Final Technical Report

REPORT TITLE: **Validation of Highly-Specific Protein Markers for the Identification of Biological Stains**

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ABSTRACT

Overview **–** The advent of DNA profiling has transformed the field of forensic serology by making it possible to individualize biological stains. The identification of the stain itself, however, can present a challenge for forensic serologists. For example, there is no test for vaginal secretions and tests for blood cannot distinguish peripheral from menstrual blood even though this information can be probative to an investigation. After successfully mapping and rigorously comparing the proteomes of six body fluids with clear forensic relevance (*i.e.*, peripheral and menstrual blood, vaginal secretions, semen, urine and saliva), it was possible to construct a database of candidate protein biomarkers of each target stain. These included anticipated protein biomarkers like statherin for saliva and lesser known ones like neutrophil gelatinase-associated lipocalin for vaginal secretions. Having been identified from analyses of just five people, however, it could not be overemphasized that these were candidate biomarkers.

The current research, therefore, was designed to validate the specificity of the most promising candidate biomarkers for their target body fluids and the consistency of their expression among multiple humans. This was necessary to accurately discriminate between proteins that are truly specific to a given body fluid vs. those that show interindividual variability or which are present in non-target stains. In addition to assays of an expanded study population, a second set of studies assessed the reliability of these biomarkers in a forensic context. Specifically, the ability of the biomarkers to be detected in single- and mixed-source samples recovered from a variety of substrates or exposed to environmental contaminants/insults was assessed.

Project Objectives **-** The specific aims of this research were to:

(1) Recruit volunteers and collect samples of forensically relevant body fluids (*i.e.*, peripheral and menstrual blood, vaginal secretions, seminal fluid, urine and saliva)**.**

(2) Develop a high-sensitivity Q-TOF assay which combined six panels of highly-specific protein biomarkers into a single multiplex assay for the purpose of evaluating the target stain specificity on 50 samples of each of six forensically-relevant body fluids.

(3) Evaluate the performance highly-specific protein biomarkers and the Q-TOF multiplex assay using forensic casework type samples.

Results and Conclusions **-** All core objectives have been achieved. Biomarker validation assays were conducted using a high-sensitivity mass spectrometry technology (Q-TOF). This enabled

the detection of even low-abundance candidate biomarkers while circumventing the obstacles of alternative approaches that would be prohibitively expensive and unnecessarily time consuming. The results obtained using an optimized 6-body fluid multiplex assay for the analysis of a representative seminal fluid, saliva, urine, vaginal fluid and peripheral and menstrual blood samples revealed the clear and unambiguous identification of targeted high-specificity biomarkers for each fluid. The inter-individual reproducibility of target ion detection was also found to be excellent across multiple samples.

To assess the broader applicability of each biomarker in terms of its specificity for a given body fluid, multiplexed Q-TOF analyses of single-source body fluid samples from a sample population of fifty human research participants were conducted. For the majority of the candidate protein bio markers the results of these assays confirmed both the body fluid specificity and the ability to reliably detect targeted biomarkers across a sample population of 50 individuals. The results of these assays also identified biomarkers that are co expressed in more than one body fluid, information that will have utility for the future development of interpretation guidelines for challenging samples.

Finally, a total of 37 unique casework-type samples were assayed using the Q-TOF multiplex assay. Single-source samples of human body fluids were accurately identified by the detection of one or more of the high-specificity biomarkers. Recovery of body fluid samples from a variety of substrates did not impede the accurate characterization of the body fluid being assayed. Of the potential inhibitors assayed only chewing tobacco juice appeared to preclude the identification of a target body fluid. A series of 2-component mixtures of human body fluids analyzed by the multiplex assay accurately identified both components in a single-pass. Only in the case of saliva and peripheral blood did matrix effects appear to impede the detection of salivary proteins.

In toto, the research supported by NIJ award 2009-DN-BX-K165 has made it possible employ cutting edge protein analysis technologies to identify and evaluate the specificity of an assemblage of high-specificity protein biomarkers for bodily fluids typically encountered in a forensic context. This information will help to facilitate the commercial production of such assays. This includes the development of a commercial mass spectrometry approach based on the multiplex assay described here.

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Introduction and Statement of the Problem

Blood and semen factors that once held promise as discriminatory instruments for individualizing biological stains have been supplanted by DNA markers, which can be amplified from tiny amounts of biological material. While DNA analysis of an evidentiary swab may reveal the presence of a DNA profile consistent with an alleged victim, the DNA profile cannot indicate whether the DNA came from saliva, vaginal fluid, urine or a host of other sources. The ability to confidently associate a DNA extract with a specific tissue source or to accurately characterize mixed stains, however, can provide criminal investigators with critical information.

Consider the case of an alleged sexual assault where a DNA profile consistent with the victim is found on the mouth of a bottle in the suspect's possession. The victim states that the suspect used the bottle as a foreign object to penetrate her vaginally. The suspect counters that the alleged victim had drunk from the bottle and that no sexual contact occurred. Both stories may explain the presence of the victim's DNA on the bottle. The ability to reliably detect traces of vaginal fluid or potentially a mixture of both vaginal fluid and saliva in this case could help to either confirm or refute these opposing claims.

Review of Relevant Literature

While tests for the presence of blood, semen, saliva and urine exist[1-4], some are laborious (*e.g.,* creatinine test for urine). Others require that serologists be proficient at a variety of methodologies, some of which employ reagents that pose health and safety risks. For example, the chemical instability of picric acid (used by some labs to test for urine) presents an explosion hazard and is toxic to liver and kidney tissue. Other serological tests consume significant amounts of a valuable sample while yielding only presumptive results.

Tests for evidence of vaginal contact have proven extremely challenging. The iodine-based Lugol's test which detects glycogenated cells held promise for identifying vaginal cells[5, 6]. More rigorous studies, however, revealed that Lugol's positive cells were also present in the male urethra[7], male urine deposits[8] and on >50% of penile swabs from males who had abstained from sex for several days[9]. Modifications to improve the reliability of Lugol's test have been suggested[10] but are not conducive for use with casework.

The routine testing for blood and seminal fluid by forensic laboratories has been greatly facilitated by the development of rapid immmunochromatographic assays[4, 11, 12]. As with any antibody-based assay, however, results are "presumptive by definition" because the potential for antibody cross-reactivity with non-target molecules can never be eliminated[13]. Moreover, casework-type samples may include environmental contaminants that can interfere with antibody binding, thereby reducing assay sensitivity[14]. For a range of other body fluids, forensically-validated commercial kits based on body fluid specific antigens are lacking entirely and this often leaves the forensic analyst without the ability to make a substantive statement about the potential tissue source of a DNA profile.

Due in part to the limitations associated with existing methods of stain identification, several novel approaches to biological stain identification are now being explored. These research efforts have as their goal the development of a more sensitive and uniform strategy for analyzing body fluids capable of providing analysts with confirmatory results. Emerging approaches include biological stain identification based on messenger- and micro-RNA expression profiles[15, 16], epigenetic[17, 18] modifications, Raman spectroscopy[19] and protein-biomarker detection by mass-spectrometry[20]. Each of these proposed methods have their own strengths and weaknesses.

Accordingly, they are not so much competing or mutually exclusive technologies, rather, they are potentially complementary technologies that will make it possible for analysts to obtain useful information from a much larger range of casework samples. These emerging strategies also offer an opportunity for greater standardization and automation of biological stain analysis as well as the incorporation of additional tests for body fluids which are not covered by existing methods. The potential to bring greater uniformity, standardization and thus automation to forensic serological testing would be akin to the type of progress that has been achieved over the past couple of decades in DNA profiling.

Protein biomarkers have attracted significant interest in recent years due in large part due to the strides that have been made in the tools to identify and characterize them. It is now possible to rigorously map entire proteomes with high reproducibility using automated 2-dimensional HPLC systems or MudPIT (multidimensional protein identification technology) to identify potentially useful biomarkers. Once identified, mass-spectrometry-based targeted-ion assays can facilitate the unambiguous detection and quantitation of even low abundance proteins, against a background of other non-target molecules. This has resulted in a wealth of new opportunities to develop protein-based assays for medical and forensic applications such as body fluid identification.

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. 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[21, 22] and post-mortem tissue[23].

Core Research Objectives

Thus, the central goal of the current research project was to evaluate the stain-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 research was designed to complement the use of DNA profiling by making it possible to more accurately and confidently associate a DNA sample with a specific type of biological stain. This will be achieved through the completion of the following three Core Research Objectives.

(1) Recruit volunteers and collect samples of forensically relevant body fluids (*i.e.*, peripheral and menstrual blood, vaginal secretions, seminal fluid, urine and saliva)

(2) Develop and test a high-sensitivity Q-TOF multiplex assay to evaluate the target stain specificity of the candidate biomarkers

(3) Evaluate the performance highly-specific protein biomarkers using forensic casework type samples.

The completion of these objectives will aid forensic analysts by providing the forensic community with a validated panel of protein biomarkers which are specific to forensically relevant body fluids and which can be used to in a forensic context.

Methods

Human Subjects – All research was IRB reviewed, approved and conducted in full compliance with U.S. Federal Policy for the Protection of Human Subjects (Basic DHHS Policy for Protection of Human Research Subjects; 56 FR 28003). A total of 100 adult (>18 y.o.) human volunteers (50 males; 50 females) were recruited for this study from within the University of Denver student population.

Body Fluid Collection and Protein Extraction – A total of fifty samples of six forensically-relevant body fluids (*i.e.*, peripheral and menstrual blood, semen, saliva, vaginal secretions and urine) were collected for proteome mapping. The choice of the bodily fluids to be analyzed and the size of the study population reflected discussions with forensic practitioner at state and private caseworking laboratories including forensic serologists at the Colorado Bureau of Investigation. In addition, an expert in the forensics of sexual assault examination has helped to guide this research to best meet the needs of the forensic community. The procedures employed for sample collection were in accordance with the NIH guidelines.

Protein Concentration, Partitioning and Quantification **–** Corning Spin-X UF concentrators (3000 NMWL) (Corning, Lowell, MA) were used to concentrate low protein content body fluids such as saliva and urine while at the same time removing unwanted salts and other low molecular weight components. The Thermo Scientific Pierce Micro BCA Protein Assay (Thermo Fisher Scientific, Rockford, IL) was used to determine final protein concentration of each extracted sample. All samples were stored in a locked -70°C freezer until analyzed.

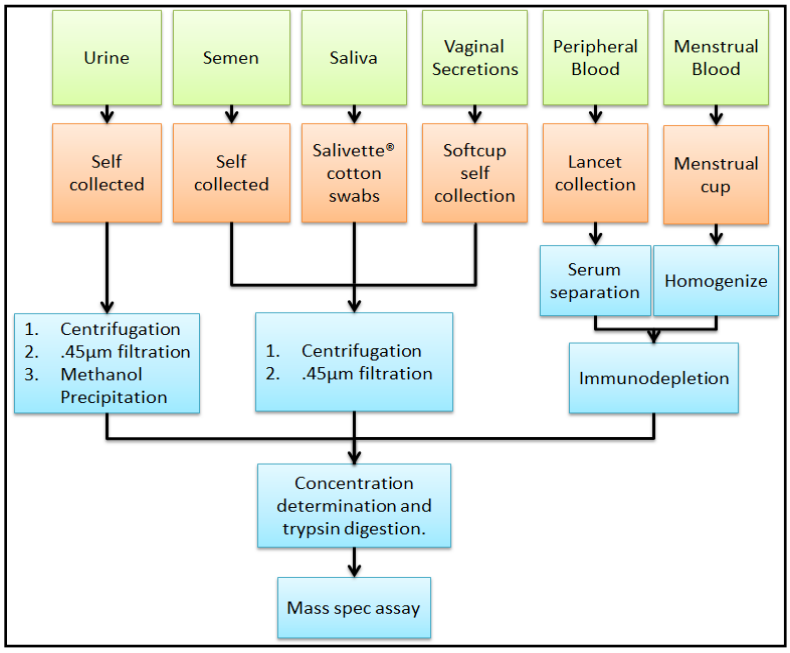
Menstrual blood samples were typically contaminated with cellular components due to the lysing of fragile erythrocytes. In addition, the presence of several high-abundance serum proteins common to both peripheral and menstrual blood made it difficult to fine tune the targeted-ion protocol for the less abundant but more specific biomarkers for these body fluids. To circumvent these problems during the initial development phase, commercially available proteome partitioning columns were employed to remove twelve highly abundant but non-specific proteins from human blood sera.

Mass Spectrometry **–** Protein extracts for analysis by mass spectrometry were sonicated and digested overnight with trypsin at 37°C. Digested samples were then purified on a C-18 spin column and analyzed using a quadrupole-time of flight mass spectrometer (Q-TOF) assay that selectively targeted specific candidate biomarker ions with femtomole (1×10^{-15}) sensitivity such that unfractionated biological stains (*e.g.*, semen, saliva or vaginal fluid) could be scanned for the presence or absence of an entire panel of biomarkers in a single run. A multiplex assay was developed to simultaneously scan for the presence of six different body fluids in 44-minutes. This assay targets a total of 45 individual precursor ions consisting of 6 peripheral blood peptides, 11 saliva peptides, 10 seminal fluid peptides, 4 urine peptides, and 14 vaginal/menstrual blood peptides. Data analysis was performed using Spectrum Mill software suite by Agilent Technologies.

Casework Type Samples **-** The applicability of a mass-spectrometry based body fluid assay to samples encountered in a forensic context was assessed using a series of casework type samples. Specifically, the ability of the biomarkers to be detected in body fluid samples recovered from a variety of substrates or containing environmental contaminants was assessed. In addition, a series of 2-component body fluid mixtures were analyzed to assess the ability of more than one body fluid to be detected simultaneously.

Results and Conclusions

Collection of Forensically Relevant Body Fluids – The first core objective of the research was the collection of samples of forensically-relevant body fluids (*i.e.*, peripheral and menstrual blood, vaginal secretions, semen, urine and saliva). The choice of these fluids reflected discussions with forensic serologists and other forensic practitioners. Executive Summary Figure 1 provides an overview of the collection and processing methods employed for the assay-development and biomarker validation phases of the project. For each body fluid, a total of 50 individuals were recruited to assess the consistency of biomarker detection across a population of humans. While there is consensus in the forensic community on the critical importance of developmental validation studies, there is less agreement on the number of subjects that should be included in a given validation study. To select a statistically suitable sample size for the current study, an important consideration was the impact of the number of samples on our ability to reliably capture the intrinsic variability present in a given population. For an infinite population, under an assumption of a standard normal distribution, the 95% confidence interval is captured within 1.96 standard deviations of the mean. By comparison, the 95% confidence interval for a sample size of 5, 50, and 100 would be 2.78, 2.01 and 1.98, respectively. Balancing the importance of capturing statistical variability with the time and financial limitations of the project, therefore, it was determined that a sample population of 50 individuals per body fluid would make it make it possible to reliably discriminate between proteins that are specific to a given body fluid *vs.* those that varied between individuals or were present in non-target stains. Finally, while the study participants reflected the ethnic and age diversity of the University of Denver student population, there were an insufficient number of study participants to enable a statistically substantive partitioning of the sample on the basis of biogeographic origin, health status or broad age cohorts. It should be emphasized, however, that while such detail was beyond the scope of the current project, these factors are important and should be addressed as a part of future validation studies.

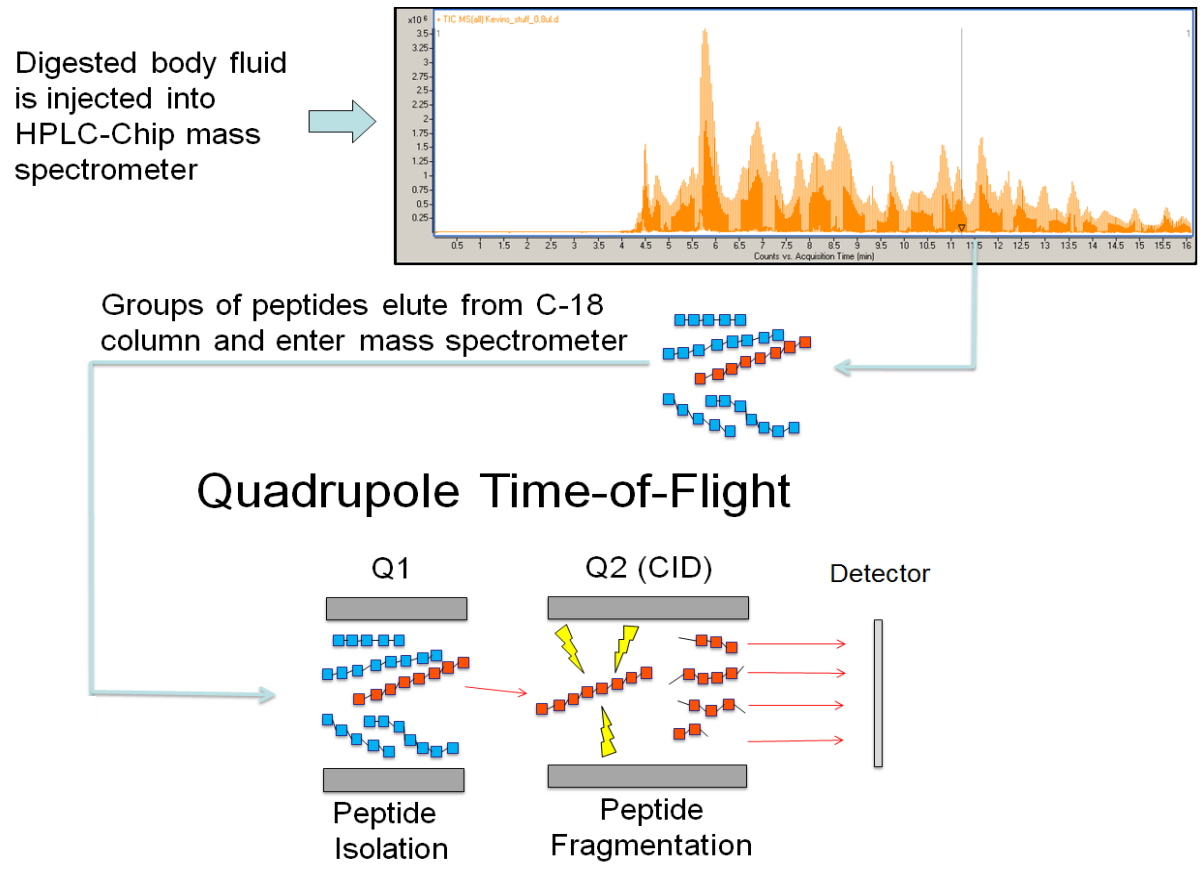


Executive Summary Figure 1 – Flow chart of the sample collection and preparation process for each of the biological stains (*i.e.*, body fluids) for which the high-specificity protein multiplex assay was developed.

Development and Testing of a High-Sensitivity Q-TOF Multiplex Assay – The second core objective of the research was the development of a high-sensitivity targeted-ion Q-TOF assay and the use of that assay to evaluate fifty samples of each body fluid for the presence or absence of individual candidate biomarkers. An important advantage of this approach was the ability to query a single sample for the presence or absence of biomarkers that are diagnostic for six different body fluids in a single pass.

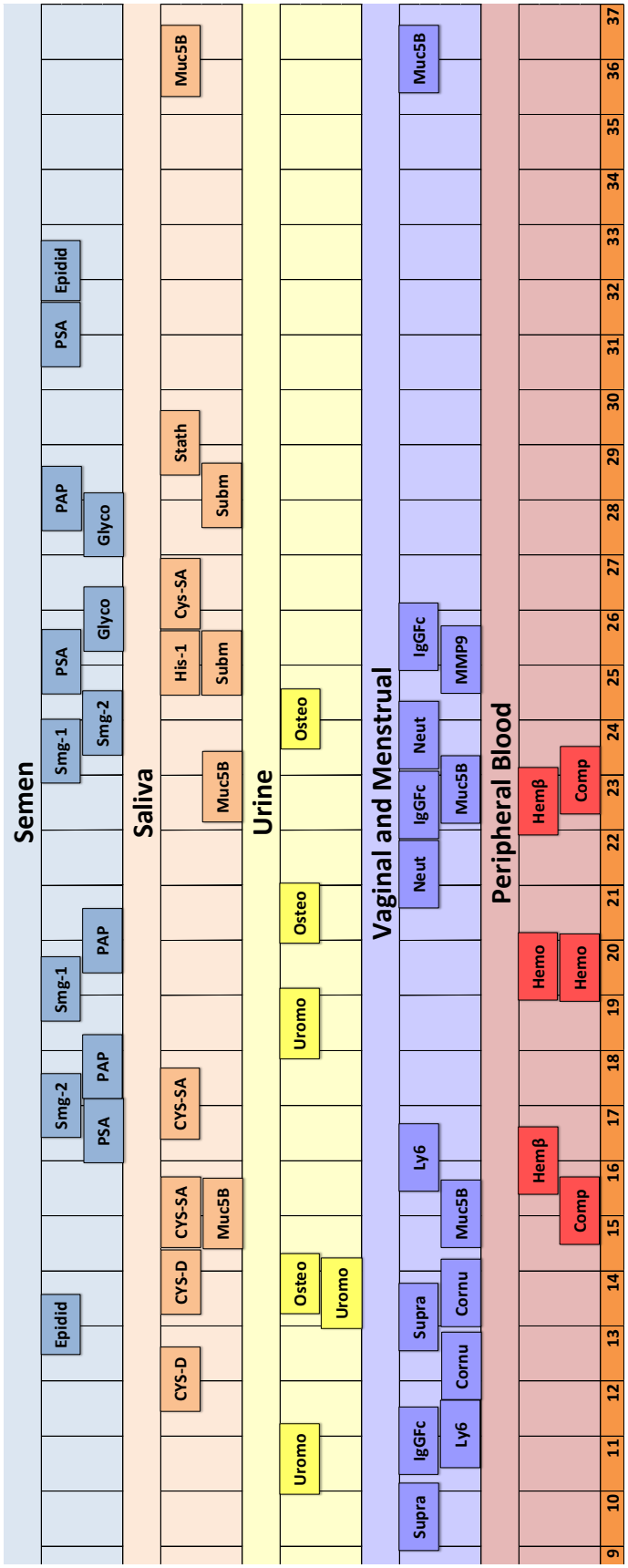
Under a prior award, six panels of proteins with potential utility as biomarkers for the identification of biological stains were identified. It is important to emphasize, however, that these protein biomarkers were identified based on the proteomes of just five individuals per bodily fluid and thus can only be considered **candidate** protein biomarkers. The ultimate applicability of a given biomarker for use with the general population necessitates a more comprehensive and thorough validation of each candidate marker for stain specificity across a larger population set.

To circumvent the limitations of antibody and other alternative approaches to biomarker validation, a targeted quadrupole-time of flight mass spectrometer (Q-TOF) strategy was employed. This strategy (Executive Summary Figure 2) allows specific ions of interest to be selected for in the first quadrupole from the background of non-targeted ions. The isolated ion is then forwarded to the collision cell where it is further fragmented. The resulting fragments then enter a TOF mass analyzer which yields highly accurate product ion spectra thereby confirming the presence and identity of the original protein biomarker. This minimized the potential for false negatives (*i.e.*, a failure to detect the presence of non-target biomarkers in the biological stains being assayed).



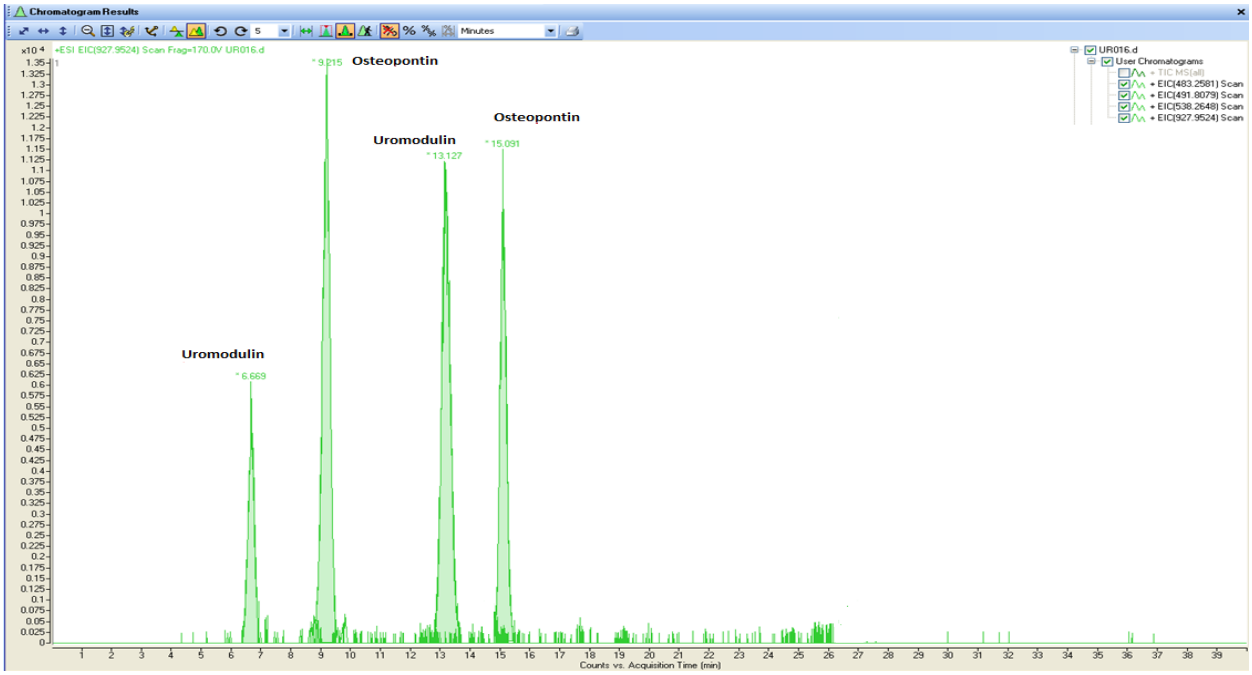
Executive Summary Figure 2 – Analysis of digested peptides using a flow-through HPLC chip MS approach. Peptides entering the Q-TOF are selectively isolated in quadrupole 1 (Q1) followed by fragmentation in the collision cell (Q2) with the resulting peptide fragments then reaching the detector. Thus, only peptides that are selected are allowed to reach the detector – even against a background of hundreds or thousands of other non-target peptides.

During Q-TOF assay development, inclusion lists of optimal peptides (ions) were compiled. Ideal peptides were those which: (1) ionized consistently across multiple experiments; (2) had no post-translational modifications that would alter the mass-charge ratio and; (3) were of high abundance so as to facilitate detection. The target ion inclusion lists for each individual body fluid were then combined into a multiplex assay. The multiplex assay was then retested with a subset of samples of each body fluid to identify possible matrix effects that could impede the performance of the assay. This process resulted in the development of a final multiplex assay for the assessment of the target stain specificity for each candidate biomarker (Executive Summary Figure 3).



Executive Summary Figure 3 – Elution times in minutes (orange row at bottom of figure) of all optimal peptides for the detection of the high-specificity biomarkers for each body fluid targeted by the Q-TOF multiplex assay. This assay targets multiple peptides that are diagnostic for each unique protein biomarker. This provides for robust internal confirmation of each biomarker that is detected in the assay. Peptides are grouped by target body fluid such that each body fluid is represented by a separate "channel". As with the multiple channels used in traditional STR analyses, data are collected from each channel simultaneously.

The results obtained using the final 6-body fluid multiplex assay for the analysis of a representative urine sample are shown in Executive Summary Figure 4. Both peptide chromatography and database search results revealed the clear and unambiguous identification of the two targeted high-specificity biomarkers for urine (*i.e.*, osteopontin and uromodulin). Underscoring the accuracy of the assay is the fact that although the assay also targets twenty other high specificity biomarkers for five biological stains other than urine, not a single non-urine associated protein was detected. The assay results obtained with representative samples of the other five body fluids of interest also proved to be of equivalent quality and specificity



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							

Executive Summary Figure 4 – Q-TOF multiplex assay results (chromatogram on top and peptide search results on bottom) from the analysis of an unfractionated urine sample. Out of a total of 45 ions being scanned for, only the targeted ions for the high-specificity urine bio-markers (uromodulin and osteopontin) were detected. None of the targeted non-urine bio-markers were detected.

The inter-individual reproducibility of target ion detection was also found to be excellent across multiple samples. As indicated by a peptide intensity distribution plots and corresponding chromatography results from multiple individuals, target peptides were consistently detected in multiple samples with a highly reproducible retention time. It should be pointed out, however, that there was significant interindividual variability in the amount of target ion detected between samples. This inter-individual variability in protein expression was not unexpected and did not appear to interfere with or compromise the accuracy of the assay.

Evaluation of Biomarker Expression Across an Expanded Sample Population **–** The forensic applicability of the candidate biomarkers necessitates a more comprehensive and validation of each candidate marker for stain specificity with a larger population set. Only when these larger-scale studies are completed, can these markers move from being candidates to serving as the foundation for a commercial multiplex assay system capable of characterizing both single source and mixed-source stains with high specificity. There are good reasons for this. For example, the possibility cannot be ignored that some candidate biomarkers might be secreted into non-target fluids in the same way that A, B, and Rh factors in blood are found in the saliva or semen of individuals termed secretors. To assess the broader applicability of each biomarker in terms of its specificity for a given body fluid, multiplexed Q-TOF analyses of single-source body fluid samples from a sample population of fifty human research participants were conducted. This made it possible to empirically assess the frequency at which target biomarkers may be detected in non-target body fluids. The results obtained are summarized below and in Executive Summary Figure 5.

Seminal Fluid: The candidate high-specificity markers of seminal fluid (semenogelin I/II, epididymal secretory protein E1, prostatic acid phosphatase and prostate specific antigen) were consistently and unambiguously detected in all semen samples. These markers were generally undetectable in non-target body fluids markers except for trace amounts of semenogelin I/II, epididymal secretory protein E1, prostatic acid phosphatase and prostate specific antigen were observed in 20-80% of male urine samples. This may represent leakage from the reproductive system or residual ejaculate. Epididymal secretory protein E1 was also detected in female urine at nearly the same frequency as in male urine samples. Moving forward, epididymal secretory protein E1 will be dropped as a high-specificity marker of seminal fluid and quantitative criteria along with the presence/absence of high-specificity urine biomarkers will be investigated as a means of discriminating between ejaculate and male urine.

Urine: The candidate high-specificity markers for urine, uromodulin and osteopontin, were unambiguously detected in all male and female urine samples. These markers were not detected in any non-target body fluids. Thus, uromodulin and osteopontin appear to be suitable high-specificity biomarkers for urine.

Saliva: Among the candidate high-specificity markers of saliva (cystatin SA, cystatin D, submaxillary gland androgen-regulated protein, histatin-1, statherin and mucin 5B) only submaxillary gland androgen-regulated protein was clearly detected in 100% saliva samples, including all female saliva samples despite the somewhat misleading descriptor of the protein as being “androgen regulated”. Three other candidate biomarkers (cystatin SA, statherin and mucin 5B), however were detected in greater than 90% of saliva samples assayed. Further improvements in assay sensitivity may make it possible to determine whether or not these proteins are present in all saliva samples – albeit at low levels. None of these markers were detected in any of the other five body fluids analyzed. Finally, although mucin 5B was ubiquitously present in most saliva samples, it was also detected in 20% and 38% of menstrual blood and vaginal fluid samples, respectively and in 4% of urine samples. Moving forward, cystatin D, histatin-1 and mucin 5B will be dropped as high-specificity markers for saliva.

	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%

Executive Summary Figure 5 – Results of targeted ion Q-TOF 6-body fluid multiplex assays of fifty samples each of saliva, seminal fluid, vaginal fluid, menstrual blood and peripheral blood and twenty-five samples each of female urine and male urine. Targeted biomarkers are listed across the top of the figure and the body fluids tested are listed along the left side of the figure. Each sample was scanned for the presence of 45 targeted ions representing 6 peripheral blood peptides, 11 saliva peptides, 10 seminal fluid peptides, 4 urine peptides, and 14 vaginal/menstrual blood peptides. 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 either detected in less than 100% of their target body fluid samples or were detected in a non-target body fluid. Red cells indicate cases where a specific biomarker was not detected in any of the samples tested.

Peripheral Blood: Among the candidate high-specificity markers of peripheral blood (hemoglobin subunit beta, complement C3, and hemopexin), hemoglobin subunit beta, complement C3 were readily detected in all peripheral blood samples and hemopexin was detected in 96% of peripheral samples analyzed. These markers were also detected in menstrual blood where it was expected that all three biomarkers would also be present since peripheral blood is a major component of menstrual blood. Among non-target body fluids, two of the candidate peripheral blood biomarkers (hemoglobin subunit beta and hemopexin) were also detected in a small number of urine and vaginal fluid samples and hemoglobin subunit beta was detected in a small number of saliva samples. It is hypothesized that these anomalous results may not be true false positives but rather samples that did contain small quantities of peripheral or menstrual blood such as from flossing teeth, urinary infections, minor vaginal abrasions or residual menstrual blood in the vaginal canal.

Vaginal Fluid and Menstrual Blood: Among the candidate high-specificity markers of vaginal fluid (cornulin, IgGFc-binding protein, neutrophil gelatinase-associated lipocalin, Ly6/PLAUR containing protein 3, suprabasin, and matrix metallo-proteinase-9), the biomarkers cornulin, neutrophil gelatinase-associated lipocalin and Ly6/PLAUR containing protein 3 were consistently and unambiguously detected in all vaginal fluid samples tested. No vaginal fluid markers were detected in saliva, seminal fluid or peripheral blood samples. The detection of cornulin and Ly6/PLAUR containing protein 3 in a single female urine sample was not anticipated based on *in silico* analyses of available proteome databases and thus may reflect inadvertent transfer of proteins from the vagina during collection.

As expected, peripheral blood markers (hemoglobin subunit beta, Complement C3, and Hemopexin) were also detected in menstrual blood samples. Additionally, the vaginal fluid biomarker, cornulin, was detected in 20% of menstrual blood samples. It is hypothesized that the overall low frequency with which vaginal markers were detected in menstrual blood may reflect matrix effects and the use of a menstrual cup which minimizes contact between menstrual blood and the vaginal canal.

Biomarker Detection with Casework-Type Samples **–** While pristine samples of biological stains can be used to validate the specificity of each candidate biomarker for a given body fluid, the applicability for use by forensic practitioners and the potential for developing a commercial platform necessitates a second more rigorous set of validation studies to assess the stability and reliability of these biomarkers in a forensic context.

A total of 37 unique casework-type samples are presented in figures 10 and 11. All single-source samples of human body fluids spotted onto sterile cotton swabs were accurately identified by the detection of one or more of the high-specificity biomarkers that were expected for each body fluid. No unexpected biomarkers for any body fluid other than that being assayed were detected. The detection of epididymal secretory protein E1 (seminal fluid biomarker detected in male urine), for example was anticipated based on the results of earlier studies.

Recovery of single-source body fluid samples from a variety of substrates ranging from a latex condom to ceiling tile and denim did not impede the accurate characterization of the body fluid being assayed with one notable exception. All three saliva swabs in the substrate studies revealed the presence of Ly6/PLAUR containing protein 3, a vaginal fluid specific biomarker. Although this has not been seen in any prior saliva samples, this protein has been reported in association with the proteome of tissues from the back of the throat. While further study of this

finding is clearly warranted, this unexpected result underscores the importance of conducting rigorous validation studies on casework-type samples.

A series of 2-component mixtures of human body fluids were analyzed by the multiplex assay to evaluate the accuracy with which both components could be accurately identified using a single-pass assay approach. In all but one case (equivalent volumes of saliva and peripheral blood), at least one high specificity biomarker for each body fluid present in the mixture was readily detected. 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.

A series of single-source body fluid samples were also assayed for the influence of potential endogenous inhibitors on biomarker detection. Of the potential inhibitors assayed only chewing tobacco juice appeared to preclude the identification of a target body fluid (*i.e.*, saliva). Clearly, however, this is another area where an internal positive control for inhibition may need to be developed.

Finally a series of dilutions were analyzed to determine the lower limit of detection for each body fluid based on the detection of at least one high-specificity protein biomarker. These results indicate the potential to detect body fluids at the nanoliter or subnanoliter scale. Estimates of the lower limit of detection should be viewed with caution since they have been calculated from data on serial dilution of a body fluid extract, rather than on data from direct extracts of actual trace samples and do not reflect the potential for matrix effects in mixed-source samples.

Implications for Policy and Practice

Funding to the principle investigator through the current NIJ award (2009-DN-BX-K165) has made it possible employ cutting edge protein analysis technologies to identify and evaluate the specificity of an assemblage of high-specificity protein biomarkers for bodily fluids typically encountered in a forensic context. This information will help to facilitate the commercial production of such assays. This includes the development of a commercial mass spectrometry approach based on the multiplex assay described here. Alternatively, as forensic technology advances, these same protein biomarkers can be readily incorporated into lab-on-a-chip or other miniaturized formats. The experiments reported here coupled with publication in peer-reviewed journals, will help to place the findings of this research on sound legal footing.

Nonetheless, some casework samples can and will still present challenges that may not necessarily be anticipated or that can complicate interpretations. The release of small quantities of blood into the oral cavity as a result of using dental floss or a minor injury to the inside of the mouth may be detected as a mixed stain – which it is. In such cases, it will fall to the experienced judgment of the serologist to make an interpretation with regard to the potential significance of the mixture.

Implications for Further Research

In the studies reported here, a quadrupole time of flight (Q-TOF) mass spectrometer was used. This instrument platform, however, proved unacceptably slow for practical applicability. Based on a series of preliminary experiments involving a three-stain (*i.e.*, saliva, semen, and vaginal fluid) multiplex assay, however, it was found that shifting to a higher-sensitivity triple quadrupole (QQQ) platform resulted in both higher-quality results and faster assay times. One promising direction for future research, therefore, would be to fully develop a QQQ multiplex by incorporating the high-specificity biomarkers identified in the current project into the assay. One

could then thoroughly assess the performance limits of an improved assay and thus its potential applicability to casework.

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FINAL TECHNICAL REPORT (MAIN BODY)

Introduction and Statement of the Problem

Blood and semen factors that once held promise as discriminatory instruments for individualizing biological stains have been supplanted by DNA markers, which can be amplified from tiny amounts of biological material. While DNA analysis of an evidentiary swab may reveal the presence of a DNA profile consistent with an alleged victim, the DNA profile cannot indicate whether the DNA came from saliva, vaginal fluid, urine or a host of other sources. The ability to confidently associate a DNA extract with a specific tissue source or to accurately characterize mixed stains, however, can provide criminal investigators with critical information.

Consider the case of an alleged sexual assault where a DNA profile consistent with the victim is found on the mouth of a bottle in the suspect's possession. The victim states that the suspect used the bottle as a foreign object to penetrate her vaginally. The suspect counters that the alleged victim had drunk from the bottle and that no sexual contact occurred. Both stories may explain the presence of the victim's DNA on the bottle. The ability to reliably detect traces of vaginal fluid or potentially a mixture of both vaginal fluid and saliva in this case could help to either confirm or refute these opposing claims. In another example, DNA consistent with the victim of an alleged sexual assault is found on a hand towel from the suspect's van where the alleged assault took place. The victim claims that the attacker wore a condom and that she had used the towel to wipe blood from her vaginal area after the assault. The suspect claims that the victim was a hitchhiker to whom he had offered a ride. He claims that no sexual contact occurred. Rather, he claims that he handed the victim the towel when she developed a nose bleed in his van. Again, the ability to reliably detect a mixture of both blood and vaginal fluid in this case could help to confirm or refute these claims. A broad variety of other scenarios can easily be imagined where the ability to differentiate between menstrual and peripheral blood, or urine and saliva would have equally important probative value. In fact, though, one needn't imagine such scenarios because they are a reality that forensic analysts currently encounter all too often.

Review of Relevant Literature: Current Approaches to Stain Identification

While tests for the presence of blood, semen, saliva and urine have long existed[1-4], some are laborious (*e.g.,* creatinine test for urine). Others require that serologists be proficient at a variety of methodologies, some of which employ reagents that pose health and safety risks. For example, the chemical instability of picric acid (used by some labs to test for urine) presents an explosion hazard and is toxic to liver and kidney tissue.

Other serological tests can consume significant amounts of a valuable sample while still failing to provide forensic practitioners with optimal sensitivity or specificity. For example, some tests for saliva may consume half of an evidentiary swab while still presenting a complex challenge. The detection of saliva is generally based on assays for the presence of the enzyme α-amylase (*i.e.*, salivary amylase)[24] activity. This requires the preservation of enzyme function – a factor that may make it difficult or impossible to test aged and weathered material or items contaminated with substances that inhibit enzyme activity. Additionally, α-amylase activity is also present in a variety of non-salivary body fluids including human blood serum, urine and cervical mucus[25-27], although normally at much lower levels than in saliva. As a result, even though a vaginal swab tests positive for amylase, the analyst is not able to tell a jury that the presence of saliva on that vaginal swab has been confirmed. Being well aware of the presumptive

nature of this test, forensic analysts are cautious and typically limit their interpretation and courtroom testimony in this scenario to stating that "a positive amylase result is consistent with saliva" and by extension "perhaps consistent with oral-genital contact".

Tests for evidence of vaginal contact have proven even more challenging. Over the years, this has involved attempts to identify vaginal epithelial cells in evidentiary samples. While the use of histochemical stains (*e.g.*, Christmas Tree stain[28]) to detect sperm cells is routine in forensic laboratories, 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 which detects glycogenated cells held promise for identifying vaginal cells[5, 6]. This was based on studies suggesting that vaginal cells contained more glycogen than other epithelial cells. If so, this could facilitate the detection of vaginal cells on penile swabs from some sexual assault suspects. Rigorous studies, however, revealed that Lugol's positive cells were also present in the male urethra[7], male urine deposits[8] and on >50% of penile swabs from males who had abstained from sex for several days[9]. Lugol's positive cells have also been identified among the epithelial cells of the oral mucosa[9]. Modifications to improve the reliability of Lugol's test have been suggested[10] but are not conducive for use with casework. Similarly, a modified Dane's staining technique is able to differentiate among pure samples of vaginal, buccal and skin cells[29]. This same test, however, 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 limits the forensic utility of this approach. We are not aware of any public forensic laboratory that currently employs histological staining to reliably identify vaginal epithelial cells.

In contrast to this, the routine testing for blood and seminal fluid by forensic laboratories has been greatly facilitated by the development of commercial forensic kits based on the detection of antigen-antibody interactions. These one-step immunoassay tests have provided forensic practitioners with good specificity and excellent sensitivity. For example, the ABAcard (Abacus Diagnostics) and HemeDirect (Seratec®) kits use the protein hemoglobin while the RSID™-Blood (Independent Forensics) uses the protein glycophorin A as markers for the presence of blood[4, 11, 12]. Similarly, the p30 (Prostate-Specific Antigen) protein serves as a marker for the presence of seminal fluid[3, 11, 30]. However, p30 can also be found in female ejaculate[31], breast milk[32], urine[33] and other non-target fluids (albeit at lower concentrations). Semenogelin is employed as a high-specificity marker by the RSID™-Semen kit[34]. As with any immunoassay, however, results are "presumptive by definition" because the potential for antibody cross-reactivity with non-target molecules (although remote) can never be eliminated[13]. Moreover, casework-type samples may include environmental contaminants or other factors related to sample processing that can interfere with antibody binding, thereby reducing assay sensitivity[14]. Only the direct visual identification of sperm cells by light microscopy enables an analyst to report a confirmatory result. It is often difficult and laborious, however, to locate sperm cells in close association with epithelial cells or non-cellular debris. Fluorescence microscopy can facilitate sperm identification [35] but microscopy in general is useless for analyzing samples from vasectomized or otherwise aspermatic males or with degraded material lacking detectible sperm heads.

For a range of other body fluids, forensically-validated commercial kits based on body fluid specific antigens are lacking entirely and this often leaves the forensic analyst without the ability to make a substantive statement about the potential tissue source of a DNA profile. Part of the reason for this is that unlike hemoglobin, p30 and semenogelin (which are abundant and relatively specific antigenic markers), much less has historically been known about the diversity,

relative abundance and stability of proteins that might have potential utility as markers for other forensically-relevant body fluids. Moreover, traditional protein detection methods often lacked the sensitivity required to detect low-abundance biomarkers in casework samples.

Review of the Relevant Literature: Emerging Approaches to Stain Identification

While existing methods of forensic body fluid identification are presumptive and rely on a variety of antibody-, enzyme activity- and chemical reaction-based tests, several novel approaches to biological stain identification are now being explored. These research efforts have as their goal the development of a more sensitive and uniform strategy for analyzing body fluids capable of providing analysts with confirmatory results. Emerging approaches include biological stain identification based on messenger- and micro-RNA expression profiles, epigenetic modifications, Raman spectroscopy and protein-biomarker detection by mass-spectrometry. Each of these proposed methods have their own strengths and weaknesses. Accordingly, they are not so much competing or mutually exclusive technologies, rather, they are potentially complementary technologies that will make it possible for analysts to obtain useful information from a much larger range of casework samples[36]. These emerging strategies also offer an opportunity for greater standardization and automation of biological stain analysis as well as the incorporation of additional tests for body fluids which are not covered by existing methods. The potential to bring greater uniformity, standardization and thus automation to forensic serological testing would be akin to the type of progress that has been achieved over the past couple of decades in DNA profiling.

The "biomolecular profile" of a specific body fluid is a function of the subset of genes that are transcribed into mRNA and then translated into protein. Among the thousands of molecules present in any given body fluid, many will be common to several body fluids while others will be highly-specific markers of a single body fluid. By rigorously comparing the full complement of biological molecules of different body fluids, it is possible to generate a comprehensive database of molecules with potential forensic utility as unique markers of specific body fluids.

mRNA Markers amplified by reverse transcription PCR and detected by capillary electrophoresis are being studied as a means of identifying body fluids on the basis of differential expression profiles[15]. For example, matrix metalloproteinase mRNA transcripts from the endometrium have been investigated as a marker for menstrual blood[37]. In 2007, a multiplex assay for identifying blood, saliva, semen, and menstrual blood was developed based on mRNA markers[38]. Because of its compatibility with existing DNA amplification technology, mRNA profiling as a means of identifying body fluids has attracted significant research interest in recent years[39]. The presumed sensitivity of mRNA to degradation has often been raised as potential concern with this approach. An in-depth study of RNA recovery under a variety of conditions, however, found that RNA remained stable in environmental samples that had been kept dry and could be recovered after 180 days of storage[40] while samples exposed to rain were unrecoverable after one to seven days.

microRNA Markers are non-coding molecules involved in post-transcriptional regulation of gene expression. Because of their short lengths (generally <25nt) and evidence of tissue-specific expression patterns[41], they have been explored as promising markers for the characterization of more highly degraded samples where longer mRNA targets might be difficult to amplify. Subsequent studies of candidate miRNA markers have often revealed low-level expression in non-target tissues or lack of tissue-specific reproducibility between studies[16, 41], researchers have shifted their attention to the use of quantitative PCR combined with mathematical approaches that may allow a target stain to be identified on the basis of a broader miRNA

expression profile rather than on the absolute presence or absence of a given marker[42]. While continuing to hold promise, it has also been pointed out that the use of miRNAs for multiplex biological stain assays may be technically difficult due to limitations on the number of fluorescent tags currently available for quantitative PCR assays.

Epigenetic Markers rely on tissue-associated differences in DNA methylation patterns[43, 44] as a means of identifying different biological stains. 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[17, 18]. Potentially complicating the use of epigenetic assays, though, is the observation that while tissue-specific methylation differences can be identified within an individual, significant inter-individual epigenetic variation in these tissue-specific patterns also exists[45]. In addition, global changes in DNA methylation are known to be associated with cancers[46] and other diseases[47] and an emerging body of evidence points to environmental factors that may impact DNA methylation patterns[48]. Finally, similarities in such developmentally related tissues as the male prostate and female periurethral glands[31] could complicate efforts to accurately interpret some assay results.

Raman Spectroscopy is an approach to body fluid identification based on the inelastic scattering of laser light as it interacts with proteins and other molecules present in a sample. In an effort to accommodate sample heterogeneity, a multidimensional "spectroscopic signature" is created and advanced statistical analysis is used to search for the best match between an expected "spectroscopic signature" and that of a questioned sample. Promising results with single source stains have been reported using this approach[19] which has the advantage of being non-destructive and rapid. Because of its reliance on statistical pattern fitting, however, it is unclear to what extent this strategy can accommodate more challenging mixed stains such as those containing contaminants or that are degraded, *i.e.*, any forensic type sample that deviates substantially from the reference "spectroscopic signature".

Protein biomarkers have attracted significant interest in recent years due in large part to the strides that have been made in the tools to identify and characterize them. It is now possible to rigorously map entire proteomes with high reproducibility using automated 2-dimensional HPLC systems or MudPIT (multidimensional protein identification technology) to identify potentially useful biomarkers. Once identified, mass-spectrometry-based targeted-ion assays facilitate the unambiguous detection and quantitation of even low abundance proteins, against a background of other non-target molecules. This has resulted in a wealth of new opportunities to develop protein-based assays for medical and forensic applications such as body fluid identification.

In addition to the protein biomarker-based multiplex assay described in this application, other researchers have also reported success using protein biomarkers. Using a panel of biomarkers identified through a literature search and empirical studies, a multiplex assay for blood, saliva and semen was developed on a MALDI-TOF mass spectrometer platform. The assay was able to characterize single and mixed biological stains in the nanoliter range and worked well with forensic type samples aged up to 20 months[20]. Unfortunately, the candidate biomarkers for detection of menstrual blood and vaginal fluid were not detected by MALDI-TOF. Ongoing efforts are directed at identifying new candidate markers for these fluids. As with other types of markers, it is also recognized that protein profiles may be altered by biological perturbations due to disease and interindividual variability.

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 epigenetic 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[21, 22]. 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[23]. 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[49].

Statement of Hypotheses and Core Research Objectives

The central goal of the current research project was to evaluate the stain-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 can complement the use of DNA profiling by making it possible to more accurately and confidently associate a DNA sample with a specific type of biological stain. The lack of such biomarkers can present forensic serologists with a significant challenge in many criminal cases.

Fundamental Hypotheses – The successful achievement of this goal rested upon four major hypotheses. Specifically it was hypothesized that:

1) sufficient differences exist in the proteomes of individual body fluids so as to allow for the identification of individual body fluids with a high degree of specificity – ideally to the exclusion of all other body fluids.
2) sufficient similarities exist across human populations that proteins specific to a given body fluid would be expressed in most if not all humans; thereby ensuring the broad applicability of stain identification assays based on the use of high-specificity protein biomarkers.
3) a multiplex Quadrupole Time-of-Flight (Q-TOF) mass spectrometry assay will enable the reliable and reproducible single-pass validation of candidate biomarker specificity across six body fluids and a sample population of 50 human research subjects.
4) a multiplex Q-TOF mass spectrometry assay will facilitate the accurate characterization of both single and mixed biological stains in casework-type forensic samples.

Core Research Objectives – The current proposal seeks to improve and provide new tools for bodily fluid identification through the completion of three Core Research Objectives. These are to:

(1) Recruit volunteers and collect samples of forensically relevant body fluids (*i.e.*, peripheral and menstrual blood, vaginal secretions, seminal fluid, urine and saliva) from a study population of 50 individuals/bodily fluid.

(2) Develop and test a high-sensitivity Q-TOF assay which combines six panels of

highly-specific protein biomarkers into a single multiplex assay to evaluate the target stain specificity of the candidate biomarkers identified under NIJ 2006-DN-BX-K001 across 50 samples of each of six forensically-relevant body fluids.

(3) Evaluate the performance highly-specific protein biomarkers and the Q-TOF multiplex assay using forensic casework type samples to determine the extent to which the biomarkers evaluated under Core Objective #2 can be used to reliably identify specific biological fluids in a forensic context.

The completion of these objectives will aid forensic analysts by providing the forensic community with a validated panel of protein biomarkers which are specific to forensically relevant body fluids and which can be used to in a forensic context. This will facilitate the subsequent development of a high-throughput multiplex commercial assay system capable of accurately characterizing biological stains from which DNA is extracted.

Methods

Human Subjects – The University of Denver Institution review Board for Research Involving Human Subjects (IRB) reviews all research involving human subjects, regardless of funding source, to ascertain that the rights and welfare of subjects are being protected. The IRB is responsible for assuring that recruitment advertising is not misleading or coercive to the research subject. All projects using human subjects are reviewed no less than annually.

All research conducted under DNA Research and Development Award 2009-DN-BX-K165 was IRB reviewed, approved and conducted in full compliance with U.S. Federal Policy for the Protection of Human Subjects (Basic DHHS Policy for Protection of Human Research Subjects; 56 FR 28003). A total of 100 adult (>18 y.o.) human volunteers (50 males; 50 females) were recruited for this study from within the University of Denver student population. An important consideration in determining an appropriate sample size was the impact of the number of individuals sampled on our ability to reliably capture the intrinsic variability present in a given population. For an infinite population under an assumption of a standard normal distribution, the 95% confidence interval is 1.96 standard deviations. By comparison, that the 95% confidence interval for a sample size of 5, 50, and 100 would be 2.78, 2.01 and 1.98, respectively. Balancing the importance of capturing statistical variability with the time and financial limitations of the project, therefore, it was determined that a sample population of 50 individuals per body fluid would make it make it possible to reliably discriminate between proteins that are specific to a given body fluid *vs.* those that varied between individuals or were present in non-target stains. While the study participants reflected the ethnic and age diversity of the University of Denver student population, there were an insufficient number of study participants to enable a statistically substantive partitioning of the sample on the basis of biogeographic origin or broad age cohorts. It should be emphasized, however, that while such detail was beyond the scope of the current project, it will be addressed as a part of future validation studies.

The purpose and significance of the research and the methods that would be used to collect body fluid samples was thoroughly explained to each volunteer. All participants then signed a statement of informed consent to participate in the research. Recruitment notices were posted in campus science buildings to attract interested volunteers. The student traffic in these buildings consists primarily of science-oriented graduate and undergraduate students. As no health care associated information was collected, HIPPA authorization was not required.

Body Fluid Collection and Protein Extraction – A total of fifty samples of each of six forensically-relevant body fluids (*i.e.*, peripheral and menstrual blood, semen, saliva, vaginal secretions and urine) were collected for protein analysis. The choice of the bodily fluids to be analyzed and the size of the study population reflected discussions with forensic practitioner at state and private caseworking laboratories including forensic serologists at the Colorado Bureau of Investigation. In addition, an expert in the forensics of sexual assault examination has helped to guide this research to best meet the needs of the forensic community. The procedures employed for sample collection were in accordance with the NIH guidelines.

Salvia: Donors were directed to gently brush their teeth and 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. 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 which was transferred to 15 ml conical vial and centrifuged again at 13,000 x g for 20 minutes at 4°C. Supernatant-containing proteins were filtered through a .45 µm filter to remove remaining debris prior to concentration.

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 at the donor's earliest convenience (within 1 hour). Semen was then incubated at room temperature for at least 30 minutes to allow it to liquefy. After transfer to a 15 ml conical vial and dilution with 1/3 volume PBS, the sample was centrifuge at 13,000 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.

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. Blood serum was removed to a 15 ml conical vial and then passed through a .45 µm filter to remove cellular material prior to immunodepletion and protein concentration.

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 concentrated to ensure a sufficient quantity of protein for proteome mapping. After transfer to 50 ml conical vials, the urine was centrifuged at 13,000 x g for 20 minutes at 4°C and passed through a .45 µm filter to ensure cellular removal prior to concentration.

Vaginal Secretions: Following clinically accepted procedures, vaginal secretions were self-collected by study participants in the privacy of their home. Subjects were financially compensated for their participation. The collection protocol employed an FDA-approved over-the-counter latex-free, hypoallergenic cup (SoftCup™). The device is similar to the hypoallergenic menstrual cup which is used as a tampon replacement during menses. For the collection of vaginal secretions, donors were instructed to insert the Softcup™ for periods of up to 12 hours and then deposit the secretions into a 50 mL sterile collection container. Donors were directed to refrigerate the sample until it could be transported to the lab at their earliest

convenience (typically within 1 hour). Upon receipt, the liquid was transferred to 50 ml conical vials and centrifuged at 13,000 x g for 20 minutes at 4°C. The resulting supernatant was passed through a .45 µm filter to ensure removal of the cellular component prior to concentration.

Menstrual Blood: Following clinically accepted procedures, menstrual blood was self-collected by study participants in the privacy of their home. Subjects were financially compensated for their participation. 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 for the first evening of menses in addition to the day before and the day after. After menses had started, the cup remained in place for up to one hour at a time. The cup was then gently removed; the contents were poured into a sterile 50ml conical tube and refrigerated until delivered to lab (within 1 hour). Blood serum was removed to a 15 ml conical vial and then passed through a .45 µm filter to remove cellular material prior to hemoglobin removal, immunodepletion and protein concentration. It should be noted that while some menstrual blood samples were still liquid and relatively uncoagulated upon their delivery to the lab, other samples contained significant clots. These samples were homogenized to break up the clots so as to facilitate sample processing. The disaggregation of the clotted material did not have an effect on the outcome of the assay.

Protein Concentration, Partitioning and Quantification **–** Corning Spin-X UF concentrators (3000 NMWL) (Corning, Lowell, MA) were used to concentrate low protein content body fluids such as saliva and urine while at the same time removing unwanted salts and other low molecular weight components.

Serum obtained from menstrual blood samples was typically contaminated with erythrocyte cellular components due to the lysing of fragile red blood cells that are abundant in the endometrial lining during menses. In addition, the presence of several high-abundance serum proteins common to both peripheral and menstrual blood made it difficult to fine tune the targeted-ion protocol for the less abundant but more specific biomarkers for these body fluid. To circumvent this problem during the initial development phase, commercially available IgY-12 Proteome Partitioning columns were employed. These antibody-based columns made it possible to remove twelve highly abundant proteins 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.

The Thermo Scientific Pierce Micro BCA Protein Assay (Thermo Fisher Scientific, Rockford, IL) was used to determine final protein concentration of each extracted sample. All samples were stored in a locked -70°C freezer until analyzed.

Mass Spectrometry **–** Protein extracts for analysis by mass spectrometry were transferred to 1.5 ml low retention microcentrifuge tubes and 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-HCl (Tris(2-carboxyethyl)phosphine hydrochloride) reducing agent and then shaken for 20 minutes at room temperature. The proteins were then alkylated by the addition of 0.88ul of 500 mM IAA (Iodoacetamide) and the sample was shaken in the dark for 15 minutes. The proteins were sonicated and digested overnight with trypsin at 37°C. Digested samples were then purified on a C-18 spin column, dried and resuspended in 3% acetonitrile and 0.1% formic acid. It should be noted that because disulfide bonds may be more efficiently alkylated under denaturing conditions, proteins were denatured in 8M urea. If the proteins had not been denatured, the efficiency of protein cleavage by trypsin would also have been reduced.

A quadrupole-time of flight (Q-TOF) mass spectrometer assay was developed to "selectively target" specific candidate biomarker ions with femtomole (1×10^{-15}) sensitivity such that unfractionated biological stains (*e.g.*, semen, saliva or vaginal fluid) could be scanned for the presence or absence of an entire panel of biomarkers in a single run. Using data generated from samples of individual body fluids and data generated during the initial biomarker discovery project, optimal precursor ions for each candidate protein biomarker were selected for a multiplex body fluid assay. Ideal precursor ions were those that ionized consistently; did not co-elute with >5 peptides present in other candidate biomarkers; and had high signal intensity.

After carefully selecting optimal precursor ions for each candidate biomarker, these were combined to develop body fluid specific assay inclusion lists. These inclusion lists direct the "targeting and isolation" of specific sets of precursor ions at specific time points during the Q-TOF run, The "isolated" precursor ions are then fragmented to produce "product ions" - the detection of which confirms the presence of the original biomarker**.**

A multiplex assay was developed to simultaneously scan for the presence of six different body fluids in 44-minutes. This assay targets a total of 45 individual precursor ions consisting of 6 peripheral blood peptides, 11 saliva peptides, 10 seminal fluid peptides, 4 urine peptides, and 14 vaginal/menstrual blood peptides.

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. Run conditions employed 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 reequilibration at 3% A.

Data Analyses - 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 peptide scores >16 were considered confident matches. In accordance with the manufacturer's specifications, peptide scores of 15 or greater, in combination with a percent scored peak intensity (%SPI) of 70 or greater, are almost certain to represent valid results. Peptide scores less than 6 seldom represent valid interpretations unless the spectra originated with an instrument capable of accurate mass measurements (*e.g.*, Agilent Q-TOF). The peptide score is calculated by comparing the observed MS/MS fragmentation spectra to that of the theoretical fragmentation of the peptide. The software adds points to the score for fragments which correlate with what is expects and subtracts points for the detection of peaks which are "unassigned" compared to the theoretical spectrum. Thus, the %SPI is the percentage of peaks which are assigned compared to total number of peaks. Accordingly, the higher the SPI and scores the more the observed data can be assigned/explained. The values of 16 and SPI of 70% are the common identification benchmarks used throughout the professional literature. In addition, the current research also used a decoy database to assess the potential false discovery rate. This made it possible to determine if the identification results were due to random matches rather than true identification. The % false discovery rate for all our ID's were less than 1%.

Casework Type Samples **-** The applicability of a mass-spectrometry based body fluid assay to samples encountered in a forensic context was assessed using a series of casework type samples. Specifically, the ability of the biomarkers to be detected in body fluid samples recovered from a variety of substrates including as nylon carpeting, cotton cloth, leather, blue denim fabric,

concrete, latex, cigarette butts, and plastic surfaces consistent with foreign objects that might be used in a sexual assault was tested. Similarly the impact of exposure to environmental contaminants/insults was assessed. For these assays, aliquots of bodily fluids applied to sterile cotton tipped applicators that had previously been dipped in such agents as spermicidal lubricants, Bluestar®, soil, chewing tobacco and coffee were used. Finally, a series of 2-component body fluid mixtures were analyzed to assess the ability of more than one body fluid to be detected simultaneously.

Results, Discussion and Conclusions

Collection of Forensically Relevant Body Fluids **–** The first core objective of the research was the collection of samples of forensically-relevant body fluids (*i.e.*, peripheral and menstrual blood, vaginal secretions, semen, urine and saliva). For each body fluid, a total of 50 individuals were recruited. This sample size was selected to make it possible to discriminate between proteins that are specific to a given body fluid *vs.* those that varied between individuals or were present in non-target stains. The choice of these fluids reflected discussions with forensic serologists and other forensic practitioners.

Samples of urine and semen were self-collected by the study participant before being applied to sterile cotton tip applicators and dried. Saliva was collected on Salivette® pads. Small volume peripheral blood samples were obtained by automatic lancets (*e.g.*, Fingerstix™) and larger volumes by venipuncture at the University of Denver Health Center by a certified professional. Menstrual blood was self-collected using a hypoallergenic menstrual cup (*e.g.*, Diva Cup®). In accordance with the advice of Professor Patricia Speck, DNSc who has extensive experience as a sexual assault nurse examiner, the collection of vaginal secretions employed the Softcup™ menstrual solution. Centrifugation and/or filtration were used to enrich samples for the protein-rich extracellular fraction. This made it possible to overcome some of the obstacles that had been encountered early in the assay development phase of the project.

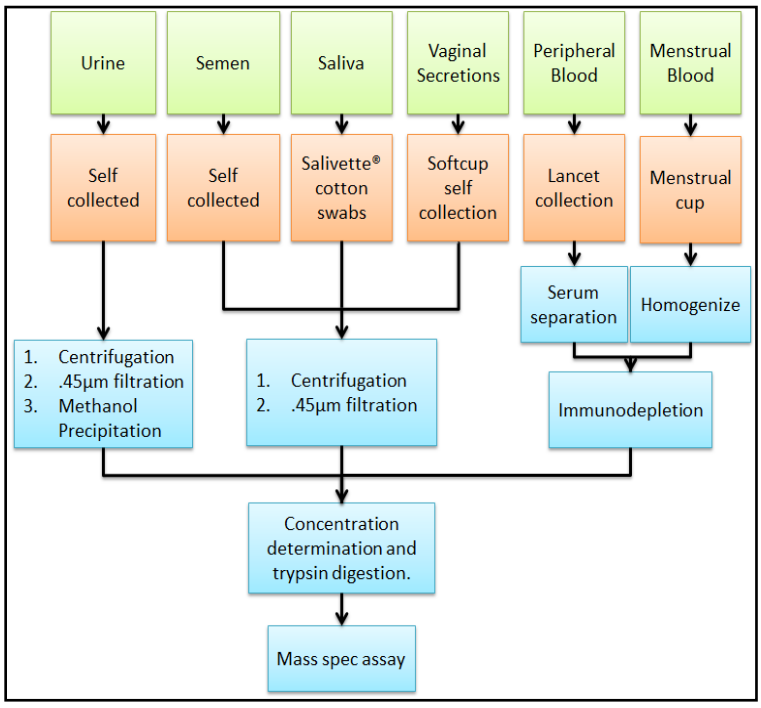


Figure 2 – Flow chart of the sample collection and preparation process for each of the biological stains (*i.e.*, body fluids) for which the high-specificity protein multiplex assay was developed. The various pretreatments used with each fluid were designed to facilitate the unambiguous identification of target proteins in the assay development phase of the project.

All samples were assigned alpha-numeric codes to ensure the anonymity of the donors and then stored in a locked freezer. These research methods have been approved by the University of Denver's Institutional Review Board for Research Involving Human Subjects and all samples were collected without incident. Figure 1 provides an overview of the collection and processing methods

employed for the assay-development and biomarker validation phases of the project.

Development and Testing of a High-Sensitivity Q-TOF Multiplex Assay – The second core objective of the research was the development of a high-sensitivity targeted-ion Q-TOF assay and the use of that assay to evaluate fifty samples of each body fluid for the presence or absence of individual candidate biomarkers. An important advantage of this approach was the ability to query a single sample for the presence or absence of biomarkers that are diagnostic for six different body fluids in a single pass.

Under a prior award, comparative "whole proteome" consensus mapping and electrospray ionization ion trap mass spectrometry was used to identify proteins with potential utility as biomarkers for the identification of biological stains. A major advantage of this "whole-proteome" approach is that it required no *a priori* assumptions with respect to the specific proteins expressed in any body fluid. Rather, the approach enabled a rigorous evaluation of the entire complement of proteins detected each proteome for their potential utility as bodily fluid specific biomarkers. ProteinMiner©, a bioinformatic software application written specifically for this purpose, was used to compare proteome profiles to look for potentially unique protein biomarkers. This approach enabled the identification of numerous candidate protein biomarkers that appeared to be highly-specific for individual body fluids including vaginal secretions. It is important to emphasize, however, that these protein biomarkers were identified by mapping the protein profiles of just five individuals per bodily fluid and thus can only be considered **candidate** protein biomarkers. While the use of even a relatively small sample group can help to reduce the potentially misleading impact of interindividual differences in protein expression, the ultimate applicability of a given biomarker for use with the general population necessitates a more comprehensive and thorough validation of each candidate marker for stain specificity across a larger population set.

In searching for an accurate and efficient means of validating the specificity of numerous candidate biomarkers across multiple body fluids, several approaches were considered. These included the use of antibodies and mass spectrometry. Though antibodies have a long history of robust reliability, their use as a means of validating the specificity of candidate protein biomarkers would have introduced several critical challenges. First would have been the need to obtain relatively large quantities of purified protein (most of which are not commercially available) for the immunization process. While pure proteins could have been prepared by heterologous expression, this is a laborious process that often fails to yield proteins in their native conformation – an essential requirement if an antibody is to be used to screen human samples. A second major challenge would have been the need to rigorously characterize the binding specificity of each of the resulting antibodies. Again, this is an extremely time, cost and labor intensive process. In short, the use of antibodies for the biomarker validation work outlined in this proposal would have been prohibitively expensive and could have easily added years to the project.

To circumvent these obstacles, a targeted quadrupole-time of flight mass spectrometer (Q-TOF) approach to biomarker validation was employed. As illustrated in Figure 2, this instrument allows specific ions of interest to be selected for in the first quadrupole (based on m/z ratio and retention time). A targeted Q-TOF approach employs ion "inclusion lists" that are generated based on the specific candidate biomarkers being validated. Each target ion in an inclusion list represents a trypsin digest product (*i.e.*, a peptide) which serves as a diagnostic fragment/ion of the candidate biomarker of interest. This targeted ion is selectively isolated in the first quadrupole from the background of non-targeted ions. The isolated ion is then forwarded to the

collision cell where it is further fragmented. The resulting fragments then enter a TOF mass analyzer which yields highly accurate product ion spectra thereby confirming the presence and identity of the original protein biomarker. If a candidate biomarker is not present in a given bodily fluid, then the corresponding diagnostic fragments of the biomarker will not be present in the first quadrupole and no protein will be detected by the TOF analyzer. Thus, Q-TOF-based targeted ion assays allows unprocessed biological stains to be directly injected and scanned for biomarkers of interest. The femtomole (1 $x10^{-15}$) detection capability of Q-TOF assays helped to ensure that even low-abundance candidate biomarkers could be reliably detected in a background of hundreds to thousands of higher-abundance proteins in complex samples such as semen, saliva or vaginal secretions, *etc.*. This minimized the potential for false negatives (*i.e.*, a failure to detect the presence of non-target biomarkers in the biological stains being assayed). Depending on the number of target biomarkers being scanned for in a given body fluid, a multiplexed assay generally takes less than 60 minutes to complete. An added benefit of employing this mass spectrometry-based approach was that it helped to establish a foundation for the possible future use of mass spectrometry by forensic practitioners to rapidly and accurately determine the identity of questioned biological stains from evidentiary material containing sub-microliter and even sub-nanoliter quantities of biological fluids. The overall development process for this approach is illustrated in Figure 3.

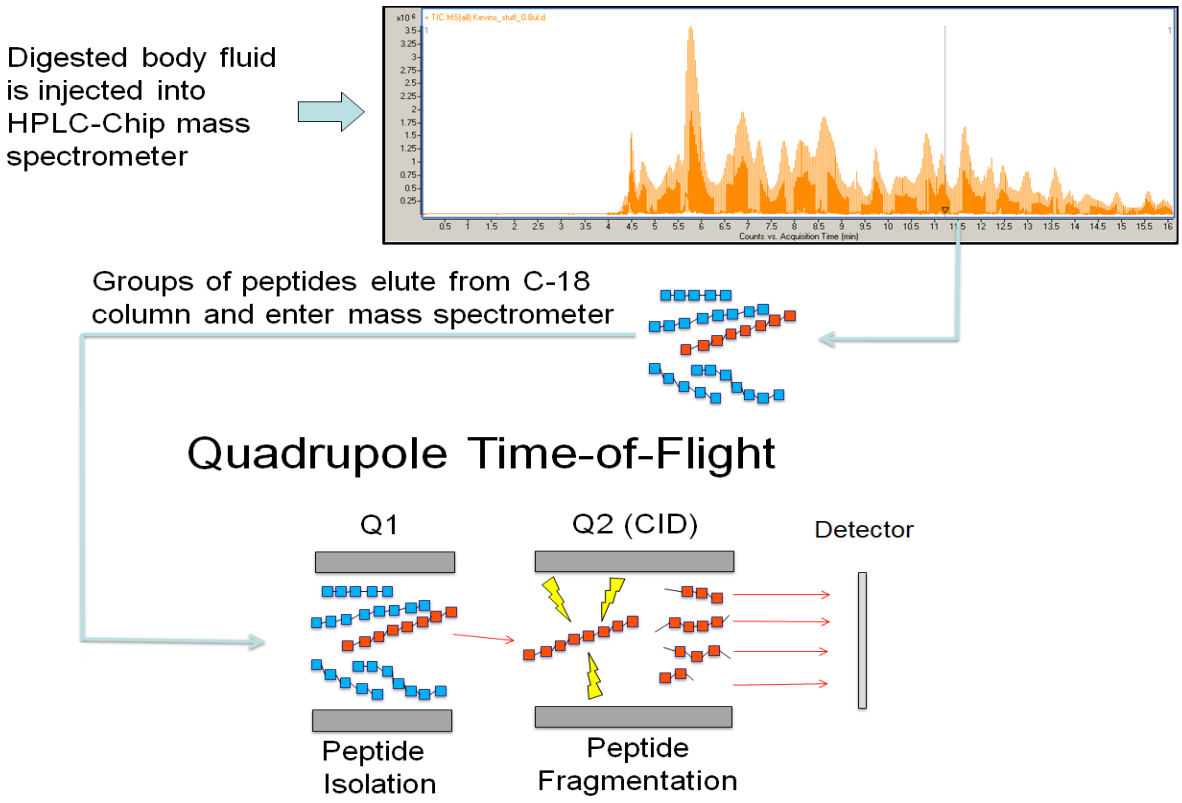


Figure 2 – Analysis of digested peptides using a flow-through HPLC chip MS approach. (Top) Post-digestion peptides bind to C-18 HPLC chip and elute into the Q-TOF mass spectrometer. (Middle) Peptides entering the Q-TOF are selectively isolated in quadrupole 1 (Q1) based on an assay-specific targeted ion inclusion list. Fragmentation of isolated target ions takes place in the collision cell (Q2) with the resulting peptide fragments then reaching the detector. Using inclusion lists. Only peptides that are included in the list are allowed to reach the detector – even against a background of hundreds or thousands of other non-target peptides.

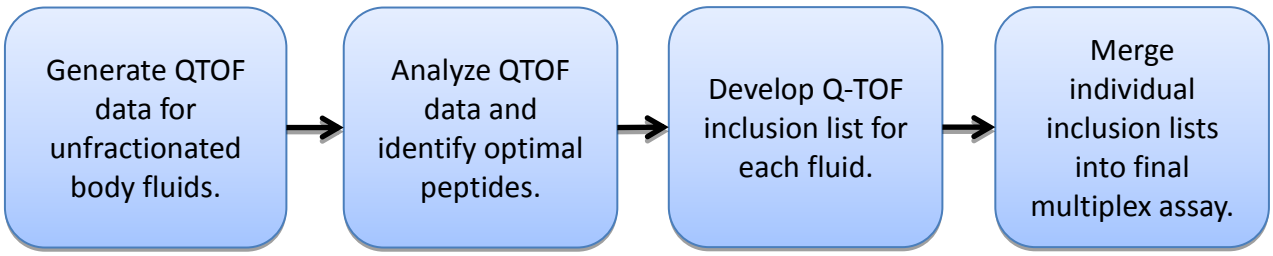


Figure 3 – Flow chart illustrating the biomarker assay development process. Based on the panels of candidate biomarkers identified previously from comparative proteome mapping, unfractionated biological stains were individually analyzed by Q-TOF to identify trypsin digest products (*i.e.*, peptides) which were optimal as "diagnostic" ions for each candidate biomarker. These "optimal peptides" for each body fluid were then used to construct an inclusion list for a singleplex targeted-ion assay for each body fluid. After confirming the ability of each singleplex to detect its target ions, the inclusion lists for multiple body fluids are merged to produce the final multiplex assay.

For assay development purposes, these candidate protein biomarkers for each body fluid were organized in order of priority with those most likely to be successfully validated at the top of the list. Several weighting factors were used to determine this ranking. First, based on information from publically available proteome databases, protein biomarkers for which there was evidence of possible expression in non-target body fluids were assigned a lower priority. Second, a higher priority was given to more abundantly expressed candidate biomarkers. Finally, a higher priority was given to biomarkers for which functional information indicated a greater relative potential for stain specificity.

During assay development, Q-TOF assays were run with only a limited number of samples for each target fluid because the objective was to compile inclusion lists of optimal ions and to fine tune their separation and detection. Ideal peptides were those which: (1) ionized consistently across multiple experiments; (2) had no post-translational modifications that would alter the mass-charge ratio and; (3) were of high abundance so as to facilitate detection. Using data from "unfractionated" samples run on the Q-TOF, optimal "diagnostic peptides" for each protein biomarker were selected and compiled into an inclusion list for each body fluid, (Tables 1A-1E). For each body fluid, the inclusion list delineates 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 combined into a multiplex assay. Based on the same principle illustrated in Figure 2 above, the multiplex Q-TOF-assay allowed unprocessed biological stains to be rapidly and efficiently "scanned" for specific biomarkers of interest such that it was possible to simultaneously test for multiple body fluids and to characterize even complex mixtures of body fluids. The multiplex assay was then retested with a subset of samples of each body fluid to identify possible matrix effects that could impede the performance of the assay. This process resulted in the development of the final multiplex assay illustrated in figure 4.

Table 1A Peripheral Blood Peptide Inclusion List

Protein Biomarker	**Target Biomarker Peptide**	**Prec m/z**	**Charge**	**Retention Time (min)**
Hemoglobin subunit beta	LLVVYPWTQR	637.8732	2	16.69
	GTFATLSELHCDK	493.577	2	10.79

Protein Biomarker	Target Biomarker Peptide	Prec m/z	Charge	Retention Time (min)
Complement C3	AAVYHHFISDGVR	491.2528	3	9.41
	VFLDCCNYITELR	851.9012	2	16.78
Hemopexin	NFPSPVDAAFR	610.8107	2	13.9
	YYCFQGNQFLR	748.474	2	13.98

Table 1B Saliva Peptide Inclusion List

Protein Biomarker	Target Biomarker Peptide	Prec m/z	Charge	Retention Time (min)
Cystatin SA	IIEGGIYDADLNDER	846.9146	2	11.89
	QLCSFQIYEVPWEDR	985.4583	2	19.77
Mucin 5B	GYQVCPVLADIECR	840.3965	2	16.51
	AAYEDFNVQLR	663.33	2	13
	AAGGAVCEQPLGLECR	844.3968	2	9.88
Cystatin D	SQPNLDNCPFNDQPK	887.3956	2	9.06
	LKEEEFCSFQINEVPWEDK	809.7150	3	17.8
Submaxillary gland androgen regulated protein	GPYPPGPLAPPQPFGPGFVPPPPPPPYGPGR	776.1541	4	21.6
	IPPPPPAPYGPGIFPPPPPQP	710.7205	3	19.9
Statherin	FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF	1215.8982	3	22.05
Histatin-1	EFPFYGDYGSNYLYDN	982.4056	2	20.093

Table 1C Seminal Fluid Peptide Inclusion List

Protein Biomarker	Target Biomarker Peptide	Prec m/z	Charge	Retention Time (min)
Semenogelin 1	KQGGSQSSYVLQTEELVANK	722.7071	3	13.91
	DIFTTQDELLVYNK	842.9264	2	17.87
Semenogelin 2	DVSQSSISFQIEK	734.3714	2	12.65
	DIFTTQDELLVYN	842.9264	2	17.87
PSA	VMDLPTQEPALGTTCYASGWGSIEPEEFLTPK	1175.5551	3	24.02
	LSEPAELTDAVK	636.8399	2	11.42
Prostatic Acid Phosphatase	ELSELSLLSLYGIHK	567.9866	3	21.549
	SPIDTFPTDPIK	665.8475	2	14.248
Epididymal secretory	AVVHGILMGVPVPFPIPEPDGCK	810.7632	3	25.327
	EVNVSPCPTQPCQLSK	922.4355	2	8.333

protein E1				

Table 1D Urine Peptide Inclusion List

Protein Biomarker	Target Biomarker Peptide	Prec m/z	Charge	Retention Time (min)
Osteopontin	GDSVVYGLR	927.9524	2	8.95
	AIPVAQDLNAPSDWDSR	927.9533	2	15.088
Uromodulin	VLNLGPITR	491.8078	2	13.173
	DGPCGTVLTR	538.2658	2	6.6

Table 1E 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	16.51
	AAYEDFNVQLR	663.33	2	13.5
	AAGGAVCEQPLGLECR	844.3968	2	10
Cornulin	ISPQIQLSGQTEQTQK	893.4706	2	8.53
	TLSESAEGACGSQESGSLHSGASQELGEGQR	1036.125	3	7.6
IgGFc-binding protein	APGWDPLCWDECR	831.3529	2	19.32
	AGCVAESTAVCR	640.7912	2	5.31
Ly6/PLAUR containing protein 3	DGVTGPGFTLSGSCCQGSR	971.925	2	11
	GCVQDEFCTR	636.2653	2	6.63
Matrix metallo-proteinase-9	GSRPQGPFLIADKWPALPR	527.2981	4	19.64
Neutrophil gelatinase-associated lipocalin	SYPGLTSYLVR	628.3402	2	15.79
	TFVPGCQPGEFTLGNIK	622.3172	3	16.92
Suprabasin	ALDGINSGITHAGR	461.2476	3	7.46
	LGQGVNHAADQAGKEVEK	617.652	3	5.35

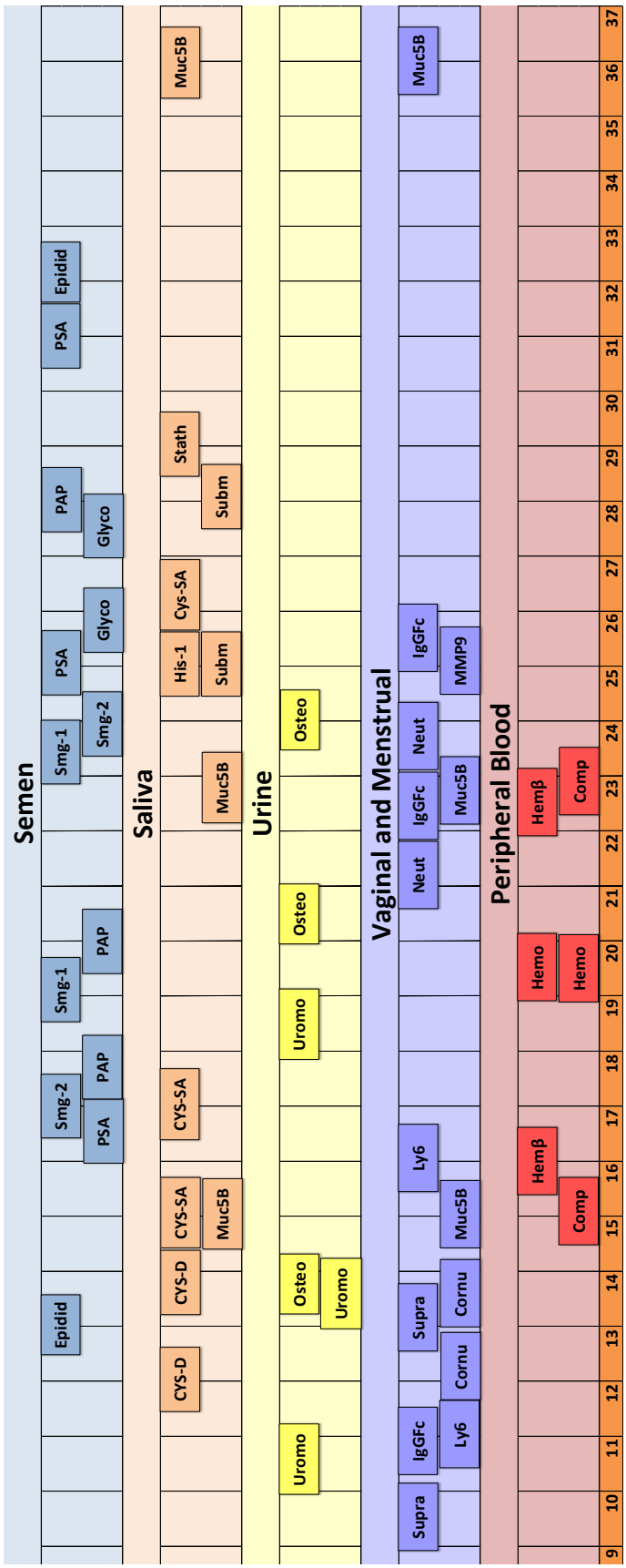
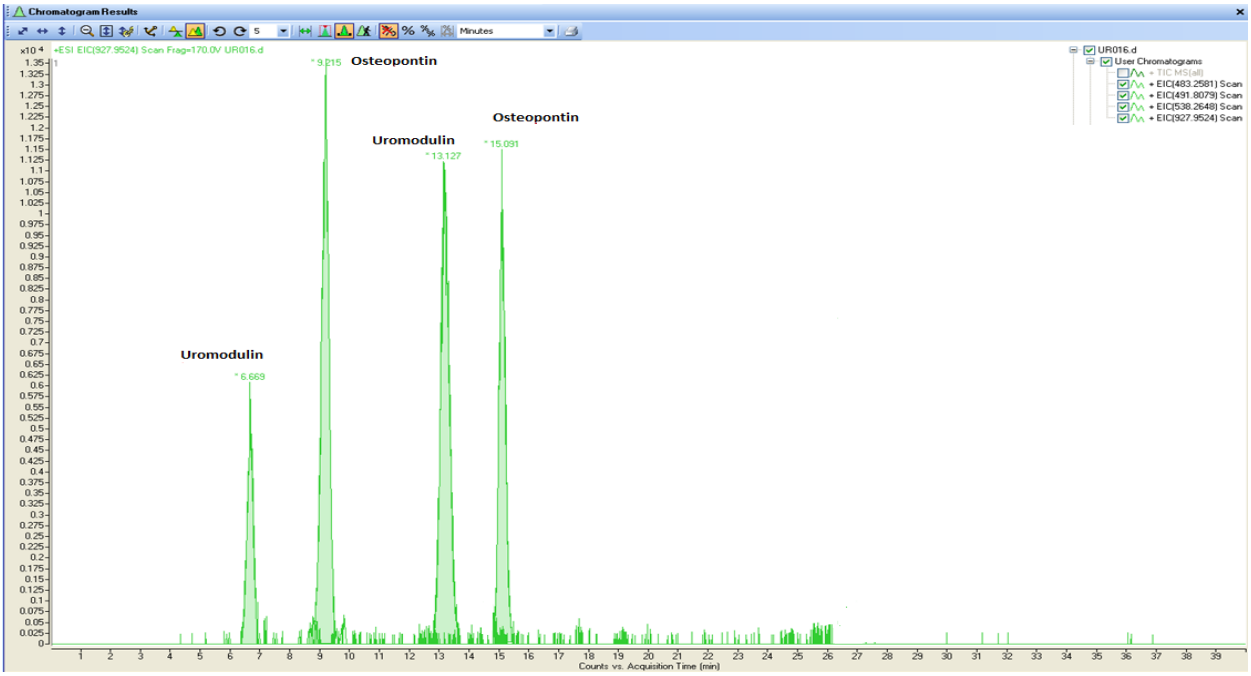


Figure 4 – Elution times in minutes (orange row at bottom of figure) of all optimal peptides for the detection of the high-specificity biomarkers for each body fluid targeted by the Q-TOF multiplex assay. This assay targets multiple peptides that are diagnostic for each unique protein biomarker. This provides for robust internal confirmation of each biomarker that is detected in the assay. Peptides are grouped by target body fluid such that each body fluid is represented by a separate "channel". As with the multiple channels used in traditional STR analyses, data are collected from each channel simultaneously.

The results obtained using the final 6-body fluid multiplex assay for the analysis of a representative urine sample are shown in Figure 5. Both peptide chromatography and database search results revealed the clear and unambiguous identification of the two targeted high-specificity biomarkers for urine (*i.e.*, osteopontin and uromodulin). Underscoring the accuracy of the assay is the fact that although the assay also targets twenty other high specificity biomarkers for five biological stains other than urine, not a single non-urine associated protein was detected. Figure 6 provides additional detail underscoring the accuracy of target peptide identification.



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 5 – Q-TOF multiplex assay results (chromatogram on top and peptide search results on bottom) from the analysis of an unfractionated urine sample. Out of a total of 45 ions being scanned for, only the targeted ions for the high-specificity urine bio-markers (uromodulin and osteopontin) were detected. None of the targeted non-urine bio-markers were detected.

The assay results obtained with representative samples of the other five body fluids of interest also proved to be of equivalent quality and specificity (Figures 7A-E). Specifically, the analysis of a representative seminal fluid sample resulted in the unambiguous detection of semenogelin I/II, prostate specific antigen, epididymal secretory protein E1, and prostatic acid phosphatase. Although the assay also targeted high-specificity biomarkers for five biological stains other than seminal fluid, no non-seminal fluid associated proteins were detected (Figures 7A). The multiplex assay of a representative saliva sample produced results that unambiguously revealed the presence of mucin 5B, cystatin SA, cystatin D, submaxillary gland androgen regulated protein, statherin and histatin-1. Here again, not a single targeted non-saliva associated protein was detected (Figures 7B). It should be noted that while mucin 5B is a saliva-associated

protein, it is also expressed in vaginal secretions. Analysis of a representative vaginal fluid

Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
2	33.30	8	1.33e+005	35422.9	HUMAN	2	Osteopontin

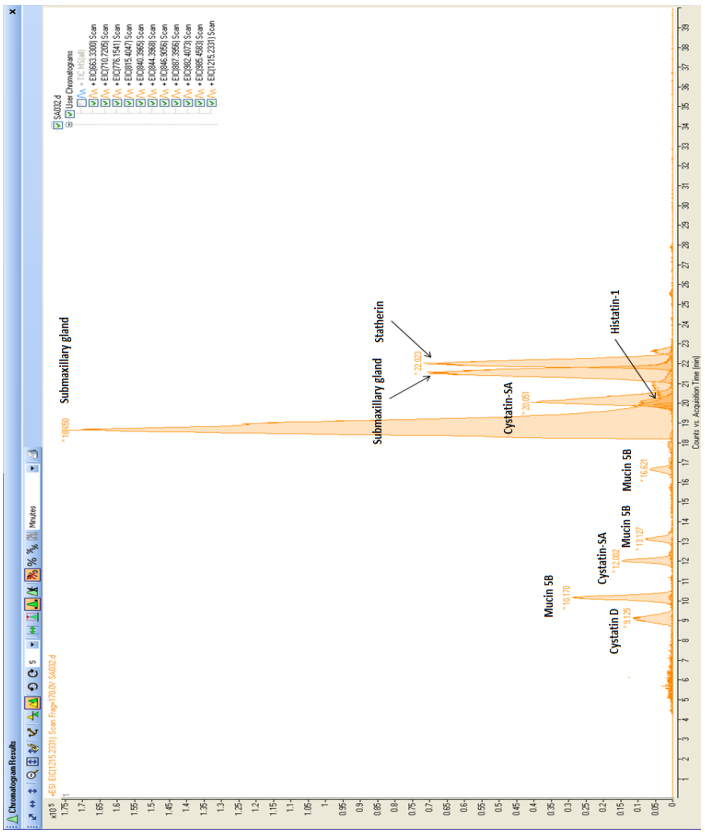
	z	Score	SPI (%)	Spectrum Intensity	Variable Sites	Start AA Position	Sequence	Modifications	RT (min)	m/z Measured (Da)	MH+ Matched (Da)	MH+ Mass Shift (Da)	MH+ Error (ppm)
0	2	21.42	53.1	1.82e+005		204	(K)AIPVAQDLNAPSDWDSR(G)		15.22	927.9524	1854.898	-0.0005	-0.3
0	2	11.88	53.9	8.46e+004		160	(R)GDSVVYGLR(S)		8.71	483.2581	965.505	0.0039	4.0

Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
2	26.52	2	8.64e+004	69761.4	HUMAN	1	Uromodulin

	z	Score	SPI (%)	Spectrum Intensity	Variable Sites	Start AA Position	Sequence	Modifications	RT (min)	m/z Measured (Da)	MH+ Matched (Da)	MH+ Mass Shift (Da)	MH+ Error (ppm)
0	2	12.03	60.1	6.34e+004		386	(R)DGPCGTVLTR(N)	C:Carbamidomethylation	6.62	538.2658	1075.520	0.0043	4.0
0	2	9.90	62.0	9.91e+004		598	(R)VLNLGPITR(K)		12.71	491.8078	982.604	0.0039	4.0
0	2	14.49	91.0	9.68e+004		598	(R)VLNLGPITR(K)		13.22	491.8078	982.604	0.0039	4.0

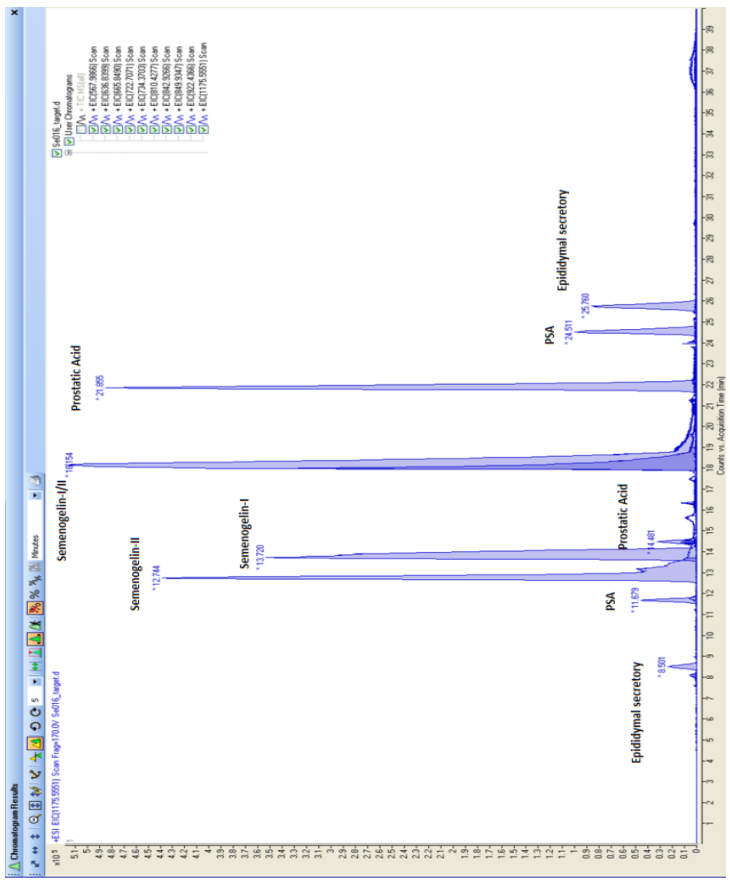
Figure 6 – Details (*e.g.*, amino acid sequence, signal intensity, retention time, mass-to-charge ratio and ms/ms search score etc.) pertaining to peptide detection and identi-fication of the urine-specific peptide ions (Top: osteopontin; Bottom: uromodulin) that are targeted in the Q-TOF multiplex assay.

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 (Figures 7C). As noted above, mucin 5B is a vaginal secretion- associated protein, it is coexpressed in saliva. Similarly, cornulin, also appears to be co-expressed in menstrual blood. The significance and potential utility of such coexpressed proteins will be addressed later in this document. 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 (Figures 7D). 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 markers, however, the protein 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. This will be addressed in greater detail later in this document. Finally, the analysis of a representative peripheral blood sample resulted in the unambiguous detection of complement C3, hemoglobin subunit beta and hemopexin (Figures 7E). Aside from the expected occurrence of these proteins in menstrual blood, these proteins were not detected in any other body fluid.



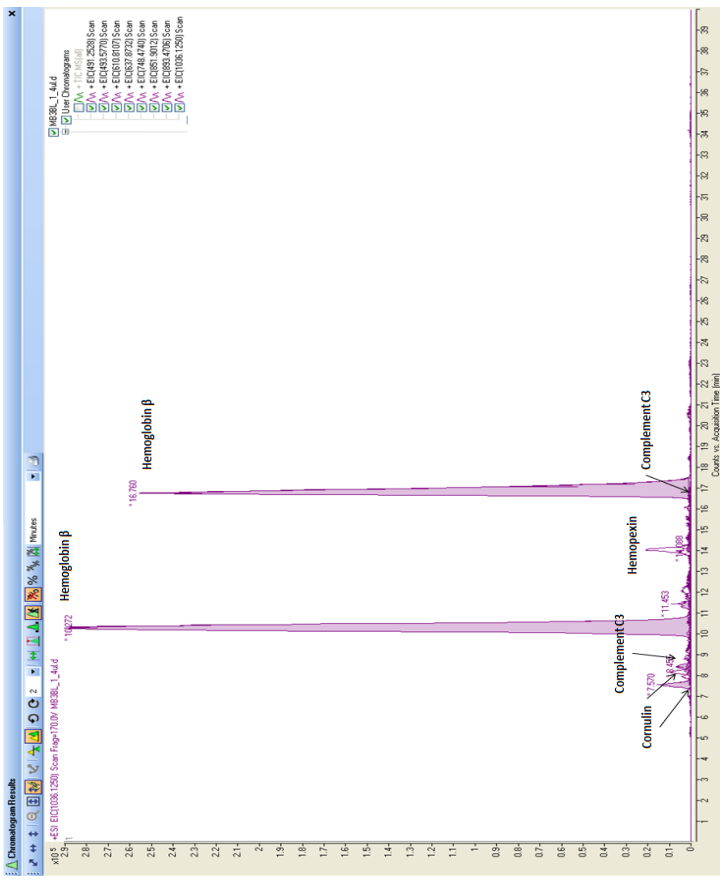
Group (#)	Subgroup (#)	Spectra (#)	Distinct Peptides (#)	Summed MS/MS Search Score	Group (#)	Subgroup (#)	Spectra (#)	Distinct Peptides (#)	Summed MS/MS Search Score	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
1	1.1	8	3	45.14	1	1.1	8	3	45.14	6.63e+004	59634.8	HUMAN	Q9H1B4	Mucin-5B OS=Homo sapiens GN=MUC5B PE=1 SV=3
2	2.1	7	2	43.40	2	2.1	7	2	43.40	5.00e+005	81871	HUMAN	P02814	Submaxillary gland androgen-regulated protein 3B OS=Homo sapiens
3	3.1	3	2	36.72	3	3.1	3	2	36.72	8.62e+004	16000.6	HUMAN	P30325	Cystatin-D OS=Homo sapiens GN=CST5 PE=1 SV=1
4	4.1	6	2	28.38	4	4.1	6	2	28.38	7.67e+004	16444.8	HUMAN	P01029	Cystatin-SA OS=Homo sapiens GN=CST2 PE=1 SV=1
5	5.1	3	1	14.39	5	5.1	3	1	14.39	3.39e+005	7304.5	HUMAN	P02808	Statherin OS=Homo sapiens GN=STATH PE=1 SV=2
6	6.1	2	1	9.33	6	6.1	2	1	9.33	7.01e+004	6962.9	HUMAN	P15515	Histatin-1 OS=Homo sapiens GN=HTN1 PE=1 SV=2
Totals:		29	11		Totals:		29	11						

Figure 7A: Representative Saliva Sample



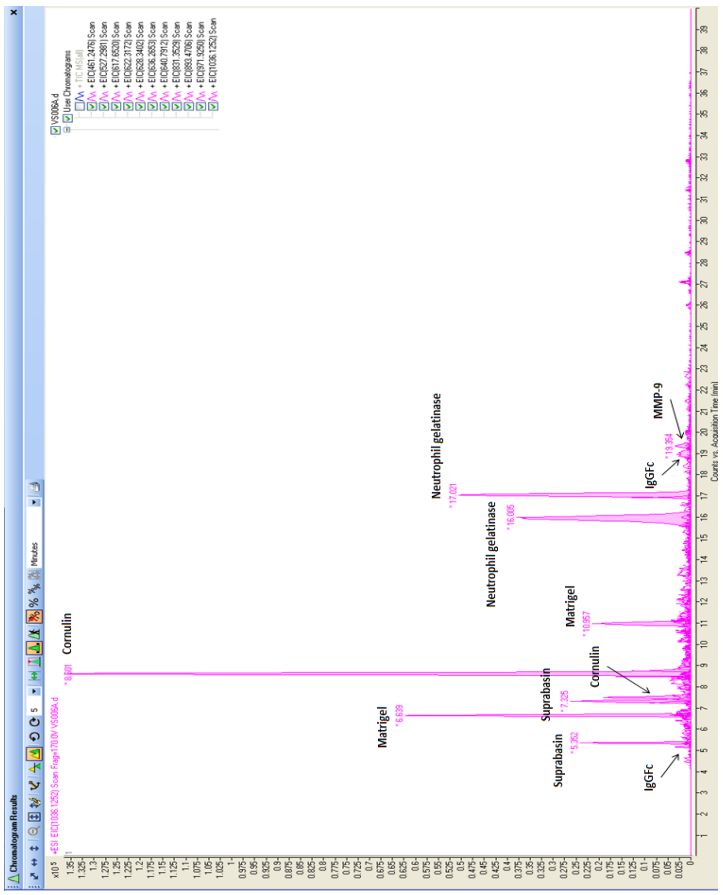
Group (#)	Subgroup (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	Mean Peptide Spectral Intensity	Protein MW (Da)	Species	Database Accession #	Protein Name
1	1.1	5	2	48.50	1.09e+007	65444.7	HUMAN	Q7	Semenogelin-2
2	2.1	6	2	42.59	1.04e+007	52131.1	HUMAN	Q6	Semenogelin-1
3	3.1	2	2	39.91	3.66e+005	28741.5	HUMAN	Q3	Prostate-specific antigen
4	4.1	3	2	39.41	1.29e+006	16570.3	HUMAN	Q5	Epididymal secretory protein E1
5	5.1	4	2	32.63	1.32e+006	44566.4	HUMAN	Q4	Prostatic acid phosphatase
Totals:		20	10						

Figure 7B: Representative Seminal Fluid Sample



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						

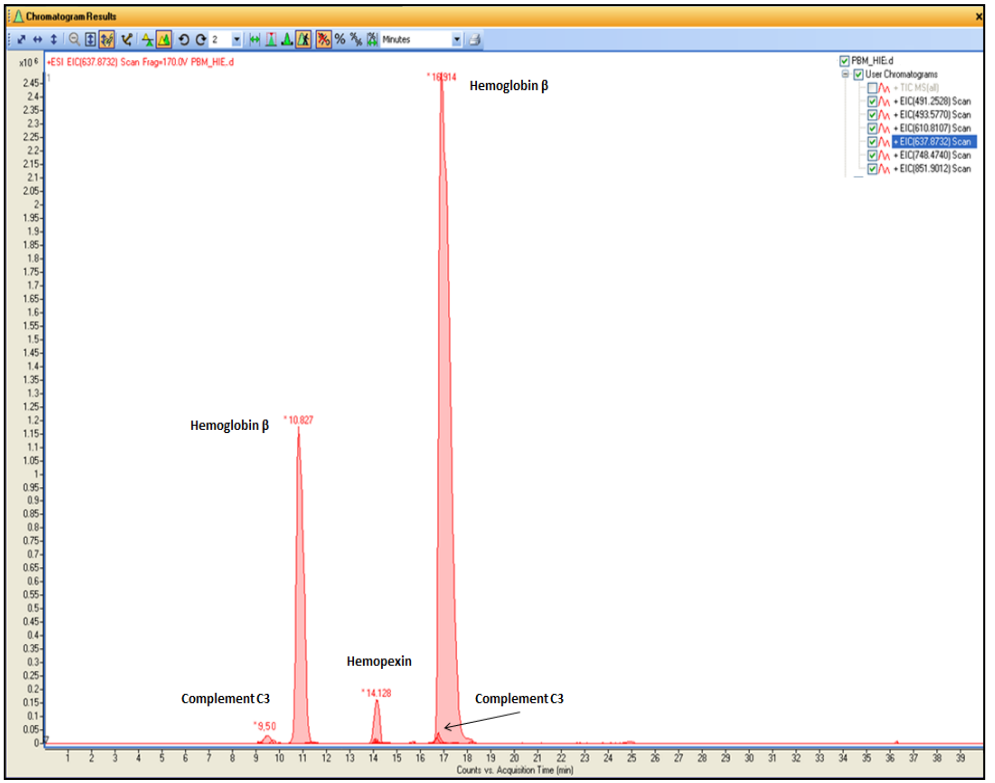
Figure 7D: Representative Menstrual Blood Sample



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 7C: Representative Vaginal Fluid Sample

Figure 7A-E – Q-TOF multiplex assay results (chromatogram on top and peptide search results on bottom) from the analysis of unfractionated body fluid samples. Out of a total of 45 ions being scanned for, only the targeted ions for the high-specificity seminal fluid biomarkers (Semenogelin I/II, Prostate Specific Antigen, Epididymal Secretory Protein E1, and Prostatic Acid Phosphatase) were detected in a representative seminal fluid sample (A). Only the targeted ions for the high-specificity saliva biomarkers (Mucin 5B, Cystatin SA, Cystatin D, Sub-maxillary gland androgen regulated protein, Statherin and Histatin-1) were detected in a representative saliva sample (B). Only the targeted ions for the vaginal fluid-specific biomarkers (*i.e.*, Cornulin, IgGFc-binding protein, Matrigel-induced gene C4 protein, Suprabasin, and Matrix metallo-proteinase-9) were detected in a representative vaginal fluid sample (C). Only the targeted ions for the peripheral blood biomarkers (Complement C3, Hemoglobin Subunit Beta, and Hemopexin) and the vaginal fluid biomarker (Cornulin) were detected in a representative menstrual blood sample (D). Only the targeted ions for the peripheral blood biomarkers (Complement C3, Hemoglobin Subunit Beta and Hemopexin) were detected in a representative sample of peripheral blood (E).



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 75E: Representative Peripheral Blood Sample

The inter-individual reproducibility of target ion detection was also found to be excellent across multiple samples. This is illustrated for the peptide FGYGYGPYQPVPEQPLYPQ-PYQPQYQQYTF which is diagnostic for the detection of statherin in saliva samples (Figure 8). As indicated by a peptide intensity distribution plot and an overlay of the corresponding chromatography results from multiple individuals, the target peptide was consistently detected in multiple samples with a highly reproducible retention time. It should be pointed out, however, that there was significant inter-individual variability in the amount of target ion detected between samples. This inter-individual variability in protein expression was not unexpected and did not appear to interfere with or compromise the accuracy of the assay since, in all cases, a sufficient amount of target protein (statherin) was present in the individual assayed to allow for detection and identification with high analytical confidence.

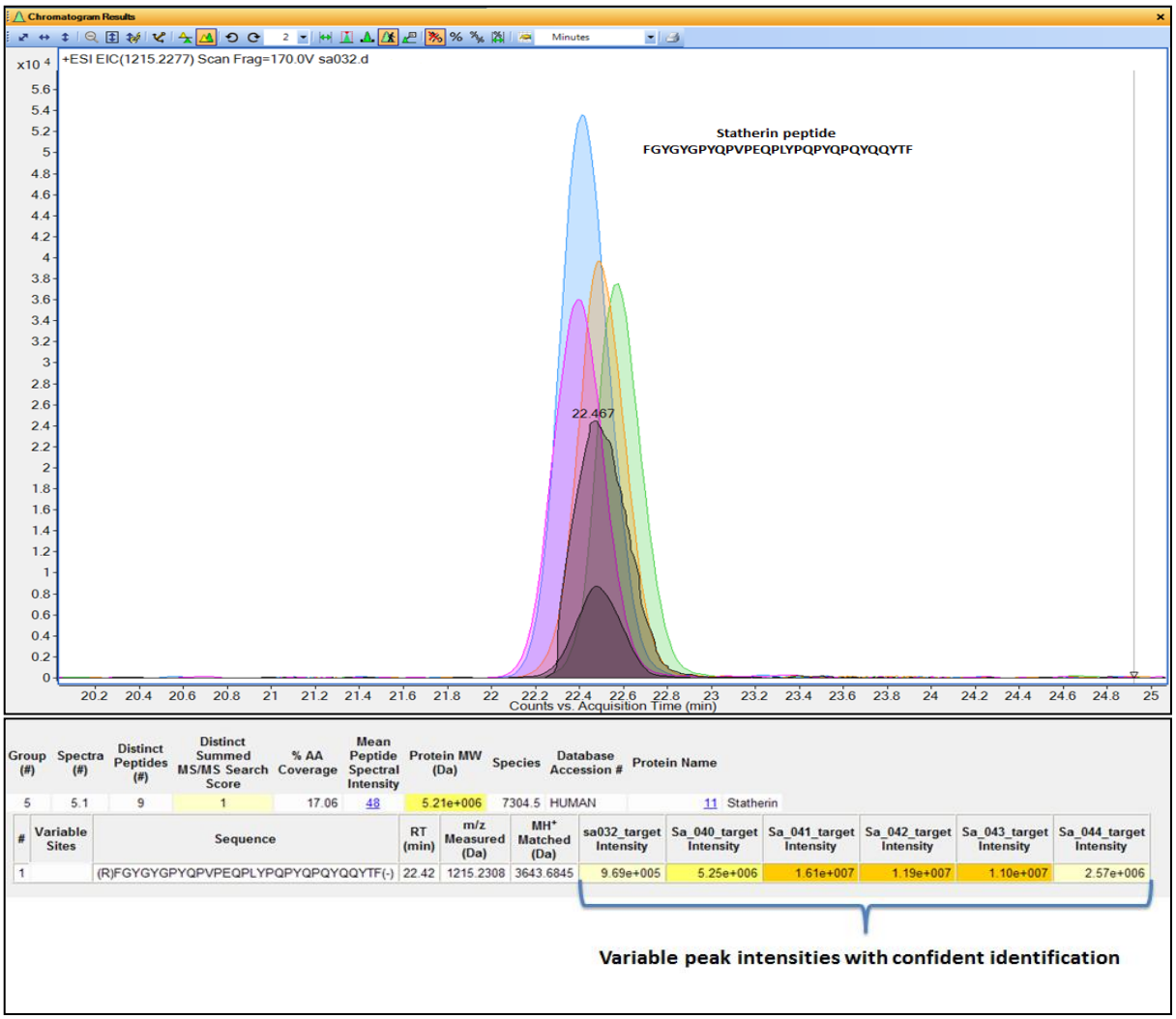


Figure 8 - Peptide intensity distribution plot (top) and overlayed chromatographic results (bottom) for statherin target ion detection in saliva samples collected from six different individuals. Results demonstrate excellent reproducibility of detection in spite of significant interindividual variability in protein expression levels.

Evaluation of Biomarker Expression Across an Expanded Sample Population – The eventual forensic applicability of the candidate biomarkers necessitates a more comprehensive and validation of each candidate marker for stain specificity with a larger population set. Only when these larger-scale studies are completed, can these markers move from being candidates to serving as the foundation for a commercial multiplex assay system capable of characterizing both single source and mixed-source stains with high specificity. There are good reasons for this. For example, the possibility cannot be ignored that some candidate biomarkers might be secreted into non-target fluids in the same way that A, B, and Rh factors in blood are found in the saliva or semen of individuals termed secretors. Confounding factors such as this might be missed in datasets derived from small sample sizes. The results obtained during the assay development phase of the project, for example, were based on a very limited number of samples and were intended to serve only in the optimization the detection of targeted ions rather than the evaluation of the specificity of candidate biomarker expression across the human population. To assess the latter, 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 empirically assess the frequency at which target biomarkers may be detected in non-target body fluids. The results obtained are summarized below and in Figure 9.

Seminal Fluid: The candidate high-specificity markers of seminal fluid (semenogelin I/II, epididymal secretory protein E1, prostatic acid phosphatase and prostate specific antigen) were consistently and unambiguously detected in all semen samples. These markers were generally undetectable in non-target body fluids markers with only a couple of notable exceptions. First, trace amounts of semenogelin I/II, epididymal secretory protein E1, prostatic acid phosphatase and prostate specific antigen were observed in 20-80% of male urine samples. Urine-specific biomarkers, however, were undetectable in seminal fluid samples. While it is not known if the presence of seminal fluid markers in male urine represents leakage from the reproductive system or residual ejaculate, the low quantity of semenogelin relative to the other seminal fluid markers is not consistent with that observed in known samples of seminal fluid. This quantitative difference suggests that leakage of secretions from the male reproductive tissues may be the more likely source of these proteins (Note: seminal fluid donors were instructed to refrain from ejaculation for 5 days prior to urine collection, although it was impossible to confirm compliance with this other than by self-reporting). Second, despite its name, epididymal secretory protein E1 was also detected in female urine at nearly the same frequency as in male urine samples. Finally, trace amounts of epididymal secretory protein was detected in a single sample of vaginal fluid. Here too, it was not possible to determine if this was an endogenous component of vaginal fluid or if the detected protein represented carry over from urine or 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. Moving forward, epididymal secretory protein E1 will be dropped as a high-specificity marker of seminal fluid and quantitative criteria along with the presence/absence of high-specificity urine biomarkers will be investigated as a means of discriminating between ejaculate and male urine.

Urine: The candidate high-specificity markers for urine, uromodulin and osteopontin, were unambiguously detected in all male and female urine samples. These markers were not detected in any non-target body fluids. Thus, uromodulin and osteopontin appear to be suitable high-specificity biomarkers for urine.

	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%

Figure 9 – Results of targeted ion Q-TOF 6-body fluid multiplex assays of fifty samples each of saliva, seminal fluid, vaginal fluid, menstrual blood and peripheral blood and twenty-five samples each of female urine and male urine. Targeted biomarkers are listed across the top of the figure and the body fluids tested are listed along the left side of the figure. Each sample was scanned for the presence of 45 targeted ions representing 6 peripheral blood peptides, 11 saliva peptides, 10 seminal fluid peptides, 4 urine peptides, and 14 vaginal/menstrual blood peptides. 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 either detected in less than 100% of their target body fluid samples or were detected in a non-target body fluid. Red cells indicate cases where a specific biomarker was not detected in any of the samples tested.

Saliva: Among the candidate high-specificity markers of saliva (cystatin SA, cystatin D, submaxillary gland androgen-regulated protein, histatin-1, statherin and mucin 5B) only submaxillary gland androgen-regulated protein was clearly detected in 100% saliva samples. Three other candidate biomarkers (cystatin SA, statherin and mucin 5B), however were detected in greater than 90% of saliva samples assayed. Further improvements in assay sensitivity may make it possible to determine whether or not these proteins are present in all saliva samples – albeit at low levels. More importantly, however, none of these markers were detected in any of the other five body fluids analyzed. Only histatin-1 and cystatin D were found to underperform to a significant degree, being detected in just 30% and 76% of saliva samples, respectively. Finally, although mucin 5B was ubiquitously present in most saliva samples, it was also detected in 20% and 38% of menstrual blood and vaginal fluid samples, respectively and in 4% of urine samples. Moving forward, cystatin D, histatin-1 and mucin 5B will be dropped as high-specificity markers for saliva and further improvements in the multiplex assay will be investigated in an effort to improve the detection of cystatin SA and statherin. Improved detection sensitivity may facilitate biomarker redundancy for saliva detection.

Peripheral Blood: Among the candidate high-specificity markers of peripheral blood (hemoglobin subunit beta, complement C3, and hemopexin), hemoglobin subunit beta, complement C3 were readily detected in all peripheral blood samples and hemopexin was detected in 96% of peripheral samples analyzed. These markers were also detected in menstrual blood where it was expected that all three biomarkers would also be present since peripheral blood is a major component of menstrual blood. In menstrual blood samples, hemoglobin subunit beta, complement C3, and hemopexin were detected in 100%, 54% and 76% of samples, respectively. The lower rate of detection in menstrual relative to peripheral blood samples may reflect unavoidable matrix effects due to the presence of high levels of cellular proteins released as a result of degradation-associated cell lysis. Among non-target body fluids, two of the candidate peripheral blood biomarkers (hemoglobin subunit beta and hemopexin) were also detected in a small number of urine and vaginal fluid samples and hemoglobin subunit beta was detected in a small number of saliva samples. Because of the small number of non-blood samples in which these markers were detected, it is hypothesized that these anomalous results may not be true false positives but rather samples that did contain small quantities of peripheral or menstrual blood such as from flossing teeth, urinary infections, minor vaginal abrasions or residual menstrual blood in the vaginal canal. Moving forward, additional controlled testing of saliva, urine and vaginal fluid samples will be analyzed to better define those conditions under which these samples are likely to yield positive results with peripheral blood biomarkers.

Vaginal Fluid and Menstrual Blood: Among the candidate high-specificity markers of vaginal fluid (cornulin, IgGFc-binding protein, neutrophil gelatinase-associated lipocalin, Ly6/PLAUR containing protein 3, suprabasin, and matrix metallo-proteinase-9), the biomarkers cornulin, neutrophil gelatinase-associated lipocalin and Ly6/PLAUR containing protein 3 were consistently and unambiguously detected in all vaginal fluid samples tested. The three remaining markers (IgGFc-binding protein, matrix metallo-proteinase-9 and suprabasin) appeared to be unique if not ubiquitous in vaginal fluid samples being detected in 68%, 20% and 22% of samples assayed, respectively. No vaginal fluid markers were detected in saliva, seminal fluid or peripheral blood samples. The detection of cornulin and Ly6/PLAUR containing protein 3 in a single female urine sample was not anticipated based on *in silico* analyses of available proteome databases and thus may reflect inadvertent transfer of proteins from the vagina during the collection of that specific urine sample.

As expected and mentioned previously, peripheral blood markers (hemoglobin subunit beta, Complement C3, and Hemopexin) were also detected in menstrual blood samples. Additionally, the vaginal fluid biomarker, cornulin, was detected in 20% of menstrual blood samples. It is hypothesized that the overall low frequency with which vaginal markers were detected in menstrual blood may reflect a combination of interference from matrix effects and the additional vaginal fluid markers may be detected in menstrual blood depending on how the samples are collected. This minimizes contact between menstrual blood and the vaginal canal. If this hypothesis is confirmed, following further testing using more realistic sexual assault type evidentiary material, it may provide a basis for the use of some vaginal fluid biomarkers as a way to discriminate between peripheral and menstrual blood samples. Positive results would have to be interpreted with caution, however, since it may be impossible to distinguish single-source menstrual blood sample from a mixture of vaginal fluid and peripheral blood.

Biomarker Detection with Casework-Type Samples **–** While pristine samples of biological stains can be used to validate the specificity of each candidate biomarker for a given body fluid, the applicability for use by forensic practitioners and the potential for developing a commercial platform necessitates a second more rigorous set of validation studies to assess the stability and reliability of these biomarkers in a forensic context. Specifically, the ability of the biomarkers to be detected in samples recovered from a variety of substrates such as nylon carpeting, cotton cloth, leather, blue denim fabric, cigarette butts or foreign objects used in a sexual assault must be tested. Similarly the impact of exposure to environmental contaminants/insults must be assessed along with sensitivity and species specificity studies *etc*.. For the preparation of casework-type samples, therefore, 10µL aliquots of undiluted bodily fluids were applied either directly to sterile cotton tipped swabs as single-source samples or to cotton tipped swabs that had previously been dipped in such agents as spermicidal lubricant, Bluestar®, soil, chewing tobacco juice, and coffee and allowed to dry. Given that the ultimate goal of the current research was to facilitate the development of a multiplex assay system capable of identifying multiple bodily fluids a single pass, the performance of the assay with mixed samples must also be evaluated. While an exhaustive forensic developmental validation study was beyond the scope of the current research, a set of preliminary validation experiments were performed on a diversity of casework-type samples as a proof of concept exercise to determine whether more rigorous developmental validation would be warranted.

The results obtained from the analysis of 37 unique casework-type samples are presented in figures 10 and 11. All single-source samples of human body fluids spotted onto sterile cotton swabs were accurately identified by the detection of one or more of the high-specificity biomarkers that were expected for each body fluid. In addition, no unexpected biomarkers for any body fluid other than that being assayed were detected. The detection of epididymal secretory protein E1 (seminal fluid biomarker detected in male urine), for example was anticipated based on the results of earlier studies. As was observed during the studies to assess the specificity and interindividual variability of target biomarker expression, those biomarkers that dropped out of a given panel were those that displayed low levels of expression and or variable expression across the expanded sample population of 50 individuals.

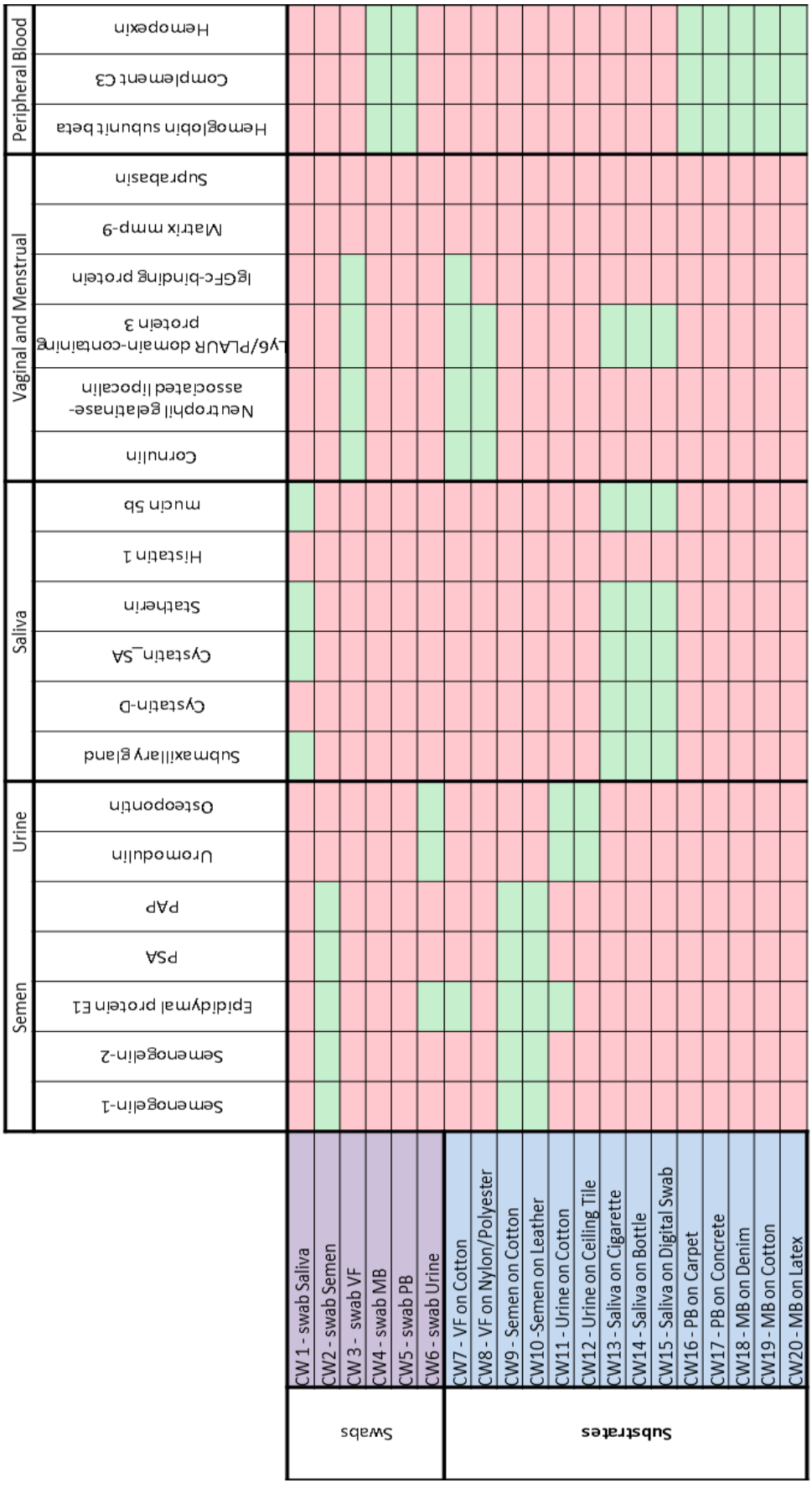


Figure 10 –Results of targeted ion Q-TOF 6-body fluid multiplex assays of casework-type samples. Targeted biomarkers are listed across the top of the figure and the description of the type of body fluid and substrate are provided along the left side of the figure. Each sample was scanned for the presence of 45 targeted ions representing 6 peripheral blood peptides, 11 saliva peptides, 10 seminal fluid peptides, 4 urine peptides, and 14 vaginal/menstrual blood peptides. 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)

Figure 10 –Results of targeted ion Q-TOF 6-body fluid multiplex assays of casework-type samples deposited on sterile cotton swabs, recovered from various substrates or mixed with potentially inhibitor contaminants. 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. Each sample was scanned for the presence of 45 targeted ions representing 6 peripheral blood peptides, 11 saliva peptides, 10 seminal fluid peptides, 4 urine peptides, and 14 vaginal/menstrual blood peptides. Green cells indicate protein biomarkers that were detected. Red cells indicate that a specific biomarker was not detected.

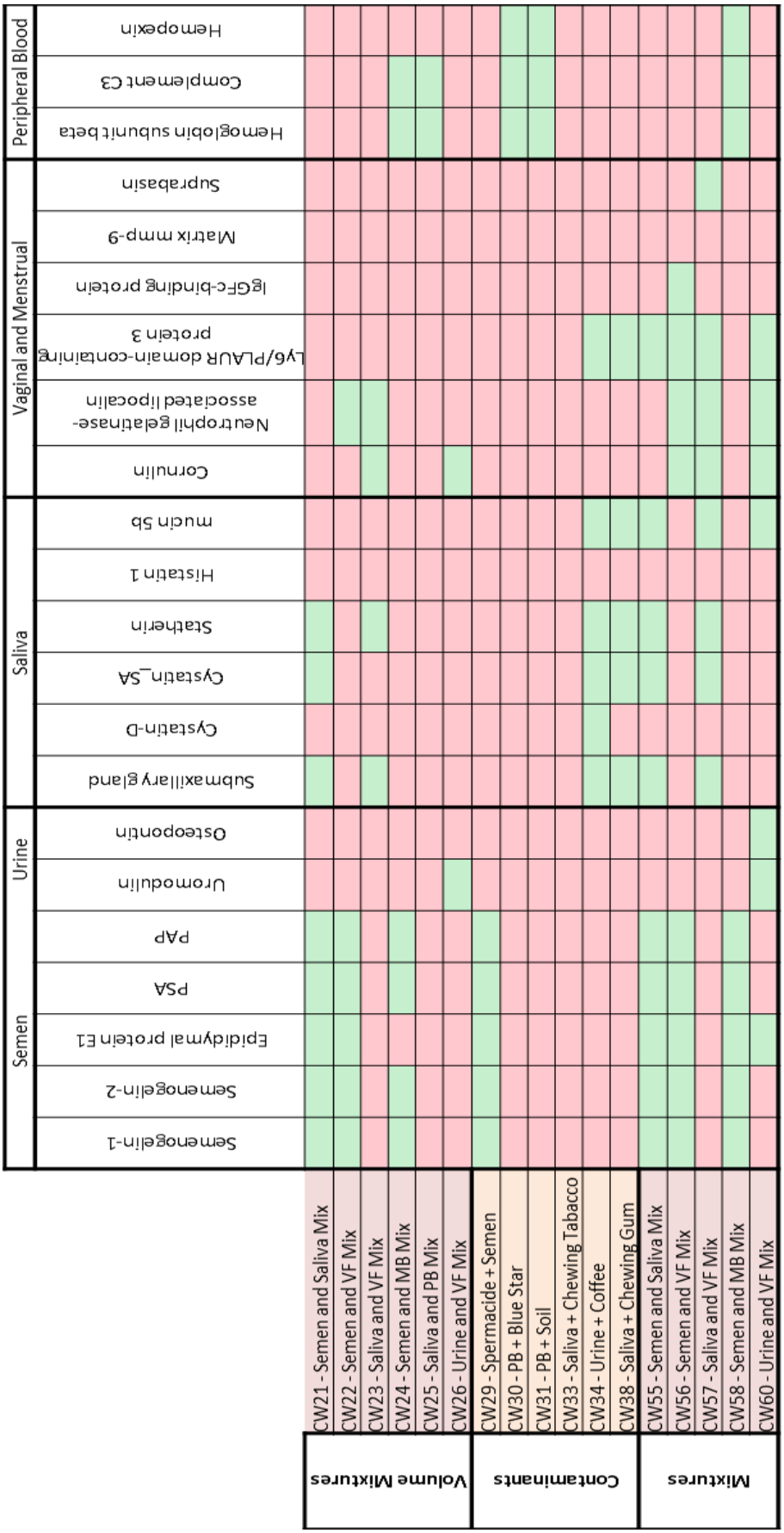


Figure 11 –Results of targeted ion Q-TOF 6-body fluid multiplex assays of casework-type mixed-source samples and samples containing potential mixtures. Targeted biomarkers are listed across the top of the figure and the description of the type of body fluid mixture and/or inhibitor are provided along the left side of the figure. Each sample was scanned for the presence of 45 targeted ions representing 6 peripheral blood peptides, 11 saliva peptides, 10 seminal fluid peptides, 4 urine peptides, and 14 vaginal/menstrual blood peptides. 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)

Recovery of single-source body fluid samples from a variety of substrates ranging from a latex condom to ceiling tile and denim did not impede the accurate characterization of the body fluid being assayed with one notable exception. All three saliva swabs in the substrate studies revealed the presence of Ly6/PLAUR containing protein 3, a vaginal fluid specific biomarker. Although this has not been seen in any prior saliva samples, this protein has been reported in association with the proteome of tissues from the back of the throat. A key difference between the preparation of the casework type samples and all other studies is that saliva was directly deposited onto the substrate from the mouth of the donor. In all other studies, saliva was directly wicked away from the salivary glands; thereby minimizing the potential mixing of proteins from other areas of the oral-pharyngeal area. While further study of this finding is clearly warranted, this unexpected result underscores the importance of conducting rigorous validation studies on casework-type samples.

A series of 2-component mixtures of human body fluids were analyzed by the multiplex assay to evaluate the accuracy with which both components could be accurately identified using a single-pass assay approach. In all but one case (equivalent volumes of saliva and peripheral blood), at least one high specificity biomarker for each body fluid present in the mixture was readily detected. 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. Matrix effects may also account for the observation that the rate of biomarker dropout tended to be higher in body fluid mixtures than in single-source samples.

A series of single-source body fluid samples were also assayed for the influence of potential endogenous inhibitors on biomarker detection. Of the potential inhibitors assayed only chewing tobacco juice appeared to preclude the identification of a target body fluid (*i.e.*, saliva).This was not entirely unexpected given that tobacco juice is also know to act as a potent inhibitor of DNA typing chemistries. Clearly, however, this is another area where additional studies will be needed and possible tools such as an internal positive control for inhibition may need to be developed.

Finally a series of dilutions were prepared and analyzed to determine the lower limit of detection for each body fluid based on the detection of at least one high-specificity protein biomarker. These results are provided in Table 2. It should be emphasized that the lower limit of detection has been calculated on the basis of the average protein content for each body fluid. Due to significant interindividual differences in protein expression levels, therefore, not all samples will necessarily yield equivalent results. Moreover, estimates of the lower limit of detection should be viewed with caution since they have been calculated from data on serial dilution of a body fluid extract, rather than on data from direct extracts of actual trace samples and do not reflect the potential for matrix effects in mixed-source samples.

Table 2 Lower Limit of Detection by Body Fluid Type

Body Fluid	Average Total Protein (mg/mL)	Lower Limit of Detection (nL)
Seminal Fluid	30	0.83
Saliva	1.5	16.67
Urine	0.88	28.41
Vaginal Fluid	1.2	20.83
Peripheral Blood	120	0.21
Menstrual Blood	120	0.21

Implications for Policy and Practice

Excellent working relationships with forensic practitioners in the US and abroad have been essential in productively guiding the current R&D efforts. Their advice has played an important role in shaping our experimental design. They have repeatedly stressed that the identification of biological stains can still be a significant challenge for forensic serologists. Practitioners have no means of readily or reliably identifying stains such as vaginal secretions or differentiating between peripheral vs. menstrual blood.

Commercial kits that have been developed for the identification of blood, semen and saliva, use proteins as diagnostic markers of these forensically important substances. While these protein markers have proven useful, they were selected at a time when the field of proteomics was in its infancy. Funding to the principle investigator through the current NIJ award (2009-DN-BX-K165) has made it possible employ cutting edge protein analysis technologies to identify an assemblage of high-specificity protein biomarkers for bodily fluids typically encountered in a forensic context. This information will help to facilitate the commercial production of such assays. This includes the development of a commercial mass spectrometry approach based on the multiplex assay described here. Alternatively, as forensic technology advances, these same protein biomarkers can be readily incorporated into lab-on-a-chip or other miniaturized formats.

The availability of highly-specific protein biomarkers for biological stains of forensic interest has significant potential to assist forensic serologist linking DNA profiles to specific biological fluids. Nonetheless, some casework samples can and will still present challenges that may not necessarily be anticipated or that can complicate interpretations. The release of small quantities of blood into the oral cavity as a result of using dental floss or a minor injury to the inside of the mouth may be detected as a mixed stain – which it is. In such cases, it will fall to the experienced judgment of the serologist to make an interpretation with regard to the potential significance of the mixture. Similarly, even with the most accurate of protein biomarkers markers, it may still not be possible in some cases (*e.g.,* mixtures) to definitively say that a DNA profile came from a specific type of epithelial cell. This would be true even if the biomarker were a cell surface molecule. In such challenging cases, however, high specificity biomarkers used in combination with technologies such as Laser Capture Microdissection may enable an analyst to make a definitive statement on the source of a DNA profile.

Finally, it is recognized that Daubert v. Merrell Dow Pharmaceuticals, Inc., 509 U.S. 579,

593-94 (1993), Frye's "general acceptance" test, Frye v. United States, 293 F. 1013, 1014 (D.C. Cir. 1923) and federal rules of evidence specifically Rules CRE 403 and CRE 702 provide the standard for admitting scientific evidence in the federal courts. The experiments reported here coupled with publication in peer-reviewed journals, will help to place the findings of this research on sound legal footing.

Implications for Further Research

This proposal builds on the principle investigator's successful completion of previous NIJ funded projects that have charted a course from basic research to practical application. This work began with the rigorous comparative proteomic mapping of thousands of proteins from six forensically relevant human body fluids. This made it possible to identify a panel of candidate high-specificity protein biomarkers for each stain. Following biomarker discovery, the specificity of each candidate protein, the consistency with which it can be detected and the degree of interindividual variability in its expression was evaluated across a larger population of human subjects. For these studies, a quadrupole time of flight (Q-TOF) mass spectrometer was used but proved unacceptably slow for practical applicability. Based on a series of preliminary experiments involving a three-stain (*i.e.*, saliva, semen, and vaginal fluid) multiplex assay, however, it was found that shifting to a higher-sensitivity triple quadrupole (QQQ) platform resulted in both higher-quality results and faster assay times. One promising direction for future research, therefore, would be to fully develop a QQQ multiplex by incorporating the high-specificity biomarkers identified in the current project into the assay. One could then thoroughly assess the performance limits of an improved assay and thus its potential applicability to casework. This goal could be readily achieved through four core research objectives:

1) **Select diagnostic target ions for existing biomarkers** to produce a six-stain multiplex QQQ assay and demonstrate their accurate detection using single source reference samples.
2) **Thoroughly optimize the performance** of the six-stain multiplex QQQ assay using synthetic standards as well as single-source and mixed-source reference samples.
3) **Conduct a rigorous developmental validation** of the multiplex assay that meets Standard 8.2 of the FBI's "Quality Assurance Standards for Forensic DNA Testing Laboratories".
4) **Develop appropriate and reliable** Standard Operating Procedures and Interpretation Guidelines for use of the multiplex QQQ assay for casework samples.

The successful completion of these objectives would not only represent the culmination of work completed under awards 2006-DN-BX-K001, 2009-DN-BX-K165 but would also help pave the way for commercial development, interlaboratory evaluations and eventual adoption by forensic practitioners.

References Cited

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Dissemination of Research Findings

The central deliverable of this project was a panel of high-specificity protein biomarkers for the identification of six human body fluids with direct forensic utility. With the completion of this project, each of the biomarkers has been rigorously assessed across a sample population sufficiently large to discriminate between optimal high-specificity protein biomarkers and those that displayed unacceptable interindividual variability in expression such that their forensic utility is compromised. While it was not the objective of this research to develop a commercial assay system for human stain identification, it was our goal to provided information to potential commercial partners to facilitate the eventual development of such systems. This includes information on all methods and the detailed analyses of each biomarker (e.g., optimal target ions, matrix effects, interindividual variability in expression levels etc.). During the course of the project, the principle investigator strived to provide the professional forensic and the broader scientific communities along with the general public with information on the progress and potential benefits this research. This was achieved through ongoing forensic science workshops and conferences. During the period of the award, the following presentations were made:

- March 2010 "Isolation and Validation of Highly Specific Protein Markers for the Identification of Biological Stains: Adapting Comparative Proteomics to Forensics", Poster Presentation, 6th Annual US Human Proteomics Conference, Denver, CO
- June 2010, "Validation of Highly-Specific Protein Markers for the Multiplexed Identification of Biological Stains" Poster presentation, NIJ Annual Meeting, Arlington, VA
- August 2010: Research conducted under this award (2009-DN-BX0-K165) was featured in a professional video publicized by the University of Denver. The video may be viewed at http://vimeo.com/15056394
- October 2010, "Validation of Highly-Specific Protein Markers for the Multiplexed Identification of Biological Stains" Invited Seminar, Split Screen Public Science Seminar, Lakewood, CO
- November 2010, "Validation of Highly-Specific Protein Markers for the Multiplexed Identification of Biological Stains" Invited Seminar, Forensic Science First Year Seminar, University of Denver, Denver, CO
- June 2011: "Highly-Specific Protein Biomarker Assays for the Identification of Biological Stains" poster presented at the annual NIJ conference in Arlington, VA.
- June 2011, "Highly-Specific Protein Markers for the Confirmatory Identification of Biological Stains" Invited Seminar, National Medical Services Labs, Willow Grove, PA.
- March 2012 "Highly-Specific Protein Biomarker Assays for the Identification of Biological Stains" poster presented at the annual End Violence Against Women International conference in San Diego, CA.
- June 2012: "Highly-Specific Protein Biomarker Assays for the Identification of Biological Stains" poster presented at the annual NIJ conference in Arlington, VA.
- August 2012: "Validation of Highly-Specific Protein Markers for the Identification of Biological Stains - Adapting Proteomics to Forensics", poster and invited talk presented

at the annual meeting of the Colorado Biological Mass Spectrometry Society, Boulder, CO

- September 2012: "Development and Testing of a Rapid Multiplex Assay for the Identification of Biological Stains", invited talk presented at the Green Mountain DNA Conference, Burlington, VT
- An invitation to contribute a chapter on advances in forensic serology and the work completed under award 2009-DN-BX-K165 to the book "Sexual Assault across the Life Span" has been accepted. Draft manuscripts are due in December of 2012.
- Additional manuscripts are being prepared submission to peer-reviewed forensic science and proteomics journals.
- The dataset produced under award 2009-DN-BX-K165 is being used as the foundation for a mock Daubert hearing as part of an experiential learning capstone project for law students at the University of Denver's Sturm College of Law.