61.55	6.3	8.98E+04	P10253	Lysosomal alpha-glucosidase
3	60.07	7.3	5.36E+05	Q14624	Inter-alpha-trypsin inhibitor heavy chain H4
3	58.76	9.1	9.14E+05	P01042	Kininogen-1
3	55.54	12.1	4.82E+05	P10451	Osteopontin
3	55.36	8.9	1.16E+06	Q6EMK4	Vasorin
3	52.4	11.4	1.32E+05	P05155	Plasma protease C1 inhibitor
3	45.54	6.5	1.86E+07	P07911	Uromodulin
2	42.82	17.3	7.73E+07	P41222	Prostaglandin-H2 D-isomerase
2	36.83	0.6	1.34E+05	P98160	Basement membrane-specific heparan sulfate proteoglycan core protein

2	35.66	3.1	1.14E+05	P01133	Pro-epidermal growth factor
2	35.28	18	1.35E+05	P24855	Deoxyribonuclease-1
2	34.83	9.3	1.83E+05	P10153	Non-secretory ribonuclease
2	33.29	22.2	9.56E+04	Q7Z5L0	Vitelline membrane outer layer protein 1 homolog
2	31.03	6.5	1.20E+05	P00734	Prothrombin
2	30.6	7.8	5.70E+04	P01009	Alpha-1-antitrypsin
2	26.74	10.1	1.34E+05	P01859	Ig gamma-2 chain C region
2	25.26	9.2	8.00E+04	Q9UNN8	Endothelial protein C receptor
1	21.72	5.3	6.21E+04	Q12907	Vesicular integral-membrane protein VIP36
1	19.54	33.8	1.35E+05	P60022	Beta-defensin 1
1	19.14	2.3	1.40E+05	P14543	Nidogen-1
1	18.11	9.5	4.57E+04	P09564	T-cell antigen CD7
1	17.52	7.6	1.90E+05	Q8WVN6	Secreted and transmembrane protein 1
1	17.21	3	2.76E+04	P02671	Fibrinogen alpha chain
1	16.82	6.3	1.81E+05	Q16769	Glutaminyl-peptide cyclotransferase
1	16.52	3.8	6.66E+04	P15309	Prostatic acid phosphatase
1	16.28	7.6	2.03E+04	O75594	Peptidoglycan recognition protein
1	16.27	4.6	1.71E+05	P02774	Vitamin D-binding protein
1	16.11	24.2	1.68E+05	P55000	Secreted Ly-6/uPAR-related protein 1
1	15.73	18.4	4.54E+05	P61769	Beta-2-microglobulin
1	15.1	15.7	3.48E+04	P01593	Ig kappa chain V-I region AG
1	15.1	15.7	3.48E+04	P01594	Ig kappa chain V-I region AU

1	15.1	15.7	3.48E+04	P01607	Ig kappa chain V-I region Rei
1	15.1	15.7	3.48E+04	P01610	Ig kappa chain V-I region WEA
1	15.1	15.7	3.48E+04	P01599	Ig kappa chain V-I region Gal
1	15.1	15.7	3.48E+04	P01608	Ig kappa chain V-I region Roy
1	15.1	15.7	3.48E+04	P01609	Ig kappa chain V-I region Scw
1	15.1	15.7	3.48E+04	P80362	Ig kappa chain V-I region WAT
1	15.1	15.7	3.48E+04	P01600	Ig kappa chain V-I region Hau
1	14.56	15.5	2.53E+04	P04206	Ig kappa chain V-III region GOL
1	14.56	15.5	2.53E+04	P01622	Ig kappa chain V-III region Ti
1	14.56	15.5	2.53E+04	P01620	Ig kappa chain V-III region SIE
1	14.56	15.5	2.53E+04	P01623	Ig kappa chain V-III region WOL
1	14.23	3.7	2.73E+04	P36896	Activin receptor type-1B
1	13.25	21	2.99E+04	P62988	Ubiquitin

Saliva PF2D 1D Protein Identification Results

Distinct Peptides	MS/MS Score	% AA Coverage	Spectral Intensity	SwissProt Accession	Protein Name
16	277.08	35.3	1.89E+09	P02768	Serum albumin
15	246.71	40.7	3.29E+09	P04745	Alpha-amylase 1
11	173.74	31.7	1.96E+09	P04746	Pancreatic alpha-amylase
13	207.25	40.5	2.42E+09	P19961	Alpha-amylase 2B
11	180.57	21.7	9.85E+08	P01833	Polymeric immunoglobulin receptor
11	166.48	19.7	3.50E+08	P02787	Serotransferrin
8	147.41	63.1	1.60E+10	P01036	Cystatin-S
2	31.47	20.5	5.75E+08	P01037	Cystatin-SN
3	52.95	31.2	2.16E+08	P09228	Cystatin-SA
9	143.58	43.3	4.77E+08	P25311	Zinc-alpha-2-glycoprotein
1	13.24	4.9	6.41E+07	A8MT79	Putative zinc-alpha-2-glycoprotein-like 1
7	123.98	60.9	3.74E+09	P12273	Prolactin-inducible protein
7	116.69	36.2	1.92E+09	P01876	Ig alpha-1 chain C region
5	83.89	27.3	9.92E+08	P01877	Ig alpha-2 chain C region
4	68.01	60.3	8.79E+08	P01834	Ig kappa chain C region
4	50.31	11.7	6.90E+07	P01871	Ig mu chain C region
3	39.91	10.4	6.41E+07	P04220	Ig mu heavy chain disease protein
3	48.98	11.4	2.03E+08	P68133	Actin, alpha skeletal muscle
3	48.98	11.4	2.03E+08	P68032	Actin, alpha cardiac muscle 1
2	34.68	8.4	1.99E+08	P62736	Actin, aortic smooth muscle
2	34.68	8.5	1.99E+08	P63267	Actin, gamma-enteric smooth

					muscle
2	33.23	2.5	1.00E+08	A5A3E0	POTE ankyrin domain family member F
2	33.23	2.5	1.00E+08	Q6S8J3	POTE ankyrin domain family member E
2	33.23	7.2	1.00E+08	Q9BYX7	Beta-actin-like protein 3
2	33.23	7.2	1.00E+08	P63261	Actin, cytoplasmic 2
2	33.23	7.2	1.00E+08	P60709	Actin, cytoplasmic 1
1	18.93	4.2	9.56E+07	Q562R1	Beta-actin-like protein 2
3	48.65	51.4	2.30E+08	P01842	Ig lambda chain C regions
3	38.1	28.4	6.94E+07	P01591	Immunoglobulin J chain
2	36.67	11.5	2.05E+07	Q6P5S2	Uncharacterized protein C6orf58
2	35.37	6.6	7.89E+07	P06733	Alpha-enolase
2	33.81	7.8	6.97E+07	P20061	Transcobalamin-1
2	29.06	10.3	3.61E+07	P06870	Kallikrein-1
2	27.57	7.1	2.38E+07	P02790	Hemopexin
2	25.62	7.8	1.40E+07	P01857	Ig gamma-1 chain C region
1	18.53	10.7	1.10E+07	P18510	Interleukin-1 receptor antagonist protein
1	17.65	10	4.12E+07	Q96DA0	Zymogen granule protein 16 homolog B
1	17.1	15.7	3.11E+07	P01593	Ig kappa chain V-I region AG
1	17.1	15.7	3.11E+07	P01594	Ig kappa chain V-I region AU
1	17.1	15.7	3.11E+07	P01607	Ig kappa chain V-I region Rei
1	17.1	15.7	3.11E+07	P01610	Ig kappa chain V-I region WEA
1	17.1	15.7	3.11E+07	P01599	Ig kappa chain V-I region Gal

1	17.1	15.7	3.11E+07	P01608	Ig kappa chain V-I region Roy
1	17.1	15.7	3.11E+07	P01609	Ig kappa chain V-I region Scw
1	17.1	15.7	3.11E+07	P80362	Ig kappa chain V-I region WAT
1	17.1	15.7	3.11E+07	P01600	Ig kappa chain V-I region Hau
1	16.67	13.1	2.26E+07	P06702	Protein S100-A9
1	16.48	15	2.84E+07	P01766	Ig heavy chain V-III region BRO
1	16.48	15.1	2.84E+07	P01777	Ig heavy chain V-III region TEI
1	16.4	12.3	5.09E+07	P10599	Thioredoxin
1	15.76	1.9	9.35E+06	Q02487	Desmocollin-2
1	15.41	6.3	6.50E+06	Q9Y5Z4	Heme-binding protein 2
1	15.27	12.2	2.39E+07	P04080	Cystatin-B OS=Homo sapiens
1	14.43	4.5	1.48E+08	P23280	Carbonic anhydrase 6
1	13.87	1.5	5.71E+06	P02671	Fibrinogen alpha chain
1	13.16	11.8	8.05E+06	P05109	Protein S100-A8
1	13.09	15.3	1.57E+07	P01776	Ig heavy chain V-III region WAS
1	13.09	15.1	1.57E+07	P01774	Ig heavy chain V-III region POM
1	13.09	15.5	1.57E+07	P01779	Ig heavy chain V-III region TUR
1	13.09	15.6	1.57E+07	P01765	Ig heavy chain V-III region TIL
1	13.06	2.7	7.01E+06	Q9BR84	Zinc finger protein 559

Vaginal fluid PF2D 1D Protein Identification Results

Distinct Peptides	MS/MS Score	% AA Coverage	Spectral Intensity	SwissProt Accession	Protein Name
22	403.3	89.9	5.74E+10	Q9UBC9	Small proline-rich protein 3
16	304.09	39.5	9.17E+09	P02768	Serum albumin
16	285.9	45.3	8.96E+09	P29508	Serpin B3
7	128.07	17.6	5.01E+09	P48594	Serpin B4
18	269.92	22	4.13E+09	A8K2U0	Alpha-2-macroglobulin-like protein 1
15	245.67	4.6	2.76E+09	Q09666	Neuroblast differentiation-associated protein AHNAK
13	244.73	33.1	5.31E+09	P07476	Involucrin OS=Homo sapiens
13	239.57	30.3	8.02E+09	P19013	Keratin, type II cytoskeletal 4
1	21.92	2.2	2.71E+08	Q86Y46	Keratin, type II cytoskeletal 73
1	15.99	2.3	6.07E+08	P08729	Keratin, type II cytoskeletal 7
1	15.99	2.4	6.07E+08	Q6KB66	Keratin, type II cytoskeletal 80
1	15.99	2.5	6.07E+08	P14136	Glial fibrillary acidic protein
12	212.68	43.3	2.54E+09	P07355	Annexin A2
8	138.64	26.8	1.95E+09	A6NMY6	Putative annexin A2-like protein
10	184	48.6	3.76E+09	Q9UBG3	Cornulin
9	157.66	48.4	8.12E+09	P01857	Ig gamma-1 chain C region
4	61.96	19.5	8.33E+08	P01861	Ig gamma-4 chain C region
4	59.82	15.3	1.17E+09	P01860	Ig gamma-3 chain C region
8	131.52	50.5	3.14E+09	P80188	Neutrophil gelatinase-associated lipocalin

6	111.71	23.6	1.87E+09	P04083	Annexin A1
7	104.24	14.5	4.00E+08	P01833	Polymeric immunoglobulin receptor
6	92.53	52.6	5.02E+09	P06702	Protein S100-A9
5	88.78	79.2	5.17E+09	P01834	Ig kappa chain C region
4	74.83	15.5	1.72E+09	Q16610	Extracellular matrix protein 1
4	67.62	44.4	3.24E+09	Q01469	Fatty acid-binding protein, epidermal
1	16.43	20.7	5.17E+08	A8MUU1	Putative fatty acid-binding protein 5-like protein 3
4	62.36	66.3	1.96E+09	P01040	Cystatin-A
3	58.81	51.6	6.70E+09	P22528	Cornifin-B
2	38.13	34.8	5.23E+09	P35321	Cornifin-A
4	56.17	18	5.14E+08	P01859	Ig gamma-2 chain C region
3	52.1	39.7	1.56E+08	P32320	Cytidine deaminase
3	50.58	61.1	8.14E+08	P35326	Small proline-rich protein 2A
2	36.01	43	6.15E+08	P22531	Small proline-rich protein 2E
3	50.53	61.1	1.04E+09	P35325	Small proline-rich protein 2B
2	46.86	11.2	3.75E+09	O95274	Ly6/PLAUR domain-containing protein
3	46.73	10	2.30E+08	O60235	Transmembrane protease, serine 11D
3	43.3	29.5	3.28E+08	P10599	Thioredoxin
3	37.77	51.4	8.17E+08	P01842	Ig lambda chain C regions
2	37.38	16.2	2.01E+08	P61626	Lysozyme C
2	35.26	23.6	8.17E+08	P05109	Protein S100-A8

2	32.49	7.8	3.08E+07	P13796	Plastin-2
2	31.89	9.6	6.21E+07	P31947	14-3-3 protein sigma
2	30.36	3.5	5.40E+07	P02787	Serotransferrin
2	28.37	30.6	1.49E+08	P31151	Protein S100-A7
2	27.88	43	4.32E+08	P22532	Small proline-rich protein 2D
1	13.36	25	1.04E+07	Q96RM1	Small proline-rich protein 2F
2	24.97	2.7	2.03E+07	P12110	Collagen alpha-2(VI) chain
1	21.35	29.2	1.50E+07	Q9UGL9	Cysteine-rich C-terminal protein 1
1	21.21	23.3	6.07E+08	P00441	Superoxide dismutase [Cu-Zn]
1	20.94	3.1	2.34E+08	P00751	Complement factor B
1	20.1	6.5	1.31E+08	P54108	Cysteine-rich secretory protein 3
1	19.71	3.3	2.17E+08	P01009	Alpha-1-antitrypsin
1	19.67	15.2	5.67E+07	P31949	Protein S100-A11
1	19.35	4.9	7.22E+07	P53634	Dipeptidyl-peptidase 1
1	17.69	7.7	1.65E+07	P09488	Glutathione S-transferase Mu 1
1	17.69	7.7	1.65E+07	P46439	Glutathione S-transferase Mu 5
1	17.69	7.7	1.65E+07	Q03013	Glutathione S-transferase Mu 4
1	17.04	7.8	4.45E+08	P04792	Heat shock protein beta-1
1	17.02	0.6	1.79E+08	Q8TD57	Dynein heavy chain 3, axonemal
1	16.82	12.2	8.06E+07	P04080	Cystatin-B
1	16.22	0.9	2.71E+07	O60437	Periplakin
1	16.07	22.9	7.02E+07	P07108	Acyl-CoA-binding protein
1	16.02	0.4	1.14E+08	P20930	Filaggrin
1	15.91	10.7	6.72E+07	P18510	Interleukin-1 receptor antagonist protein
1	14.99	5.7	3.15E+08	O75191	Xylulose kinase

1	14.92	18.8	3.43E+07	P19957	Elafin
1	14.77	1.6	3.67E+07	P05164	Myeloperoxidase
1	14.73	10.3	9.42E+06	P17900	Ganglioside GM2 activator
1	14.66	9.4	4.78E+07	P01764	Ig heavy chain V-III region VH26
1	14.64	7.1	1.61E+08	P25311	Zinc-alpha-2-glycoprotein
1	14.54	10.5	2.22E+08	Q8N4C7	Syntaxin-19
1	14.51	3.7	1.70E+08	P08238	Heat shock protein HSP 90-beta
1	14.37	1.2	8.55E+07	Q9BXL7	Caspase recruitment domain-containing protein 11
1	14.3	7	2.15E+08	Q8IUQ0	Retinaldehyde-binding protein 1-like protein 1
1	14.12	4.2	8.12E+07	P01876	Ig alpha-1 chain C region
1	13.93	17.5	3.83E+07	P60903	Protein S100-A10
1	13.88	3.4	2.86E+08	Q02108	Guanylate cyclase soluble subunit alpha-3
1	13.79	1.4	9.01E+07	Q8WWQ8	Stabilin-2
1	13.75	2.4	2.26E+07	P10909	Clusterin
1	13.73	4.5	2.88E+07	Q8N4F0	Bactericidal/permeability-increasing protein-like 1
1	13.7	1.3	8.77E+07	Q96C24	Synaptotagmin-like protein 4
1	13.66	3	1.54E+08	Q8IYU2	E3 ubiquitin-protein ligase HACE1
1	13.6	2.1	5.14E+06	P00450	Ceruloplasmin
1	13.56	9.5	2.72E+07	P09211	Glutathione S-transferase P
1	13.51	3.9	2.42E+07	Q9UBX7	Kallikrein-11
1	13.44	2.7	8.82E+07	Q6AI39	Uncharacterized protein KIAA0240

1	13.35	3.4	1.97E+07	Q8IYM0	Protein FAM186B
1	13.33	6.3	4.96E+07	P02649	Apolipoprotein E
1	13.23	1	1.78E+08	P21333	Filamin-A
1	13.21	15	2.83E+07	P01766	Ig heavy chain V-III region BRO
1	13.21	15.1	2.83E+07	P01777	Ig heavy chain V-III region TEI
1	13.12	4.2	9.18E+07	P38646	Stress-70 protein, mitochondrial
1	13.09	1	1.29E+08	Q8N3P4	Vacuolar protein sorting-associated protein 8 homolog

Menstrual Blood PF2D 1D Protein Identification Results

Distinct Peptides	MS/MS Score	% AA Coverage	Spectral Intensity	SwissProt Accession	Protein Name
48	911.81	76.1	1.95E+11	P02768	Serum albumin
39	665.17	36.3	6.03E+09	P01024	Complement C3
33	528.17	35.6	5.75E+09	P01023	Alpha-2-macroglobulin
4	59.92	3.5	7.58E+08	P20742	Pregnancy zone protein
26	467.91	46.7	8.70E+09	P02787	Serotransferrin
19	310.25	62.5	1.42E+10	P02647	Apolipoprotein A-I
16	266.69	50.4	5.98E+09	P01009	Alpha-1-antitrypsin
16	263.75	37.6	2.14E+09	P02675	Fibrinogen beta chain
17	262.95	29.4	1.96E+09	P00450	Ceruloplasmin
13	247.81	89.7	1.45E+11	P68871	Hemoglobin subunit beta
1	16.36	6.8	1.89E+10	P02100	Hemoglobin subunit epsilon
1	16.36	6.8	1.89E+10	P69891	Hemoglobin subunit gamma-1
1	16.36	6.8	1.89E+10	P69892	Hemoglobin subunit gamma-2
11	204.24	80.9	6.30E+10	P02042	Hemoglobin subunit delta
15	234.68	15.1	7.06E+08	P0C0L5	Complement C4-B
15	234.68	15.1	7.06E+08	P0C0L4	Complement C4-A
10	186.53	83.8	7.38E+10	P69905	Hemoglobin subunit alpha
11	186.08	44.5	6.53E+09	P01857	Ig gamma-1 chain C region
8	123.09	41.1	2.35E+09	P01859	Ig gamma-2 chain C region
6	95.06	20.4	1.44E+09	P01860	Ig gamma-3 chain C region
6	86.13	24.1	1.75E+09	P01861	Ig gamma-4 chain C region
10	163.96	34.6	3.44E+09	P02790	Hemopexin

9	145.45	31.2	1.53E+09	P02774	Vitamin D-binding protein
9	139.82	28.9	2.53E+09	P02679	Fibrinogen gamma chain
8	137.61	30.7	8.06E+08	P00738	Haptoglobin OS=Homo sapiens
4	73.02	14.6	4.86E+08	P00739	Haptoglobin-related protein
9	130.79	11.3	3.68E+08	P08603	Complement factor H
8	121.8	3.1	3.25E+08	P04114	Apolipoprotein B-100
6	101.04	32.4	4.84E+08	P02749	Beta-2-glycoprotein 1
7	98.1	16.4	1.91E+08	P00751	Complement factor B
5	96.27	17.1	5.22E+08	P01019	Angiotensinogen
5	80.58	59	1.47E+09	P02652	Apolipoprotein A-II
5	80.28	17.1	5.16E+08	P04217	Alpha-1B-glycoprotein
4	73.77	66	2.94E+09	P01834	Ig kappa chain C region
5	70.31	19	2.28E+08	P01871	Ig mu chain C region
3	38.64	11.5	1.59E+08	P04220	Ig mu heavy chain disease protein
4	67.01	21.5	1.01E+09	P02765	Alpha-2-HS-glycoprotein
4	64.87	6.5	3.41E+08	P02671	Fibrinogen alpha chain
4	63.4	10.5	3.09E+08	P01042	Kininogen-1
4	60.11	6.5	1.88E+08	P19823	Inter-alpha-trypsin inhibitor heavy chain H2
3	54.3	47.1	5.24E+08	P0CG04	Ig lambda-1 chain C regions
3	54.3	47.1	5.24E+08	P0CG05	Ig lambda-2 chain C regions
3	54.3	47.1	5.24E+08	P0CG06	Ig lambda-3 chain C regions
2	33.65	28.3	3.26E+08	P0CF74	Ig lambda-6 chain C region
1	15.99	14.1	1.43E+08	A0M8Q6	Ig lambda-7 chain C region
3	50.98	18.9	7.13E+08	P02763	Alpha-1-acid glycoprotein 1
2	32.08	12.9	3.84E+08	P19652	Alpha-1-acid glycoprotein 2

3	49.71	19.2	2.62E+08	P01876	Ig alpha-1 chain C region
2	34.03	15.5	1.77E+08	P01877	Ig alpha-2 chain C region
3	47.6	9.6	1.06E+08	P04004	Vitronectin
3	47.03	5.1	2.99E+08	P19827	Inter-alpha-trypsin inhibitor heavy chain H1
3	44.64	21	2.01E+08	P00915	Carbonic anhydrase 1
3	43.49	11.5	1.81E+08	P10909	Clusterin
3	42.4	12.5	6.99E+07	P01008	Antithrombin-III
2	39.68	4.8	1.80E+08	P01833	Polymeric immunoglobulin receptor
2	35.13	7.8	2.03E+08	P01011	Alpha-1-antichymotrypsin
2	31.36	5.6	1.30E+08	P00734	Prothrombin
2	30.6	27.2	4.04E+08	P02656	Apolipoprotein C-III
2	30	19.5	7.72E+07	Q9UBC9	Small proline-rich protein 3
2	28.62	16.1	8.17E+07	P80188	Neutrophil gelatinase-associated lipocalin
2	28.39	6.5	1.05E+08	P02760	Protein AMBP
2	25.2	19.1	6.42E+07	P59666	Neutrophil defensin 3
2	25.2	19.1	6.42E+07	P59665	Neutrophil defensin 1
2	24	1.9	2.58E+08	Q7Z442	Polycystic kidney disease protein 1-like 2
1	20.65	2.3	6.86E+07	Q14624	Inter-alpha-trypsin inhibitor heavy chain H4
1	19.59	14.9	4.97E+07	P02766	Transthyretin O
1	18.06	0.9	2.62E+07	Q86VH2	Kinesin-like protein KIF27
1	16.65	9	6.14E+07	P03973	Antileukoproteinase

1	15.75	3.2	1.56E+07	P06727	Apolipoprotein A-IV
1	15	15.7	2.98E+07	P22528	Cornifin-B
1	15	15.7	2.98E+07	P35321	Cornifin-A
1	14.83	4.2	3.25E+07	P68133	Actin, alpha skeletal muscle
1	14.83	4.2	3.25E+07	P68032	Actin, alpha cardiac muscle 1
1	14.83	4.2	3.25E+07	P62736	Actin, aortic smooth muscle
1	14.83	4.2	3.25E+07	P63267	Actin, gamma-enteric smooth muscle
1	14.47	18.4	1.49E+07	P61769	Beta-2-microglobulin
1	14.38	1.7	2.12E+07	P04196	Histidine-rich glycoprotein
1	13.64	7.1	9.06E+07	P25311	Zinc-alpha-2-glycoprotein
1	13.49	3.3	2.92E+08	Q8NB16	Mixed lineage kinase domain-like protein
1	13.26	1.4	6.21E+06	P01031	Complement C5
1	13.06	6.5	1.82E+08	P14384	Carboxypeptidase M

Appendix C: Unfractionated Protein Identification Results

Unfractionated Seminal Fluid Protein Identification Results

Distinct Peptides	MS/MS Score	% AA Coverage	Spectral Intensity	SwissProt Accession	Protein Name
16	281.63	35.6	6.65E+06	P02788	Lactotransferrin
10	190.82	22.6	1.35E+08	Q02383	Semenogelin-2
10	179.8	28.5	1.24E+08	P04279	Semenogelin-1
10	161.67	31.3	8.09E+06	P15309	Prostatic acid phosphatase
8	132.47	20.6	5.82E+06	P02768	Serum albumin
7	127.65	5.9	1.05E+06	P02751	Fibronectin
6	108.85	16.4	2.74E+06	P10909	Clusterin OS=Homo sapiens
6	98.9	49.3	4.40E+06	P12273	Prolactin-inducible protein
4	87.07	36.7	6.20E+06	P07288	Prostate-specific antigen
2	38.88	6.7	8.03E+04	P49221	Protein-glutamine gamma-glutamyltransferase 4
2	35.24	25.8	1.25E+06	P61916	Epididymal secretory protein E1
1	22.49	4.6	9.85E+05	P16870	Carboxypeptidase E
1	21.67	5.4	1.06E+06	P25311	Zinc-alpha-2-glycoprotein
1	15.48	5	1.34E+05	P80303	Nucleobindin-2
1	15.33	7.1	1.06E+05	P04406	Glyceraldehyde-3-phosphate dehydrogenase
1	14.87	18.4	4.47E+05	P61769	Beta-2-microglobulin
1	13.96	8	7.35E+04	P54107	Cysteine-rich secretory protein 1

Unfractionated Urine Protein Identification Results

Distinct Peptides	MS/MS Score	% AA Coverage	Spectral Intensity	SwissProt Accession	Protein Name
33	609.38	62.5	1.48E+08	P02768	Serum albumin
13	267.7	49.1	6.65E+07	P02760	Protein AMBP
13	200.77	22.6	7.21E+06	P04264	Keratin, type II cytoskeletal 1
2	34.77	3.2	2.07E+06	Q7Z794	Keratin, type II cytoskeletal 1b
5	72.43	8.5	1.40E+06	P04259	Keratin, type II cytoskeletal 6B
12	186.83	24.6	9.41E+06	P07911	Uromodulin
9	162.52	44.8	5.12E+06	P01857	Ig gamma-1 chain C region
3	55.76	14.6	3.30E+06	P01861	Ig gamma-4 chain C region
3	48.19	9	2.63E+06	P01860	Ig gamma-3 chain C region
4	57.21	15.6	2.86E+06	P01859	Ig gamma-2 chain C region
9	154.59	34.2	2.03E+06	P04745	Alpha-amylase 1
8	137.56	29.5	2.14E+06	P04746	Pancreatic alpha-amylase
9	147.8	15.3	9.84E+06	P01042	Kininogen-1
8	144.38	12.2	8.22E+06	Q14624	Inter-alpha-trypsin inhibitor heavy chain H4
8	126.26	36.3	1.38E+07	P10451	Osteopontin
7	120.82	2.4	3.48E+06	P98160	Basement membrane-specific heparan sulfate proteoglycan core protein
6	119.93	24.6	3.38E+06	P01009	Alpha-1-antitrypsin
7	114.17	16.7	6.05E+06	Q6EMK4	Vasorin
7	110.66	13.3	2.39E+06	P35527	Keratin, type I cytoskeletal 9

6	110.11	15.3	1.75E+06	P01833	Polymeric immunoglobulin receptor
5	94.22	79.2	2.30E+07	P01834	Ig kappa chain C region
5	85.37	24.4	1.26E+06	P25311	Zinc-alpha-2-glycoprotein OS=Homo sapiens
1	13.26	4.9	1.46E+05	A8MT79	Putative zinc-alpha-2-glycoprotein-like 1
5	79.53	16.6	4.32E+05	P05155	Plasma protease C1 inhibitor
5	79.32	24.7	1.91E+06	Q12907	Vesicular integral-membrane protein VIP36
5	75.62	5.9	6.02E+05	P01133	Pro-epidermal growth factor
4	73.04	11	1.97E+06	P00734	Prothrombin
4	72.69	23.4	2.73E+05	P02749	Beta-2-glycoprotein 1
5	70.77	8.5	1.08E+06	P02538	Keratin, type II cytoskeletal 6A
5	70.77	8.5	1.08E+06	P48668	Keratin, type II cytoskeletal 6C
3	44.03	4.9	9.16E+05	P13647	Keratin, type II cytoskeletal 5
3	43.6	5.2	7.31E+05	O95678	Keratin, type II cytoskeletal 75
2	29.6	3.5	6.07E+05	Q5XKE5	Keratin, type II cytoskeletal 79
3	42.21	5.6	2.63E+05	P19013	Keratin, type II cytoskeletal 4
5	70.49	13.7	6.84E+05	P08779	Keratin, type I cytoskeletal 16
3	46.09	6.7	4.59E+05	P02533	Keratin, type I cytoskeletal 14
3	46.09	7	4.59E+05	P19012	Keratin, type I cytoskeletal 15
2	30.35	4.6	3.04E+05	Q04695	Keratin, type I cytoskeletal 17
2	27.71	5	2.62E+05	P13646	Keratin, type I cytoskeletal 13
1	18.38	2.2	1.97E+05	P08727	Keratin, type I cytoskeletal 19
4	69.37	36.6	3.25E+05	Q7Z5L0	Vitelline membrane outer layer

					protein 1 homolog
4	67.81	27.7	1.13E+06	P02765	Alpha-2-HS-glycoprotein
4	65.5	17.2	1.17E+06	P01876	Ig alpha-1 chain C region OS=Homo sapiens GN=IGHA1 PE=1 SV=2
3	48.28	14.4	7.96E+05	P01877	Ig alpha-2 chain C region
3	63.27	32.6	1.67E+07	P41222	Prostaglandin-H2 D-isomerase
3	61.55	6.3	1.21E+05	P10253	Lysosomal alpha-glucosidase
3	59.66	11.6	1.99E+06	P15309	Prostatic acid phosphatase
4	59.07	6.3	1.54E+05	P00450	Ceruloplasmin
3	56.69	16.3	1.12E+06	Q9UNN8	Endothelial protein C receptor
3	55.51	4.1	1.13E+06	P16070	CD44 antigen
3	49.93	16.4	5.06E+05	P02750	Leucine-rich alpha-2-glycoprotein
3	49.34	21.4	3.08E+05	O75594	Peptidoglycan recognition protein
3	43.97	19.5	7.77E+06	P13987	CD59 glycoprotein
2	41.46	30.2	1.01E+06	P04206	Ig kappa chain V-III region GOL
2	41.46	30.2	1.01E+06	P01622	Ig kappa chain V-III region Ti
2	41.46	30.2	1.01E+06	P01620	Ig kappa chain V-III region SIE
2	41.46	30.2	1.01E+06	P01623	Ig kappa chain V-III region WOL
1	22.69	12.4	8.99E+05	P18135	Ig kappa chain V-III region HAH
1	22.69	12.4	8.99E+05	P18136	Ig kappa chain V-III region HIC
2	39.17	28.3	1.72E+06	P0CG04	Ig lambda-1 chain C regions
2	39.17	28.3	1.72E+06	P0CG05	Ig lambda-2 chain C regions
2	39.17	28.3	1.72E+06	P0CF74	Ig lambda-6 chain C region
2	39.17	28.3	1.72E+06	P0CG06	Ig lambda-3 chain C regions
1	17.36	14.1	2.73E+05	A0M8Q6	Ig lambda-7 chain C region

1	21.26	14.2	4.83E+05	P01842	Ig lambda chain C regions
2	38.98	14.5	6.68E+05	Q8WVN6	Secreted and transmembrane protein 1
2	38.92	21.8	3.00E+05	P19652	Alpha-1-acid glycoprotein 2
2	37.92	9.6	1.35E+05	P08571	Monocyte differentiation antigen CD14
2	37.2	13.8	3.99E+05	Q16270	Insulin-like growth factor-binding protein 7
2	37.12	14.2	2.14E+05	Q9UBC9	Small proline-rich protein 3
2	36.8	5	3.68E+05	P02671	Fibrinogen alpha chain
2	35.97	18	2.17E+05	P24855	Deoxyribonuclease-1
2	34.83	9.3	2.09E+05	P10153	Non-secretory ribonuclease
2	34.28	31.8	2.29E+05	P01617	Ig kappa chain V-II region TEW
1	15.86	9.7	1.52E+05	P06310	Ig kappa chain V-II region RPMI 6410
1	15.86	11.3	1.52E+05	P01614	Ig kappa chain V-II region Cum
1	15.86	11.1	1.52E+05	P06309	Ig kappa chain V-II region GM607 (Fragment)
2	33.11	10.2	6.24E+05	Q16769	Glutaminyl-peptide cyclotransferase
2	32.82	3.4	8.09E+05	P14543	Nidogen-1
2	32.44	13.2	5.00E+05	P05090	Apolipoprotein D
2	32.41	37.8	7.26E+05	P55000	Secreted Ly-6/uPAR-related protein 1
2	31.79	5.8	6.08E+04	P02787	Serotransferrin
2	31.52	4.8	7.40E+04	P04217	Alpha-1B-glycoprotein

2	28.05	4.9	4.15E+05	P05154	Plasma serine protease inhibitor
2	27.98	30.1	1.75E+06	P07998	Ribonuclease pancreatic
2	26.79	8	2.99E+05	P07288	Prostate-specific antigen
1	21.58	15.7	1.56E+05	P01593	Ig kappa chain V-I region AG
1	21.58	15.7	1.56E+05	P01594	Ig kappa chain V-I region AU
1	21.58	15.7	1.56E+05	P01607	Ig kappa chain V-I region Rei
1	21.58	15.7	1.56E+05	P01610	Ig kappa chain V-I region WEA
1	21.58	15.7	1.56E+05	P01599	Ig kappa chain V-I region Gal
1	21.58	15.7	1.56E+05	P01608	Ig kappa chain V-I region Roy
1	21.58	15.7	1.56E+05	P01609	Ig kappa chain V-I region Scw
1	21.58	15.7	1.56E+05	P80362	Ig kappa chain V-I region WAT
1	21.58	15.7	1.56E+05	P01600	Ig kappa chain V-I region Hau
1	20.23	14.9	3.51E+04	P01625	Ig kappa chain V-IV region Len
1	19.71	3	4.28E+04	P04279	Semenogelin-1
1	19.67	9.5	1.56E+05	P09564	T-cell antigen CD7
1	19.54	33.8	1.94E+06	P60022	Beta-defensin 1
1	19.32	1.5	1.06E+05	P55290	Cadherin-13
1	18.39	4.6	4.84E+05	P02774	Vitamin D-binding protein
1	18.24	6.9	2.85E+05	P02763	Alpha-1-acid glycoprotein 1
1	17.92	18.4	1.00E+06	P61769	Beta-2-microglobulin
1	17.82	11.2	2.53E+05	Q14508	WAP four-disulfide core domain protein 2
1	17.23	2.9	6.61E+04	Q08380	Galectin-3-binding protein
1	16.1	5.1	5.26E+04	P10909	Clusterin
1	15.81	15.5	3.80E+04	Q01469	Fatty acid-binding protein, epidermal

1	15.81	20.7	3.80E+04	A8MUU1	Putative fatty acid-binding protein 5-like protein 3
1	15.12	3.1	1.00E+05	Q8WZ75	Roundabout homolog 4
1	15.05	3.8	2.65E+04	P22792	Carboxypeptidase N subunit 2
1	14.51	13.1	6.25E+04	P06702	Protein S100-A9
1	14.33	1.4	1.67E+05	Q8IWU5	Extracellular sulfatase Sulf-2 OS=Homo sapiens
1	14.23	3.7	2.73E+04	P36896	Activin receptor type-1B
1	14.2	20.4	3.57E+04	Q9H299	SH3 domain-binding glutamic acid-rich-like protein 3
1	14.04	2	2.60E+04	P12830	Cadherin-1
1	13.87	29.6	6.38E+04	P11684	Uteroglobin
1	13.82	3.8	4.82E+04	Q9BRK3	Matrix-remodeling-associated protein 8
1	13.36	1.7	6.71E+04	P39060	Collagen alpha-1(XVIII) chain
1	13.25	21	2.99E+04	P62988	Ubiquitin
1	13.1	4.5	3.27E+04	P02790	Hemopexin

Unfractionated Saliva Protein Identification Results

Distinct Peptides	MS/MS Score	% AA Coverage	Spectral Intensity	SwissProt Accession	Protein Name
41	689.84	11.3	2.50E+07	Q9HC84	Mucin-5B
3	48.8	0.7	3.84E+05	P98088	Mucin-5AC (Fragments)
33	532.82	58.9	2.97E+07	P02768	Serum albumin
25	507.56	67.1	3.71E+08	P04745	Alpha-amylase 1
18	363.52	46.7	2.23E+08	P19961	Alpha-amylase 2B
17	344.83	45.4	2.07E+08	P04746	Pancreatic alpha-amylase
19	333.39	35.8	1.41E+07	P01833	Polymeric immunoglobulin receptor
11	198.38	61.7	3.00E+07	P01876	Ig alpha-1 chain C region
8	139.51	39.4	2.56E+07	P01877	Ig alpha-2 chain C region
9	151.85	39.2	3.66E+06	P25311	Zinc-alpha-2-glycoprotein
3	39.44	13.2	1.98E+05	A8MT79	Putative zinc-alpha-2-glycoprotein-like 1
9	147.94	32.5	4.69E+06	Q8N4F0	Bactericidal/permeability-increasing protein-like 1
8	124.34	34.1	2.50E+06	Q96DR5	Short palate, lung and nasal epithelium carcinoma-associated protein 2
7	118.85	45.1	7.10E+06	Q96DA0	Zymogen granule protein 16 homolog
6	115.61	61.7	4.01E+07	P01036	Cystatin-S
6	115.35	60.2	2.77E+07	P01037	Cystatin-SN
4	69.47	42.5	1.23E+07	P09228	Cystatin-SA

6	106.13	43.1	6.81E+06	P12273	Prolactin-inducible protein
6	102.13	22	3.40E+06	P23280	Carbonic anhydrase 6
6	101.26	79.2	5.33E+06	P01834	Ig kappa chain C region
6	93.21	61.2	7.04E+06	P28325	Cystatin-D
5	80.69	44.3	3.12E+06	P31025	Lipocalin-1
2	26.49	11.1	1.40E+06	Q5VSP4	Putative lipocalin 1-like protein 1
4	75.43	65	3.70E+06	P0CG05	Ig lambda-2 chain C regions
4	75.43	65	3.70E+06	P0CG06	Ig lambda-3 chain C regions
3	60.73	46.2	1.91E+06	P0CF74	Ig lambda-6 chain C region
3	56.91	47.1	3.02E+06	P0CG04	Ig lambda-1 chain C regions
2	38.67	32	1.47E+06	A0M8Q6	Ig lambda-7 chain C region
4	73.04	39.8	3.17E+06	P61626	Lysozyme C
4	70.51	14.4	2.94E+06	P13646	Keratin, type I cytoskeletal 13
2	37.45	3.8	2.24E+06	Q99456	Keratin, type I cytoskeletal 12
1	19.06	4	4.88E+05	P08727	Keratin, type I cytoskeletal 19
4	67.27	7.3	5.10E+05	P02788	Lactotransferrin
3	60.54	14.8	9.72E+05	P01857	Ig gamma-1 chain C region
1	16.99	4.2	4.50E+05	P01860	Ig gamma-3 chain C region
1	16.99	4.8	4.50E+05	P01861	Ig gamma-4 chain C region
4	59.36	29.5	8.11E+05	P01591	Immunoglobulin J chain
2	45.17	65.8	9.07E+07	P02814	Submaxillary gland androgen-regulated protein 3B
3	43.96	9	1.60E+06	Q8TAX7	Mucin-7 OS=Homo sapiens
3	42.2	37.7	1.99E+05	P06702	Protein S100-A9
2	38.83	26.8	6.19E+05	P61769	Beta-2-microglobulin
2	37.37	12.2	3.15E+05	P63261	Actin, cytoplasmic 2

2	37.37	12.2	3.15E+05	P60709	Actin, cytoplasmic 1
1	16.64	1.4	1.27E+05	A5A3E0	POTE ankyrin domain family member F
1	16.64	1.4	1.27E+05	Q6S8J3	POTE ankyrin domain family member E
1	16.64	4.2	1.27E+05	P68133	Actin, alpha skeletal muscle
1	16.64	4.2	1.27E+05	P68032	Actin, alpha cardiac muscle 1
1	16.64	4.2	1.27E+05	Q9BYX7	Putative beta-actin-like protein 3
1	16.64	4.2	1.27E+05	P62736	Actin, aortic smooth muscle
1	16.64	4.2	1.27E+05	Q562R1	Beta-actin-like protein 2
1	16.64	4.2	1.27E+05	P63267	Actin, gamma-enteric smooth muscle
2	36.36	11.2	7.75E+05	Q6P5S2	UPF0762 protein C6orf58
2	33.2	19.1	6.37E+05	P01034	Cystatin-C
2	33.02	9.7	7.40E+05	P54108	Cysteine-rich secretory protein 3
2	32.09	3.3	2.58E+05	P22079	Lactoperoxidase
2	29.81	33.6	8.38E+05	P04080	Cystatin-B
2	28.67	20.4	9.55E+04	P03973	Antileukoproteinase
1	19.48	2.8	1.63E+05	P19013	Keratin, type II cytoskeletal 4
1	19.19	0.5	9.45E+05	Q9UGM3	Deleted in malignant brain tumors 1 protein
1	18.7	4.7	1.68E+05	P04259	Keratin, type II cytoskeletal 6B
1	18.7	4.7	1.68E+05	P02538	Keratin, type II cytoskeletal 6A
1	18.7	4.7	1.68E+05	P48668	Keratin, type II cytoskeletal 6C
1	18.34	5	1.10E+05	P80303	Nucleobindin-2
1	18.21	2.7	1.86E+05	P20061	Transcobalamin-1

1	17.17	12.3	1.10E+05	P10599	Thioredoxin
1	16.77	9.7	1.72E+05	P06310	Ig kappa chain V-II region RPMI 6410
1	16.77	11.3	1.72E+05	P01614	Ig kappa chain V-II region Cum
1	16.77	11.1	1.72E+05	P06309	Ig kappa chain V-II region GM607
1	16.77	11.5	1.72E+05	P01617	Ig kappa chain V-II region TEW
1	16.09	48.3	3.41E+07	P02808	Statherin
1	15.83	28	2.29E+05	P15515	Histatin-1
1	15.7	15	1.94E+05	P01766	Ig heavy chain V-III region BRO
1	15.7	15.1	1.94E+05	P01777	Ig heavy chain V-III region TEI
1	15.03	12.4	7.14E+04	P18135	Ig kappa chain V-III region HAH
1	15.03	12.4	7.14E+04	P18136	Ig kappa chain V-III region HIC
1	15.03	14.6	7.14E+04	P04206	Ig kappa chain V-III region GOL
1	15.03	14.6	7.14E+04	P01622	Ig kappa chain V-III region Ti
1	15.03	14.6	7.14E+04	P01620	Ig kappa chain V-III region SIE
1	15.03	14.6	7.14E+04	P01623	Ig kappa chain V-III region WOL
1	13.68	2	1.34E+05	P02787	Serotransferrin
1	13.37	2.4	8.73E+04	P01871	Ig mu chain C region
1	13.37	2.8	8.73E+04	P04220	Ig mu heavy chain disease protein

Unfractionated Vaginal Fluid Protein Identification Results

Distinct Peptides	MS/MS Score	% AA Coverage	Spectral Intensity	SwissProt Accession	Protein Name
52	847.87	13.3	4.79E+07	Q09666	Neuroblast differentiation-associated protein AHNAK
42	753.16	70.9	1.05E+09	P02768	Serum albumin
25	423.88	88.7	4.89E+08	Q9UBC9	Small proline-rich protein 3
24	419.66	68.4	5.78E+07	P07355	Annexin A2
17	285.75	46	3.90E+07	A6NMY6	Putative annexin
24	414.07	58.9	7.78E+07	P13646	Keratin, type I cytoskeletal 13
3	57.83	5.4	1.85E+07	Q99456	Keratin, type I cytoskeletal 12
3	49.5	4.2	5.12E+06	P35900	Keratin, type I cytoskeletal 20
1	19.31	3	1.49E+06	Q8N1A0	Keratin-like protein KRT222
1	15.11	2	6.39E+05	Q2M2I5	Keratin, type I cytoskeletal 24
8	123.93	12.5	1.41E+07	P19012	Keratin, type I cytoskeletal 15
4	69.46	6.7	6.11E+06	P02533	Keratin, type I cytoskeletal 14
4	69.46	6.7	6.11E+06	P08779	Keratin, type I cytoskeletal 16
3	50.82	4.6	4.56E+06	Q04695	Keratin, type I cytoskeletal 17
5	80.89	9.5	2.32E+06	P13645	Keratin, type I cytoskeletal 10
23	412.11	47.1	2.68E+07	P02788	Lactotransferrin
22	389.74	39.9	2.19E+07	P02787	Serotransferrin
21	376.19	52.3	6.90E+07	P29508	Serpin B3
12	194.04	33.8	3.89E+07	P48594	Serpin B4
18	314.26	53.1	5.37E+07	P04083	Annexin A1
19	309.55	34.5	4.13E+07	P02538	Keratin, type II cytoskeletal 6A

3	41.32	3.6	1.55E+06	P12035	Keratin, type II cytoskeletal 3
2	29.48	1.8	1.32E+06	Q01546	Keratin, type II cytoskeletal 2 oral
2	29.48	2.1	1.32E+06	O95678	Keratin, type II cytoskeletal 75
2	29.48	2.2	1.32E+06	Q5XKE5	Keratin, type II cytoskeletal 79
1	18.81	2.9	1.08E+05	Q14CN4	Keratin, type II cytoskeletal 72
17	263.55	29.9	2.63E+07	P04259	Keratin, type II cytoskeletal 6B
11	170.1	19.4	1.74E+07	P13647	Keratin, type II cytoskeletal 5
3	50.1	3.7	3.24E+06	P35908	Keratin, type II cytoskeletal 2
2	32.47	4.1	3.98E+06	P05787	Keratin, type II cytoskeletal 8
20	304.68	21.3	7.53E+06	P01023	Alpha-2-macroglobulin
2	31.14	2	9.51E+05	P20742	Pregnancy zone protein
17	283.75	27.3	3.56E+07	P04264	Keratin, type II cytoskeletal 1
1	20.62	2	1.92E+06	Q7Z794	Keratin, type II cytoskeletal 1b
1	18.57	2.3	2.95E+06	P08729	Keratin, type II cytoskeletal 7
1	18.57	2.4	2.95E+06	Q6KB66	Keratin, type II cytoskeletal 80
1	18.57	2.5	2.95E+06	P14136	Glial fibrillary acidic protein
12	189.75	23	2.30E+07	P19013	Keratin, type II cytoskeletal 4
12	244.23	88.4	5.64E+07	P68871	Hemoglobin subunit beta
1	18.47	6.8	2.44E+06	P02100	Hemoglobin subunit epsilon
1	18.47	6.8	2.44E+06	P69891	Hemoglobin subunit gamma-1
1	18.47	6.8	2.44E+06	P69892	Hemoglobin subunit gamma-2
7	146.42	48.9	3.33E+07	P02042	Hemoglobin subunit delta
13	237.08	48.8	5.43E+07	Q9UBG3	Cornulin OS=Homo sapiens
12	233.38	53	9.33E+07	P01857	Ig gamma-1 chain C region
8	131.82	35.8	1.62E+07	P01859	Ig gamma-2 chain C region
7	125.84	33.9	2.10E+07	P01861	Ig gamma-4 chain C region

13	233.3	70.3	6.72E+07	Q01469	Fatty acid-binding protein, epidermal
2	42.47	26.7	1.63E+07	A8MUU1	Putative fatty acid-binding protein 5-like protein 3
16	233.19	15.8	6.26E+06	A8K2U0	Alpha-2-macroglobulin-like protein 1
13	218	31.4	4.20E+06	P13796	Plastin-2
3	46.9	5.5	1.17E+06	P13797	Plastin-3
2	32.04	3.4	7.47E+05	Q14651	Plastin-1
15	207.5	9.8	2.86E+06	P01024	Complement C3
11	204.17	62.3	3.94E+07	P31151	Protein S100-A7
3	57.35	23.7	1.86E+07	Q86SG5	Protein S100-A7A
12	194.91	4.7	6.12E+06	Q9Y6R7	IgGFc-binding protein
11	174.31	27.6	1.50E+07	P07476	Involucrin
10	168.05	61.1	3.26E+07	P80188	Neutrophil gelatinase-associated lipocalin
7	124.77	64	5.57E+07	P69905	Hemoglobin subunit alpha
8	123.28	29.3	1.48E+07	P63261	Actin, cytoplasmic 2
8	123.28	29.3	1.48E+07	P60709	Actin, cytoplasmic 1
4	57.89	9.5	4.11E+06	P68133	Actin, alpha skeletal muscle
4	57.89	9.5	4.11E+06	P68032	Actin, alpha cardiac muscle 1
4	57.89	9.5	4.11E+06	P62736	Actin, aortic smooth muscle
4	57.89	9.5	4.11E+06	P63267	Actin, gamma-enteric smooth muscle
3	47.44	2.7	4.58E+06	A5A3E0	POTE ankyrin domain family member F

3	47.44	2.7	4.58E+06	Q6S8J3	POTE ankyrin domain family member E
3	47.44	2.7	4.58E+06	P0CG38	POTE ankyrin domain family member I
2	27.54	1.8	1.70E+06	P0CG39	POTE ankyrin domain family member J
1	17.22	3.4	1.42E+06	Q9BYX7	Putative beta-actin-like protein 3
7	116.59	36.2	1.05E+07	P01876	Ig alpha-1 chain C region
4	63.78	21.4	4.12E+06	P01877	Ig alpha-2 chain C region
7	111.57	14.3	2.67E+06	P01833	Polymeric immunoglobulin receptor
7	106.6	19.9	1.91E+06	Q9UIV8	Serpin B13
5	103.33	64.1	6.95E+07	P01834	Ig kappa chain C region
6	97.2	87.6	7.92E+07	P22528	Cornifin-B
5	80.41	78.6	7.53E+07	P35321	Cornifin-A
6	96.59	45.6	6.72E+07	P06702	Protein S100-A9
6	93.59	23.2	2.58E+06	P12429	Annexin A3
7	93.18	11.3	2.73E+06	P14780	Matrix metalloproteinase-9
5	88.11	21.6	2.36E+06	P02790	Hemopexin
4	85.66	65	2.77E+07	P0CG05	Ig lambda-2 chain C regions
4	85.66	65	2.77E+07	P0CG06	Ig lambda-3 chain C regions
3	65.99	47.1	2.00E+07	P0CG04	Ig lambda-1 chain C regions
3	65.55	46.2	2.49E+07	P0CF74	Ig lambda-6 chain C region
2	40.77	32	1.03E+07	A0M8Q6	Ig lambda-7 chain C region
6	85.62	18.5	1.63E+06	P02675	Fibrinogen beta chain
5	85.06	44	2.09E+07	P05109	Protein S100-A8

4	83.58	64.7	1.07E+07	P31949	Protein S100-A11
5	81.66	5.1	1.73E+06	Q9NQ38	Serine protease inhibitor Kazal-type 5
4	77.65	21.9	1.10E+07	O95274	Ly6/PLAUR domain-containing protein
5	76.99	24.3	1.94E+06	P02647	Apolipoprotein A-I
4	74.65	32.3	3.95E+06	P02763	Alpha-1-acid glycoprotein 1
2	33.8	12.9	1.94E+06	P19652	Alpha-1-acid glycoprotein 2
4	72.92	66.6	3.61E+07	P35326	Small proline-rich protein 2A
1	17.82	17.8	2.63E+07	Q9BYE4	Small proline-rich protein 2G
4	66.38	66.6	3.09E+07	P35325	Small proline-rich protein 2B
3	48.95	54.1	3.04E+07	P22532	Small proline-rich protein 2D
3	51.55	66.6	3.28E+07	P22531	Small proline-rich protein 2E
4	65.82	75.5	6.95E+06	P01040	Cystatin-A
4	62.89	6.3	1.36E+06	P05164	Myeloperoxidase
4	62.47	39.7	3.70E+06	P04080	Cystatin-B
3	61.15	38	6.01E+06	P10599	Thioredoxin
3	60.69	35.1	7.39E+06	P61626	Lysozyme C OS=Homo sapiens
4	56.7	14	1.85E+06	P00738	Haptoglobin OS=Homo sapiens
2	28.75	6.8	3.41E+05	P00739	Haptoglobin-related protein
3	55.79	13.7	1.95E+06	P04406	Glyceraldehyde-3-phosphate dehydrogenase
3	55.66	22.4	4.64E+05	P30086	Phosphatidylethanolamine-binding protein 1
3	51.45	20.5	2.98E+06	P08246	Neutrophil elastase
2	50.87	17.9	1.62E+06	P54108	Cysteine-rich secretory protein 3

3	49.79	24.2	7.85E+05	P18510	Interleukin-1 receptor antagonist protein
3	49.27	4.1	1.67E+05	P00450	Ceruloplasmin
3	48.99	17.1	4.44E+05	P32119	Peroxiredoxin-2
3	48.92	9.2	4.89E+05	P01871	Ig mu chain C region
2	26.68	6.6	1.83E+05	P04220	Ig mu heavy chain disease protein
3	48.41	11.9	8.05E+05	Q9UKR3	Kallikrein-13
3	46.49	52.8	2.51E+06	P07108	Acyl-CoA-binding protein
3	42.8	29	1.18E+06	P19957	Elafin
2	41.75	30.2	1.82E+06	P04206	Ig kappa chain V-III region GOL
2	41.75	30.2	1.82E+06	P01622	Ig kappa chain V-III region Ti
2	41.75	30.2	1.82E+06	P01620	Ig kappa chain V-III region SIE
2	41.75	30.2	1.82E+06	P01623	Ig kappa chain V-III region WOL
1	20.34	12.4	4.86E+05	P18135	Ig kappa chain V-III region HAH
1	20.34	12.4	4.86E+05	P18136	Ig kappa chain V-III region HIC
2	39.18	0.7	5.01E+05	Q9HC84	Mucin-5B
2	37.04	13.1	3.13E+05	Q92876	Kallikrein-6
2	36.24	24.1	3.13E+06	P01766	Ig heavy chain V-III region BRO
1	21.57	15.1	2.96E+06	P01777	Ig heavy chain V-III region TEI
1	14.67	9.5	1.77E+05	P01767	Ig heavy chain V-III region BUT
1	14.67	9.6	1.77E+05	P01763	Ig heavy chain V-III region WEA
2	36.2	19.1	1.07E+07	P59666	Neutrophil defensin 3
2	36.2	19.1	1.07E+07	P59665	Neutrophil defensin 1
2	33.97	22.9	7.76E+05	P04792	Heat shock protein beta-1
2	33.78	5.9	6.52E+05	O95171	Sciellin
2	33.78	11.7	6.76E+05	P08311	Cathepsin G

2	32.92	36.1	6.55E+06	Q96RM1	Small proline-rich protein 2F
2	32.56	4.6	1.25E+06	Q16610	Extracellular matrix protein 1
2	32.25	4.9	3.17E+05	P29401	Transketolase
2	31.65	5.9	1.95E+05	P22894	Neutrophil collagenase
2	31.62	4.5	4.08E+05	P14618	Pyruvate kinase isozymes M1/M2
2	30.83	7.1	5.92E+05	P37837	Transaldolase
2	29.54	20.4	2.69E+06	P03973	Antileukoproteinase
2	28.69	7	6.36E+05	P07951	Tropomyosin beta chain
2	28.69	8	6.36E+05	P67936	Tropomyosin alpha-4 chain
1	14.42	3.5	5.55E+05	P06753	Tropomyosin alpha-3 chain
1	14.42	3.5	5.55E+05	P09493	Tropomyosin alpha-1 chain
2	28.26	12.9	6.82E+05	Q6UWP8	Suprabasin
2	27.75	6.6	2.74E+05	P24158	Myeloblastin
2	27.02	3	1.20E+06	P0CG48	Polyubiquitin-C
2	27.02	9.1	1.20E+06	P0CG47	Polyubiquitin-B
2	27.02	13.4	7.91E+05	P62979	Ubiquitin-40S ribosomal protein S27a
2	27.02	16.4	7.91E+05	P62987	Ubiquitin-60S ribosomal protein L40
2	26.63	7.4	1.34E+06	P04075	Fructose-bisphosphate aldolase A
2	25.38	10.4	9.06E+05	P16402	Histone H1.3
2	25.38	10.5	9.06E+05	P10412	Histone H1.4
2	25.38	10.7	9.06E+05	P16403	Histone H1.2
1	23.21	15.7	6.14E+05	P01593	Ig kappa chain V-I region AG
1	23.21	15.7	6.14E+05	P01594	Ig kappa chain V-I region AU
1	23.21	15.7	6.14E+05	P01607	Ig kappa chain V-I region Rei

1	23.21	15.7	6.14E+05	P01610	Ig kappa chain V-I region WEA
1	23.21	15.7	6.14E+05	P01599	Ig kappa chain V-I region Gal
1	23.21	15.7	6.14E+05	P01608	Ig kappa chain V-I region Roy
1	23.21	15.7	6.14E+05	P01609	Ig kappa chain V-I region Scw
1	23.21	15.7	6.14E+05	P80362	Ig kappa chain V-I region WAT
1	23.21	15.7	6.14E+05	P01600	Ig kappa chain V-I region Hau
1	21.86	15.7	2.71E+06	Q9NZT1	Calmodulin-like protein 5
1	21.51	4.2	1.35E+05	Q9UL52	Transmembrane protease serine 11E
1	21.09	19.5	5.04E+05	P80511	Protein S100-A12
1	18.83	29.2	3.42E+05	Q9UGL9	Cysteine-rich C-terminal protein 1
1	18.52	2.6	3.35E+05	P02679	Fibrinogen gamma chain
1	18.36	1.9	3.29E+05	P22735	Protein-glutamine gamma-glutamyltransferase K
1	18.2	15.3	5.87E+04	P01776	Ig heavy chain V-III region WAS
1	18.2	15.1	5.87E+04	P01774	Ig heavy chain V-III region POM
1	18.2	15.5	5.87E+04	P01779	Ig heavy chain V-III region TUR
1	18.2	15.6	5.87E+04	P01765	Ig heavy chain V-III region TIL
1	17.97	8.8	1.83E+05	P02766	Transthyretin
1	17.54	11.4	3.54E+05	P62158	Calmodulin
1	17.24	4.6	1.18E+06	P02774	Vitamin D-binding protein
1	16.68	11.4	9.20E+05	P07737	Profilin-1
1	16.59	0.8	5.16E+04	P0C0L5	Complement C4-B
1	16.59	0.8	5.16E+04	P0C0L4	Complement C4-A
1	16.28	13.6	8.12E+05	P32320	Cytidine deaminase
1	16.25	7.8	2.71E+05	P14174	Macrophage migration inhibitory

					factor
1	16.08	21.4	2.51E+05	P00441	Superoxide dismutase [Cu-Zn]
1	15.75	15.7	3.70E+05	Q5VTM1	Protein FAM25
1	15.61	9.7	2.63E+05	P06310	Ig kappa chain V-II region RPMI 6410
1	15.61	11.3	2.63E+05	P01614	Ig kappa chain V-II region Cum
1	15.61	11.1	2.63E+05	P06309	Ig kappa chain V-II region GM607 (Fragment)
1	15.61	11.5	2.63E+05	P01617	Ig kappa chain V-II region TEW
1	15.48	7.8	2.93E+05	P22392	Nucleoside diphosphate kinase B
1	15.48	7.8	2.93E+05	P15531	Nucleoside diphosphate kinase A
1	14.9	12.2	7.46E+04	P35754	Glutaredoxin-1
1	14.82	9.4	9.36E+04	P47914	60S ribosomal protein L29
1	14.45	3	1.37E+04	Q6XPR3	Repetin
1	14.43	43.1	2.55E+05	Q08EQ4	Putative thymosin beta-4-like protein
1	14.43	43.1	2.55E+05	A8MW06	Thymosin beta-4-like protein 3
1	14.43	43.1	2.55E+05	P62328	Thymosin beta-4
1	14.43	43.1	2.55E+05	A9Z1Y9	Putative thymosin beta-4-like protein 6
1	14.4	4.6	6.36E+04	Q9BW04	Specifically androgen-regulated gene protein
1	14.27	0.6	2.15E+05	P48552	Nuclear receptor-interacting protein 1
1	14.18	6.4	1.36E+05	P49913	Cathelicidin antimicrobial peptide
1	14.09	9.7	4.38E+05	P62805	Histone H4

1	13.86	2.3	1.28E+05	Q05639	Elongation factor 1-alpha 2
1	13.86	2.3	1.28E+05	Q5VTE0	Putative elongation factor 1-alpha-like 3
1	13.86	2.3	1.28E+05	P68104	Elongation factor 1-alpha 1
1	13.67	4.3	4.41E+05	P52209	6-phosphogluconate dehydrogenase, decarboxylating
1	13.65	12.2	3.73E+04	P61019	Ras-related protein Rab-2A
1	13.64	27.4	3.10E+05	Q14508	WAP four-disulfide core domain protein 2

Unfractionated Menstrual Blood Protein Identification Results

Distinct Peptides	MS/MS Score	% AA Coverage	Spectral Intensity	SwissProt Accession	Protein Name
28	488.77	54.5	6.82E+07	P02768	Serum albumin
28	482.75	56.4	2.10E+07	P02788	Lactotransferrin
22	408.31	56.3	1.32E+08	P13646	Keratin, type I cytoskeletal 13
6	108.41	10.5	6.22E+07	P19012	Keratin, type I cytoskeletal 15
2	42.5	3.8	7.40E+06	Q99456	Keratin, type I cytoskeletal 12
2	40.79	6.2	8.84E+06	P08727	Keratin, type I cytoskeletal 19
2	35.98	2.5	1.33E+07	P35900	Keratin, type I cytoskeletal 20
2	35.98	2.5	1.33E+07	Q04695	Keratin, type I cytoskeletal 17
1	18.14	3	1.55E+06	Q8N1A0	Keratin-like protein KRT222
1	17.64	2	2.26E+06	Q2M2I5	Keratin, type I cytoskeletal 24
9	147.59	21.2	7.75E+06	P13645	Keratin, type I cytoskeletal 10
5	90.22	10.7	3.07E+07	P08779	Keratin, type I cytoskeletal 16
4	75.25	6.7	3.00E+07	P02533	Keratin, type I cytoskeletal 14
19	336.92	35.1	7.22E+07	P02538	Keratin, type II cytoskeletal 6A
5	84.44	7.4	1.87E+07	O95678	Keratin, type II cytoskeletal 75
4	65.21	4.7	9.41E+06	P12035	Keratin, type II cytoskeletal 3
4	64.74	5.7	1.36E+07	Q5XKE5	Keratin, type II cytoskeletal 79
3	48.6	2.9	6.21E+06	Q01546	Keratin, type II cytoskeletal 2 oral
18	309.07	33.3	6.65E+07	P48668	Keratin, type II cytoskeletal 6C
17	298.89	30.3	6.09E+07	P04259	Keratin, type II cytoskeletal 6B
11	183.65	17.7	3.00E+07	P13647	Keratin, type II cytoskeletal 5
6	107.08	7.9	2.16E+07	P35908	Keratin, type II cytoskeletal 2

					epidermal
3	54.81	5.5	8.64E+06	P05787	Keratin, type II cytoskeletal 8
13	282.18	89.7	8.56E+08	P68871	Hemoglobin subunit beta
1	18.52	6.8	7.69E+07	P02100	Hemoglobin subunit epsilon
10	203.79	72.1	2.24E+08	P02042	Hemoglobin subunit delta
3	57.89	23.8	7.80E+07	P69891	Hemoglobin subunit gamma-1
3	57.89	23.8	7.80E+07	P69892	Hemoglobin subunit gamma-2
16	258.57	16.9	1.75E+07	P01023	Alpha-2-macroglobulin
1	16.96	0.8	1.27E+06	P20742	Pregnancy zone protein
16	253.88	48.3	2.71E+07	P02647	Apolipoprotein A-I
13	249.46	26.5	2.48E+07	P04264	Keratin, type II cytoskeletal 1
2	41.05	3.2	8.46E+06	Q7Z794	Keratin, type II cytoskeletal 1b
1	22.48	2.4	1.55E+06	Q6KB66	Keratin, type II cytoskeletal 80
1	22.48	2.5	1.55E+06	P14136	Glial fibrillary acidic protein
8	133.15	20.2	9.51E+06	P19013	Keratin, type II cytoskeletal 4
3	52.38	5.7	3.87E+06	P08729	Keratin, type II cytoskeletal 7
16	244.61	30.7	8.24E+06	P05164	Myeloperoxidase
2	24.86	2.9	4.77E+05	P11678	Eosinophil peroxidase
14	233.13	37.6	9.93E+06	P02675	Fibrinogen beta chain
14	213.77	11.4	5.14E+06	P01024	Complement C3
10	197.45	45.8	2.29E+07	P63261	Actin, cytoplasmic 2
10	197.45	45.8	2.29E+07	P60709	Actin, cytoplasmic 1
5	111.44	6.8	1.21E+07	Q6S8J3	POTE ankyrin domain family member E
5	91.69	15.1	1.26E+07	P68133	Actin, alpha skeletal muscle

5	91.69	15.1	1.26E+07	P68032	Actin, alpha cardiac muscle 1
4	87.98	4.7	1.10E+07	A5A3E0	POTE ankyrin domain family member F
3	69.95	10.6	7.68E+06	Q9BYX7	Putative beta-actin-like protein 3
4	68.59	12.2	9.30E+06	P62736	Actin, aortic smooth muscle
4	68.59	12.2	9.30E+06	P63267	Actin, gamma-enteric smooth muscle
3	65.99	3.2	8.02E+06	P0CG38	POTE ankyrin domain family member I
2	47.96	2.3	4.71E+06	P0CG39	POTE ankyrin domain family member J
2	35.17	6.3	4.97E+06	Q562R1	Beta-actin-like protein 2
8	168	83.8	4.49E+08	P69905	Hemoglobin subunit alpha
10	152.37	26.8	5.61E+06	P08670	Vimentin OS=Homo sapiens
9	147.8	20	9.36E+06	P02787	Serotransferrin
8	133.99	36.7	3.98E+06	P04083	Annexin A1
7	125.13	27.8	1.07E+07	Q9UBG3	Cornulin
8	122.74	6.8	1.04E+06	P35579	Myosin-9
1	13.54	0.5	9.82E+04	P35580	Myosin-10
1	13.4	0.8	1.75E+05	Q7Z406	Myosin-14
1	13.4	0.8	1.75E+05	P35749	Myosin-11
6	114.3	55.2	3.59E+07	P06702	Protein S100-A9
7	106.99	23.8	4.43E+06	P02679	Fibrinogen gamma chain
7	105.63	25.1	2.68E+06	P00738	Haptoglobin OS=Homo sapiens
3	45.95	12.3	7.37E+05	P00739	Haptoglobin-related protein
6	100.79	20.5	1.87E+06	Q5VTE0	Putative elongation factor 1-alpha-

					like 3
6	100.79	20.5	1.87E+06	P68104	Elongation factor 1-alpha 1
3	49.92	6.6	7.25E+05	Q05639	Elongation factor 1-alpha 2
6	96.79	18.4	7.44E+06	P01009	Alpha-1-antitrypsin
6	94.82	33	2.56E+06	P01857	Ig gamma-1 chain C region
2	28.63	11.6	6.41E+05	P01861	Ig gamma-4 chain C region
3	45.88	15.1	2.11E+06	P01860	Ig gamma-3 chain C region
7	93.14	19.7	1.23E+06	P68363	Tubulin alpha-1B chain
6	81.25	16.9	1.07E+06	Q9BQE3	Tubulin alpha-1C chain
5	71.76	15.6	7.07E+05	P68366	Tubulin alpha-4A chain
5	65.28	13.7	1.00E+06	Q71U36	Tubulin alpha-1A chain
3	38.44	9.7	5.31E+05	Q13748	Tubulin alpha-3C/D chain
3	38.44	9.7	5.31E+05	Q6PEY2	Tubulin alpha-3E chain
2	30.95	7.1	2.97E+05	Q9NY65	Tubulin alpha-8 chain
1	15.97	5.8	6.47E+04	Q9H853	Putative tubulin-like protein alpha-4B
1	15.92	2	1.44E+05	A6NHL2	Tubulin alpha chain-like 3
4	83.3	37.4	6.36E+06	P08246	Neutrophil elastase
5	80.88	41.7	1.66E+07	P62805	Histone H4
5	75.16	40.3	1.23E+06	P60660	Myosin light polypeptide 6
2	30.32	11.5	8.20E+05	P14649	Myosin light chain 6B
5	74.41	21.5	1.34E+06	Q9BVA1	Tubulin beta-2B chain
5	74.41	21.5	1.34E+06	Q13885	Tubulin beta-2A chain
5	74.41	21.5	1.34E+06	P68371	Tubulin beta-2C chain
5	74.41	21.6	1.34E+06	P07437	Tubulin beta chain

5	74.41	21.6	1.34E+06	P04350	Tubulin beta-4 chain
2	28.84	6.8	3.84E+05	Q13509	Tubulin beta-3 chain
2	28.27	6.2	1.41E+05	Q9BUF5	Tubulin beta-6 chain
1	14.58	3.1	7.63E+04	Q3ZCM7	Tubulin beta-8 chain
1	14.58	3.1	7.63E+04	A6NNZ2	Tubulin beta-8 chain B
1	14.58	3.7	7.63E+04	A6NKZ8	Putative tubulin beta chain-like protein ENSP00000290377
5	72.14	28.6	2.01E+06	P01876	Ig alpha-1 chain C region
3	39.18	18.5	1.22E+06	P01877	Ig alpha-2 chain C region
4	69.78	39.8	1.59E+06	P20160	Azurocidin
5	69.64	22.8	1.13E+06	P63104	14-3-3 protein zeta/delta
2	21.96	7	6.17E+05	P62258	14-3-3 protein epsilon
2	21.96	7.2	6.17E+05	P61981	14-3-3 protein gamma
2	21.96	7.3	6.17E+05	Q04917	14-3-3 protein eta
2	21.96	7.3	6.17E+05	P31946	14-3-3 protein beta/alpha
2	21.96	7.3	6.17E+05	P27348	14-3-3 protein theta
4	55.74	16.9	1.26E+06	P31947	14-3-3 protein sigma
4	69.26	6.9	5.07E+05	P02730	Band 3 anion transport protein
3	58.1	24.7	2.19E+07	P05109	Protein S100-A8
4	56.99	19.6	5.29E+06	P02790	Hemopexin
3	55.36	43.8	2.68E+06	P04908	Histone H2A type 1-B/E
3	55.36	43.8	2.68E+06	Q7L7L0	Histone H2A type 3
3	55.36	43.8	2.68E+06	P20671	Histone H2A type 1-D
3	55.36	43.8	2.68E+06	Q93077	Histone H2A type 1-C
3	55.36	43.8	2.68E+06	P0C0S8	Histone H2A type 1
3	55.36	44.1	2.68E+06	Q9BTM1	Histone H2A.J

3	55.36	44.5	2.68E+06	Q99878	Histone H2A type 1-J
3	55.36	44.5	2.68E+06	Q96KK5	Histone H2A type 1-H
2	35.35	26.5	1.89E+06	P16104	Histone H2A.x
2	35.35	29.2	1.89E+06	Q8IUE6	Histone H2A type 2-B
1	13.09	6.8	1.05E+06	Q96QV6	Histone H2A type 1-A
1	13.09	7	1.05E+06	P0C0S5	Histone H2A.Z
1	13.09	7	1.05E+06	Q71UI9	Histone H2A.V
3	52.39	43.8	2.14E+06	Q6FI13	Histone H2A type 2-A
3	52.39	44.1	2.14E+06	Q16777	Histone H2A type 2-C
3	53.14	8.3	2.17E+05	P06576	ATP synthase subunit beta, mitochondrial
3	52.82	50.9	4.15E+06	P01834	Ig kappa chain C region
3	51.35	10.1	6.28E+05	P02774	Vitamin D-binding protein
4	50.97	17.9	8.53E+05	P27797	Calreticulin
3	50.61	6.6	5.93E+05	P00747	Plasminogen
3	50.57	27.8	1.93E+06	P04792	Heat shock protein beta-1
3	50.16	18.9	6.03E+06	P02763	Alpha-1-acid glycoprotein 1
3	47.5	4.5	4.72E+06	P00450	Ceruloplasmin
3	47.49	20.3	6.51E+05	P00915	Carbonic anhydrase 1
4	47.44	2	5.83E+05	P15924	Desmoplakin
3	45.57	9.6	1.06E+06	P04004	Vitronectin
3	45.33	27.1	4.21E+05	P07737	Profilin-1
3	44.18	11.3	1.23E+06	P24158	Myeloblastin
3	41.93	8.4	3.51E+05	P07237	Protein disulfide-isomerase
3	41.01	45.9	7.97E+05	P04080	Cystatin-B
3	39.68	32.3	6.20E+05	P47929	Galectin-7

2	38.53	19	1.20E+07	Q99879	Histone H2B type 1-M
2	38.53	19	1.20E+07	Q99880	Histone H2B type 1-L
2	38.53	19	1.20E+07	P33778	Histone H2B type 1-B
2	38.53	19	1.20E+07	P57053	Histone H2B type F-S
2	38.53	19	1.20E+07	P58876	Histone H2B type 1-D
2	38.53	19	1.20E+07	Q99877	Histone H2B type 1-N
2	38.53	19	1.20E+07	Q16778	Histone H2B type 2-E
2	38.53	19	1.20E+07	Q5QNW6	Histone H2B type 2-F
2	38.53	19	1.20E+07	Q8N257	Histone H2B type 3-B
2	38.53	19	1.20E+07	P23527	Histone H2B type 1-O
2	38.53	19	1.20E+07	P62807	Histone H2B type 1-C/E/F/G/I
2	38.53	19	1.20E+07	P06899	Histone H2B type 1-J
2	38.53	19	1.20E+07	Q93079	Histone H2B type 1-H
2	38.53	19	1.20E+07	O60814	Histone H2B type 1-K
1	17.34	7	8.48E+06	Q96A08	Histone H2B type 1-A
3	38.23	6.5	3.58E+05	P11021	78 kDa glucose-regulated protein
2	36.68	33	5.46E+05	P0CG04	Ig lambda-1 chain C regions
2	36.68	33	5.46E+05	P0CG05	Ig lambda-2 chain C regions
2	36.68	33	5.46E+05	P0CG06	Ig lambda-3 chain C regions
1	19.96	14.1	3.21E+05	A0M8Q6	Ig lambda-7 chain C region
1	19.96	14.1	3.21E+05	P0CF74	Ig lambda-6 chain C region
2	36.25	4.6	3.23E+05	Q8N1N4	Keratin, type II cytoskeletal 78
2	35.43	11.9	1.27E+06	P02765	Alpha-2-HS-glycoprotein
2	34.24	27	1.39E+06	P61626	Lysozyme C
2	33.75	4.8	4.12E+05	P01042	Kininogen-1

2	33.68	21.7	3.00E+05	P29373	Cellular retinoic acid-binding protein 2
2	33.67	5.7	7.04E+05	P01871	Ig mu chain C region OS=Homo sapiens
2	33.67	6.6	7.04E+05	P04220	Ig mu heavy chain disease protein
2	33.03	25.9	6.00E+05	Q01469	Fatty acid-binding protein, epidermal
1	13.44	20.7	2.45E+05	A8MUU1	Putative fatty acid-binding protein 5-like protein 3
2	32.55	1.6	9.04E+05	P02751	Fibronectin
2	32.28	8.6	3.25E+05	P10909	Clusterin OS=Homo sapiens
2	29.64	25.7	4.30E+05	P31949	Protein S100-A11
2	29.23	19.1	6.54E+05	P59666	Neutrophil defensin 3
2	29.23	19.1	6.54E+05	P59665	Neutrophil defensin 1
2	29.01	24.8	3.06E+05	P62937	Peptidyl-prolyl cis-trans isomerase A
1	14.45	8.5	6.49E+04	Q9Y536	Peptidylprolyl cis-trans isomerase A-like 4A/B/C O
2	28.71	7.3	2.17E+05	P14923	Junction plakoglobin
2	27.57	1.7	2.79E+05	P08603	Complement factor H
2	27.27	18.9	9.31E+05	Q9UBC9	Small proline-rich protein 3
2	25.94	9.5	3.09E+05	P32119	Peroxiredoxin-2
1	21.94	5.1	8.46E+04	P30101	Protein disulfide-isomerase A3
1	20.67	19.5	7.80E+04	P80511	Protein S100-A12
1	20.36	20	1.43E+06	P02652	Apolipoprotein A-II
1	20	3	2.65E+05	P04217	Alpha-1B-glycoprotein

1	19.46	8.8	2.04E+05	P02766	Transthyretin
1	19.4	3.4	1.15E+05	P08697	Alpha-2-antiplasmin
1	19.2	3.7	1.01E+05	P25705	ATP synthase subunit alpha, mitochondrial
1	18.67	8.2	1.73E+05	P08311	Cathepsin G
1	18.27	6.6	3.63E+05	P02749	Beta-2-glycoprotein 1
1	17.87	1.1	7.94E+04	P11215	Integrin alpha-M
1	17.68	0.6	4.41E+04	P21333	Filamin-A
1	17.63	23.5	3.44E+05	P68431	Histone H3.1
1	17.52	5.3	5.58E+04	P02649	Apolipoprotein E
1	16.05	1.3	1.07E+05	O43707	Alpha-actinin-4
1	16.05	1.3	1.07E+05	P12814	Alpha-actinin-1
1	16.02	5	2.64E+05	P28676	Grancalcin
1	15.97	1.7	1.11E+05	P41218	Myeloid cell nuclear differentiation antigen
1	15.64	0.7	4.17E+04	P11277	Spectrin beta chain, erythrocyte
1	15.47	6.2	5.33E+04	A6NL28	Putative tropomyosin alpha-3 chain-like protein
1	15.39	8.4	2.15E+05	P52566	Rho GDP-dissociation inhibitor 2
1	15.13	2	5.26E+04	P17213	Bactericidal permeability-increasing protein
1	15.05	5.4	2.49E+05	P04075	Fructose-bisphosphate aldolase A
1	15.03	4.1	9.02E+04	P23284	Peptidyl-prolyl cis-trans isomerase B
1	14.47	9	3.21E+05	P03973	Antileukoproteinase
1	13.97	4.9	1.37E+05	P01859	Ig gamma-2 chain C region

1	13.83	1.6	1.87E+05	P27824	Calnexin
1	13.67	16.8	1.77E+05	P23528	Cofilin-1
1	13.5	5.3	1.19E+05	P04406	Glyceraldehyde-3-phosphate dehydrogenase
1	13.46	10.5	1.46E+05	P80723	Brain acid soluble protein 1
1	13.24	3.4	2.09E+05	P11142	Heat shock cognate 71 kDa protein
1	13.1	19.1	8.10E+05	P35321	Cornifin-A

Unfractionated Peripheral Blood Protein Identification Results

Distinct Peptides	MS/MS Score	% AA Coverage	Spectral Intensity	SwissProt Accession	Protein Name
22	370.36	19.8	1.29E+07	P01024	Complement C3
21	355.21	20.7	2.37E+07	P01023	Alpha-2-macroglobulin
4	64.9	3.5	5.04E+06	P20742	Pregnancy zone protein
18	307.59	39.1	2.89E+07	P02787	Serotransferrin
12	255.67	89.7	1.38E+09	P68871	Hemoglobin subunit beta
1	19.69	6.8	2.68E+08	P02100	Hemoglobin subunit epsilon
1	19.69	6.8	2.68E+08	P69891	Hemoglobin subunit gamma-1
1	19.69	6.8	2.68E+08	P69892	Hemoglobin subunit gamma-2
9	173.13	66.6	6.05E+08	P02042	Hemoglobin subunit delta
13	221.45	32.8	1.35E+08	P02768	Serum albumin
12	201.37	46.8	2.75E+07	P02647	Apolipoprotein A-I
8	157.1	83.8	8.68E+08	P69905	Hemoglobin subunit alpha
9	153.12	26.1	6.47E+06	P02774	Vitamin D-binding protein
9	147.79	26.5	1.24E+07	P01009	Alpha-1-antitrypsin
8	132.96	32.6	1.00E+07	P02790	Hemopexin
5	97.64	34.3	6.67E+06	P02765	Alpha-2-HS-glycoprotein
6	86.67	8.7	3.65E+06	P00450	Ceruloplasmin
4	76.94	48	2.10E+07	P02652	Apolipoprotein A-II
5	76.48	4.7	1.02E+06	P08603	Complement factor H
4	72.03	40.1	1.92E+06	P02766	Transthyretin
4	68.78	66	7.39E+06	P01834	Ig kappa chain C region
4	68.57	21.2	2.91E+06	P01876	Ig alpha-1 chain C region

3	52.32	18.5	2.50E+06	P01877	Ig alpha-2 chain C region
4	65.34	10.5	1.07E+06	P01008	Antithrombin-III
3	48.64	7.8	8.94E+05	P00751	Complement factor B
3	46.44	2	2.02E+06	P02751	Fibronectin
3	45.92	4.2	4.63E+05	P19823	Inter-alpha-trypsin inhibitor heavy chain H2
2	40.61	15.7	5.59E+06	P00915	Carbonic anhydrase 1
3	40.32	18.9	2.40E+06	P02763	Alpha-1-acid glycoprotein 1
2	33.48	12.9	2.22E+06	P19652	Alpha-1-acid glycoprotein 2
2	38.21	10.6	3.23E+06	P01857	Ig gamma-1 chain C region
1	17.32	4.2	5.15E+05	P01860	Ig gamma-3 chain C region
1	17.32	4.8	5.15E+05	P01861	Ig gamma-4 chain C region
2	34.22	2.1	4.16E+05	P0C0L4	Complement C4-A
2	34.22	2.1	4.16E+05	P0C0L5	Complement C4-B
2	30.5	0.6	3.68E+05	P04114	Apolipoprotein B-100
2	29.14	3.6	2.94E+05	P00734	Prothrombin
2	24.93	7.3	8.85E+05	P01011	Alpha-1-antichymotrypsin
2	24.9	5.5	5.18E+05	P06727	Apolipoprotein A-IV
1	18.85	0.9	1.65E+05	Q14624	Inter-alpha-trypsin inhibitor heavy chain H4
1	17.77	3.1	2.92E+05	P06396	Gelsolin
1	17.72	3.3	1.59E+05	P01871	Ig mu chain C region
1	17.72	3.8	1.59E+05	P04220	Ig mu heavy chain disease protein
1	16.73	3.1	3.56E+05	P02760	Protein AMBP
1	16.42	3.1	4.24E+05	P04004	Vitronectin
1	15.88	4.9	2.84E+05	P02749	Beta-2-glycoprotein 1

1	14.86	2	5.00E+05	P05155	Plasma protease C1 inhibitor
1	14.53	5.1	3.01E+05	P10909	Clusterin
1	14.49	3	4.87E+05	P04217	Alpha-1B-glycoprotein
1	13.29	3.4	2.13E+06	P04040	Catalase