

REPORT DOCUMENTATION PAGE			Form Approved OMB NO. 0704-0188		
The public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA, 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 01-12-2016		2. REPORT TYPE Monthly Progress Report		3. DATES COVERED (From - To) 30-Sep-2016 - 30-Oct-2016	
4. TITLE AND SUBTITLE Validatoin of a Mass Spectrometry Based Serological Assay			5a. CONTRACT NUMBER W911NF-15-2-0132		
			5b. GRANT NUMBER		
			5c. PROGRAM ELEMENT NUMBER		
6. AUTHORS Kevin M Legg			5d. PROJECT NUMBER		
			5e. TASK NUMBER		
			5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAMES AND ADDRESSES Fredric Rieders Family Renaissance Founda 2300 Stratford Ave. Willow Grove, PA 19090 -4123			8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS (ES) U.S. Army Research Office P.O. Box 12211 Research Triangle Park, NC 27709-2211			10. SPONSOR/MONITOR'S ACRONYM(S) ARO		
			11. SPONSOR/MONITOR'S REPORT NUMBER(S) 67484-LS-RIF		
12. DISTRIBUTION AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES The views, opinions and/or findings contained in this report are those of the author(s) and should not contrued as an official Department of the Army position, policy or decision, unless so designated by other documentation.					
14. ABSTRACT DNA testing can link an item of evidence to an individual but it cannot identify the body fluid that is the source of the DNA. Such basic information, however, is often critical to providing a probative context for the evidence at issue in a criminal investigation. Consider a case where swabs of a suspect's fingers reveal DNA consistent with that of an alleged sexual assault victim. Multiple explanations can be offered for this result. One possibility is the claim by the alleged victim that the suspect sexually assaulted her by digitally penetrating her. Alternatively, the suspect may claim that the female innocently licked food from his fingers, leaving her DNA behind. The ability to					
15. SUBJECT TERMS Forensic Serology, Forensic Biology, Mass Spectrometry					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	15. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON Kevin Legg
a. REPORT UU	b. ABSTRACT UU	c. THIS PAGE UU			19b. TELEPHONE NUMBER 215-366-1769

Report Title

ABSTRACT

DNA testing can link an item of evidence to an individual but it cannot identify the body fluid that is the source of the DNA. Such basic information, however, is often critical to providing a probative context for the evidence at issue in a criminal investigation. Consider a case where swabs of a suspect's fingers reveal DNA consistent with that of an alleged sexual assault victim. Multiple explanations can be offered for this result. One possibility is the claim by the alleged victim that the suspect sexually assaulted her by digitally penetrating her. Alternatively, the suspect may claim that the female innocently licked food from his fingers - leaving her DNA behind. The ability to reliably identify trace amounts of saliva and/or vaginal fluids would enable investigators to resolve such contradictory claims. It is not currently possible, however, for forensic scientists to unambiguously identify forensically-relevant body fluids with existing assays which yield only presumptive results and are often not amenable to trace evidence. In fact, for some body fluids (e.g., vaginal fluid) there are no commercially available tests at all. Thus, there is clear value in the proposed development of an accurate and sensitive technology for unambiguously identifying human body fluids encountered in a forensic context.

Validation of a Mass Spectrometry Based Serological Assay

W911NF-15-2-0132

A Progress Report Submitted to:

United States Department of Defense

U.S. Army Research Office

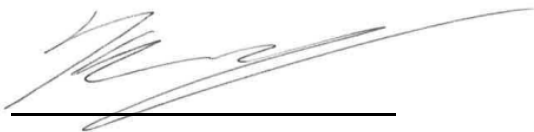
Monthly Progress Report Submitted on: November 30th 2016

Award Period: September 25th 2015 to September 24th 2017

Reporting Period: October 1st 2016 to October 31st 2016

Kevin M. Legg, PhD
Center for Forensic Science Research and Education
EIN:35-2221255
DUNS: 798820150
2300 Stratford Ave.
Willow Grove, PA 19090

Phone: 215-657-1769
Email: Kevin.Legg@frfoundation.org



Kevin M. Legg, PhD

11/30/16

Date

Accomplishments

Project Overview and Goals:

DNA testing can link an item of evidence to an individual but it cannot identify the body fluid that is the source of the DNA. Such basic information, however, is often critical to providing a probative context for the evidence at issue in a criminal investigation. Consider a case where swabs of a suspect's fingers reveal DNA consistent with that of an alleged sexual assault victim. Multiple explanations can be offered for this result. One possibility is the claim by the alleged victim that the suspect sexually assaulted her by digitally penetrating her. Alternatively, the suspect may claim that the female innocently licked food from his fingers - leaving her DNA behind. The ability to reliably identify trace amounts of saliva and/or vaginal fluids would enable investigators to resolve such contradictory claims. It is not currently possible, however, for forensic scientists to unambiguously identify forensically-relevant body fluids with existing assays which yield only presumptive results and are often not amenable to trace evidence. In fact, for some body fluids (*e.g.*, vaginal fluid) there are no commercially available tests at all. Thus, there is clear value in the proposed development of an accurate and sensitive technology for unambiguously identifying human body fluids encountered in a forensic context.

The overarching goal of this project is to develop, validate, and assess a high-specificity forensic serology assay. The assay will enable the simultaneous identification of up to six human body fluids including those most commonly encountered when investigating/prosecuting sexual assaults. This assay will combine automated sample preparation with two well-established technologies, Ultra Performance Liquid Chromatography and Triple Quadrupole (UPLC-QQQ) Mass Spectrometry to produce an integrated system for body fluid identification. This work is being accomplished in four phases as follows:

- **Phase 1: Method Transfer and Optimization** – Standards for targeted biomarkers will be synthesized and used to optimize the chromatographic separation, and operational parameters for transfer of the prototype assay to a high speed UPLC-QQQ platform.
- **Phase 2: Development of Automated Sample Processing** – Sample processing chemistry will be transferred from a single tube manual format to a 96-well fully automated format.
- **Phase 3: Validation and Standard Operating Procedures** – A rigorous series of developmental validation studies will be conducted to establish the performance limits of the assay and to support the creation of detailed standard operating procedures (SOPs) and interpretation guidelines.
- **Phase 4: Implementation Assessment** – The automated sample processing and multiplex assay will be implemented in a forensic laboratory. Critical functionalities will be assessed to ensure that the technology is "mission qualified" in its operational environment.

Accomplishment Overview:

The timeline for the 24-month award period (September 2015 – September 2017) is shown in the Gantt Chart below. During the current reporting period (September 1st 2016 to September 30th 2016) the following events have been completed or initiated:

1. Phase 3 of the project is currently in progress
2. The reproducibility/repeatability studies have been completed.
3. The sensitivity and stability studies are underway.

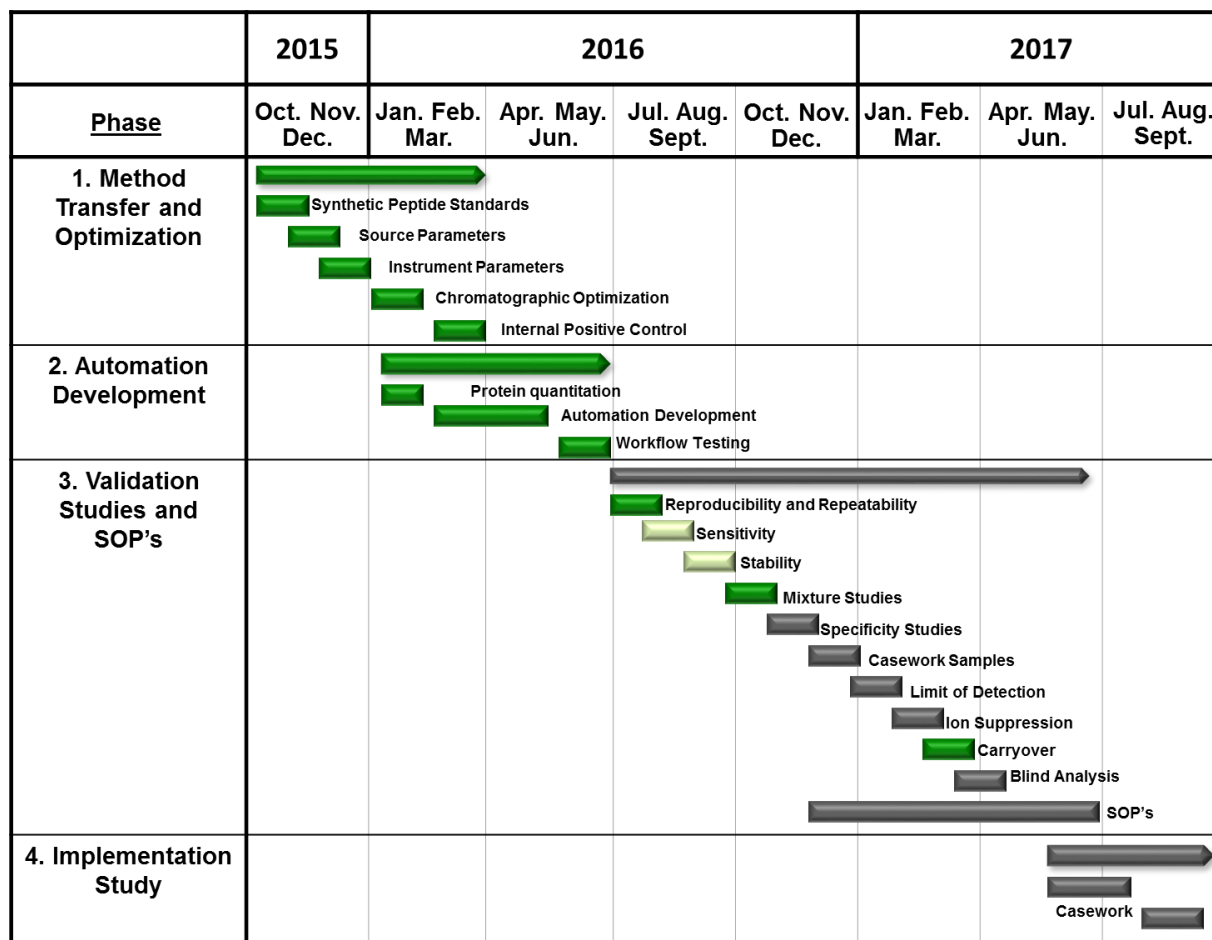


Figure 1: Gantt chart of 24-month project timeline.

Accomplishment Details:

Phase 1: Method Transfer and Optimization (100% Complete)

The core objective of this project phase, was the design and optimization of the UPLC-QQQ method. In particular, human body fluid-specific biomarkers (*i.e.*, proteins and peptide sequences) were selected; peptide standards were synthesized; the internal control was designed; and the Agilent 6495 QQQ LC/MS system's operating parameters (source, instrumental and chromatographic conditions) were determined.

Aim 1.1 Peptide targets and standards (100% Complete)

To maximize the sensitivity of the fluid identification assay, every effort was made to first identify and then target the most abundant proteins, peptides, and peptide fragment ions during data acquisition. This was carried out through a review of previously acquired data; synthesis of peptide standards; and product ion selection through fragmentation of the peptide standards. Once the product ions were selected, authentic body fluids were used to fine tune the assay by selecting the most abundant and interference-free peptide targets.

Product ion selection was carried out using a Quadrupole Time-of-flight (Q-TOF) mass spectrometer. This system was employed to empirically identify product ions for each potential peptide target. These targets were then transferred to the Agilent 6495 QQQ LC/MS system running in Multiple Reaction Monitoring (MRM) mode for integration into the final assay. Finally, each product ion was tested using authentic samples on the Agilent 6495 QQQ LC/MS system and high-purity isotopically-labeled peptides have been purchased for use in later validation studies.

Aim 1.2: Source Parameters (100% Complete)

In order to maximize ionization efficiency and thereby assay performance, source conditions including drying gas flow; nebulizing gas flow; sheath gas flow; nozzle voltage and gas temperatures were optimized for the Agilent Jet Stream source. Source optimization was carried out by infusing peptide standards and manually modifying source parameters until a signal maximum was reached. This aim has been successfully completed and the optimized source parameters are presented in **Table 1**.

Table 1: Optimized Agilent Jet Stream Source Parameters.

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

Aim 1.3: Instrument Parameters (100% Complete)

Collision energy was optimized to achieve maximum ion transmission and fragmentation. As was done under Aim 1.2, instrument settings were incrementally modified to maximize the signal from the target ion. Skyline Proteomics Environment Software (MacCoss Lab Software, University of Washington) was used to initiate the optimization by making *in silico* predictions of the optimal collision energy. Synthetic peptides were then used to detect and resolve possible matrix effects associated with the biological fluids of interest. All collision energy experiments have been completed and optimal collision energy values are provided in **Appendix 1**.

Aim 1.4: Chromatographic Optimization (100% Complete)

Optimization of the chromatographic separation was achieved by analyzing a “master mixture” of synthetic standards representing all targeted peptides while modifying the slope of the HPLC acetonitrile gradient. The detection of each peptide target was then assessed as a function of the changes made in the HPLC gradient. Once optimized, the selected gradient was then tested using authentic fluid samples to ensure acceptable peak morphology, signal intensity, and peak-to-peak resolution. A 10-minute gradient was developed on a 3 mm x 100 mm PeptideMap analytical column with a flow rate of 1.0 mL/min. The specific analytical parameters for the gradient is listed in **Table 2**.

Table 2: Chromatographic Gradient Parameters.

Parameter	Value	
Column	Agilent PeptideMap 3x100 mm C18	
Column Temperature	45°C	
Mobile Phase	A: H2O 0.1% FA B: ACN 0.1% FA	
Flow Rate	1 mL/min	
Gradient	Time	%B
	initial	5
	8.5	32
	8.7	80
	9.7	80
	9.9	5
	10	5
Stop time	10 min	
Post Time	3 min	
Injection Volume	10 µL	

Aim 1.5: Internal Positive Control (IPC) (100% Complete)

Under this aim, an IPC was designed and tested to monitor for inhibition of the proteolytic digestion process which might be associated with sample-specific contaminants or inhibitors. The IPC consisted of two protein components. The first is a bovine-specific “intact protein” that is cleaved during sample preparation to yield a “control peptide”. The second is a “heavy peptide standard” having the same sequence as the bovine control peptide but being labeled with a non-radioactive “heavy” carbon isotope. Inhibition in protein cleavage efficiency is indicated by a reduction in the signal intensity of the control vs. the heavy peptide. Bovine myelin basic protein was selected to serve as the internal control protein. A high-purity isotopically-labeled peptide was synthesized concurrently with the peptides ordered under aim 1.1. To each sample, a 100ng 1:1 ratio of isotopically-labeled peptide to intact myelin basic protein will be added to each sample.

Phase 2: Development of Automated Sample Processing Workflow (100% Complete)

Under this phase, a high-throughput sample processing protocol was developed on the AssayMAP Bravo Platform to automate all sample preparation steps aside from the initial swabbing/cutting of the evidentiary material. This project phase has been divided into three individual aims. They include an accelerated protein quantitation technique, development and testing of the automated sample preparation system, and a comparison of the automation workflow to the previously employed manual preparation method.

Aim 2.1: Accelerated Protein Quantitation (100% complete)

The bicinchoninic acid assay (BCA) is a standard technique for the reliable and specific quantitation of protein content. While analytically reliable and conservative with regard to

consumption of evidentiary material, newer instrumentation employing micro spectrophotometry (e.g., Thermo Scientific NanoDrop™ spectrophotometer) have been promoted by some practitioners and manufacturers. If the system works as well as its proponents suggest, the built in 280 nm quantitation function of the NanoDrop™ could provide a potentially faster alternative to obtaining protein concentration data while consuming as little as 1µl of extract.

The manufacturer agreed to provide a demonstration model on loan for the purpose of evaluating the performance of this system. However, the protein quantitation estimates provided by the NanoDrop™ in the 280nm absorbance mode were determined to be inconsistent and unreliable. This was most likely due to the presence of non-protein matrix components that absorb light in the 280nm range. In addition, the standard spectrophotometer proved to be the faster of the two methods. In conclusion, the BCA assay using a standard spectrophotometer will remain the method of choice for protein quantitation for this study.

Aim 2.2: Automation Development (100% complete)

Manual single-tube trypsin digestion and Solid Phase Extraction (SPE) cleanup have been replaced by a 96-well automated workflow on the AssayMAP Bravo Platform. The reagent volumes and robotic processes have been developed using existing methodologies. This platform deposits purified tryptic digest products directly into the mass spectrometer autosampler plate, thereby eliminating the final lyophilization step. To date, all digestion and SPE cleanup steps including specific reagents, concentrations, liquid flow rates, and volume transfers have been designed and tested. Specific automated liquid handling parameters (*i.e.*, sample process settings and work deck layout) for the trypsin digestion and SPE cleanup are shown in **Figures 2** and **3**, respectively.

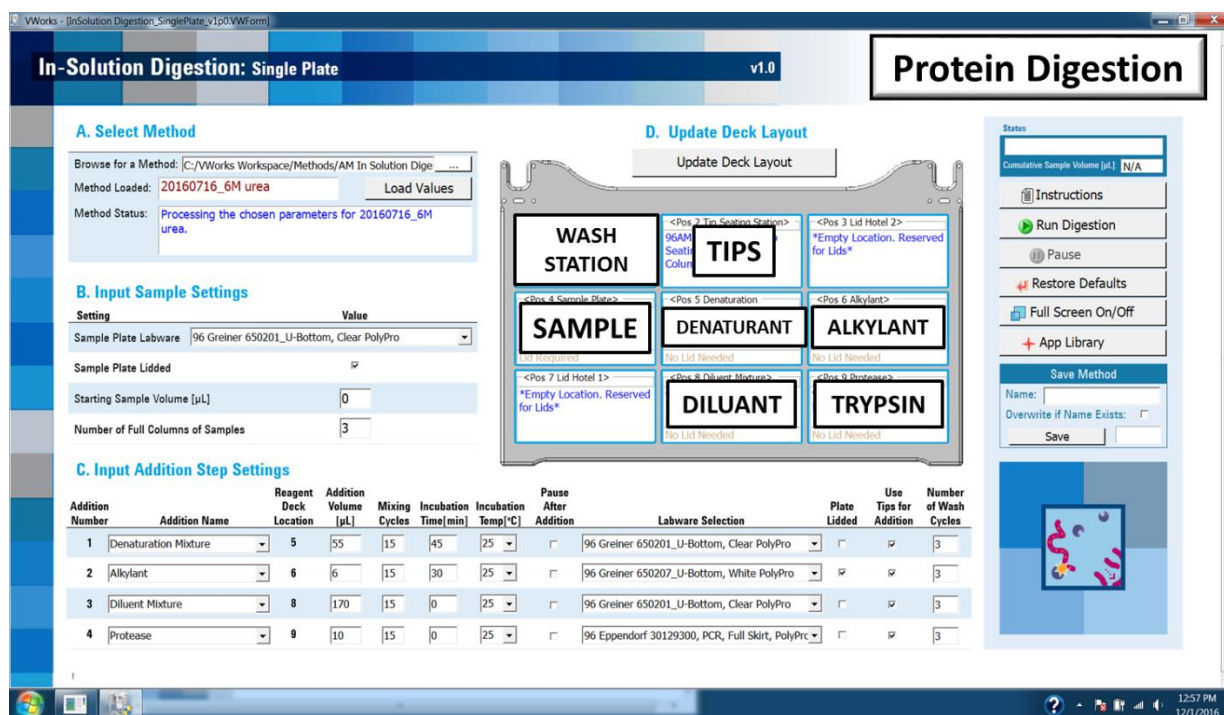


Figure 2: Sample process settings and work deck layout for automated trypsin digestion in a 96-well microplate format on the AssayMAP Bravo Platform.

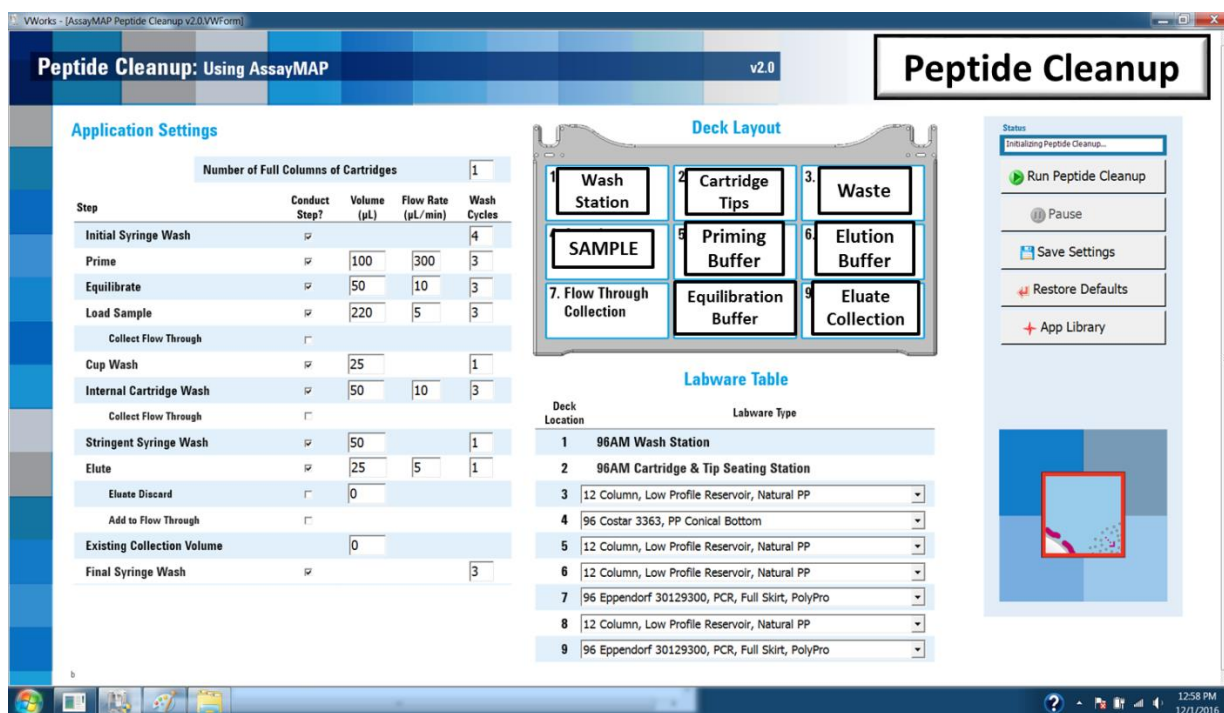


Figure 3: Sample process settings and work deck layout for automated SPE cleanup in a 96-well microplate format on the AssayMAP Bravo Platform.

Aim 2.3: Workflow Testing and Optimization (100%)

A comparison between the previously employed manual preparation protocol and the automation platform for digestion and SPE cleanup served as the final assessment of the performance parameters automated approach. This was carried out with three single-source samples of each body fluid spiked with “heavy-labeled” internal standards. The heavy-labeled internal standard mix was added to both workflows to enable comparisons of the efficiency (*i.e.*, digestion, SPE recovery, etc.) between the two methods. As indicated, some automation parameters (*e.g.*, sample volumes, C18 elution volumes, *etc.*) were modified to improve performance. This assessment was performed using the optimized digestion and solid phase extraction parameters determined under Aim 2.2. Samples were prepared in triplicate and contained single-use aliquots of body fluids collected from three individuals. IPC was spiked at the previously determined 100 ng prior to protein digestion. The heavy-labeled internal standard was spiked at a final concentration of 50 fmol/μL prior to solid phase extraction. Digestion efficiency was determined by calculating the ratio of the digested peptide in relation to the response of the heavy-labeled internal standard. From the data, equivalent responses were seen across all biofluids between the automation and manual workflows.

Phase 3: Validation and Standard Operating Procedures (10% Complete)

Validation of the automated multiplex assay will follow Standard 8.2 of the FBI’s “Quality Assurance Standards for Forensic DNA Testing Laboratories” as well as the recently released guidelines for the Collection and Serological Examination of Biological Evidence published by the Scientific Working Group on DNA Analysis Methods (SWGDM). In addition, because this workflow employs liquid chromatography and mass spectrometry techniques, appropriate guidelines from the Scientific Working Group for Forensic Toxicology (SWGTOX) will also be followed.

Aim 3.1 Reproducibility and Repeatability (100% complete)

Reproducibility and repeatability were assessed to ensure that reliable/robust results can be generated over multiple analysis batches prepared by single and multiple individuals. Over a three-day period, two batches of five pooled body fluid samples were processed individually and assayed by two different analysts in triplicate on the mass spectrometer. This includes extraction of each sample, BCA quantification, robotic digestion, SPE cleanup, and QQQ analysis. Reproducibility and repeatability were assessed based on the total protein quant found via the BCA assay as well as MS results including signal response/area, retention time, and qualitative response ratio between each transition (**Figure 4**). In addition, the performance of the internal positive control was evaluated.

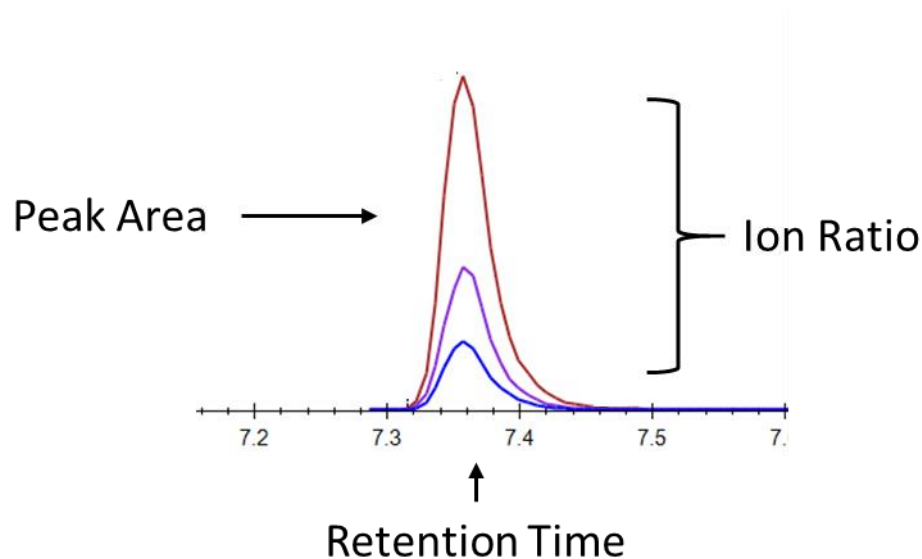


Figure 4: Parameters to be assess for the reproducibility/repeatability study.

BCA quantitation is required to normalize the total amount of protein prior to the robotic preparation. This is to ensure the proper ratios of enzyme:protein as well as loading amounts onto the SPE cartridges and mass spectrometer. The average protein concentration as well as the measured combined and individual variability of the BCA assay for each body fluid can be found in **Table 3**.

Table 3: BCA Quantitation and Variability Expressed as %CV

Fluid	Average Protein Concentration (mg/mL)	Analyst 1	Analyst 2	Combined
Menstrual Blood	35.457	5.562	7.300	6.188
Peripheral Blood	43.993	4.021	6.315	5.056
Seminal Fluid	26.100	5.437	8.342	6.861
Vaginal Fluid	2.830	6.899	8.370	8.557
Saliva	2.740	4.095	7.830	7.385

Peak Area: The peak area of each peptide in the assay was assessed for all replicates as well as across the replicates for the two analysts individually. Of the 46 peptides included in the assay, 41 showed CV values of <25% with the majority of these targets showing <10%. An illustrative example of these results for saliva is shown in **Table 4**. The remainder of the data is presented in Appendix 2.

As previously stated, a small fraction of peptides did display higher degrees of peak area variability. High %CV values were typically associated with either small hydrophilic (early eluting

peptides) or large hydrophobic peptides (later eluting peptides). For example, Semenogelin 2 GSISIQTEEK is one of the first eluting peptides in the assay at 1.9 minutes and had a peak area CV of approximately 50%. It is hypothesized that some of this target is not being captured efficiently during the SPE cleanup - resulting in more variable recovery across replicates. Likewise, it is hypothesized that more hydrophobic peptides will be bound (essentially irreversibly) to plastics during digestion and to the the SPE cartridges during cleanup. In addition, there may also be reduced resolubilization issues after any lyophilization steps. At the outset, it was expected that some peptides would display more variability in recovery, solubility, and nonspecific absorption than others, simply due to the specific chemistries associated with each peptide target. As a counterweight to this potential challenge, the ability to target multiple proteins per fluid and multiple peptides for each protein (*i.e.*, a fundamental concept underlying the design of the assay), will help to ensure that there are reliable peptides which can be consistently detected within each fluid. For example, other Semenogelin peptides (*e.g.* DVSQSSISFQIEK) show consistent CV values of approximately 8%, albeit at lower overall peak areas.

Table 4: Variability in Peak Areas for Individual Saliva Peptides. Calculated Values are Expressed as %CV.

Fluid	Protein	Peptide	Analyst 1	Analyst 2	Combined
Saliva	Alpha Amylase	LSGLLDLALGK	6.434	7.201	9.067
		IAEYMNHLIDIGVAGFR	3.954	9.962	9.079
	Stathrin	FGYGYGPYQPVPEQPLYQPYPQYQQYTF	12.736	11.058	13.988
	Submax	GPYPGGLAPPQFPGPGFVPPPPPTGPGR	7.189	9.944	10.139
		IPPPPPAPYGPFGIFPPPPQP	5.941	8.414	9.873
	Cystatin	IIEGGIYDADLNDER	5.811	10.255	12.909
		ALHFVISEYNK	5.991	8.555	10.719

Retention Time: The retention time of each peptide in the assay was assessed for all replicates as well as across the three replicates for the two analysts individually. Retention time averages for each analyst were used to calculate the variability. %CV values for this dataset were all below 0.5%. An illustrative example of these results for saliva is shown in **Table 5**. The remainder of the data is presented in Appendix 2.

Table 5: Variability in Retention Times for Individual Saliva Peptides. Calculated Values are Expressed as %CV.

Fluid	Protein	Peptide	Analyst 1	Analyst 2	Combined
Saliva	Alpha Amylase	LSGLLDLALGK	0.044	0.036	0.040
		IAEYMNHLIDIGVAGFR	0.058	0.036	0.047
	Stathrin	FGYGYGPYQPVPEQPLYQPYPQYQQYTF	0.044	0.032	0.039
	Submax	GPYPGGLAPPQFPGPGFVPPPPPTGPGR	0.076	0.055	0.064
		IPPPPPAPYGPFGIFPPPPQP	0.072	0.073	0.074
	Cystatin	IIEGGIYDADLNDER	0.053	0.053	0.051
		ALHFVISEYNK	0.000	0.000	0.000

Response Ratio: The ion ratio for each peptide was assessed by dividing the peak area response of the quantifier ion by the peak area response of the qualifier ion(s). Ion ratios were assessed for all replicates as well as across the two analysts individually. The ion ratios for all peptides were found to be highly consistent with most showing CV values of <5%. Only NGAL in menstrual blood showed higher variability in the qualitative response ratio. In this case the proteins were of very low abundance in the sample. An illustrative example of these results for saliva is shown in

Table 6. The remainder of the data is presented in Appendix 2.

Table 6: Variability in Ion Ratios for Individual Saliva Peptides. Calculated Values are Expressed as %CV.

Fluid	Protein	Peptide	Analyst 1	Analyst 2	Combined
Saliva	Alpha Amylase	LSGLLDLALGK	3.647	2.038	3.099
		IAEYMNHLIDIGVAGFR	4.826	4.782	4.867
	Stathrin	FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF	3.617	6.615	5.362
	Submax	GPYPPGPLAPPQPFPGFVPPPPPPPTGPGR	0.864	1.209	1.044
		IPPPPPAPYGPGFPPPPPPQP	0.511	0.588	0.586
	Cystatin	IIEGGIYDADLNDER	4.112	4.217	4.541
		ALHFVISEYNK	2.928	4.286	3.695

Internal Positive Control: The performance of the internal positive control was assessed by comparing the response ratio of the digested myelin basic protein to that of a spiked, isotopically labelled internal standard. The response ratio was found to be highly consistent across all body fluids. The variability observed both among different samples of individual body fluids and between different body fluids consistently displayed CV values of <8% (**Figure 5 and Table 7**). Across all samples assayed, the average $\pm$ SD myelin heavy:light ratio was 13.7 $\pm$ 0.999.

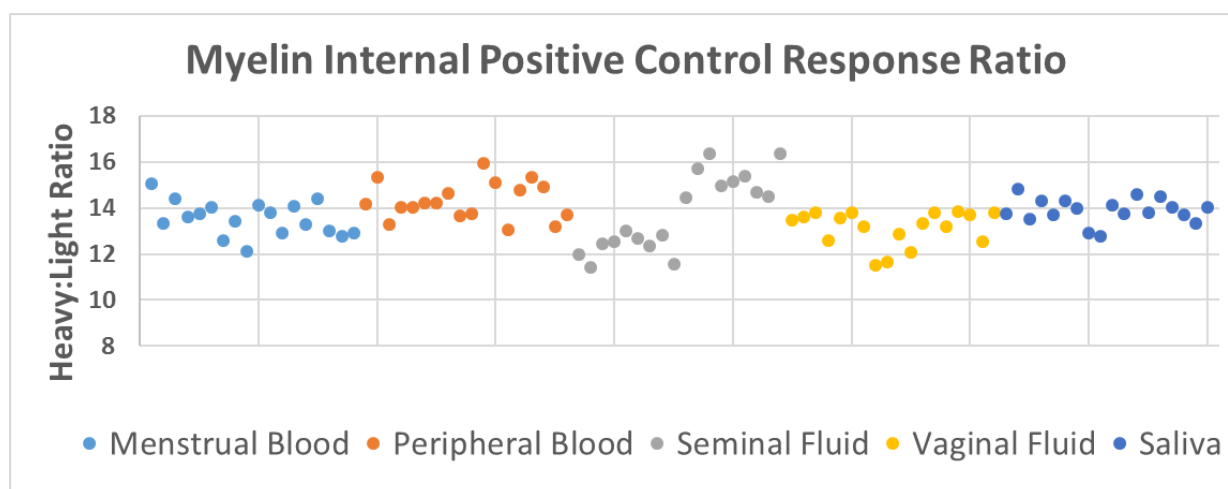


Figure 5: Internal positive control performance across Day 1 of the seminal fluid and saliva reproducibility/repeatability study. The y-axis shows response ratio of the digested myelin basic protein to that of a spiked, isotopically labelled internal standard. The x-axis reflects individual samples assayed for each body fluid. Samples of individual body fluids are randomly ordered.

Table 7: Variability in the Performance of the Internal Positive Control as Reflected by the Myelin heavy:light ratio. Calculated Values are Expressed as %CV.

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

Aim 3.2: Sensitivity (5% complete) - A series of fifteen 2-fold dilutions (1:1 through 1:16,384) will be prepared for each body using pooled material from multiple individuals. The samples will be assayed to establish the minimal protein quantity above which, reliable results can be obtained. It is expected that total protein concentrations will vary by body fluid. Seminal fluid, for example, has an approximate total protein concentration of 30 mg/mL. A series of fifteen 2-fold dilutions, therefore, would correspond on the lower end to the pg/mL range when analyzed by the mass spectrometer. The samples for this study have already been prepared and are scheduled to be run in November 2016.

Aim 3.3: Stability (50% complete) - Stability is being evaluated to ensure that samples remain viable if an instrumentation error or power outage were to occur that causes samples either: (1) to remain in the autosampler overnight without being processed or (2) to be subjected to additional freeze-thaw cycles. This aim will assess two aspects of samples stability. First, autosampler stability was evaluated by storing processed samples in the chilled autosampler and analyzing them over a period of 3 days. Second (in progress), freeze/thaw stability will be assessed using nine aliquots of pooled samples. Three samples will be assayed immediately and will serve as a baseline. The remaining six samples will be frozen at -80°C. These will be all be thawed the following day. Three of the thawed samples will be analyzed on the second day and the remaining three will be refrozen for subsequent thawing and analysis on the following day.

The autosampler stability was completed by assessing three replicates of the five target body fluids over a period of 3 days. Samples were analyzed on the Day 1 (*i.e.*, 0 hours.) and stored on the instrument's chilled autosampler which was set at 8°C. This study was designed to determine whether or not there would be a significant drop in the detected signal for samples stored on the autosampler. A significant drop in signal strength or quality would indicate that any samples stored in excess of 24 hours on the autosampler would need to be re-extracted, digested and cleaned up. Fortunately, it was shown that this is not the case. There was no significant drop in peak area response for any target peptides in the assay. On the contrary, a slight increase in peak area response was often seen. This was evident across all fluids; however, this increase is not significant (**Figure 6**). This was attributed to sample evaporation and concentration of target peptides that occurred after the 96-well plate cover was punctured for initial injection. This results in the evaporation. Results from this portion of the study can be seen in Appendix 3. In addition, ion ratios for the myelin internal positive control were assessed for autosampler stability. There was no significant difference between ion ratios for 0 and 24 hours ($t_s=0.404$, $df=58$; $P>0.05$) or for 0 and 48 hours ($t_s=1.084$, $df=58$, $P>0.05$). %CV values for the internal positive control are listed in **Table 9**. Overall, these results indicate that samples plated on the autosampler can be analyzed within two days of being processed without any significant change in signal strength.

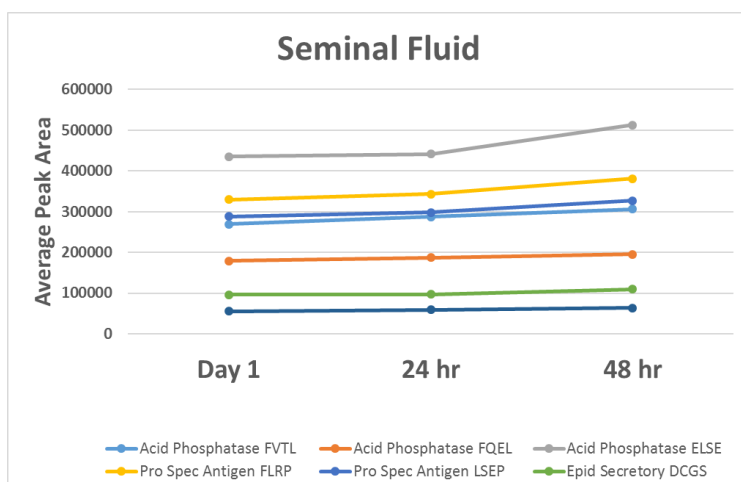


Figure 6: Peak area for seminal fluid targets stored on the instrument autosampler for two days post-analysis.

Table 8: Myelin internal positive control %CV values for Day 1, +24 hours, and +48 hours in the autosampler.

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

Aim 3.4: Mixture Studies (100% complete)

Pooled samples from ten individuals were prepared in triplicate as 1:1 v/v mixtures among each of the five fluids. The primary purpose of these mixture studies is to identify potential cross-fluid interference and ion suppression that could produce a false positive/negative result. The pairwise matrix of five body fluids includes all possible combinations of body fluid interactions. These studies are very similar to the interference studies that are typically performed as part of LC-MS validations for toxicological assays. It is important to emphasize that the objective of the mixture studies under this aim is fundamentally different from mixture studies used in forensic DNA testing. In the context of a DNA assay validation the goal of a mixture study is typically to detect and resolve major and minor contributors.

In several cases, cross fluid interferences were detected which could potentially lead to difficulties in the interpretation of results. These have been identified, and using an abundance of caution, the interfering transition have either been removed or replaced. **Figure 7** provides an illustrative example of this. A major interference peak can be detected with the prostatic acid phosphatase peptide FVTLVFR when seminal fluid is mixed with blood. In this specific case, the transition 534.3398 generates the interference. By replacing transition 534.3398 with transition 247.11, the problem is readily eliminated in all subsequent analyses.

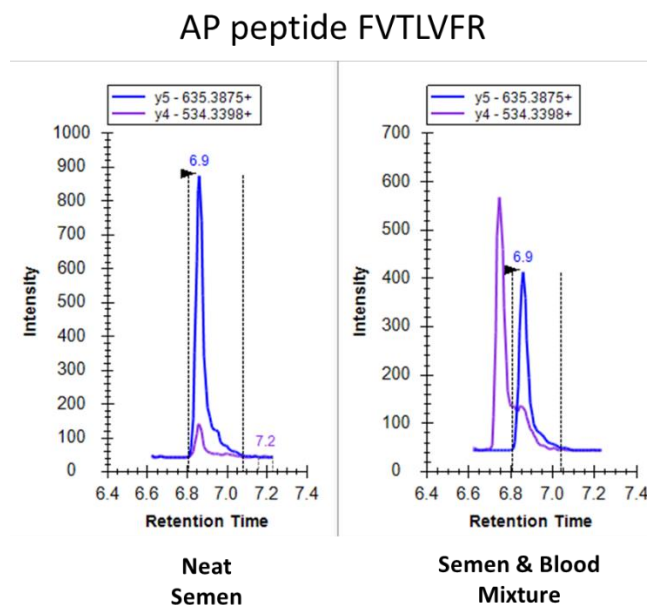


Figure 7: Acid Phosphatase peptide FVTLVFR. Interferences can be observed when seminal fluid is mixed with blood.

Aim 3.9: Carryover (100% complete)

Autosampler carryover has been reassessed during this reporting period after making more stringent injector and column washing steps. Furthermore, additional lower quantities of injected protein has been evaluated to see if carryover could be minimized or eliminated.

To assess carryover, triplicate injections with variable quantities of protein have been loaded onto the system (.5 µg, 1 µg, 2.5 µg, 5 µg, 10 µg, 20 µg, and 30 µg protein quantities). Following each injection, blank injections immediately followed to determine whether any carryover interference are detectable above the assay's lower limit of detection. Results of this study can be found in **Table 9**. Of the fifty peptide targets present in the assay, only two peptides (Hemoglobin peptide LLVYYPWTQR and Semenogelin 1 peptide DIFSTQDELLVYNK) showed an indication of carryover between the different loading quantities. Low levels of Semenogelin 1 can be detected after a 5 µg injection, whereas low levels of Hemoglobin can be detected across all levels tested (**Figure 8**). In all cases, the amount of carryover was extremely small (e.g., 0.005% of the total peak area in the blank vs. analyzed sample).

There are some additional steps that could be taken to eliminate the traces of carryover. The Agilent 1290 Infinity Flexible Cube is an auxiliary module for the 1290 autosampler with a built-in pump to facilitate enhanced flushing capabilities and to reduce carry-over. This could be acquired and used for the remainder of the project or evaluated later as an add-on option. Second, HemogloBind™, which is capable of selectively depleting ≈ 80% of the hemoglobin from protein samples, is another solution for the hemoglobin carryover but would require additional sample preparation steps prior to protein quantification and digestion.

Consistent biomarker detection across all five fluids is possible using lower levels of injected protein (e.g. 1 µg or 2.5 µg) while also limiting the potential for carryover. However, when an unknown sample is processed with high levels of visible hemoglobin, a blank should be

run to ensure that no carryover is detected in subsequent samples. As another option, a cutoff threshold could be implemented in the interpretation guidelines where the response of the hemoglobin peak must be > 1000 counts when analyzing $1\ \mu\text{g}$ or $2.5\ \mu\text{g}$ of suspected blood.

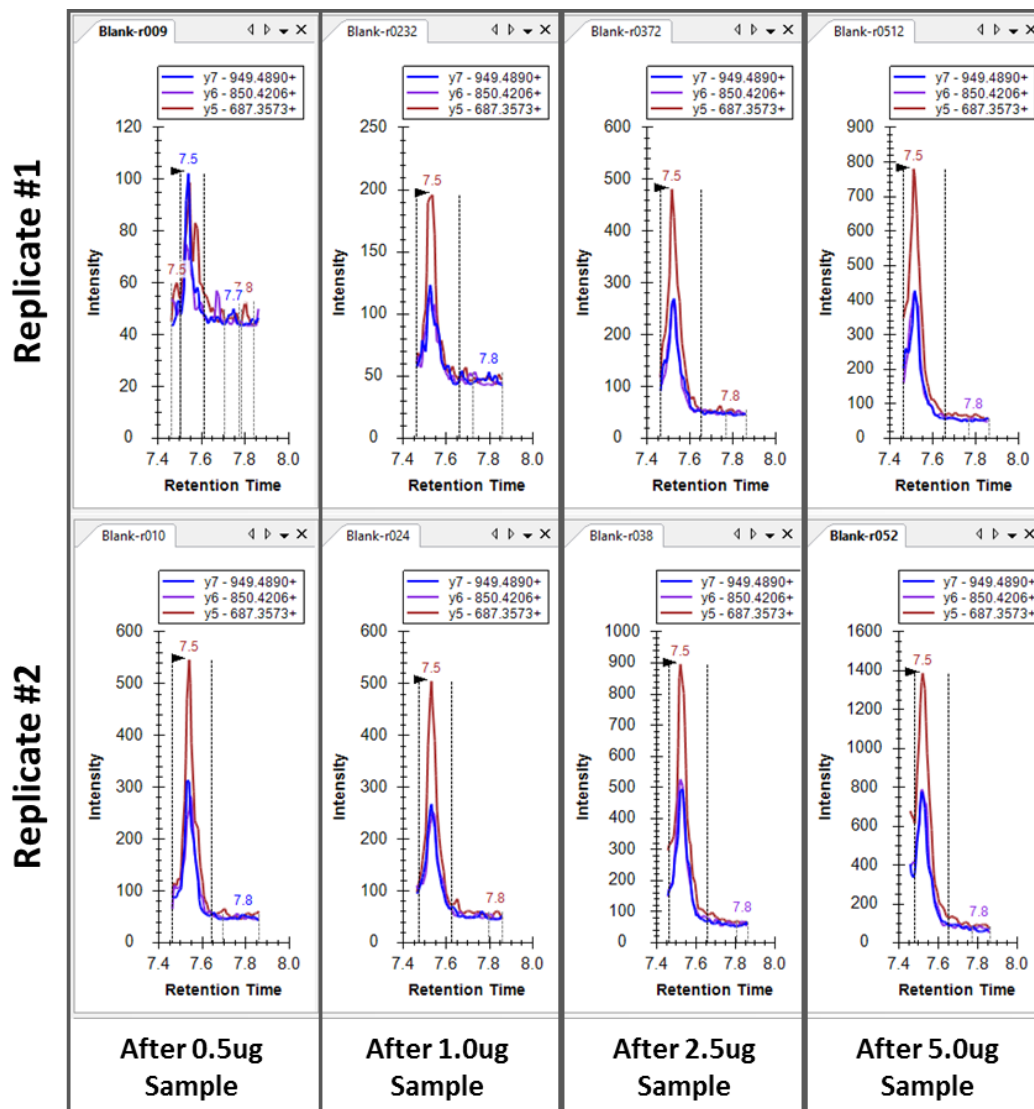


Figure 8: Hemoglobin carryover across low levels of injected whole blood.

Table 9: Carryover Study Results

		Blank (ug)						
Fluid	Protein	0.5	1.0	2.5	5.0	10.0	20.0	30.0
Peripheral Blood	Alpha-1 Antitrypsin							
	Hemoglobin							
	Hemopexin							
	Apolipoprotein							
	Serotransferrin							
Fluid	Protein	0.5	1.0	2.5	5.0	10.0	20.0	30.0
Saliva	Alpha Amylase							
	Stathrin							
	Submax							
	Cystatin							
Fluid	Protein	0.5	1.0	2.5	5.0	10.0	20.0	30.0
Vaginal Fluid	Cornulin							
	Neutrophil Gelatinase							
	Ly6/PLAUR							
	Suprabasin							
	Periplakin							
	Small PRP 3							
	Involucrin							
Fluid	Protein	0.5	1.0	2.5	5.0	10.0	20.0	30.0
Seminal Fluid	PPAP							
	PSA							
	Semenogelin 2							
	Semenogelin 1							
	Epididymal Secretory							
Fluid	Protein	0.5	1.0	2.5	5.0	10.0	20.0	30.0
Menstrual Blood	Cornulin							
	Neutrophil Gelatinase							
	Ly6/PLAUR							
	Suprabasin							
	Periplakin							
	Involucrin							
	Small PRP 3							
	Alpha-1 Antitrypsin							
	Hemoglobin							
	Hemopexin							
	Apolipoprotein							
	Serotransferrin							

Green = Target Protein Detected. Red = No Protein Detected.

Training and Professional Development Opportunities:

Nothing to report.

Dissemination:**Goals for Next Reporting Period:**

During the next reporting period (November 1st to November 31st) effort on phase 3 of the collaborative agreement will continue.

It is anticipated that the sensitivity and stability studies will be completed during the next reporting period.

PRODUCTS:

Nothing to report.

PARTICIPANTS and OTHER COLLABORATING ORGANIZATIONS:

Nothing to report.

IMPACT:

Nothing to report.

CHANGES/PROBLEMS:

Nothing to report.

Appendix 1:

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

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

Bio Fluid	Protein	Peptide Sequence	Precursor ion (m/z)	Product ions (m/z)	Collision Energy (V)	Retention Time (min)
Semen	Semenogelin-2	GSISIQTEEQIHGK	509.5985 +++	691.8673 ++	7.5	2.5
				635.3253 ++	19.5	
				461.5807 +++	10.5	
		DVSQSSISFQIEK	734.3699 ++	1038.5466 +	23.8	5
				951.5146 +	26.8	
				751.3985 +	20.8	
		GSISIQTEEK	546.2826 ++	517.2980 +	20.8	2
				834.4203 +	14.9	
				747.3883 +	20.9	
	Semenogelin-1	DIFSTQDELLVYNK	842.9251 ++	634.3042 +	13.9	7.4
				1309.6634 +	27.1	
				523.2875 +	24.1	
		QTIPSQEQEHSQK	551.6128 +++	424.2191 +	24.1	
				706.3442 ++	12.1	
				599.2784 ++	18.1	
	Prostate-specific antigen	LSEPAELTDAVK	636.8677 ++	1072.5521 +	17.7	4.4
				943.5095 +	20.7	
				646.3770 +	23.7	
		FLRPGDDSSHDLMLLR	468.7413 ++++	472.2584 ++	20.7	5.9
				760.4386 +	15.1	
				536.2844 ++	15.1	
	Prostatic acid phosphatase	FQEESETLK	612.3113 ++	575.6298 +++	18.1	3.5
				948.4884 +	20.0	
				819.4458 +	23.0	
		ELSELSLSLYGIHK	567.9856 +++	276.1343 +	20.0	8.8
				930.5407 +	18.6	
				817.4567 +	18.6	
		FVTLVFR	441.2658 ++	730.4246 +	18.6	6.9
				635.3875 +	14.7	
				247.1441 +	14.7	
	Epididymal secretory protein E1	SGINCP1QK	508.7633 ++	421.2558 +	14.7	1.7
				759.3831 +	16.8	
				645.3389 +	16.8	
		DCGSVDGV1K	525.2502 ++	485.3082 +	16.8	2.4
				774.4356 +	14.3	
				630.3821 +	14.3	
				276.0649 +	17.3	

Bio Fluid	Protein	Peptide Sequence	Precursor ion (m/z)	Product ions (m/z)	Collision Energy (V)	Retention Time (min)
Vaginal Fluid/Menstrual Blood	Cornulin	AHQTGETVTGSGTQTQAGATQTV EQDSSSHQTGR	839.8888 +++	1001.4647 ++	28.4	1.4
				886.9115 ++	22.4	
				851.3930 ++	25.4	
		ISPQIQLSGQTEQTQK	893.4707 ++	1247.6226 +	31.7	3.4
				1006.4800 +	31.7	
				793.4127 ++	31.7	
		LLDEDHTGTVEFK	501.9157 +++	294.1812 +	13.3	3.6
				695.8279 ++	16.3	
				639.2859 ++	16.3	
	Neutrophil gelatinase-associated lipocalin	WYVVGLAGNAILR	716.4090 ++	884.5312 +	23.2	8.8
				350.1499 +	20.2	
				449.2183 +	23.2	
		MYATIYELK	566.2914 ++	1000.5350 +	18.6	5.4
				837.4716 +	21.6	
				766.4345 +	15.6	
		GCVQDEFCTR	636.2608 ++	295.1111 +	15.6	2.3
				1054.4622 +	23.7	
				955.3938 +	20.7	
	Ly6/PLAUR domain-containing protein 3	GLDLHGLLAFIQLQQCAQDR	766.0671 +++	827.3352 +	23.7	10.3
				905.3894 +	22.8	
				649.2722 +	25.8	
		ALDGINSGITHAGR	461.2443 +++	1038.5778 +	19.8	2.8
				541.2841 +	14.8	
				599.3022 ++	11.8	
		FGQGVHHGLSEGWK	513.5881 +++	696.3444 ++	13.7	2.3
				603.8044 ++	16.7	
				659.8775 ++	10.3	
	Involucrin	GEVLLPVEHQQQK	502.2755 +++	610.3433 ++	13.3	3.3
				553.8013 ++	13.3	
				505.7669 ++	14.1	
		QEAQLELPEQVGVQPK	607.9829 +++	699.3308 +	17.1	4.2
				812.4149 +	14.1	
				689.3213 +	15.9	
		HLVQQEGQLEQQER	574.6237 +++	560.2787 +	12.9	1.5
				689.3213 +	15.9	
				920.4585 +	12.9	
	Periplakin	NQGPQESVVR	557.2860 ++	871.4632 +	24.3	1.4
				814.4417 +	18.3	
				243.1088 +	18.3	
		NLLDEIASR	515.7800 ++	803.4258 +	17.0	6.2
				690.3417 +	17.0	
				228.1343 +	14.0	
		AQSLQSAK	416.7298 ++	633.3566 +	10.9	0.9
				546.3246 +	16.9	
				200.1030 +	13.9	
	Small Proline Rich Protein 3	VPEPGCTK	444.2182 ++	788.3607 +	17.8	1.1
				562.2654 +	17.8	
				394.6840 ++	17.8	
		VPVPGYTK	430.7475 ++	664.3665 +	17.4	2.3
				565.2980 +	14.4	
				381.2132 ++	14.4	

Appendix 2:

Peak Area Response calculated %CV.

Fluid	Protein	Peptide	Analyst 1	Analyst 2	Combined
Peripheral Blood	Alpha-1 Antitrypsin	LSITGYDLK	11.845	10.800	11.530
		SVLGQLGITK	9.366	9.210	9.336
		SAVTALWGK	7.054	6.768	6.792
	Hemoglobin	LLVVYPWTQR	7.457	5.374	6.463
	Hemopexin	NFPSPVDAAFR	10.990	9.664	10.756
	Apolipoprotein	LLDNWDSVTSTFSK	13.664	9.755	11.945
		VSFLSALEEYTK	7.843	15.313	12.289
		DGAGDVAFVK	12.350	10.574	11.615
	Serotransferrin	SASDLTWDNLK	6.351	7.044	7.045
Fluid	Protein	Peptide	Analyst 1	Analyst 2	Combined
Saliva	Alpha Amylase	LSGLLDLALGK	6.434	7.201	9.067
		IAEYMNHLIDIGVAGFR	3.954	9.962	9.079
		FGYGYGPYPVPEQPLYPQYPQYQQYTF	12.736	11.058	13.988
	Submax	GPYPGGLAPPQPFPGFVPPPPPTGPGR	7.189	9.944	10.139
		IPPPPPAPYGPGFPPPPQP	5.941	8.414	9.873
	Cystatin	IEGGIYDADLNDER	5.811	10.255	12.909
Vaginal Fluid	Cornulin	LLDEDHTGTVEFK	6.647	15.880	13.902
		ISPQIQLSGQTEQTQK	6.635	17.521	14.595
		AHQGTGETVTGSGTQTQAGATQTVEQDSSHQTGR	4.567	10.743	8.064
		WYVVGLAGNAILR	7.926	11.252	10.790
	Neutrophil Gelatinase	MYATIYELK	9.313	12.546	12.265
		GLDHGLLAFIQLQCAQDR	10.027	7.458	8.874
	Ly6/PLAUR	GCVQDEFCTR	7.244	16.604	14.520
	Suprabasin	ALGDINSGITHAGR	4.790	12.886	11.096
		FGQGVHHLSEGWK	10.922	13.427	13.655
		AQSLQSAK	5.933	11.403	11.056
	Periplakin	NLLDEIASR	9.246	15.735	16.460
		NQGPQESVVR	7.738	13.654	12.771
	Small Proline Rich Protein 3	VPEPGCTK	8.700	7.030	8.070
		VPVPGYTK	9.925	11.226	10.284
		HLVQQEGQLEQQR	5.846	18.955	15.417
	Involucrin	QEAQLELPEQQVGQPK	4.357	15.600	12.361
		GEVLLPVEHQQK	9.393	10.984	10.677
Fluid	Protein	Peptide	Analyst 1	Analyst 2	Combined
Seminal Fluid	Acid Phosphatase	FVTLVFR	87.638	41.252	95.688
		FQELESETLK	19.405	14.717	26.483
		ELSELILLSYGIHK	5.787	10.988	8.773
	Prostate Specific Antigen	FLRPGDDSSHDLMILLR	54.820	21.388	62.553
		LSEPAELTDAVK	34.849	17.358	45.393
	Semenogelin 2	GSISIQTEEK	38.678	19.467	51.764
		GSISIQTEEQIHGK	10.208	10.259	15.247
		DVSQSSISFQIEK	2.677	11.744	8.304
	Semenogelin 1	DIFSTQDELLVYNK	5.340	10.368	8.063
		QITIPSQEQEHSQK	6.822	7.261	7.747
	Epididymal Secretory	DCGSVDGVK	6.243	11.758	9.125
		SGINCPQK	9.318	9.948	9.425
Fluid	Protein	Peptide	Analyst 1	Analyst 2	Combined
Menstrual Blood	Cornulin	LLDEDHTGTVEFK	14.904	12.570	14.500
		ISPQIQLSGQTEQTQK	N/D	N/D	N/D
		AHQGTGETVTGSGTQTQAGATQTVEQDSSHQTGR	N/D	N/D	N/D
	Neutrophil Gelatinase	WYVVGLAGNAILR	24.821	15.549	20.181
		MYATIYELK	22.655	24.441	23.949
	Ly6/PLAUR	GLDHGLLAFIQLQCAQDR	N/D	N/D	N/D
		GCVQDEFCTR	N/D	N/D	N/D
	Suprabasin	ALGDINSGITHAGR	N/D	N/D	N/D
		FGQGVHHLSEGWK	N/D	N/D	N/D
	Periplakin	AQSLQSAK	N/D	N/D	N/D
		NLLDEIASR	N/D	N/D	N/D
		NQGPQESVVR	N/D	N/D	N/D
	Involucrin	HLVQQEGQLEQQR	N/D	N/D	N/D
		QEAQLELPEQQVGQPK	N/D	N/D	N/D
		GEVLLPVEHQQK	N/D	N/D	N/D
	Small Proline Rich Protein 3	VPEPGCTK	8.312	10.234	10.224
		VPVPGYTK	8.304	4.714	8.635
	Alpha-1 Antitrypsin	LSITGYDLK	19.303	11.382	16.432
		SVLGQLGITK	13.169	11.973	12.729
	Hemoglobin	SAVTALWGK	9.186	6.718	8.131
		LLVVYPWTQR	8.966	5.508	7.739
	Hemopexin	NFPSPVDAAFR	15.337	12.862	14.875
	Apolipoprotein	LLDNWDSVTSTFSK	21.725	13.442	19.061
		VSFLSALEEYTK	22.803	17.151	21.686
	Serotransferrin	DGAGDVAFVK	15.868	11.908	14.349
		SASDLTWDNLK	12.424	11.779	12.704

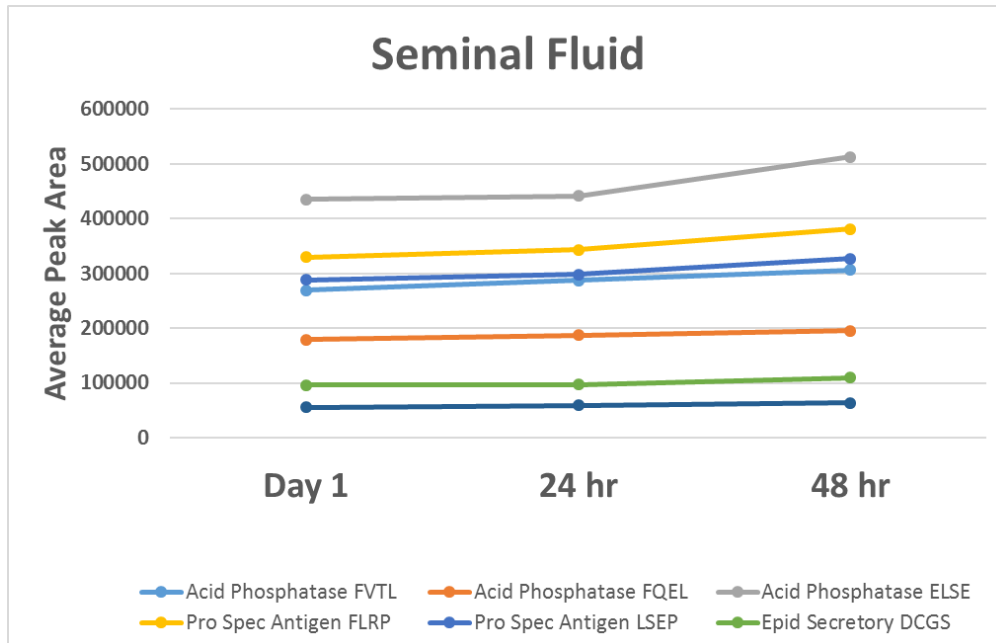
Retention Time calculated %CV.

Fluid	Protein	Peptide	Analyst 1	Analyst 2	Combined
Peripheral Blood	Alpha-1 Antitrypsin	LSITGTYDLK	0.093	0.093	0.090
		SVLGQLGITK	0.077	0.043	0.060
	Hemoglobin	SAVTALWGK	0.106	0.132	0.118
		LLVVYPWTQR	0.184	0.124	0.157
	Hemopexin	NFSPVDAAFR	0.062	0.059	0.059
		LLDNWDSVTSTFSK	0.109	0.070	0.089
	Apolipoprotein	VSFLSALEETK	0.106	0.025	0.075
		DGAGDVAFK	0.000	0.000	0.000
	Serotransferrin	SASDLTWDNLK	0.102	0.076	0.088
Fluid	Protein	Peptide	Analyst 1	Analyst 2	Combined
Saliva	Alpha Amylase	LSGLLDLALGK	0.044	0.036	0.040
		IAEYMNHLIDIGVAGFR	0.058	0.036	0.047
	Stathrin	FGYGYGPYQPVPEQLYPQPYQPQQYTF	0.044	0.032	0.039
	Submax	GPYPPGLAPPQPFPGPGFVPPPPPTGPGR	0.076	0.055	0.064
		IPPPPPAPYVGGIFPPPPQP	0.072	0.073	0.074
	Cystatin	IIEGGIYDADLNDER	0.053	0.053	0.051
		ALHFVISEYNK	0.000	0.000	0.000
Fluid	Protein	Peptide	Analyst 1	Analyst 2	Combined
Vaginal Fluid	Cornulin	LLDEHTGTVEFK	0.118	0.065	0.093
		ISPQQLSGQTEQTQK	0.128	0.107	0.115
		AHQTGETVTGSGTQTQAGATQTVQDSSHQTGR	0.176	0.264	0.226
	Neutrophil Gelatinase	WYVVGLAGNAILR	0.064	0.036	0.052
		MYATIYELK	0.066	0.066	0.064
	Ly6/PLAUR	GLDHGLLAFIQLQCAQDR	0.029	0.022	0.025
		GCVQDEFCTR	0.193	0.170	0.185
	Suprabasin	ALGDINSGITHAGR	0.135	0.171	0.152
		FGQGVHHGLSEGWK	0.248	0.159	0.215
	Periplakin	AQSLSQSAK	0.000	0.269	0.190
		NLLDEIASR	0.038	0.061	0.053
	Small Proline Rich Protein 3	NQGPQESVVR	0.186	0.295	0.258
		VPEPGCTK	0.324	0.342	0.331
	Involucrin	VPVPGYTK	0.172	0.163	0.163
		HLVQQEGQLEQQR	0.000	0.225	0.165
		QEAQLELPEQQVGPQK	0.100	0.118	0.107
		GEVLLPVEHQQQK	0.155	0.110	0.136
Fluid	Protein	Peptide	Analyst 1	Analyst 2	Combined
Seminal Fluid	Acid Phosphatase	FVTLVFR	0.049	0.059	0.054
		FQLESETLK	0.069	0.069	0.067
		ELSELSLLSLYGIHK	0.058	0.059	0.058
	Prostate Specific Antigen	FLRPGDDSSHDLMLLR	0.081	0.081	0.078
		LSEPAELTDAVK	0.085	0.071	0.078
	Semenogelin 2	GSISIQTEEK	0.124	0.124	0.120
		GSISIQTEEQIHGK	0.134	0.160	0.147
		DVSQSSISFQIEK	0.075	0.079	0.076
	Semenogelin 1	DIFSTQDELLVYNK	0.063	0.042	0.052
		QITIPSQEQEHSQK	0.174	0.132	0.151
	Epididymal Secretory	DCGSVDGVK	0.138	0.157	0.148
		SGINCPIQK	0.147	0.000	0.104
Fluid	Protein	Peptide	Analyst 1	Analyst 2	Combined
Menstrual Blood	Cornulin	LLDEHTGTVEFK	0.104	0.118	0.113
		ISPQQLSGQTEQTQK	N/D	N/D	N/D
		AHQTGETVTGSGTQTQAGATQTVQDSSHQTGR	N/D	N/D	N/D
	Neutrophil Gelatinase	WYVVGLAGNAILR	0.089	0.083	0.084
		MYATIYELK	0.066	0.114	0.093
	Ly6/PLAUR	GLDHGLLAFIQLQCAQDR	N/D	N/D	N/D
		GCVQDEFCTR	N/D	N/D	N/D
	Suprabasin	ALGDINSGITHAGR	N/D	N/D	N/D
		FGQGVHHGLSEGWK	N/D	N/D	N/D
	Periplakin	AQSLSQSAK	N/D	N/D	N/D
		NLLDEIASR	N/D	N/D	N/D
		NQGPQESVVR	N/D	N/D	N/D
	Involucrin	HLVQQEGQLEQQR	N/D	N/D	N/D
		QEAQLELPEQQVGPQK	N/D	N/D	N/D
		GEVLLPVEHQQQK	N/D	N/D	N/D
	Small Proline Rich Protein 3	VPEPGCTK	0.000	0.000	0.000
		VPVPGYTK	0.231	0.163	0.202
	Alpha-1 Antitrypsin	LSITGTYDLK	0.078	0.082	0.078
		SVLGQLGITK	0.064	0.085	0.073
	Hemoglobin	SAVTALWGK	0.066	0.128	0.099
		LLVVYPWTQR	0.176	0.094	0.137
	Hemopexin	NFSPVDAAFR	0.114	0.071	0.094
	Apolipoprotein	LLDNWDSVTSTFSK	0.106	0.043	0.078
		VSFLSALEETK	0.054	0.062	0.057
	Serotransferrin	DGAGDVAFK	0.100	0.136	0.122
		SASDLTWDNLK	0.102	0.051	0.078

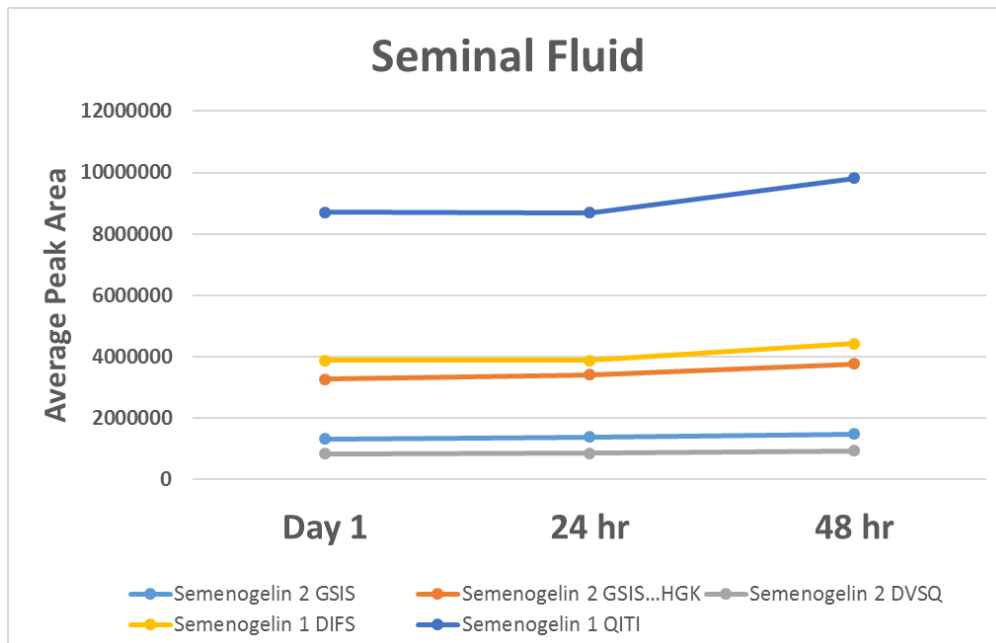
Ion Ratio calculated %CV.

Fluid	Protein	Peptide	Analyst 1	Analyst 2	Combined
Peripheral Blood	Alpha-1 Antitrypsin	LSITGTYDLK	7.520	5.011	6.315
		SVLGQLGITK	3.103	3.745	3.341
	Hemoglobin	SAVTALWVK	4.820	4.842	4.743
		LLVVPWTQR	1.963	1.824	1.969
	Hemopexin	NFPSVDAAFR	3.156	3.490	3.496
		LLDNWDSVTSTFSK	1.830	3.817	3.088
	Apolipoprotein	VSFLSALEEYTK	2.973	2.785	2.796
		DGAGDVAFVK	3.095	2.746	2.853
	Serotransferrin	SASDLTWDNLK	4.843	5.640	5.469
Fluid	Protein	Peptide	Analyst 1	Analyst 2	Combined
Saliva	Alpha Amylase	LSGLLDLALGK	3.647	2.038	3.099
		IAEYMNHLIDIGVAGFR	4.826	4.782	4.867
	Stathrin	FGYGYGPYQPVPEQPLYPQPYQPQQYTF	3.617	6.615	5.362
		GPYPPGPLAPPQFPGPGFVPPPPPPPTGPR	0.864	1.209	1.044
	Submax	IPPPPPAPYVGGIFPPPPPPQ	0.511	0.588	0.586
		IEGGIYDADLNDER	4.112	4.217	4.541
	Cystatin	ALHFVISEYNK	2.928	4.286	3.695
Fluid	Protein	Peptide	Analyst 1	Analyst 2	Combined
Vaginal Fluid	Cornulin	LLDEDHTGTVEFK	4.193	3.883	4.534
		ISPQQLSGQTEQTQK	2.287	2.340	2.333
		AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR	7.412	6.244	7.175
	Neutrophil Gelatinase	WYVVGLAGNAILR	6.804	5.743	6.108
		MYATIYELK	4.088	3.308	3.753
	Ly6/PLAUR	GLDHGLLAFIQLQCCAQDR	2.913	1.805	2.364
		GCVQDEFCTR	5.220	4.690	5.500
	Suprabasin	ALGDINSGITHAGR	3.605	5.569	4.568
		FGQGVHHGLSEGWK	6.678	6.419	6.370
	Periplakin	AQSLQSAK	2.477	2.819	2.896
		NLLDEIASR	4.053	3.911	4.113
		NQGPGQESVVR	8.886	3.902	7.232
		VPEPGCTK	4.396	3.693	3.954
	Small Proline Rich Protein 3	VPVPGYTK	5.035	2.408	4.044
		HLVQKEGQLEQQR	5.630	7.285	6.368
	Involucrin	QEAQLELPEQQVQPK	1.731	2.578	2.309
		GEVLLPVEHQQQK	2.940	2.489	2.651
Fluid	Protein	Peptide	Analyst 1	Analyst 2	Combined
Seminal Fluid	Acid Phosphatase	FVTLVFR	1.503	1.240	1.342
		FQELESETLK	4.248	3.127	3.636
		ELSELSLSLYGIHK	4.844	2.577	4.455
	Prostate Specific Antigen	FLRPGDDSSHDLMLLR	3.828	3.360	3.677
		LSEPAELTDAVK	1.918	1.032	1.601
	Semenogelin 2	GSISIQTEEK	4.360	3.249	4.025
		GSISIQTEEQIHGK	3.749	3.127	3.505
		DVSQSSISFQIEK	6.209	3.842	5.066
	Semenogelin 1	DIFSTQDELLVYNK	3.455	4.174	3.801
		QITIPSQEQEHSQK	2.578	3.036	2.810
	Epididymal Secretory	DCGSVDGVIK	5.835	5.021	5.501
		SGINCPIQK	3.971	3.225	3.863
Fluid	Protein	Peptide	Analyst 1	Analyst 2	Combined
Menstrual Blood	Cornulin	LLDEDHTGTVEFK	5.145	7.970	6.519
		ISPQQLSGQTEQTQK	N/D	N/D	N/D
		AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR	N/D	N/D	N/D
	Neutrophil Gelatinase	WYVVGLAGNAILR	11.812	14.081	12.682
		MYATIYELK	8.859	29.230	20.998
	Ly6/PLAUR	GLDHGLLAFIQLQCCAQDR	N/D	N/D	N/D
		GCVQDEFCTR	N/D	N/D	N/D
	Suprabasin	ALGDINSGITHAGR	N/D	N/D	N/D
		FGQGVHHGLSEGWK	N/D	N/D	N/D
	Periplakin	AQSLQSAK	N/D	N/D	N/D
		NLLDEIASR	N/D	N/D	N/D
		NQGPGQESVVR	N/D	N/D	N/D
		HLVQKEGQLEQQR	N/D	N/D	N/D
	Involucrin	QEAQLELPEQQVQPK	N/D	N/D	N/D
		GEVLLPVEHQQQK	N/D	N/D	N/D
	Small Proline Rich Protein 3	VPEPGCTK	4.830	1.963	3.579
		VPVPGYTK	5.886	7.259	6.432
	Alpha-1 Antitrypsin	LSITGTYDLK	4.598	4.323	4.418
		SVLGQLGITK	3.147	2.257	2.661
	Hemoglobin	SAVTALWVK	4.661	3.597	4.200
		LLVVPWTQR	3.446	2.150	2.820
	Hemopexin	NFPSVDAAFR	3.964	2.758	3.320
		LLDNWDSVTSTFSK	3.863	2.246	3.070
	Apolipoprotein	VSFLSALEEYTK	3.316	3.242	3.805
		DGAGDVAFVK	2.730	3.418	3.001
	Serotransferrin	SASDLTWDNLK	3.875	6.952	5.488

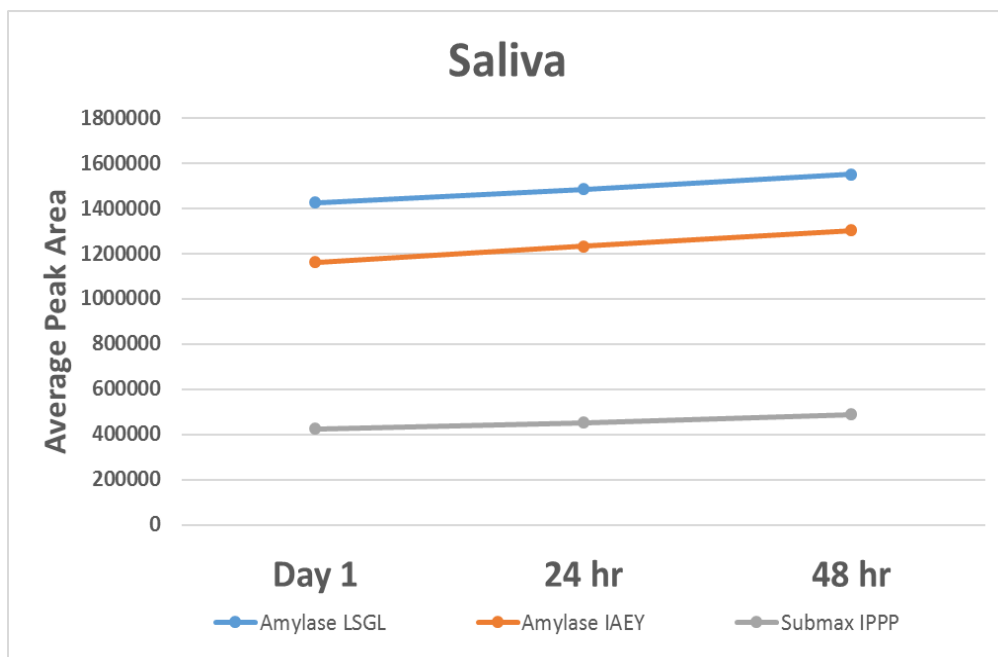
Appendix 3:



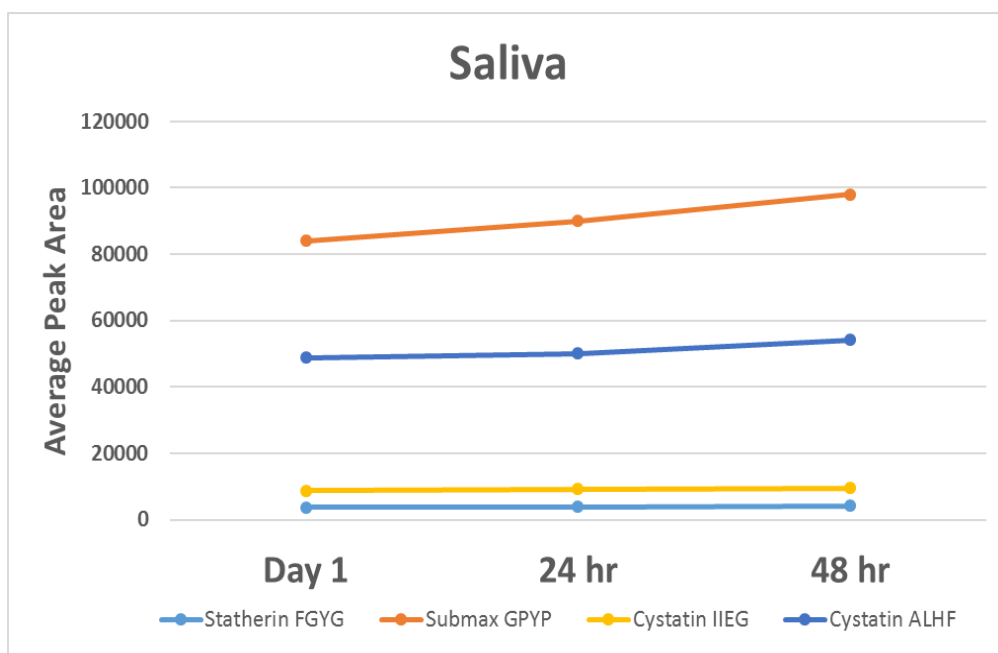
Seminal fluid autosampler stability data for high abundant peptide markers.



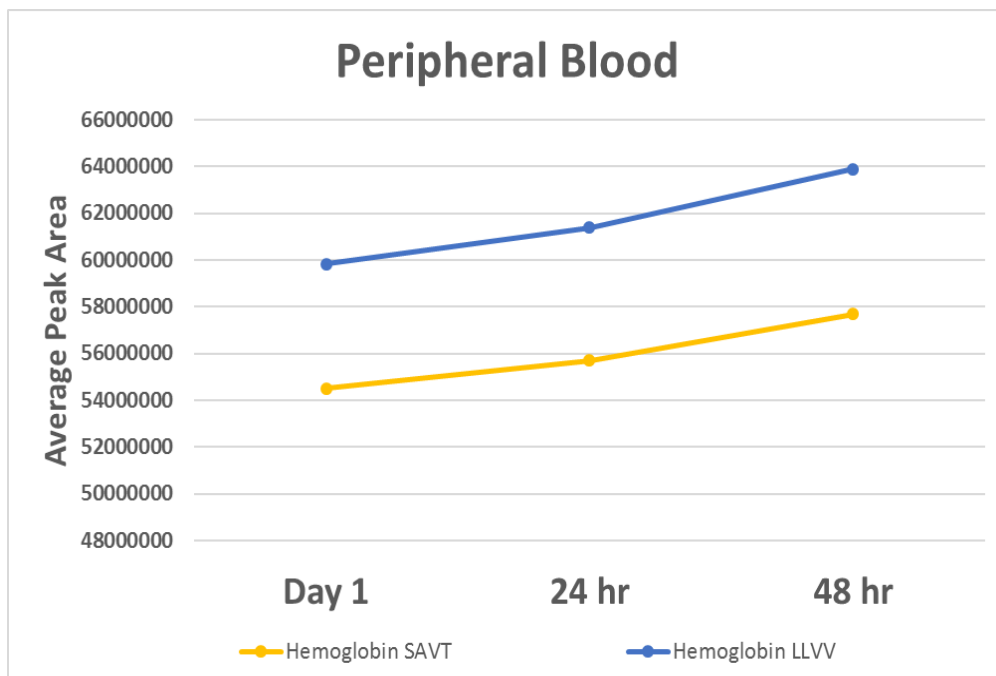
Seminal fluid autosampler stability data for less abundant peptide markers.



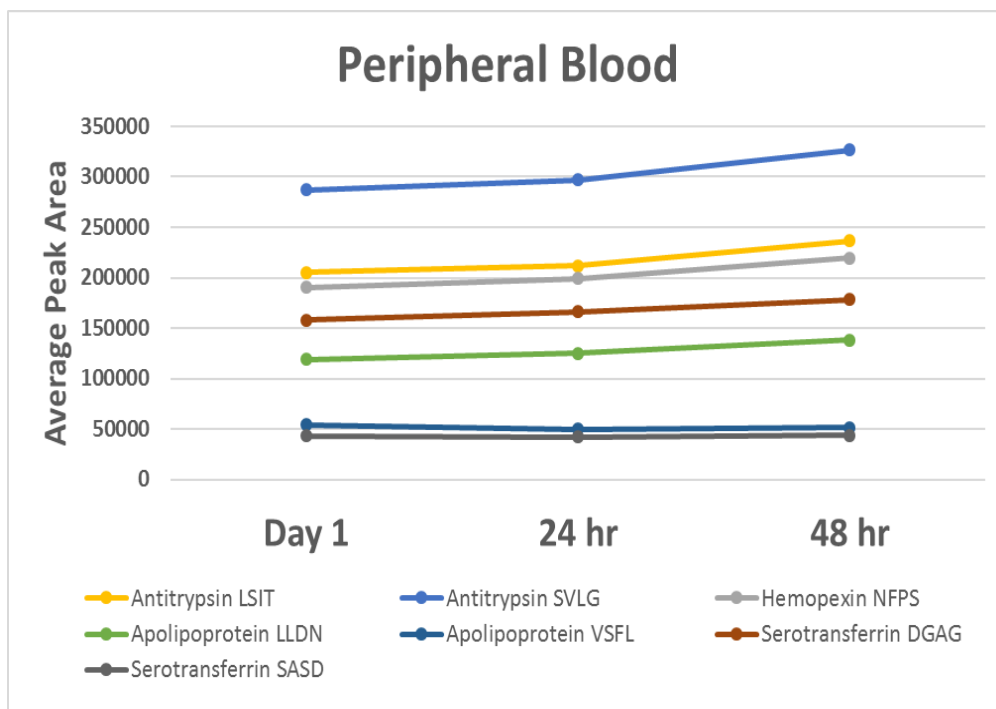
Saliva autosampler stability data for high abundant peptide markers.



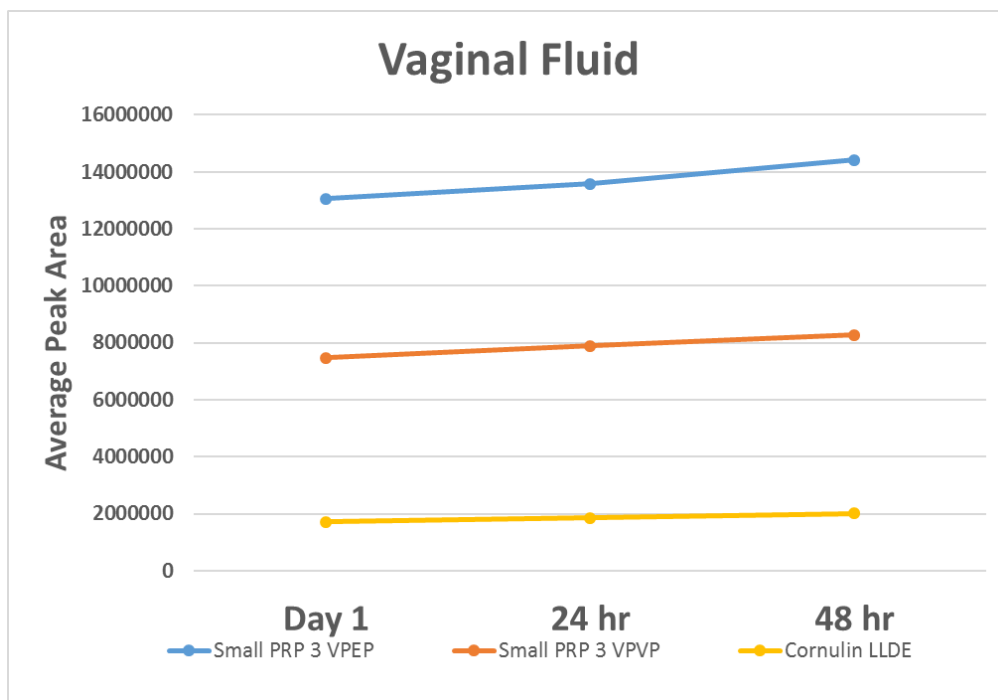
Saliva autosampler stability data for less abundant peptide markers.



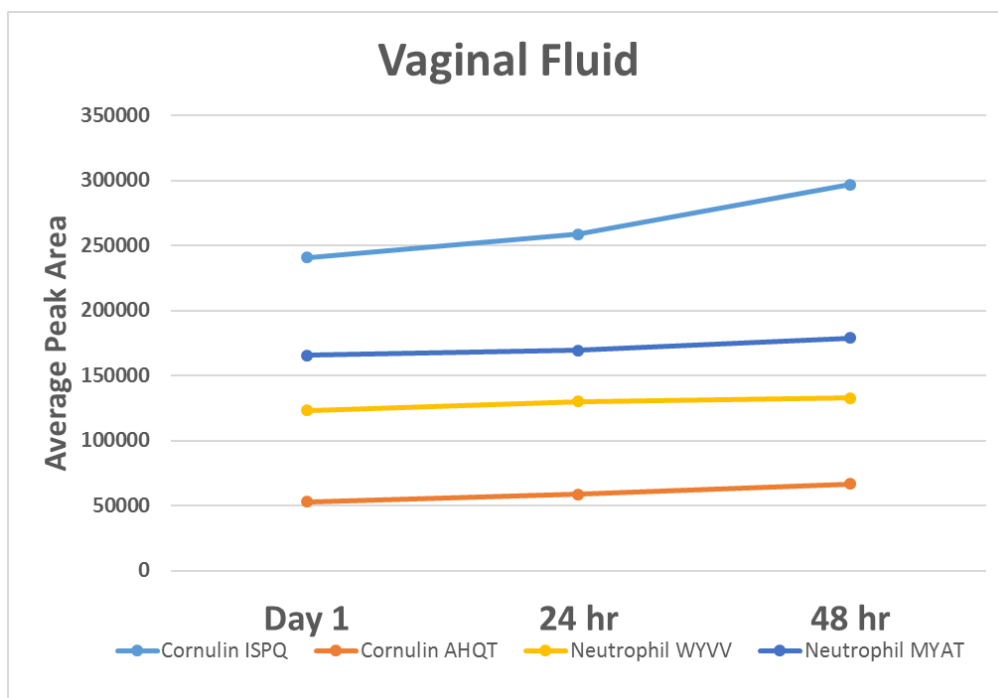
Peripheral blood autosampler stability data for hemoglobin peptide markers.



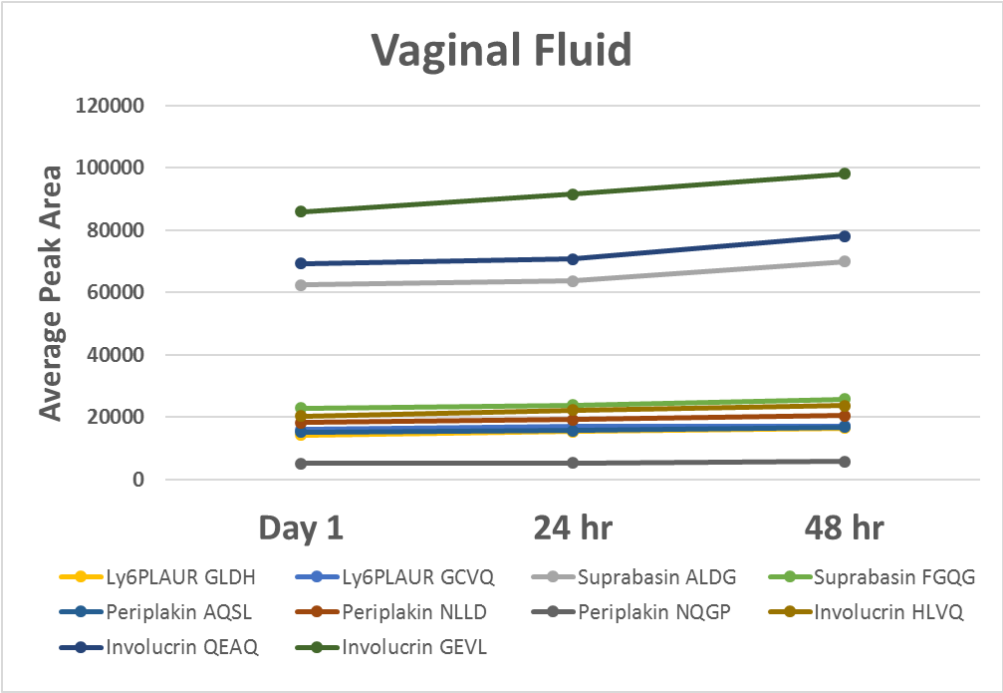
Peripheral blood autosampler stability data for less abundant peptide markers.



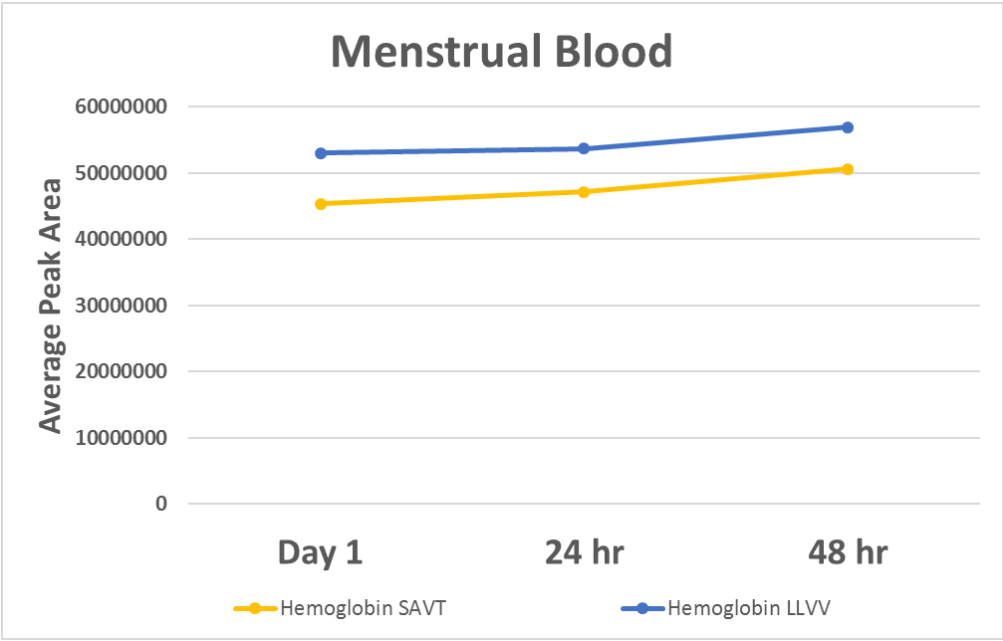
Vaginal fluid autosampler stability data for high abundant peptide markers.



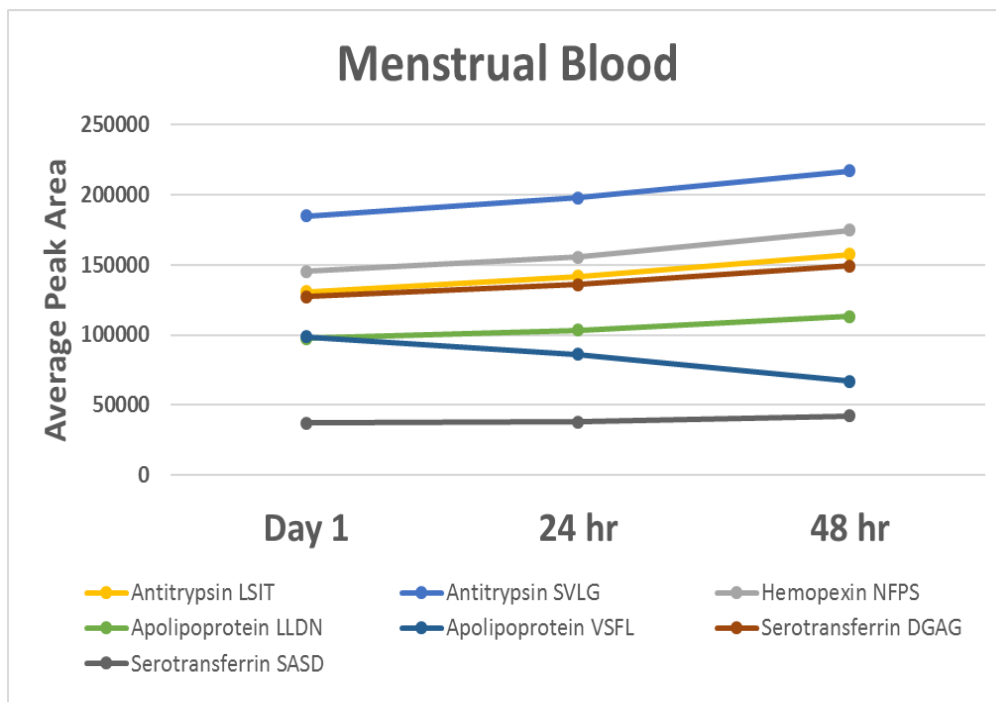
Vaginal fluid autosampler stability data for less abundant peptide markers.



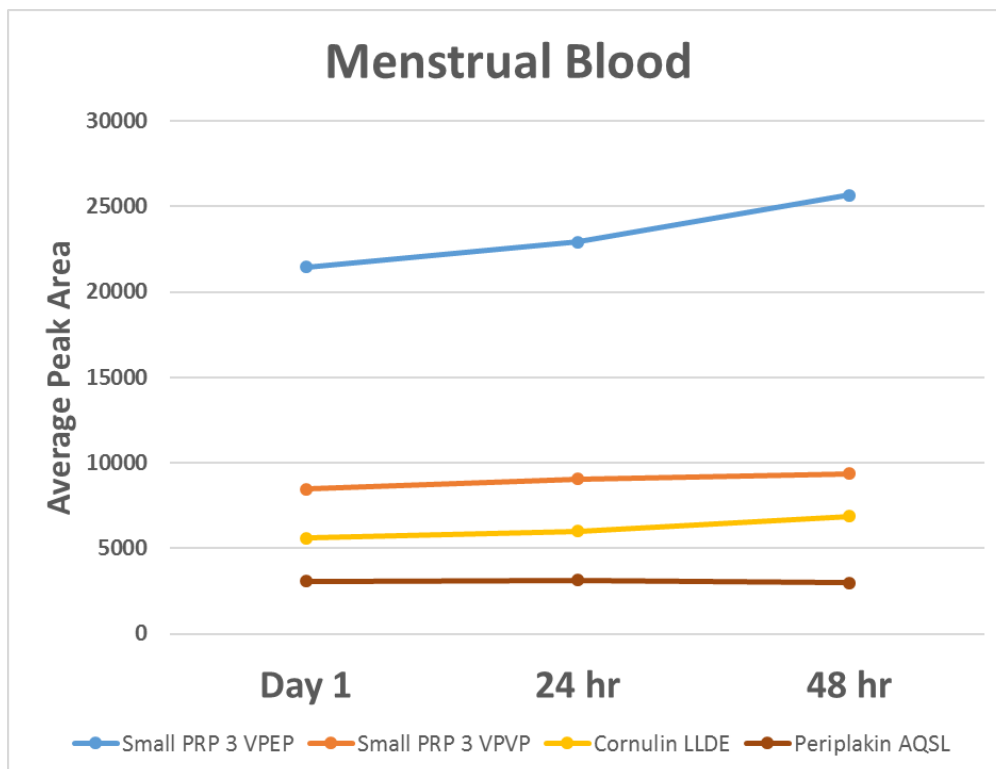
Vaginal fluid autosampler stability data for low abundance peptide markers.



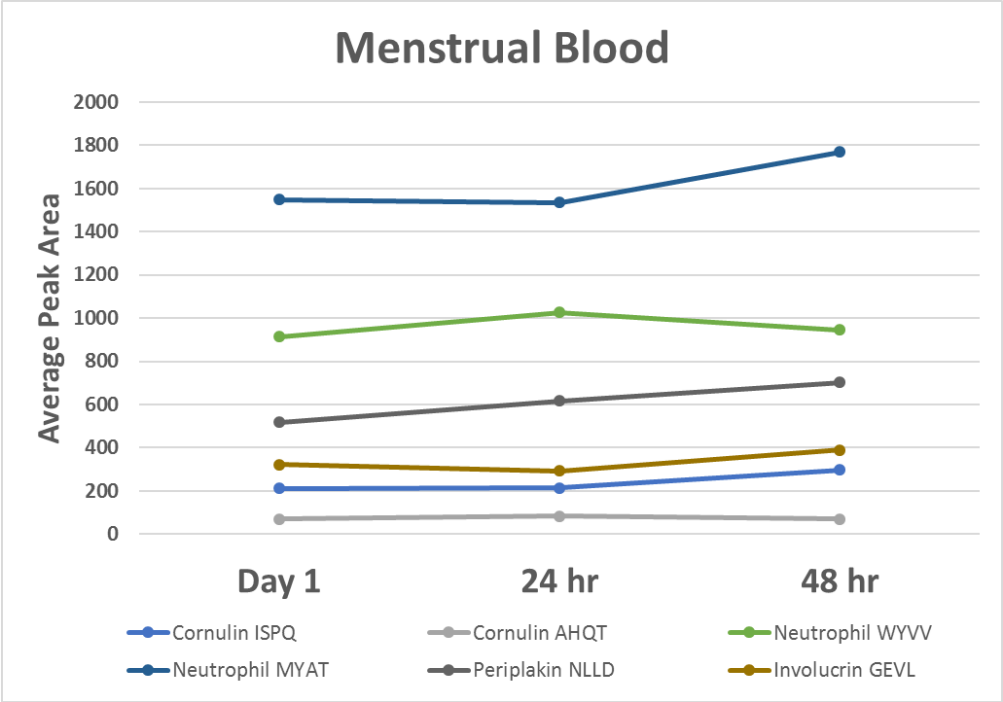
Menstrual blood autosampler stability data for hemoglobin peptide markers.



Menstrual blood autosampler stability data for blood peptide markers (apart from hemoglobin).



Menstrual blood autosampler stability data for high abundant vaginal peptide markers.



Menstrual blood autosampler stability data for less abundant vaginal peptide markers.

ROUTING AND ACTION

MEMORANDUM

ROUTING

TO:(1) Life Sciences Branch (McElhinny, Stephanie)

Report is available for review

(2) Proposal Files Report No.: -RIF

Proposal Number: 67484-LS-RIF.1

DESCRIPTION OF MATERIAL

CONTRACT OR GRANT NUMBER: W911NF-15-2-0132

INSTITUTION: Fredric Rieders Family Renaissance Foundation

PRINCIPAL INVESTIGATOR: Kevin Legg

TYPE REPORT: Final Report

DATE RECEIVED: 1/13/22 1:45PM

PERIOD COVERED: 9/25/15 12:00AM through 6/24/21 12:00AM

TITLE: Final Report: Validatoin of a Mass Spectrometry Based Serological Assay

ACTION TAKEN BY DIVISION

(x) Report has been reviewed for technical sufficiency and IS [x] IS NOT [] satisfactory.

(x) Based on my technical review, I have identified no OPSEC or Technology Protection concerns that need to be addressed regarding this report.

(x) Perfomance of the research effort was accomplished in a satisfactory manner and all other technical requirements have been fulfilled.

(x) Based upon my knowledge of the research project, I agree with the patent information disclosed.

Approved by SSL\STEPHANIE.MCELHINNY on 1/18/22 11:59AM

ARO FORM 36-E

REPORT DOCUMENTATION PAGE			Form Approved OMB NO. 0704-0188		
The public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA, 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 13-01-2022		2. REPORT TYPE Final Report		3. DATES COVERED (From - To) 25-Sep-2015 - 24-Jun-2021	
4. TITLE AND SUBTITLE Final Report: Validatoin of a Mass Spectrometry Based Serological Assay			5a. CONTRACT NUMBER W911NF-15-2-0132		
			5b. GRANT NUMBER		
			5c. PROGRAM ELEMENT NUMBER		
6. AUTHORS			5d. PROJECT NUMBER		
			5e. TASK NUMBER		
			5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAMES AND ADDRESSES Fredric Rieders Family Renaissance Founda 2300 Stratford Ave. Willow Grove, PA 19090 -4123			8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS (ES) U.S. Army Research Office P.O. Box 12211 Research Triangle Park, NC 27709-2211			10. SPONSOR/MONITOR'S ACRONYM(S) ARO		
			11. SPONSOR/MONITOR'S REPORT NUMBER(S) 67484-LS-RIF.1		
12. DISTRIBUTION AVAILABILITY STATEMENT Approved for public release; distribution is unlimited.					
13. SUPPLEMENTARY NOTES The views, opinions and/or findings contained in this report are those of the author(s) and should not contrued as an official Department of the Army position, policy or decision, unless so designated by other documentation.					
14. ABSTRACT					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	15. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON Kevin Legg
a. REPORT UU	b. ABSTRACT UU	c. THIS PAGE UU			19b. TELEPHONE NUMBER 215-366-1769

RPPR Final Report

as of 18-Jan-2022

Agency Code: 21XD

Proposal Number: 67484LSRIF

Agreement Number: W911NF-15-2-0132

INVESTIGATOR(S):

Name: Kevin Legg
Email: kevin.legg@frfoundation.org
Phone Number: 2153661769
Principal: Y

Organization: **Fredric Rieders Family Renaissance Foundation**

Address: 2300 Stratford Ave., Willow Grove, PA 190904123

Country: USA

DUNS Number: 798820150

EIN: 352221255

Report Date: 24-Jul-2021

Date Received: 13-Jan-2022

Final Report for Period Beginning 25-Sep-2015 and Ending 24-Jun-2021

Title: Validation of a Mass Spectrometry Based Serological Assay

Begin Performance Period: 25-Sep-2015

End Performance Period: 24-Jun-2021

Report Term: 0-Other

Submitted By: Kevin Legg

Email: kevin.legg@frfoundation.org

Phone: (215) 366-1769

Distribution Statement: 1-Approved for public release; distribution is unlimited.

STEM Degrees: 8

STEM Participants: 8

Major Goals:

1. Develop and fully validate an automated mass spectrometry-based assay for the identification of saliva, seminal fluid, peripheral blood, vaginal and menstrual fluid.
2. Complete a comprehensive comparison of the validated mass spectrometry assay to traditional serological techniques as well as STR typing technologies.
3. Assess and develop a solution for the inhibitory impacts of personal lubricants on fluid identification by mass spectrometry.
4. Develop and evaluate a protease-free strategy for the identification of saliva, seminal fluid, and vaginal fluid for higher throughput fluid identification.
5. Develop and evaluate an automated immuno-purification extraction strategy coupled with intact mass analysis for the identification of saliva in forensic samples.

Accomplishments: All objectives were successfully completed during the project award period. Utilizing the Agilent AssayMap Bravo, complex proteomic sample preparation was automated to a user friendly 96-well plate format. In parallel, the multiplex assay targeting 5 forensically-relevant body fluids including saliva, seminal fluid, peripheral blood, vaginal and menstrual fluid was developed on the Agilent 6495 Triple Quadrupole Mass Spectrometer. The multiplex assay was successfully validated (Section 4.1) and compared to traditional serological assays and STR typing technologies (Section 4.2). Based off sensitivity and robustness the mass spectrometry platform, the technology provided superior results compared to traditional serological tests. Moreover, it was demonstrated that the ability to produce serological information at sensitivity levels consistent with STR/Y-STR typing is achievable with protein mass spectrometry. The inhibitory impact of samples subjected to personal lubricants (Section 4.3) was successfully mitigated through an additional pre-digestion cleanup protocol. Finally, two expedited workflows were developed and assessed with a variety of case-type samples. This included a protease-free strategy coupled to high resolution mass spectrometry for the identification of saliva, seminal fluid, and vaginal fluid (Section 4.4) as well as immuno-purification coupled with intact mass analysis for saliva identification (Section 4.5). In toto, this work successfully developed, validated, and exhaustively assessed the use of protein mass spectrometry for the confirmatory identification of human body fluids.

Training Opportunities: International Symposium on Human Identification Workshop, "Reliable and Sensitive Body Fluid Identification by Mass Spectrometry with Robotic Sample Preparation", 2017 ISHI Conference. Seattle, WA.

RPPR Final Report as of 18-Jan-2022

Results Dissemination: Publications:

Davidovics, R, Saw, Y.L., Brown, C.O., Prinz, M, McKiernan, H.E., Danielson, P.B., Legg, K.M. High throughput seminal fluid identification by automated immunoaffinity mass spectrometry. J Forensic Sci, <https://doi.org/10.1111/1556-4029.14975>

Glendon J. Parker, G.J, McKiernan, H.M., Legg, K.M, Goecker, Z.C., Forensic proteomics, Forensic Science International: Genetics, VOLUME 54, 102529, SEPTEMBER 01, 2021. <https://doi.org/10.1016/j.fsigen.2021.102529>

McKiernan, H.M., Danielson, P.B., Brown, C.O., Signaevskya, S, Westringd, C.G., Legg, K.M, Developmental validation of a multiplex proteomic assay for the identification of forensically relevant biological fluids, Forensic Science International, 2021 Jul 17;326:110908. doi: 10.1016/j.forsciint.2021.110908

Brown, C, Robbins, B, McKiernan, H.M., Danielson P.B., Legg, K.M, Direct seminal fluid identification by protease-free high-resolution mass spectrometry, J Forensic Sci, 66(3):1017-1023, 2020. <https://doi.org/10.1111/1556-4029.14646>

McKiernan, H.M, Brown, C, Arantes, L.C., Danielson, P.B. & Legg, K.M., NextGen Serology: Leveraging Mass Spectrometry for Protein-Based Human Body Fluid Identification, ACS Symposium Series, Applications in Forensic Proteomics, Ch 4, 47-80

Presentations:

American Academy of Forensic Sciences, "Assessment of Robotic Solid Phase and Immunoaffinity Microextraction with Intact Mass Analysis for Saliva Identification in Forensic Samples. 2022 American Academy of Forensic Sciences Annual Scientific Meeting.

American Society for Mass Spectrometry, "Assessment of a Protease-free Method for Body Fluid Identification in Sexual Assault Evidence by Directed MS/dd-MS2 HRMS". 2021 ASMS Conference on Mass Spectrometry and Allied Topics

American Society for Mass Spectrometry, "Development of an Automated Immunoaffinity Mass Spectrometry Assay for Seminal Fluid Identification in Sexual Assault Samples". 2021 ASMS Conference on Mass Spectrometry and Allied Topics

American Academy of Forensic Sciences, "Assessing Potential Inhibitory Effects of Personal Lubricant on the Ability to Detect Biomarkers Consistent with Semen, Saliva, and Vaginal Fluid", 2019 American Academy of Forensic Sciences Annual Scientific Meeting.

American Academy of Forensic Sciences, "Evaluating In Vitro Seminal Fluid and Saliva Biomarker Degradation using a Mass Spectrometry-Based Serological Assay". 2019 American Academy of Forensic Sciences Annual Scientific Meeting.

American Academy of Forensic Sciences, "Comparative Study of a Mass Spectrometry Based Serological Assay with Existing Casework Models". 2018 American Academy of Forensic Sciences Annual Scientific Meeting.

American Chemical Society, "NextGen Serology: Leveraging Mass Spectrometry for Protein Based Human Body Fluid Identification," 2018 256th National Meeting of the American Chemical Society

SciX, "NextGen Serology: Leveraging Mass Spectrometry for Protein Based Human Body Fluid Identification," 2017 Federation of Analytical Chemistry and Spectroscopy Societies (SciX) meeting

International Symposium on Human Identification, "The Development of a Top-Down Proteomic Method for the Confirmatory Identification of Saliva and Semen by Q-TOF Mass Spectrometry." 2017 ISHI Conference

International Symposium on Human Identification, "Reliable and Sensitive Body Fluid Identification by Mass Spectrometry with Robotic Sample Preparation", 2017 ISHI Conference

RPPR Final Report

as of 18-Jan-2022

Green Mountain DNA Conference, "Body Fluid Identification by Mass Spectrometry", 2016 9th Green Mountain DNA Conference

Eastern Analytical Symposium, "The Absolute Quantitation of Semen Specific Biomarkers from Post-Coital Samples." 2015 Eastern Analytical Symposium

Green Mountain DNA Conference, "Body Fluid Identification by Mass Spectrometry: Ready for Primetime", 2015 8th Green Mountain DNA Conference

Honors and Awards: Nothing to Report

Protocol Activity Status:

Technology Transfer: Nothing to Report

PARTICIPANTS:

Participant Type: PD/PI

Participant: Kevin Legg

Person Months Worked: 3.00

Project Contribution:

National Academy Member: N

Funding Support:

Participant Type: Co-Investigator

Participant: Phillip Danielson

Person Months Worked: 1.00

Project Contribution:

National Academy Member: N

Funding Support:

Participant Type: Graduate Student (research assistant)

Participant: Catherine Brown

Person Months Worked: 9.00

Project Contribution:

National Academy Member: N

Funding Support:

RPPR Final Report
as of 18-Jan-2022

Partners

,

I certify that the information in the report is complete and accurate:

Signature: Kevin M. Legg

Signature Date: 1/13/22 1:45PM

Final Technical Report

A Mass Spectrometry Based Serological Assay

W911NF-15-2-0132

Award period:

September 25th, 2015 to June 24th, 2021

Prepared for:

United States Army Research Office

Prepared by:

Catherine O Brown, PhD
Phillip B. Danielson, PhD
Kevin M. Legg, PhD

Submitted by:

Kevin M. Legg, PhD



The Center for Forensic Science Research and Education
EIN:35-2221255
DUNS: 798820150
2300 Stratford Ave.
Willow Grove, PA 19090

Abstract

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

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

Multi-stage workflows associated with proteomic analysis remain a major hurdle towards the adoption of the technique in caseworking laboratories. Two streamlined sample-to-results workflow has been developed via peptidome analysis as well as intact mass spectrometry. These workflows allowed for straightforward preparation versus bottom up methodologies. Both methods were assessed using case-type samples.

In conclusion, the implementation of the protein MS approach offers an advantageous relationship between a positive identification and downstream DNA testing, including the capacity to deliver confirmatory contextual information in a criminal investigation.

Table of Contents

Abstract	i
Table of Contents	ii
List of Figures	ii
1 Original sample preparation strategy using vaginal swabs fortified with lubricant types	22
2 Modified sample preparation strategy developed	23
3 Preparation strategy for control and experimental samples containing semen	24
4 Preparation strategy for control and experimental samples containing saliva	25
5 Preparation strategy for control and experimental samples containing both semen and saliva	25
6 Internal positive control response ratio	44
7 Small proline rich protein 3 peptide VPEPGCTK peak area	47
8 Suprabasin peptide FGQGVHHGLSEGWK peak area response	51
9 Internal positive control response ratio for the freeze thaw stability study	51
10 Prostatic acid phosphatase peptide FVTLFR peak area response	53
11 Hemoglobin peptide LLVVYPWTQR peak area response	55
12 Area response of vaginal fluid markers in saliva samples	55
13 Area response of species specificity samples	56
14 Peak area response of seminal fluid biomarkers in mock sexual assault kit samples	66
15 Peak area response of seminal fluid biomarkers in mock sexual assault kit samples	67
16 Peak area response of peptide biomarkers at lowest detected sensitivity sample	76
17 Peak area response of peptide biomarkers at 50 fmol limit	80
18 AQUA response of hemoglobin peptide LLVVYPWTQR	84
19 Carryover response of hemoglobin peptide LLVVYPWTQR	86
20 Carryover response of semenogelin 1 peptide DIFSTQDELLVYNK	86
21 Carryover response of hemoglobin and semenogelin peptides	87
22 Biomarker profile of Blind Sample #15	90
23 Biomarker profile of Blind Sample #22	91
24 Serological screening test results on substrate samples	95
25 Serological screening test results for environmental contaminant samples	97
26 Serological screening test results for mixture samples containing saliva	99
27 Serological screening test results for mixture samples containing semen	100
28 Immunochromatographic results for simulated sexual assault samples	103
29 Immunochromatographic results for mock sexual assault samples	106
30 Serological screening test results for digital swab samples	108
31 Immunochromatographic results for degraded samples	111
32 Serological screening test results for aged samples	114
33 Serological screening test results for sensitivity samples	119
34 Total protein concentration of control and lubricated vaginal swab	126
35 Internal positive control response ratio for control and lubricated samples	127
36 Lubricant effects on on peak area response of endogenous vaginal fluid proteins	129
37 Chromatographic peak response for NGAL	130
38 Chromatographic peak response for NGAL with SPE	130
39 Effects of lubricant on saliva and seminal fluid	132
40 Effects of lubricant on saliva and seminal fluid mixtures	133
41 Semenogelin 2 peptide GSISIQTEEQIHGK with lubricant	134
42 Effects of condom lubricants	136
43 NGAL response recovered from condoms	137
44 Saliva recovered from a rectal swab	153

45	Mass spectrum of seminal fluid treated with 10% bleach	156
46	Mass spectrum of seminal fluid recovered from laundered samples	158
47	Statherin sensitivity	162

List of Tables iii

1	Dilutions analyzed for the sensitivity study of the multiplex assay validation	10
2	Dilutions analyzed for the sensitivity study within casework sample analysis	16
3	Volumes and concentrations of internal standard	17
4	Personal lubricants	21
5	Condom brands utilized for this study	26
6	Performance assessment sample preparation	29
7	Traditional serological screening tests	35
8	Average protein concentration	40
9	CV for peak area response of seminal fluid peptide markers	42
10	Calculated CV for retention time of seminal fluid peptide markers	42
11	CV for ion ratios of seminal fluid peptide markers	43
12	CV for the internal positive control peak area	43
13	Sensitivity limit of peripheral blood	45
14	Sensitivity limit of menstrual blood	46
15	Sensitivity limit of seminal fluid	46
16	Sensitivity limit of saliva	46
17	sensitivity limit of vaginal fluid	46
18	Freeze thaw stability results	49
19	Freeze thaw stability results	50
20	CV for the internal positive control for the autosampler stability study	52
21	Species specificity	54
22	Detection of body fluid markers in substrate samples	58
23	Detection of body fluid markers in samples subjected to environmental contaminants	60
24	Detection of body fluid markers in mixture samples	62
25	Detection of body fluid markers in simulated sexual assault samples	63
26	Detection of body fluid markers in sexual assault kit samples	65
27	Detection of body fluid markers in digital swab samples	69
28	Detection of body fluid markers in degraded samples	71
29	Detection of body fluid markers in aged samples	72
30	Detection of body fluid markers in casework sensitivity samples	75
31	Detection of body fluid markers in casework sensitivity samples	75
32	Detection of body fluid markers in casework sensitivity samples	75
33	Limit of detection	78
34	Limit of detection	79
35	Ion suppression and enhancement	82
36	Ion suppression and enhancement	83
37	Carryover assessment	88
38	Summary of comparative results for substrate samples	94
39	Summary of comparative results for environmental contaminant samples	96
40	Summary of comparative results for mixture samples	99
41	STR genetic results for mixture samples	100
42	Y-STR genetic results for mixture samples	101
43	Summary of comparative results for simulated sexual assault samples	102
44	STR genetic results for simulated sexual assault samples	104
45	Summary of comparative results for mock sexual assault samples	105
46	STR genetic results for simulated sexual assault samples	106
47	Summary of comparative results for digital swab samples	107
48	STR genetic results for digital swab samples	108
49	Summary of comparative results for degraded samples	110

50	STR genetic results for degraded samples	111
51	Comparative results for aged samples	113
52	Comparative results for peripheral blood sensitivity samples	117
53	Comparative results for menstrual blood sensitivity samples	117
54	Comparative results for seminal fluid sensitivity samples	118
55	Comparative results for saliva sensitivity samples	118
56	Comparative results for vaginal fluid sensitivity samples	118
57	STR genetic results for peripheral blood sensitivity samples	119
58	STR genetic results for menstrual blood sensitivity samples	120
59	STR genetic results for seminal fluid sensitivity samples	121
60	Y-STR genetic results for seminal fluid sensitivity samples	122
61	STR genetic results for saliva sensitivity samples	123
62	STR genetic results for vaginal fluid sensitivity samples	124
63	Descriptive statistics of non-vasectomized semen population	143
64	Descriptive statistics of vasectomized semen population	143
65	Descriptive statistics of saliva population	144
66	Descriptive statistics of vaginal fluid population	145
67	Peptide markers for seminal fluid	147
68	Peptide markers for saliva	148
69	Peptide markers for vaginal fluid	149
70	Sensitivity sample results for seminal fluid	150
71	Sensitivity sample results for saliva	150
72	Sensitivity sample results for vaginal fluid	150
73	Aged samples	151
74	Sexual assault kit sample results	152
75	Mixture sample results	154
76	Contaminant samples results	155
77	Substrate samples results	157
78	Population assessment of 20 individuals for the presence of statherin	159
79	Casework-type samples for statherin identification	161

1 Summary 1

2 Introduction 3

2.1 Statement of the Problem 3

2.2 Limitations of Current Technologies 3

2.3 Emerging Approaches to Serological Testing 5

2.4 Project Objectives 7

3 Methods 8

3.1 Body Fluid Identification by Automated LC-QQQ 8

3.1.1 Sample Collection and Preparation 8

3.1.2 Developmental Validation Sample Preparation 9

3.1.2.1 Repeatability and Reproducibility 9

3.1.2.2 Sensitivity 10

3.1.2.3 Stability 11

3.1.2.3.1 Freeze Thaw Stability 11

3.1.2.3.2	Autosampler Stability	11
3.1.2.4	Mixture Studies	11
3.1.2.5	Specificity Studies	12
3.1.2.6	Casework Samples	12
3.1.2.6.1	Substrates	12
3.1.2.6.2	Environmental Contaminants	12
3.1.2.6.3	Mixtures	13
3.1.2.6.4	Sexual Assault Samples	14
3.1.2.6.4.1	Simulated Sexual Assault Samples	14
3.1.2.6.4.2	Mock Sexual Assault Kit Samples	14
3.1.2.6.4.3	Digital Swabs	14
3.1.2.6.5	Degradation Samples	15
3.1.2.6.6	Aged Samples	16
3.1.2.6.7	Sensitivity Samples	16
3.1.2.7	Limit of Detection	17
3.1.2.8	Ion Suppression	17
3.1.2.9	Carryover Study	18
3.1.2.10	Blind Sample Analysis	18
3.1.3	Sample Digestion and Cleanup with the Agilent AssayMap Bravo	19
3.1.4	Data Acquisition with the Agilent 6495 LC-QQQ	20
3.1.5	Data Processing and Interpretation	20
3.2	Solid Phase Cleanup of Lubricant Contaminated Samples	20
3.2.1	Sample Collection and Materials	20
3.2.2	Lubricants and Vaginal Fluid Biomarker Detection	21
3.2.2.1	Experimental Sample Assembly	21
3.2.2.2	Pre-Digestion Cleanup	23
3.2.2.3	Sample Processing and Analysis	23
3.2.3	Lubricants and the Detection of Semen and Saliva Biomarkers	24
3.2.4	Vaginal Fluid Biomarker Detection from Condoms	25
3.3	Body fluid Identification by Protease-Free High Resolution Mass Spectrometry	26
3.3.1	Sample Collection and Preparation	26
3.3.2	Population Study and Performance Assessment	27
3.3.3	Solid Phase Extraction	29
3.3.4	Data Acquisition with the Thermo Q-Exactive HRMS	30
3.3.5	Data Processing	30
3.4	Saliva Identification by Intact High Resolution Mass Spectrometry	31
3.4.1	Sample Collection and Preparation	31

3.4.2	Solid Phase Extraction with the Agilent AssayMap Bravo	32
3.4.3	Immunoaffinity Extraction with the Agilent AssayMap Bravo	32
3.4.4	Data Acquisition with the Thermo Q-Exactive HRMS	33
3.4.5	Data Processing	33
3.5	Body fluid Identification by Traditional Casework Models	34
3.5.1	Sample Preparation	34
3.5.2	Immuno chromatographic Assays and Traditional Assays	34
3.5.3	Genetic Analysis	35
3.5.3.1	DNA Extraction	35
3.5.3.2	DNA Quantitation	36
3.5.3.3	DNA Amplification	37
3.5.3.4	DNA Separation and Analysis	37
4	Results and Discussion	39
4.1	Developmental Validation of an Automated LC-QQQ Assay	39
4.1.1	Repeatability and Reproducibility	39
4.1.1.1	BCA	40
4.1.1.2	Signal Response	41
4.1.1.3	Retention Time	42
4.1.1.4	Response Ratios	42
4.1.1.5	Internal Positive Control	43
4.1.2	Sensitivity	44
4.1.3	Stability	47
4.1.4	Freeze Thaw Stability	48
4.1.5	Autosampler Stability	51
4.1.6	Mixture Studies	52
4.1.7	Specificity Studies	53
4.1.8	Casework Samples	56
4.1.8.1	Substrates	56
4.1.8.2	Environmental Contaminants	58
4.1.8.3	Mixtures	60
4.1.8.4	Sexual Assault Samples	63
4.1.8.4.1	Simulated Sexual Assault Samples	63
4.1.8.4.2	Mock Sexual Assault Kit Samples	64
4.1.8.4.3	Digital Swabs	67
4.1.8.5	Degradation Samples	69
4.1.8.6	Aged Samples	71

4.1.8.7	Sensitivity Samples	72
4.1.9	Limit of Detection	76
4.1.10	Ion Suppression	80
4.1.11	Carryover Study	84
4.1.12	Blind Sample Analysis	88
4.2	Comparison of a Validated LC-QQQ to Existing Forensic Casework Models	92
4.2.1	Substrate Samples	93
4.2.2	Environmental Contaminants	95
4.2.3	Mixture Samples	97
4.2.4	Sexual Assault Samples	101
4.2.4.1	Simulated Sexual Assault Samples	101
4.2.4.2	Mock Sexual Assault Samples	104
4.2.4.3	Digital Swab Samples	107
4.2.5	Degradation Samples	108
4.2.6	Aged Samples	112
4.2.7	Sensitivity Samples	114
4.3	Solid Phase Cleanup of Lubricant Contaminated Samples	124
4.3.1	Lubricants and Vaginal Fluid Biomarker Detection	125
4.3.1.1	Digest Efficiency	125
4.3.1.2	Biomarker Detection	127
4.3.2	Lubricants and the Detection of Semen and Saliva Biomarkers	130
4.3.3	Vaginal Fluid Biomarker Detection from Condoms	134
4.4	Body fluid Identification by Protease-Free High Resolution Mass Spectrometry	138
4.4.1	Biomarker Selection and Population Study	139
4.4.2	Target Peptide Biomarker Selection	145
4.4.3	Performance Assessment	149
4.4.3.1	Sensitivity Samples	149
4.4.3.2	Aged Samples	150
4.4.3.3	Sexual Assault Samples	151
4.4.3.4	Mixture Samples	153
4.4.3.5	Contaminant Samples	154
4.4.3.6	Substrate Samples	156
4.5	Saliva Identification by Intact High Resolution Mass Spectrometry	159
4.5.1	Population Assessment	159
4.5.2	Performance Assessment	160
5	Conclusions	163

6	Recommendations for Further Applications	169
7	References	171
8	Appendices	185

1 Summary

For more than two decades, the forensic community has focused on improving the sensitivity and robustness of DNA profiling. While advances have made it possible to individualize biological traces on challenging types of evidentiary material, DNA alone does not readily indicate the body fluid source from which it was extracted. Serological testing to identify the body fluid from which a DNA profile has been generated, however, can provide vital contextual information to facilitate a successful prosecution. In the event of a sexual assault or rape, a Suspect Sexual Assault Evidence Collection Kit is taken by a trained nurse and submitted for forensic testing. Kits generally include vaginal, oral, and rectal swabs, in addition to articles of clothing and supplemental swabs submitted on a case-by-case basis (e.g., breast, labia, inner thigh swabs). In addition, objects such as condoms or items used for vaginal penetration can be submitted for testing. Given the persistent sexual assault kit backlogs and that the sensitivity of post-coital DNA testing now exceeds that of contemporary serological tests, many labs have adopted a “direct to DNA” workflow. While this approach has a number of advantages, serological testing is especially important when the item of evidence in question (e.g., a towel or bedding) does not readily lend itself to an interpretation of likely sexual contact to the same degree as an intimate swab or underwear, where the mere presence of suspect’s DNA may be sufficient for criminal proceedings.

The ability to confidently identify the source of a genetic profile can provide critical context during a criminal investigation. However, current testing strategies often only provide a presumptive indication of a target body fluid and there are no routine assays to identify vaginal fluid or menstrual blood. Due to limitations of historic serological assays, several emerging strategies have emerged including protein mass spectrometry, mRNA and miRNA profiling, epigenetics, and raman spectroscopy. The overall goal of this work was to develop and validate an automated, high throughput, mass spectrometry platform for the identification of saliva, semen, peripheral blood, and menstrual/vaginal fluid. In addition, two higher throughput applications were assessed to further increase turnaround time of the assay.

This work was carried out under the following specific objectives:

1. Develop and fully validate an automated mass spectrometry-based assay for the identification of saliva, seminal fluid, peripheral blood, vaginal and menstrual fluid.
2. Complete a comprehensive comparison of the validated mass spectrometry assay to traditional serological techniques as well as STR typing technologies.
3. Assess and develop a solution for the inhibitory impacts of personal lubricants on fluid identification by mass spectrometry.
4. Develop and evaluate a protease-free strategy for the identification of saliva, seminal fluid, and vaginal fluid for higher throughput fluid identification.
5. Develop and evaluate an automated immuno-purification extraction strategy coupled with intact mass analysis for the identification of saliva in forensic samples.

All objectives were successfully completed during the project award period. Utilizing the Agilent AssayMap Bravo, complex proteomic sample preparation was automated to a user friendly 96-well plate format. In parallel, the multiplex assay targeting 5 forensically-relevant body fluids including saliva, seminal fluid, peripheral blood, vaginal and menstrual fluid was developed on the Agilent 6495 Triple Quadrupole Mass Spectrometer. The multiplex assay was successfully validated (Section 4.1) and compared to traditional serological assays and STR typing technologies (Section 4.2). Based off sensitivity and robustness the mass spectrometry platform, the technology provided superior results compared to traditional serological tests. Moreover, it was demonstrated that the ability to produce serological information at sensitivity levels consistent with STR/Y-STR typing is achievable with protein mass spectrometry. The inhibitory impact of samples subjected to personal lubricants (Section 4.3) was successfully mitigated through an additional pre-digestion cleanup protocol. Finally, two expedited workflows were developed and assessed with a variety of case-type samples. This included a protease-free strategy coupled to high resolution mass spectrometry for the identification of saliva, seminal fluid, and vaginal fluid (Section 4.4) as well as immuno-purification coupled with intact mass analysis for saliva identification (Section 4.5). In toto, this work successfully developed, validated, and exhaustively assessed the use of protein mass spectrometry for the confirmatory identification of human body fluids.

2 Introduction

2.1 Statement of the Problem

Sexual assault remains one of the most serious and yet underreported crimes in the United States. According to the Federal Bureau of Investigation's Uniform Crime Reporting Program, an estimated 95,730 rapes were reported in 2016 – an increase of 4.9% over the 2015 estimates. This increased in the occurrence and the associated volume of evidence being submitted for testing has contributed to a workflow backlog at many laboratories. The development of accurate, robust, high-throughput method for analyzing sexual assault samples while maximizing the probative value of the available evidence is clearly desirable for practitioners in caseworking laboratories.

DNA testing can link an item of evidence to an individual, but it cannot identify the body fluid that is the source of the DNA. Such basic information, however, is often critical to identifying probative evidence in a criminal investigation. Consider a case where swabs of a suspect's fingers reveal DNA consistent with that of an alleged sexual assault victim. Multiple explanations can be offered for this result. One possibility is the claim by the alleged victim that the suspect sexually assaulted her by digitally penetrating her. Alternatively, the suspect may claim that the female innocently licked food from his fingers - leaving her DNA behind.

The capacity to reliably identify trace amounts of saliva and/or vaginal fluids would enable investigators to resolve such contradictory claims. It is not currently possible, however, for forensic scientists to unambiguously identify forensically relevant body fluids with existing assays which yield only presumptive results and are often not amenable to trace evidence. In fact, for body fluids (e.g., vaginal fluid) there are no commercially available tests at all. Thus, there is clear value in the proposed development of an accurate and sensitive technology for unambiguously identifying human body fluids in a forensic context.

2.2 Limitation of Current Technologies

The unambiguous identification of biological stains is a significant challenge for forensic serologists that cannot be met by existing commercial technologies. For example, practitioners have no means of readily or reliably identifying stains such as vaginal fluid

on evidentiary items (e.g., a suspect's skin or clothing, foreign objects) from an alleged sexual assault. Moreover, the available presumptive tests for other body fluids such as saliva and seminal fluid prevent experts from making definitive statements in court in regarding the biological stain from which a DNA profile was obtained.

Tests for blood, semen, saliva, and urine exist [3] but these can be laborious or require that serologists be proficient in methodologies from several different scientific disciplines. Other serological tests consume significant amounts of a valuable DNA-containing sample while providing forensic practitioners with suboptimal sensitivity and specificity. For example, some tests for saliva may consume half of an evidentiary swab while still providing only a presumptive result. The detection of saliva requires the preservation of salivary enzyme function – making it difficult to test aged/degraded samples. Additionally, such body fluids as urine and cervical mucus also contain “salivary” proteins [4]. As a result, an analyst is not able to tell a jury that the presence of saliva was confirmed on that vaginal swab even when a positive result is obtained.

Tests for evidence of vaginal contact have proven even more challenging. Effort to use histochemical stains to differentiate among skin, oral and vaginal cells have not been successful. The iodine-based Lugol's test held promise for identifying vaginal cells[5] based on studies suggesting that vaginal cells contained more glycogen than other epithelial cells. However, later studies revealed that Lugol's positive cells were also present in the male urethra, male urine deposits[6] and on penile swabs from males who had abstained from sex for several days[7]. Modifications to Lugol's test and other staining technique have been suggested [8] but the results have been disappointing. This has left the forensic scientist with no reliable method of detecting vaginal fluid even though it has significant probative value in sexual assault investigations.

Seminal fluid testing has been facilitated by forensic kits based on the detection of antigen-antibody interactions. These tests provide improved specificity and sensitivity. For example, the p30 (Prostate-Specific Antigen) protein serves as a marker for seminal fluid[9]. However, p30 can also be found in female ejaculate[10], breast milk[11], urine[12] (albeit at lower concentrations). As with any antibody-based assay, the results are “presumptive by definition” because the potential for antibody cross-reactivity with non-target molecules cannot be eliminated. Moreover, samples may contain environmental

contaminants that impede antibody binding and sensitivity [13]. Only direct visual identification of sperm by microscopy (often a labor intensive procedure) enables an analyst to report a confirmatory result. But microscopy is useless with samples from vasectomized or aspermatic males or degraded material lacking intact sperm.

2.3 Emerging Approaches to Serological Testing

Several novel approaches have been explored in recent years in an effort to address the limitations of current serological techniques including Raman spectroscopy, differentially expressed messenger- and micro-RNA markers, and epigenetics.

mRNA/miRNA Markers have attracted significant research interest as a means of identifying body fluids on the basis of tissue-specific mRNA expression profiles[12]. In 2003, pioneering work identified potential markers for saliva by searching the Cancer Genome Anatomy Project for proteins expressed only in the salivary glands. Candidate markers, including histatin 3, statherin, and PRB1/2/3, were shown to be expressed in saliva but not blood or semen[13]. By multiplexing mRNA genetic markers with a housekeeping gene to normalize for differences in mRNA expression between different body fluids it has been possible to more precisely identify expression patterns characteristic of individual body fluids [14]. The use of mRNA biomarkers for body fluid identification also has the advantage of enabling forensic practitioners to employ familiar sample preparation and PCR-based amplification methods. Thus, a single method can be used for stain individualization and body fluid identification[15].

Micro RNAs (miRNA) are small, 21-25 nucleotide long, non-coding RNA molecules which regulate gene expression[16] and which have been explored as markers for the identification of body fluids. The first study, published in 2009, used a combination of database evaluation and empirical testing to identify miRNA candidates which showed potentially promising differential expression patterns for semen, saliva, vaginal fluid, peripheral blood, and menstrual blood. In a more recent study, however, candidate miRNA markers for peripheral blood and semen were identified reliable markers for saliva, vaginal fluid or menstrual blood remained elusive[17].

Epigenetic Markers take advantage of transmissible changes in DNA methylation patterns [18]. The use of epigenetic biomarkers for body fluid identification, therefore,

relies on detecting tissue-associated differences in DNA methylation[19, 20]. 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[21, 22]. Subsequent and more extensive studies aimed at identifying methylation response ratios identified 38 loci which showed differential methylation patterns with potential utility for identifying semen, blood, saliva and skin cells[22].

Raman Spectroscopy is a non-destructive approach to body fluid identification which offers an attractive alternative to conventional methods that can be unacceptably consumptive of minimal samples. Raman spectroscopy characterizes an unknown substance [23] by measuring the inelastic scattering of light produced by the chemical constituents within the sample. The technology is robust and can achieve pictogram-level detection sensitivity [24]. This technique has been used to identify “multidimensional spectroscopic signatures” for specific body fluids. Advanced statistical algorithms are then used to help compensate for sample-to-sample heterogeneity. Encouraging results have been reported with single-source vaginal fluid, sweat, blood, and semen stains [25]. Additional studies are needed to demonstrate that the method can accommodate more challenging mixed and degraded stains as well as those containing contaminants, i.e., any forensic type sample that deviates from the reference “spectroscopic signature”.

Protein Mass Spectrometry is among the most promising of the emerging strategies for body fluid identification. This is due in large part to the significant advances that have been made in proteomics instrumentation in recent years. Protein mass spectrometry workflows rely on the combination of primary protein structure and bioinformatics. The use of high resolution mass spectrometry is common in forensic toxicology for the analysis of small molecule drugs, as this technology serves as a means of confirmatory identification via unique molecular weight and charge state. The application of proteomics to challenges in forensic serology has been the focus of research and development activities of multiple research groups [26, 27].

2.4 Project Objectives

The overarching goal of this project was to develop and rigorously validate a mass spectrometry based forensic serology assay. The assay was designed for the simultaneous identification of five forensically relevant body fluids: peripheral blood, seminal fluid, saliva, vaginal fluid, and menstrual fluid. To achieve this, two well-established technologies, ultra-performance liquid chromatography and triple quadrupole mass spectrometry (UPLC-QQQ), were selected in combination with automated sample preparation to produce an integrated system well-suited for forensic casework needs. Validation procedures were conducted in accordance with the Federal Bureau of Investigation's Quality Assurance Standard for Forensic DNA Testing Laboratories as well as guidelines published by the Scientific Working Group on DNA Analysis Methods (SWGDM). In addition, given the analytical instrumentation employed, appropriate guidelines from the Organization of Scientific Area Committees for Forensic Science (OSAC) Chemistry: Seized Drug and Toxicology Scientific Area Committee were also considered. The final deliverable presents a fully validated analytical assay and corresponding SOP that demonstrates sensitivity and specificity gains over traditional serological screening techniques and is compatible with downstream genetic testing.

Additional assessments were formulated to assess and mitigate the deleterious effects of personal lubricants and spermicidal products on the ability to accurately identify seminal fluid and saliva in cases of sexual assault. Supplementary measures were incorporated into the sample processing procedure to diminish competitive binding during sample purification and remove the interferents prior to proceeding with protein digestion. The multiplex assay was then used as a foundation for the development of a peptidomic methodology for the assessment of sexual assault evidence. This workflow aimed to expedite sample processing by omitting traditional trypsin-digestion. It was demonstrated that same-day sample preparation could be used for the identification of seminal fluid, saliva, and vaginal fluid. Finally, a top-down method was explored to identify salivary statherin using immuno-purification and intact mass analysis. The successful completion of these objectives has resulted in the development of several strategies for body fluid identification, especially in the event of sexual assault or rape.

3 Methods

3.1 Body Fluid Identification by Automated LC-QQQ

3.1.1 Sample Collection and Preparation

All research conducted under this phase of the project was reviewed and approved by the University of Denver Institutional Review Board (IRB) for research involving human subjects. Sample collection and research was conducted in full accordance with the U.S. federal policy for the protection of human subjects. In total, 60 subjects were recruited from the graduate population at Arcadia University (Glenside, PA) and staff members employed at The Center for Forensic Science Research & Education (Willow Grove, PA). All volunteers agreed and signed a letter of consent acknowledging that they had received, read, and understood all protocols involved in sample collection. Furthermore, all collected samples were assigned a random unique identifier to protect confidentiality.

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

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

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

Vaginal secretions were self-collected by consenting donors. Female participants were asked to refrain from unprotected sexual intercourse for 12 days prior to the collection of vaginal fluid. The hypoallergenic, over the counter Softdisc™ collection cup (The Flex

Company, Venice, CA) was utilized. Donors inserted the collection cup into the vagina following manufacturer's instructions. It was requested the cup remain in the vagina for a minimum of 1 hour but could be left in for a period up to 12 hours. The entire Softdisc™ was placed into a sterile collection cup and transported to the laboratory. The liquid contents were placed into the collection cup and the Softdisc™ was repeatedly washed with 1 mL of ultra-pure water to remove mucous-like secretions. The liquid contents and mucous secretions were pooled and thoroughly vortexed to create a homogenous sample. 200 µL single use aliquots were prepared and stored at -80 °C until use. In addition to vaginal secretions collected with the Softdisc™, female participants were asked to provide self-collected vaginal swabs. Consenting volunteers were provided with sterile cotton-tipped swabs. A single swab was inserted into the vaginal cavity, similar to inserting a tampon. The swab was rotated in a circular manner for 15 seconds and gently removed. Swabs were placed into sterile manila collection envelopes for transport to the laboratory. Upon receipt, swabs were dried at room temperature where the cotton tip was removed from the wooden handle and placed in a clean microcentrifuge tube. Swabs were stored at -80 °C until use.

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

3.1.2 Developmental Validation Sample Preparation

3.1.2.1 Repeatability and Reproducibility

All body fluid aliquots for sample preparation following protocols outlined in previously. Aliquots were stored at -80 °C until use. For the remainder of the study, both the research scientist and laboratory advisor performed sample processing procedures on

the prepared aliquots simultaneously, including total protein quantitation, reagent preparation, plate loading for digestion and cleanup, and sample reconstitution for instrumental analysis.

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

3.1.2.2 Sensitivity

Target body fluid from five individuals was prepared according to parameters outlined previously. A serial dilution of pooled fluid was created in deionized water. For seminal fluid, saliva, and vaginal fluid, dilutions were started at 2-fold. Knowing a priori peripheral and menstrual blood exhibit concentrated protein material, dilutions were started at 100-fold. Dilutions were not quantitated for total protein amount. 200 μ L of each dilution was lyophilized and digested followed by SPE cleanup using protocols detailed below. Sample dilutions were prepared in triplicate, with 20 μ L of sample injected on column. A select set of sample dilutions were prepared and analyzed given laboratory resources. Table 1 below details dilutions analyzed via LC-MS/MS per target body fluid.

Table 1: Dilutions analyzed for the sensitivity study of the multiplex assay validation.

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

3.1.2.3 Stability

3.1.2.3.1 Freeze Thaw Stability

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

3.1.2.3.2 Autosampler Stability

Target body fluid from five individuals was prepared according to parameters described. Samples were analyzed via LC-MS/MS over the course of 3 days, with the microplate containing processed sample remaining in the autosampler kept at 8 °C for the duration of the study. 2.5 µg of material was analyzed on column. Samples were reinjected 24 hours (Day 2) and 48 hours (Day 3) after original sample preparation.

3.1.2.4 Mixture Studies

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

3.1.2.5 Specificity Studies

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

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

3.1.2.6 Casework Samples

3.1.2.6.1 Substrates

Substrates tested include cotton, denim, leather, carpet, drywall, plastic beverage bottle, aluminum beverage can, and feminine hygiene menstrual pad. Substrates were cut into approximately 2 cm x 2 cm squares upon which 50 µL of single source body fluid was applied. Samples were dried at room temperature overnight. Substrates were solubilized in 1 mL deionized water for 30 minutes with frequent vortexing. Substrates were placed in a centrifugal basket and centrifuged at 10,000 x g for 10 minutes. Supernatant was transferred for analysis following procedures outlined in section 3.1.3-3.1.4 and cell pellets were discarded.

3.1.2.6.2 Environmental Contaminants

Environmental contaminants tested include dirt slurry, rust slurry, 10% bleach in water solution, chewing tobacco, cigarette butt, lubricated condom with spermicide additive, and water-based personal lubricant. Dirt slurry was prepared by mixing 1 gram weather-conditioned soil with 1 mL tap water. In a similar manner, the rust slurry was prepared by mixing 500 µg rust with 500 µL of tap water. Rust was obtained by scarping

the external surface of a weather-conditioned chain link fence. Neat bleach was diluted 10-fold with deionized water for a final concentration of 10% bleach. Chewing tobacco expellant was obtained from a volunteer that actively engages in using smokeless tobacco products. Water-based personal lubricant containing glycerin was purchased (CVS Pharmacy™, Woonsocket, RI).

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

3.1.2.6.3 Mixtures

Mixture samples were prepared using single source body fluid from consenting donors. A 50-fold two-component mixture solution was created by thoroughly vortexing 10 μ L of neat body fluid serving as a minor contributor with 500 μ L neat body fluid serving as the major contributor. 100 μ L of mixture solution was applied to a cotton-tipped swab and allowed to dry at room temperature overnight. Full swabs were extracted in 1 mL deionized water for 30 minutes with frequent vortexing. Substrates were placed in a centrifugal basket and centrifuged at 10,000 x g for 10 minutes. Supernatant was transferred for analysis following procedures outlined in section 3.1.3-3.1.4 and cell pellets were reserved for genetic testing.

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

3.1.2.6.4 Sexual Assault Samples

3.1.2.6.4.1 Simulated Sexual Assault Samples

Simulated sexual assault samples were prepared by thoroughly mixing 10 μ L neat semen with 1 mL neat vaginal fluid, both fluids being from a single donor. This was repeated for a total of seven samples. The semen and vaginal fluid utilized for all seven samples were donated by the same individuals to prevent inconsistency in protein expression. Samples were incubated at 37 °C for one day, three days, five days, seven days, nine days, and eleven days, with time zero samples immediately stored for future processing. When the designated time point was reached, samples were removed from the incubator and frozen at -80 °C until analysis. Samples were prepared and analyzed following procedures outlined in section 3.1.3-3.1.4.

3.1.2.6.4.2 Mock Sexual Assault Kit Samples

Vaginal, oral, and rectal swabs were obtained from a single, consenting female individual. Immediately after sampling, neat semen from a single donor was applied in order to most accurately simulate an authentic sample collection. Semen was diluted 100-fold with deionized water prior to application. 10 μ L of diluted semen was applied, for a volume equivalent of 0.1 μ L semen applied to each swab. Samples were prepared in duplicate and allowed to dry at room temperature overnight. Full swabs were extracted in 1 mL deionized water for 30 minutes with frequent vortexing. Substrates were placed in a centrifugal basket and centrifuged at 10,000 $\times$ g for 10 minutes. Supernatant was transferred for analysis following procedures outlined in section 3.1.3-3.1.4 and cell pellets were reserved for genetic testing, described previously. This preparation protocol was duplicated using semen provided by a vasectomized male individual, for a total of 12 mock sexual assault kit samples (6 non-vasectomized and 6 vasectomized male donors).

3.1.2.6.4.3 Digital Swabs

Three types of digital swab samples were prepared for this portion of the study: vaginal swabs, menstrual blood swabs, and saliva swabs. One consenting female donor was utilized. The volunteer thoroughly washed their hands prior to self-penetration. In the comfort of the individual's home, the volunteer was instructed to digitally penetrate the

vagina both during menses and in the absence of menses using the index finger for 20 seconds. The volunteer removed their finger and allowed the deposited material to dry for approximately 5 minutes. Self-collection using a dry cotton-tipped swab was performed, with the entire area of the finger swabbed for collection. Collection was repeated in duplicate for each form of penetration, with each sample taken on a separate day. Upon receipt at the laboratory, swabs were dried at room temperature overnight to ensure consistency. Full swabs were extracted in 1 mL deionized water for 30 minutes with frequent vortexing. Substrates were placed in a centrifugal basket and centrifuged at 10,000 x g for 10 minutes. Supernatant was transferred for analysis following procedures outlined in section 3.1.3-3.1.4 and cell pellets were reserved for genetic testing.

3.1.2.6.5 Degradation Samples

Degradation samples simulating laundered items of evidence were generated for this portion of the study. Peripheral blood and semen were the selected target body fluids. To a cotton bath towel and to denim jeans, 1 mL of single-source peripheral blood was applied to the substrate, outlined with permanent marker, and allowed to dry at room temperature overnight. In addition, a control region to which no body fluid was applied approximately 20 cm from the stain region was marked for analysis. To a cotton-blend bed sheet and a cotton pair of women's underwear, 1 mL of single-source semen was applied to the substrate, outlined with permanent marker, and allowed to dry at room temperature overnight. In addition, a control region to which no body fluid was applied approximately 20 cm from the stain region (bed sheet) or 8 cm from the stain region (underwear) was marked for analysis. Target body fluid was applied to simulate stained items of evidence that are commonly received in caseworking laboratories.

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

was transferred for analysis following procedures outlined in section 3.1.3-3.1.4 and cell pellets were reserved for genetic testing.

3.1.2.6.6 Aged Samples

Aged swabs were prepared by spotting 50 μ L of neat, single-source peripheral blood, menstrual blood, saliva, semen, or vaginal fluid on a full cotton swab. An additional set of samples were prepared using 150 μ L of neat, single-source vaginal fluid. Swabs were allowed to incubate at room temperature for 35 days, with collections at time zero, Day 1, Day 3, Day 7, and Day 35. Upon collection, full swabs were frozen at -80 °C until analysis. All time points were prepared in duplicate. Samples were prepared according to procedures outlined in section 3.1.3-3.1.4.

3.1.2.6.7 Sensitivity Samples

Sensitivity samples were prepared by diluting single-source, neat body fluid in deionized water in a serial manner to cover a range of final concentrations. Dilutions prepared are detailed in Table 3.2. 150 μ L of each dilution was spotted on a full cotton swab and allowed to dry at room temperature. Full swabs were solubilized in 1 mL deionized water for 30 minutes with frequent vortexing. Substrates were placed in a centrifugal basket and centrifuged at 10,000 x g for 10 minutes. Supernatant was transferred for analysis following procedures outlined in section 3.1.3-3.1.4 and cell pellets were reserved for genetic testing.

Table 2: Dilutions analyzed for the sensitivity study within casework sample analysis.

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

3.1.2.7 Limit of Detection

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

Blank matrix was prepared by pooling target body fluid from five individuals. 100 μ g total protein underwent tryptic digestion and SPE cleanup prior to AQUA fortification. The 0.5 pmol/ μ L stock solution was further diluted with 30% ACN with 0.1% FA to 50 fmol/ μ L and 5 fmol/ μ L substocks, used for creation of the serial dilution. Matrix was fortified with AQUA material following volumes and target concentrations listed in Table 3.3. Samples were analyzed following parameters outlined in section 3.1.3-3.1.4.

Table 3: Volumes and concentrations of internal standard used during the limit of detection experimentation.

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

3.1.2.8 Ion Suppression

Target body fluid from five individuals was prepared and pooled for ion suppression and enhancement analysis. 100 μ g of total protein was targeted for digestion. AQUA stock solution was prepared in the same manner as detailed in the previous section. Two sample sets were prepared for this portion of the study. First, 20 μ L of 0.5 pmol/ μ L AQUA stock solution was analyzed independently (i.e., in the absence of blank matrix). Second, pooled body fluid was fortified with 20 μ L of 0.5 pmol/ μ L AQUA stock for a

target amount of 10 pmol, with all samples prepared in triplicate. Samples were prepared according to procedures outlined in section 3.1.3-3.1.4. 2.5 µg of sample was analyzed per injection.

3.1.2.9 Carryover Study

Samples were prepared in triplicate for a preliminary carryover study by pooling fluid from five different individuals and digesting 100 µg of total protein. Following tryptic digestion and SPE cleanup parameters outlined in section 3.1.3-3.1.4, samples were reconstituted in 100 µL of 2% ACN with 0.1% FA. 10 µg, 20 µg, and 30 µg of processed sample were injected on column, with each injection followed by a blank mobile phase injection (0.1% FA in water) of the same volume. Carryover was assessed by the presence of compounds within the subsequent blank injection.

A second set of samples targeting smaller quantities of total protein were prepared in triplicate and analyzed. Following the same procedure as described above, 0.5 µg, 1 µg, 2.5 µg, and 5 µg quantities of digested protein were evaluated per body fluid. A blank mobile phase sample (0.1% FA in water) of the same volume was injected directly after each sample.

3.1.2.10 Blind Sample Analysis

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

centrifuged at 10,000 x g for 10 minutes. Supernatant was transferred for analysis following procedures outlined in section 3.1.3-3.1.4 and cell pellets were discarded.

3.1.3 Sample Digestion and Cleanup with the Agilent AssayMap Bravo

Prior to sample digestion all samples were quantified via the bicinchoninic acid (BCA) assay. Assay standards were prepared by diluting concentrated bovine serum albumin (BSA) in 100 mM tris-hydrochloride (tris-HCl) to the following concentrations: 1,500 µg/mL; 1,000 µg/mL; 750 µg/mL; 500 µg/mL; 250 µg/mL; 125 µg/mL; and 25 µg/mL. To a 96-well flat bottom plate, 25 µL of standard or sample was added in duplicate. Samples and standards were treated with 200 µL of working reagent containing 200 µL of Reagent A and 50 µL of Reagent B per sample. The plate was incubated at 37 °C for 30 minutes followed by 10 minutes at room temperature. Samples were read using a spectrophotometer at 652 nm wavelength. Following quantitation, 100 µg of total protein or a maximum of 100 µL of supernatant was consumed in the case of casework samples. Samples were fortified with 20 µL of 0.5 mg/mL intact myelin protein and lyophilized to dryness in a vacuum evaporator at 55°C.

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

The tryptic reaction was stopped with the addition of 10 µL of 25% TFA. Digested samples were manually fortified with 10 µL of 1 µg/mL isotopically-labeled IPC stock solution. The sample plate was placed back on the AssayMAP Bravo deck for SPE cleanup. C18 microextraction cartridges were primed with 100 µL 50% ACN 0.5% TFA at a flow rate of 300 µL/min. Cartridges were equilibrated with 50 µL of 0.5% TFA in deionized water at 10 µL/min. 200 µL of digested sample was passed over the cartridge at a flow rate of 15 µL/min. Cartridges were washed with 50 µL of 0.5% TFA in deionized water at a rate of 10 µL/min. Sample material was eluted from the sorbent in 25 µL of 70% ACN

0.1% FA at 5 μ L/min. Eluate was lyophilized to dryness and prepared for LC-MS/MS analysis.

3.1.4 Data Acquisition with the Agilent 6495 LC-QQQ

Lyophilized samples were reconstituted in 100 μ L of 2% ACN with 0.1% FA in deionized water. Acquisition was performed using 10 μ L of sample per injection on an Agilent 6495 mass spectrometer operating in positive dynamic MRM (dMRM) mode coupled to a 1290 series liquid chromatograph. An Agilent Poroshell 120 EC-C18 3 mm x 100 mm analytical column was used for separation. Mobile phase A consisted of water with 0.1% FA and mobile phase B consisted of ACN with 0.1% FA. Separation initiated at 5% B followed by a linear 8-32% B gradient over 8.5 minutes, a 1-minute hold at 80% B, followed by a 3-minute re-equilibration at a 1.0 mL/min flow rate and column temperature of 50 °C. Source conditions included a gas temp of 200 °C at a flow rate of 13 l/min, nebulizer pressure of 35, Sheath gas temp of 200 °C at 12 l/min, and a capillary voltage of 3500 V. High pressure ion funnel RF was 150 and low pressure ion funnel RF was 60. Protein and peptide target details can be found in Appendix A.

3.1.5 Data Processing

Results were assessed qualitatively using the peak area of the quantifier ion in the Skyline Proteomics Environment v. 20.1.0.155. (MacCoss Laboratory, University of Washington) and Agilent MassHunter software. Positive peptide identification required a retention time apex within 0.1 minutes to the target, signal-to-noise (s/n) ratio greater than 3, and an ion ratio within 20% of the target.

3.2 Solid Phase Cleanup of Lubricant Contaminated Samples

3.2.1 Sample Collection and Materials

All research conducted under this phase of the project was reviewed and approved by the University of Denver IRB for research involving human subjects. Sample collection and research was conducted in full accordance with the U.S. federal policy for the protection of human subjects. In total, 10 subjects were recruited from the graduate

population at Arcadia University (Glenside, PA) and staff members employed at The Center for Forensic Science Research & Education (Willow Grove, PA).

Five classes of personal lubricant were selected for this study: water-based lubricant with glycerin (Astroglide®), water-based lubricant without glycerin (Sliquid® H2O), hybrid lubricant (Sliquid® Silk), silicone lubricant (Swiss Navy®), and natural oil-based lubricant (Coconut Oil). Lubricant details are described in Table 4.

Table 4: Personal lubricants utilized for this study. Notes on lubricant ingredients, formula, and pH compiled from patent material and/or product descriptions.

Lubricant Class	Lubricant Make and Model	Abbreviation	Notes
Water-Based without Glycerin	Sliquid® H ₂ O Natural Intimate Lubricant	Sliquid® H ₂ O	•Glycerin and paraben free formula •Water soluble •Unflavored •Unscented •pH balanced (pH 4.0-4.4)
Water-Based with Glycerin	Astroglide® Liquid Water-Based Personal Lubricant	Astroglide®	Contains Purified Water, Glycerin, Propylene Glycol, Polyquaternium 15
Hybrid	Sliquid® Silk Intimate Hybrid Lubricant	Sliquid® Silk	•Water and silicon blend •Glycerin and paraben free formula •Unflavored •Unscented •pH balanced (4.0-4.4 pH)
Silicone	Swiss Navy® Silicone Lubricant	Swiss Navy®	Contains Cyclopentasiloxane, Dimethicone, Tocopheryl Acetate (Vitamin E).
Natural Oil	Majestic Pure™ Cosmeceuticals Fractionated Coconut Oil (Cocos Nucifera)	Coconut Oil	•Fractionation process removed long chain fatty acids •High concentration of capric acid and caprylic acid •Hexane, paraben, and sulfate free formula

3.2.2 Lubricants and Vaginal Fluid Biomarker Detection

3.2.2.1 Experimental Sample Assembly

Two experimental design plans were constructed for the processing and preparation of vaginal swab samples subjected to personal lubricants. The original experimental design consisted of full vaginal swabs that were cut in half, with one half serving as a control and the second half fortified with a set volume of personal lubricant (Figure 1). Lubricant volumes of 1 µL, 5 µL, or 15 µL were added to half swabs. Control half swabs were not fortified with lubricant. Swab halves were dried at room temperature overnight. Half swabs were solubilized in 500 µL of deionized water for 30 minutes with frequent agitation.

Swabs were placed in a centrifugal basket and centrifuged at 10,000 x g for 10 minutes. Supernatant was transferred to a clean microcentrifuge tube and cell pellets were discarded. Supernatant was reserved for analysis via LC-MS/MS.

For the second experimental design, vaginal swabs were solubilized and extract was fortified with personal lubricant (Figure 2). Swab tips were solubilized in 500 μ L of deionized water for 30 minutes at room temperature with frequent agitation. Swabs were placed in a centrifugal basket and spun for 10 minutes at 10,000 x g to remove excess liquid. Supernatant was transferred and pooled for further preparation. Processed swabs and cell pellets were discarded. Pooled vaginal extract was fortified with increasing volumes of personal lubricant. For each lubricant type evaluated, 700 μ L of pooled vaginal extract was fortified with either 7 μ L, 35 μ L, or 105 μ L of personal lubricant. These volumes simulate targeting 1 μ L, 5 μ L, and 15 μ L of personal lubricant per sample replicate. Control samples consisted of pooled vaginal extract and were not subject to any personal lubricant manipulation. Mixtures were thoroughly vortexed and 100 μ L aliquots were transferred to clean microcentrifuge tubes for triplicate analysis.

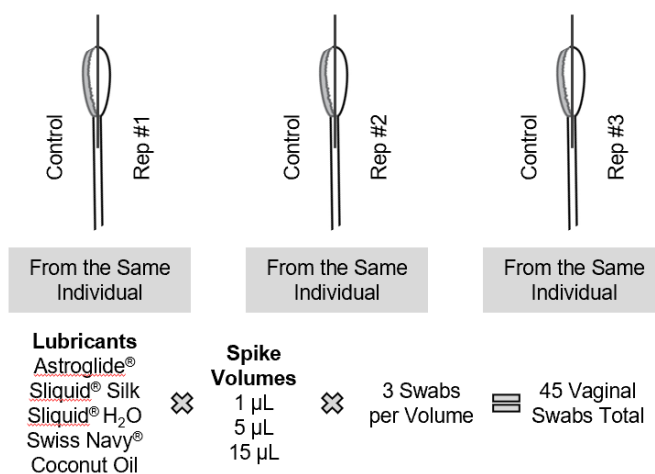


Figure 1: Original sample preparation strategy using vaginal swabs fortified with lubricant types. One vaginal swab was cut in half, with each paired half representing a control and an experimental sample.

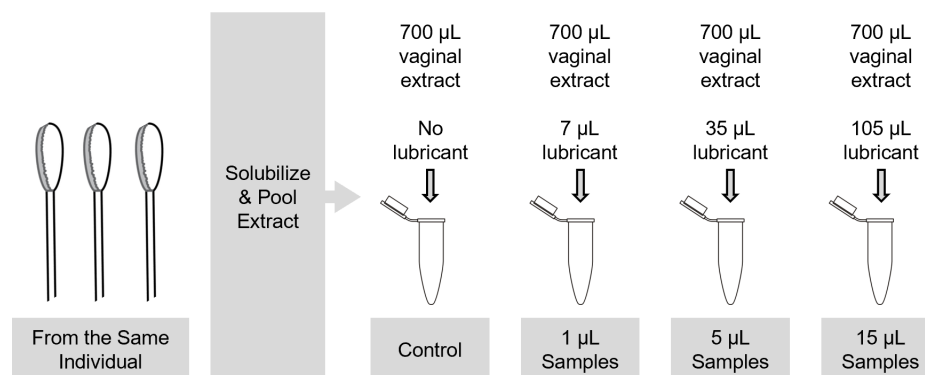


Figure 2: Modified sample preparation strategy developed to mitigate expression inconsistencies in vaginal fluid proteins. Vaginal swabs were solubilized and pooled prior to mixing with lubricant types.

3.2.2.2 Pre-Digestion Cleanup

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

3.2.2.3 Sample Processing and Analysis

Extracted samples were quantitated using the BCA assay and 100 µg total protein was lyophilized to dryness. Samples were subjected to the previously described 8 M urea tryptic digestion and SPE purification on the AssayMAP Bravo liquid handling platform. Eluate was fortified with 20 µL of 0.5 mg/mL intact myelin protein prior to tryptic digestion and 2.5 pmol of isotopically-labeled internal standard prior to SPE cleanup. Eluate was lyophilized to dryness and reconstituted in 100 µL of 2% ACN 0.1% FA in deionized water for LC-MS/MS analysis. Prepared samples were injected onto the 6495 triple quadrupole mass spectrometer coupled to a 1290 series liquid chromatograph (Agilent Technologies). Data was acquired using the validated analytical method developed, with data analysis performed in Skyline Software.

3.2.3 Lubricants and the Detection of Semen and Saliva Biomarkers

Vaginal swabs were collected as previously described. Pooled vaginal extract was fortified with various amounts of lubricant and either semen, saliva, or a mixture of semen and saliva. Personal lubricant volumes of 1 μL , 5 μL , and 15 μL were also utilized for this portion of the assessment. In addition, target volumes of 1 μL of semen and 10 μL of saliva were utilized for single fluid experimental samples. For example, 400 μL of pooled extract was fortified with either 4 μL , 20 μL , or 60 μL of personal lubricant in addition to 4 μL of semen or 40 μL of saliva (Figures 3 and 4). Mixture samples were prepared in a similar manner. Pooled vaginal extract was fortified with personal lubricant as previously described in addition to 4 μL of semen and 40 μL of saliva (Figure 5). Control samples were fortified with semen and/or saliva but absent of personal lubricant. Samples were thoroughly vortexed and 100 μL of sample was aliquoted for further processing. Samples underwent pre-digestion cleanup, tryptic digestion, and SPE purification as described previously.

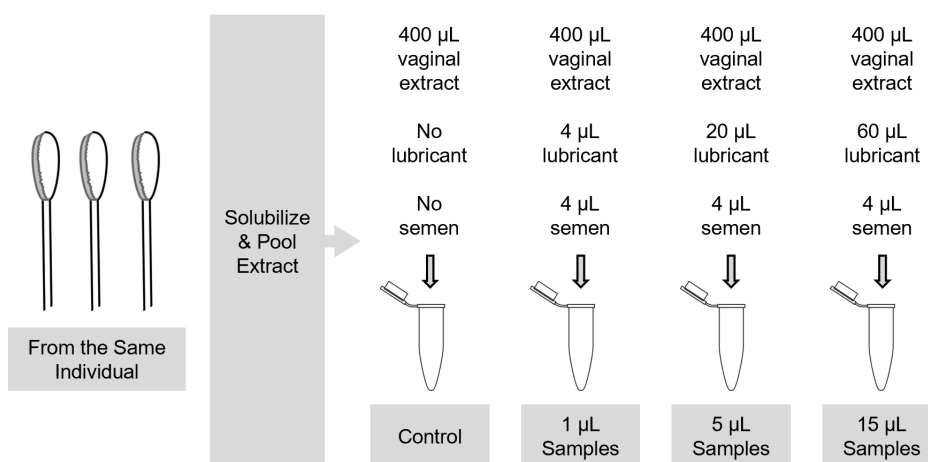


Figure 3: Sample preparation strategy for control and experimental samples containing semen.

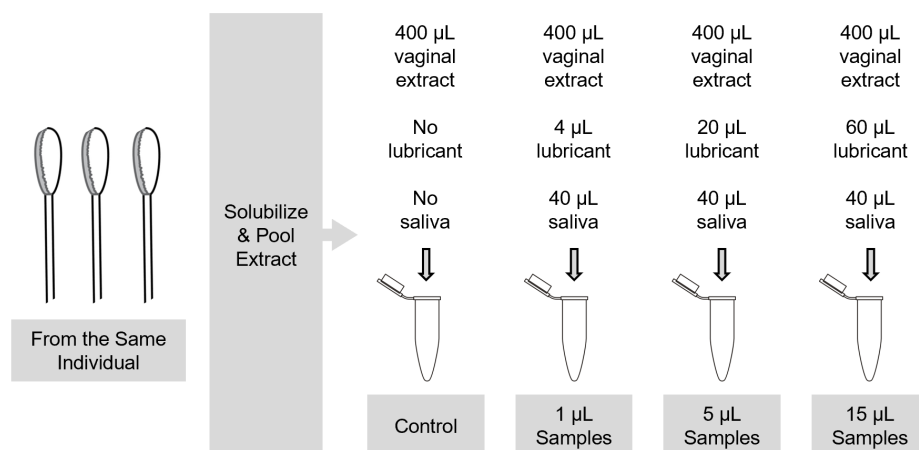


Figure 4: Sample preparation strategy for control and experimental samples containing saliva.

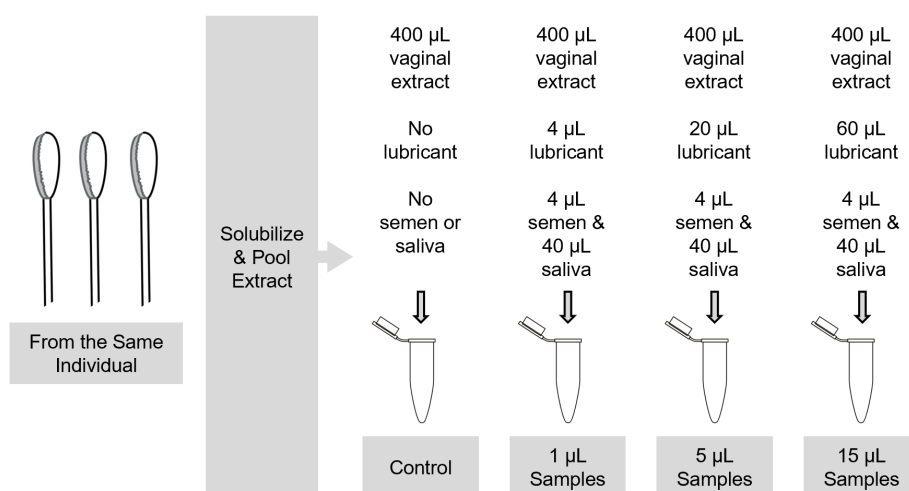


Figure 5: Sample preparation strategy for control and experimental samples containing both semen and saliva.

3.2.4 Vaginal Fluid Biomarker Detection from Condoms

Four condom types were selected for evaluation: Trojan™ ENZ™ unlubricated condoms, SKYN® Original condoms, SKYN® Extra Lube condoms, and Durex® Performax® condoms. These condoms were selected because they were marketed to cover a range of lubricant amounts. A summary of condom types and product information is outlined in Table 5.

Condoms were removed from the packaging, unraveled, and placed on a clean piece of butcher paper. Simulating authentic casework, only the external surface (i.e., the surface that contacts the vaginal cavity when inserted) was swabbed for this study. Vaginal swabs were obtained from female volunteers and dried at room temperature. A single swab was

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

Table 5: Condom brands utilized for this study. Notes on condom lubricant (if present) and material compiled from patent information and/or product descriptions.

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

3.3 Body fluid Identification by Protease-Free High Resolution Mass Spectrometry

3.3.1 Sample Collection and Preparation

Semen was self-collected from consenting donors as described previously. Collection cups were vortexed and 200 μ L single use aliquots were prepared and stored at -80 °C until use. Saliva was collected from consenting volunteers who refrained from eating or drinking for 1 hour prior to collection. Salivette® collection tubes (Sarstedt, Nümbrecht, Germany) were utilized as described in section 3.1.1. 200 μ L single use aliquots were prepared and stored at -80 °C until use. Single use body fluid aliquots (semen or saliva) were thawed at room temperature for 30 minutes then centrifuged for 10 minutes

at 10,000 x g. Supernatant was transferred to a clean microcentrifuge tube and cell pellets were discarded. For this study, vaginal secretions were collected using only the swabbing method as described in section 3.1.1. Upon receipt, swabs were dried at room temperature where the cotton tip was removed from the wooden handle and placed in a clean microcentrifuge tube. Swabs were stored at -80 °C until use. Full cotton swabs were solubilized in 600 µL of deionized water for 30 minutes with frequent agitation. Swabs were placed into centrifugal baskets and centrifuged at 10,000 x g for 10 minutes. Supernatant was pooled and transferred to a clean microcentrifuge tube.

Given laboratory and IRB restrictions in place out of caution for the SARS-CoV-2 virus, additional biological matrices were purchased from both Lee Biosolutions, Inc. (Maryland Heights, MO) and Innovative Research, Inc. (Novi, MI) for the population assessment. Upon ordering, it was requested that each sample was obtained from a unique donor, with no repetitive donors. Biological material was shipped on dry ice and immediately stored at -80 °C until use. Saliva and semen samples were thawed at room temperature for 30 minutes, vortexed, and pulse spun. 200 µL was removed for analysis and treated as described above. Supernatant was transferred to a clean microcentrifuge tube and cell pellets were discarded. Full vaginal swabs were cut and treated as described above.

Body fluid material was quantitated using the bicinchoninic acid (BCA) assay as described in section 3.1.1.

3.3.2 Population Study and Performance Assessment

Twenty semen samples were purchased from Innovative Research and thirty semen samples were purchased from Lee Biosolutions, for a total of 50 semen donors. An additional 5 semen samples from vasectomized donors were purchased from Lee Biosolutions. Twenty-five saliva samples were purchased from Innovative Research and Lee Biosolutions for a total of 50 saliva donors. Twenty-five vaginal swabs were purchased from Lee Biosolutions and seven vaginal swabs were collected under IRB, for a total of 32 vaginal swab donors. Regardless of donor source, received vaginal swabs were stored at -80 °C upon receipt. Population samples were purchased due to SARS-CoV-2 restrictions put in place for collection of biological samples under the approved IRB. Performance assessment samples were divided into six categories: sensitivity, aged, substrates,

contaminants, mixtures, and simulated sexual assault kit. All samples were prepared in triplicate as described in Table 6. Substrates were washed with Tide Natural detergent in cold water and dried prior to use to ensure consistency between samples.

Population samples were quantitated using the BCA assay and extracted using the Bond Elut Plexa plate protocol as described above. Performance assessment samples were extracted using Waters Oasis HLB cartridges (Milford, MA), the manufacturer equivalent of the Bond Elut Plexa chemistry. This switch was induced by time constraints and backordered consumables due to SARS-CoV-2 testing. 100 µg total protein was used for analysis. Extraction procedures between the two manifold cartridges were equivalent, with the exception of increasing the organic elution solvent to 35% ACN with 0.1% FA with the Waters Oasis HLB cartridges, per manufacturer recommendations. Eluate was lyophilized to dryness for LC-MS/MS analysis.

Table 6: Performance assessment sample preparation parameters. Room temperature (RT). Samples were prepared in triplicate, with the exception of laundered sheets (substrates).

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

3.3.3 Solid Phase Extraction

Agilent Bond Elut Plexa plates (Agilent Technologies) along with a positive pressure manifold was utilized for sample extraction. Wells were primed with 500 µL of

50% ACN with 0.5% TFA in deionized water and washed with 500 μ L of 0.5% TFA in deionized water. A total protein amount of 100 μ g was diluted with 0.5% TFA in deionized water for a final volume of 500 μ L and loaded onto the cartridge. Sorbent material was washed with 500 μ L of 0.5% TFA in deionized water. Sample material was eluted with 100 μ L of 30% ACN with 0.1% FA in deionized water. This step was repeated for a total of 200 μ L of eluate, which was lyophilized to dryness prior to analysis.

3.3.4 Data Acquisition with the Thermo Q-Exactive HRMS

Lyophilized samples were reconstituted in 50 μ L of 2% ACN with 0.1% FA in deionized water. Acquisition was performed using 5 μ L of sample per injection on a Q Exactive™ Quadrupole-Orbitrap™ mass spectrometer operating in positive dd-MS² mode (Thermo Scientific, Waltham, MA). A Waters® ACQUITY UPLC BEH C18 analytical column (1 x 100 mm, 1.7 μ m, 130Å) was used for separation (Waters, Milford, MA). Mobile phase A consisted of water with 0.1% FA and mobile phase B consisted of ACN with 0.1% FA. Separation initiated at 5% B followed by a linear 5-35% B gradient over 17 minutes, a 3-minute hold at 90% B, followed by a 5-minute re-equilibration at a 0.30 mL/min flow rate and column temperature of 50 °C. MS resolution was set at 70,000 with an AGC target of 3e6 over a scan range of 350 to 2,000 m/z. MSMS resolution was set to 17,500 with an AGC target of 1e5 with a loop count of 10 and a minimum AGC target of 1e3 to trigger an MSMS scan. Normalized collision energy was set to 27 with an isolation windows of 2.2 m/z. Maximum IT in MS mode was 50 ms and 100 in MSMS mode. Peptide match was set to preferred with charge exclusion set to unassigned, 6 - 8, and >8. dynamic exclusion was set to 6 seconds. Source parameters included sheath gas flow rate set to 40, aux gas to 12, sweep gas to 1, gas temp of 400, capillary temp at 320, and S-Lense RF level to 50. Directed dd-MS2 was employed for analyzing all casework type samples via an inclusion list. The inclusion list targets can be found in Appendix 2.

3.3.5 Data Processing

Acquired data was searched using Protein Metrics Byonic™ (v. 3.8) software (Protein Metrics Inc., Cupertino, CA). A no enzyme search method was created with the following criteria: for sample digestion parameters, the cleavage site was left blank, the cleavage side

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

3.4 Saliva Identification by Intact High Resolution Mass Spectrometry

3.4.1 Sample Collection and Preparation

Research was approved by the University of Denver Institutional Review Board (IRB #796971-5) with study participants provided written informed consent before. Saliva was collected by the participant spitting into sterile collection cups. Vaginal swabs, buccal swabs, and vaginal swabs collected during menses were collected from female volunteers who abstained from unprotected sexual intercourse for seven days prior to collection. All swabs were dried at room temperature overnight and stored at -80 °C until use. Semen was self-collected from male volunteers and single use aliquots were stored at -80 °C until use.

Population samples were prepared directly from liquid saliva. 500 µL of saliva was centrifuged for 10 minutes at 10,000 x g at 4°C to pellet cellular and 25 µL of supernatant transferred to NBS plates for extraction. Casework samples were prepared from cotton swabs which were cut in half, 1 mm substrate cuttings, or by a wet swabbing technique. Casework samples were resuspended in 500 µL PBS, incubated with gentle rotation for 30

minutes, solid material placed in a spin basket, and centrifuged for 10 minutes at 10,000 x g at 4°C. 125 µL of supernatant was transferred to NBS plates for extraction.

3.4.2 Solid Phase Extraction with the Agilent AssayMap Bravo

C18 samples were mixed 1:1 with 0.5% TFA in deionized water. AssayMAP C18 cartridges were primed with 100 µL 50% ACN 0.5% TFA at a flow rate of 300 µL/min, equilibrated with 50 µL of 0.5% TFA in deionized water at 10 µL/min, and 215 µL of sample was passed over the cartridge at a flow rate of 5 µL/min. Cartridges were washed with 50 µL of 0.5% TFA in deionized water at a rate of 10 µL/min and peptides eluted from the sorbent in 25 µL of 70% ACN 0.1% FA at 5 µL/min. Eluate was lyophilized to dryness, resuspended in 50 µL of 10% ACN with 0.1% FA, and transferred to Waters™ QuanRecovery Vials for analysis.

3.4.3 Immunoaffinity Extraction with the Agilent AssayMap Bravo

ProteinTech (Rosemont, IL, USA) Rabbit anti-Statherin Polyclonal antibody (cat# 19724-1-AP) was used for immunopurification. Briefly, antibody was dialyzed in Slide-A-Lyzer MINI Dialysis devices with a 10K MWCO membranes according to the manufacturers recommendation. Biotinylation labelling was carried out using EZ-Link™ Sulfo-NHS-LC-Biotin with a 25-fold molar excess of biotin to antibody for 30 minutes at room temperature. Following incubation, excess biotin was removed with 7K MWCO Zeba™ Spin Desalting Columns per the manufacturer's instructions. Biotinylated antibody was stored as single use aliquots at -80 °C until use.

For immuno-purification, samples were diluted 1:1 with 2x HALT protease inhibitor with 1 mg/mL BSA in PBS, fortified with 2 µg of biotinylated antibody, and allowed to incubate for 90 minutes with gentle rotation at room temperature. AssayMap Streptavidin (SA-W cartridge with 5 µL bed volume) extraction cartridges were purged with 250 µL of 1% FA at 300 µL/min followed by a second purge with 100 µL of 1% FA at 10 µL/min to remove unbound streptavidin. Cartridges were primed with 250 µL of PBS at 50 µL/min, equilibrated with 50 µL of PBS at 10 µL/min, and 215 µL of sample was loaded into the column at 5 µL/min, samples were then washed sequentially with 50 µL of

PBS and 50mM ABC at 10 μ L/min. Statherin was eluted with 50 μ L of 10% ACN with 5% acetic acid and transferred to QuanRecovery vials for analysis.

3.4.4 Data Acquisition with the Thermo Q-Exactive HRMS

MS was performed on the Thermo Q-Exactive mass spectrometer with Vanquish UHPLC system. Separation was carried out with a Hypersil GOLD C8 column at a flow rate of 0.3 ml/min at 55°C. Solvent A consisted of water with 0.1% FA and solvent B 0.1% FA in ACN. Gradient separation initiated at 20% B rising to 45% B over 5.9 minutes, increasing to 95% B at 6 minutes, holding at 95% B for 2 minutes, followed by a 2-minute re-equilibration at initial conditions. Solvent flow was directed to waste from 0 to 1 minutes and from 5 to 10 minutes. Source parameters included a sheath gas flow rate of 40, Aux gas flow rate of 15, Sweep gas flow rate of 1, capillary temp at 300 °C, Aux gas heater temp at 350 °C, spray voltage of 4.5 kV, and S-Lens RF at 80. Q-Exactive was operated in positive Full-MS mode with a resolution setting of 70,000 over 600 to 2000 m/z. Initial statherin confirmation and characterization was performed in data dependent mode with an MS2 resolution of 17,500, AGC target of 2e5, isolation window of 2.2 m/z, and nce of 20.

3.4.5 Data Processing

Initial protein confirmation was carried out using BioPharma 4.1 with 2x Phosphoserines as variable modifications. Intact deconvolution employed the Xtract algorithm with a s/n threshold of 5 and relative abundance threshold of 5%. MSMS confirmation was performed with the peptide mapping module. Identification employed a no enzyme search with specificity set to low, s/n threshold of 5, HCP and HDX were disabled.

Statherin identification in the population and casework studies was performed using Pinpoint 1.4 software by correlation the experimental to theoretical isotope distribution as has been performed for similarly isotopically resolved proteins. Six isotopes including the monoisopic mass from the doubly phosphorylated 4+ charge state (1345.1179, 1345.3685, 1345.6193, 1345.8700, 1346.3715, 1346.1207,) were monitored with an extraction

tolerance of 5 ppm. Identification required a correlation coefficient > 0.95 to be considered valid.

3.5 Body fluid Identification by Traditional Casework Models

3.5.1 Sample Preparation

Samples were prepared as outlined in section 3.1.2 and carried over to this portion of the research for operational testing. Three categories of laboratory prepared casework-type samples were reserved for serological comparison only: substrate samples, environmental contaminant samples, and aged samples. Six categories of casework-type samples were reserved for both serological comparison and genetic analysis: sensitivity samples, mixtures, simulated sexual assault samples, sexual assault type samples, digital swab samples, and degraded samples. Swabs or substrates were solubilized as previously described. Remaining supernatant was tested against existing serological models in tandem with proteomic analysis, eliminating an additional freeze/thaw cycle to ensure consistency in material evaluated. Cell pellets were preserved for genetic analysis at -20°C and were thawed at room temperature prior to DNA typing.

3.5.2 Immunochromatographic and Traditional Assays

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

For sensitivity samples, the maximum volume suggested by the manufacturer was used for testing (200 μL ABACard® assays, 100 μL RSID™ assays, 50 μL SALIgAE® assay). For remaining sample types, matched liquid samples were prepared as an equivalent

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

Table 7: Selected traditional serological screening tests for comparison with the developed proteomic assay.

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

3.5.3 Genetic Analysis

3.5.3.1 DNA Extraction

Two DNA extraction protocols were utilized. All samples containing semen or seminal fluid were subjected to a manual organic differential extraction procedure. All other samples (those free of semen) were subjected to a manual organic extraction protocol.

Pelleted cellular material selected for organic extraction was treated with 350 μ L of master mix containing extraction buffer and proteinase K solution. Samples were incubated at 56 °C overnight (a maximum of 18 hours) on a shaker at 850 RPM. After incubation, samples were purified and concentrated. Each sample received two iterations of purification using 300 μ L of phenol:chloroform:isoamyl alcohol reagent. An additional cleanup step was performed with the addition of 300 μ L of water saturated butanol. Purified sample was passed through a Vivacon® 500 centrifugal concentrator (Vivaproducts, Inc.,

Littleton, MA), washed with 200 μ L of TE-4 buffer (10mM Tris-HCl, 0.1mM EDTA, pH 8.0), and eluted in 100 μ L of TE-4 buffer.

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

3.5.3.2 DNA Quantitation

Once purified, all samples were quantitated using Quantifiler™ Trio DNA Quantification Kit (Applied Biosystems™, Foster City, CA). Extracts were thawed and brought to room temperature prior to quantitation. A previously validated standard operating procedure was used. A standard curve was prepared, in addition to a negative control, for each batch of samples. Standards were prepared by diluting the DNA Standard with Quantifiler™ THP DNA Dilution Buffer to the following final concentrations: 50 ng/ μ L, 5.0 ng/ μ L, 0.5 ng/ μ L, 0.05 ng/ μ L, and 0.005 ng/ μ L. TE-4 buffer was used for the negative control. Standards, controls, and samples were treated with a master mix of kit specific Primer Mix and PCR Reaction Mix. Quantitation was performed on an Applied Biosystems™ 7500 Real-Time PCR System. Thermal cycler parameters were set to heat at 95 °C for 2 minutes followed by 40 cycles of 9 seconds at 95 °C and 30 seconds at 60 °C. The quantification batch was considered valid if the standard curve slope measured between -3.0 and -3.6 and the R² value was greater than or equal to 0.98.

3.5.3.3 DNA Amplification

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

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

3.5.3.4 DNA Separation and Analysis

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

GeneMapper® ID-X software (v.1.4) was utilized for profile interpretation. Through internal validation that independently evaluated the signal-to-noise characteristics of each dye channel, the following analytical thresholds were set for a 10 second injection with GlobalFiler™: 6-FAM™, 60 relative fluorescence units (RFUs); VIC™, 75 RFUs; NED™, 50 RFUs; TAZ™, 60 RFUs; and SID™, 60 RFUs. From a separate internal

validation, the following analytical thresholds were set for a 10 second injection with Yfiler™ Plus: 6-FAM™, 50 RFUs; VIC™, 50 RFUs; NED™, 40 RFUs; TAZ™, 40 RFUs; and SID™, 40 RFUs.

4 Results and Discussion

4.1 Developmental Validation of an Automated LC-QQQ Assay

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

This section reports the validation results of the proteomic assay for the identification of peripheral blood, seminal fluid, saliva, and vaginal/menstrual fluid. Body fluid specific protein markers were previously selected under prior funding, where they underwent rigorous discovery and verification of the candidate markers within a greater population. The selected markers were reconfirmed as described in previously, with each protein marker and subsequent peptide targets assessed both in vitro with analytical identification and in silico using bioinformatics software. The successful operation of the method at each level described herein identified working conditions and associated limitations that were acknowledged to estimate the true performance of the analytical method. Finally, the full method SOP can be found in appendix B.

4.1.1 Repeatability and Reproducibility

Reproducibility was assessed to ensure robust results could be obtained over a period of multiple analysis batches prepared by a single individual. Repeatability was evaluated to ensure reliable results could be obtained between individual analysts across a

period of multiple analysis batches. For this portion of the validation, reproducibility and repeatability were assessed in tandem through the preparation, analysis, and data acquisition of samples by two analysts over the course of a three-day period. This entailed BCA quantitation, robotic digestion, SPE cleanup, and analysis via LC-MS/MS.

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

4.1.1.1 BCA

BCA quantitation is required prior to tryptic digestion in order to normalize the amount of total protein input into the robotic preparation process. This is to ensure that the proper ratio of digestion enzyme to protein substrate is met, in addition to monitoring the amount loaded onto the SPE cleanup cartridges and LC-MS/MS system. The average protein concentration, as well as the measured combined and individual variability of the BCA quantitation assay for each body fluid, is detailed in Table 8. Coefficient of variation (CV) values both within and between analysts was less than 10% across this portion of the validation.

Table 8: Calculated CV (as a percent) of average protein concentration between analysts and combined for each fluid assayed.

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

4.1.1.2 Signal Response

The peak area of each peptide in the assay was assessed for all replicates as well as across the replicates for the two analysts individually. Of the 46 peptides included in the assay, 41 showed CV values less than 25%, with the majority of these targets showing less than 10% variation. Data for this portion of the study can be found in Appendix D, with results for seminal fluid depicted in Table 9.

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

Table 9: Calculated CV (as a percent) for peak area response of seminal fluid peptide markers between analysts and combined.

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

4.1.1.3 Retention Time

The retention time of each peptide biomarker in the assay was assessed for all replicates as well as across the three replicates for the two analysts. Retention time averages for each analyst were used to calculate the variability. Percent CV values for this dataset were all below 0.5%. An illustrative example of results for seminal fluid is shown in Table 10 and in appendix E.

Table 10: Calculated CV (as a percent) for retention time of seminal fluid peptide markers between analysts and combined.

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

4.1.1.4 Response Ratios

The ion ratio for each peptide biomarker was assessed by dividing the peak area response of the quantifier ion (i.e., more abundant transitions observed during target selection) by the peak area response of the qualifier ion(s) (i.e., lower abundant transitions observed during peptide selection). Ion ratios were assessed for all replicates as well as across the two analysts. The ion ratios for all peptides were found to be highly consistent,

with a majority of calculations showing CV values less than 5%. An illustrative example of results for seminal fluid is shown in Table 11. Epididymal secretory protein peptide DCGSVDGVK exhibited a higher CV value but was consistent between the two analysts, demonstrating a high rate of precision both within-run and between-run. The remainder of the data are presented in detail in Appendix F. Only neutrophil gelatinase protein in menstrual blood exhibited higher variability in the qualitative response ratio. In this case, the proteins were of very low abundance in the pooled sample being assessed.

Table 11: Calculated CV (as a percent) for ion ratios of seminal fluid peptide markers between analysts and combined.

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

4.1.1.5 Internal Positive Control

The performance of the internal positive control (IPC) was assessed by comparing the response ratio of the digested myelin basic protein to that of an isotopically-labeled internal standard added to the sample being analyzed. The response ratio was found to be highly consistent across all body fluids. The variability observed both among different samples of individual body fluid and between different body fluids consistently displayed CV values less than 8% (Table 12 and Figure 6). Samples evaluated for this portion of the validation displayed and average peak area response ratio of 13.7 ± 0.999 .

Table 12: Combined calculated CV (as a percent) for the internal positive control peak area response ratio for the duration of repeatability and reproducibility experimentation.

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

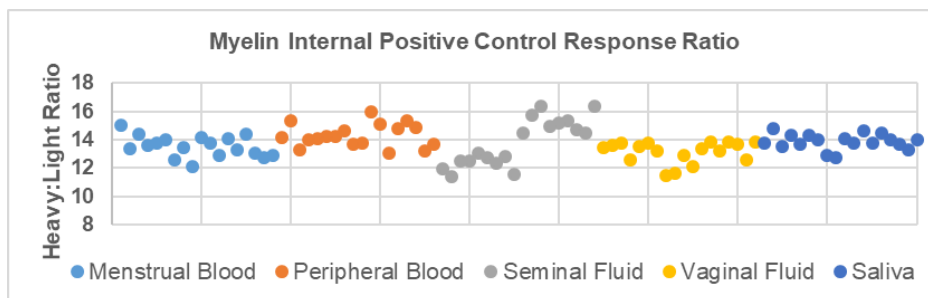


Figure 6: Internal positive control response ratio for the duration of repeatability and reproducibility experimentation.

4.1.2 Sensitivity

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

Results were evaluated for retention time consistency, chromatographic peak shape, peak area response, as well as calculated ion ratios. Overall, peripheral blood was confidently identified at a 1:6,533,600 dilution (Table 13) and menstrual blood at 1:6,400 dilution (Table 14). Although both peptide targets for hemoglobin were accurately detected at the lowest dilution in the peripheral blood matrix, a majority of the remaining protein markers exhibited dropout at the 1:204,800 dilution. Similar results were observed for peripheral blood protein markers in menstrual blood; however, vaginal fluid markers in menstrual blood were shown to exhibit less sensitivity. Four protein targets failed to be detected within the menstrual blood matrix (Ly6 PLAUR, suprabasin, periplakin, and involucrin), with remaining biomarkers exhibiting dropout at the protein level after the 6,400-fold dilution. Small proline rich protein 3 was detected in each dilution assessed,

however, peptide peak areas did not display negative linearity as the dilution series decreased. Peak area intensities for peptide VPEPGCTK remained consistent between the 25,600-fold dilution and the 6,533,600-fold dilution (Figure 7). Therefore, this marker was not considered when characterizing menstrual blood for biomarker sensitivity. Strong instances of peptide and protein dropout, particularly for the vaginal fluid biomarkers in this matrix, may be attributed to expression variability during the menstruation cycle.

Seminal fluid was accurately detected at the lowest dilution assessed, with three peptides identified at the 524,288-fold dilution (Table 15). All peptide targets were correctly detected at the 16,389-fold dilution, with linear peptide and protein dropout displayed as the dilution series decreased. Semenogelin 1, semenogelin 2, and acid phosphatase peptide targets met all acceptance criteria at the lowest dilution sample.

Saliva was characterized at the lowest dilution tested, with a single alpha amylase peptide detected at the 65,536-fold dilution (Table 16). All peptide markers were detected through the 256-fold dilution, and similar to seminal fluid, exhibited linear protein and peptide dropout as the dilution series decreased. With the exception of alpha amylase, remaining protein markers displayed complete dropout at the 8,192-fold dilution tested.

Vaginal fluid was identified in a two-tiered manner. Because the target biomarkers exhibit a wide range of specificity for vaginal fluid, the confidence level of accurately detecting and identifying vaginal fluid fluctuates. For this data set, vaginal fluid was confidently identified down to the 2,048-fold dilution by the presence of cornulin and neutrophil gelatinase, both of which exhibit strong specificity for vaginal fluid (Table 17). However, small proline rich protein 3 was detected at the 32,768-fold dilution. Given the known cross-reactivity this protein exhibits, vaginal fluid detection was made with less confidence at this lower range. The use of this protein as a presumptive biomarker has been thoroughly detailed in interpretation guidelines.

Table 13: Observed sensitivity limit of peripheral blood. Proteins that were positively identified by all of their respective peptide markers are shown in dark green. Light green indicates where at least one peptide marker for the respective protein dropped out. White indicates complete protein dropout.

[illegible]

Table 14: Observed sensitivity limit of menstrual blood. Proteins that were positively identified by all of their respective peptide markers are shown in dark green. Light green indicates where at least one peptide marker for the respective protein dropped out. White indicates complete protein dropout.

[illegible]

Table 15: Observed sensitivity limit of seminal fluid. Proteins that were positively identified by all of their respective peptide markers are shown in dark green. Light green indicates where at least one peptide marker for the respective protein dropped out. White indicates complete protein dropout.

[illegible]

Table 16: Observed sensitivity limit of saliva. Proteins that were positively identified by all of their respective peptide markers are shown in dark green. Light green indicates where at least one peptide marker for the respective protein dropped out. White indicates complete protein dropout.

[illegible]

Table 17 Observed sensitivity limit of vaginal fluid. Proteins that were positively identified by all of their respective peptide markers are shown in dark green. Light green indicates where at least one peptide marker for the respective protein dropped out. White indicates complete protein dropout.

[illegible]

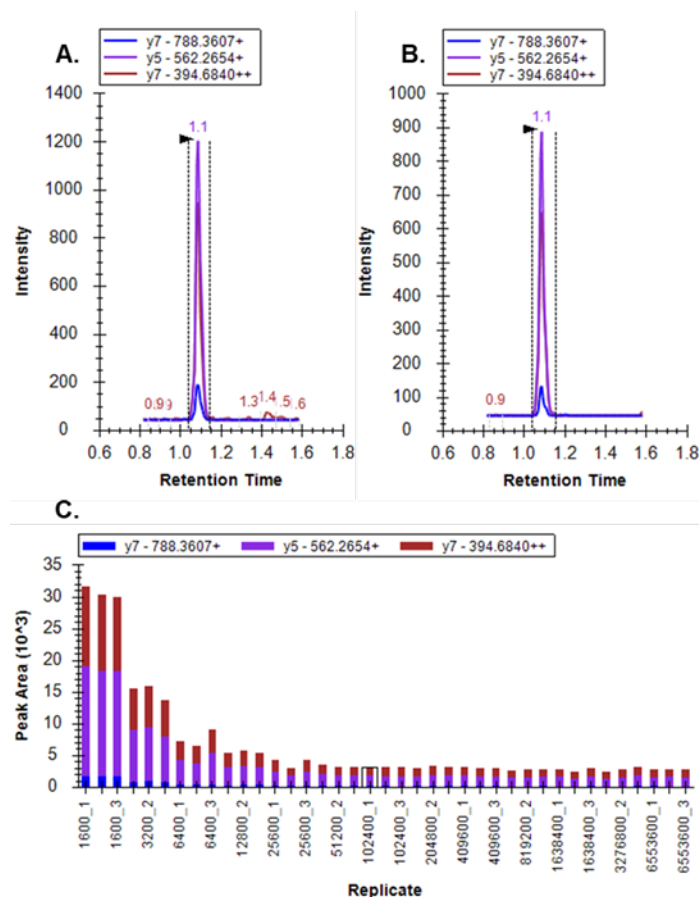


Figure 7: Small proline rich protein 3 peptide VPEPGCTK peak area response. (A) Peak area intensity at a 25,600-fold dilution. (B) Peak area intensity at a 6,553,600-fold dilution. (C) Peak area response across all replicates evaluated, progressing from 1,600-fold to 6,553,600-fold dilution.

4.1.3 Stability

Stability was evaluated to ensure that samples remain viable if an instrumentation error or power outage were to occur that causes samples either: (1) to remain in the autosampler overnight without being processed or (2) to be subjected to additional freeze-thaw cycles. This portion of the validation assessed two aspects of a sample's stability. First, freeze thaw stability was evaluated using aliquots of pooled samples. Three samples were assayed immediately and served as baseline peak area response. The remaining six samples were stored at -80°C . All six remaining aliquots were thawed the following day. Three of the thawed samples were analyzed on the second day and the remaining three were refrozen for subsequent thawing and analysis on day three. The second form of stability assessed, autosampler stability, was evaluated by storing processed samples in the chilled autosampler and analyzing them over a period of 3 days.

4.1.4 Freeze Thaw Stability

Single use aliquots of pooled body fluid were evaluated over two freeze thaw cycles to evaluate if storage would have a negative effect on the detection of a particular fluid (Tables 18 and 19). A significant reduction in signal intensity would indicate that samples are degrading during the freeze-thaw process and would require re-extraction, digestion, and cleanup prior to reliable analysis. Overall, only one vaginal fluid peptide was affected by repeated freeze thaw cycles (Table 19). Suprabasin peptide FGQGVHHGLSEGWK was detected in Day 1 samples but was absent after the first freeze thaw cycle (Figure 8). Because FGQGVHHGLSEGWK is a low abundant target, even minor degradation of the sample appears to have deleterious effects. In addition to the one suprabasin peptide in vaginal fluid, several markers (e.g., suprabasin, periplakin, etc.) in menstrual blood were not detected during this portion of the study. These results may be attributed to the low abundance of these markers in the menstrual blood matrix, which is consistent with results observed under subsequent aims. Furthermore, given that protein dropout was observed on Day 1 of the study, it is more likely due to low abundance in comparison with protein biomarker degradation due to instability. Finally, performance of the IPC was assessed to ensure complete sample preparation over the course of this study (Figure 9). Although a negative trend pattern is visible when plotting the IPC response ratio over time, all calculated ratios fell within acceptance criteria.

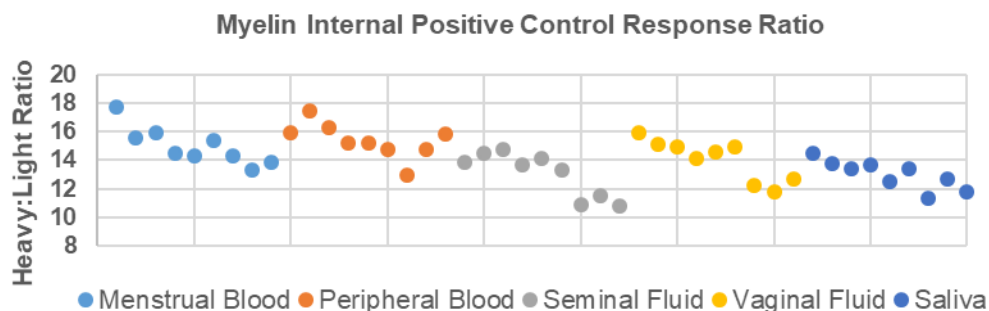
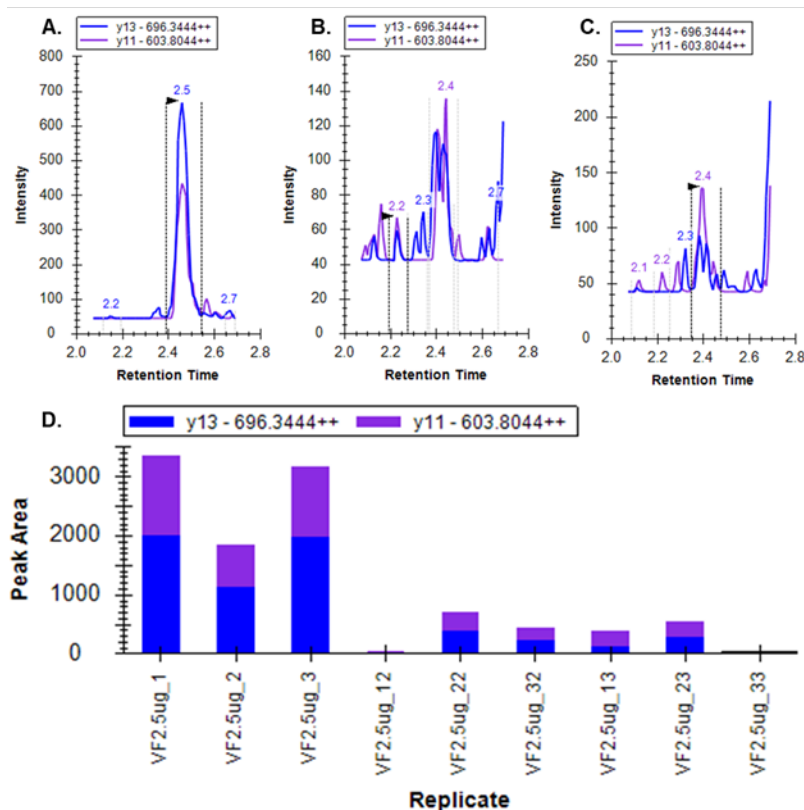
Table 18: Freeze thaw stability results for peripheral blood, saliva, and seminal fluid. Peptide markers that were positively identified are shown in dark green. No peptide or protein dropout was observed.

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

Table 19: Freeze thaw stability for vaginal fluid and menstrual blood. Peptide markers that were positively identified are shown in dark green. White indicates peptide dropout.

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

Three replicates of the five target body fluids were processed and evaluated over a period of 3 days. Samples were analyzed on Day 1 (i.e., 0 hours) and stored on the liquid chromatograph's chilled autosampler set at 8 °C. The same samples were then reinjected 24 hours (Day 2) and again at 48 hours (Day 3) after original processing to assess



fluctuations in detection signal. A significant drop in signal strength or quality would indicate that any samples stored in excess of 24 hours on the autosampler would need to be re-extracted, digested, and cleaned up. Fortunately, the resulting data confirms that no such problems occurred. There was no sufficient drop in peak area response for any target peptide in the assay. On the contrary, a slight increase in peak area response was often observed. This was evident across all fluids; however, this increase is not sufficient. This was attributed to sample evaporation and concentration of target peptides that occurred after the 96-well sample plate cover was punctured for initial injection, resulting in evaporation of reconstitution solvent. Detailed results for this portion of the study are outlined in Appendix G. In addition, IPC ion ratios across the three days were calculated, with no significant difference between ion ratios for 0 and 24 hours ($t_s=0.404$, $df=58$, $P>0.05$) or for 0 and 48 hours ($t_s=1.084$, $df=58$, $P>0.05$). Percent CV value for the IPC are listed in Table 20. Overall, these results indicate that samples plated on the autosampler can be analyzed within two days of being processed without significant change in signal strength.

Table 20: Calculated CV (as a percent) for the internal positive control for the duration of the autosampler stability study.

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

4.1.6 Mixture Studies

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

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

In several cases, cross-fluid interferences were detected, potentially leading to difficulties in the interpretation of results. These have been identified, and using an abundance of caution, the interfering transition was either removed or replaced. For example, a major interference peak was detected with prostatic acid phosphatase peptide FVTLFR when seminal fluid was mixed with peripheral blood (Figure 10). In this case, the transition m/z 534.3398⁺ (purple peak) generates the interference peak. By replacing transition m/z 534.3398⁺ with transition m/z 247.1441⁺, the problem was readily eliminated in all subsequent analyses.

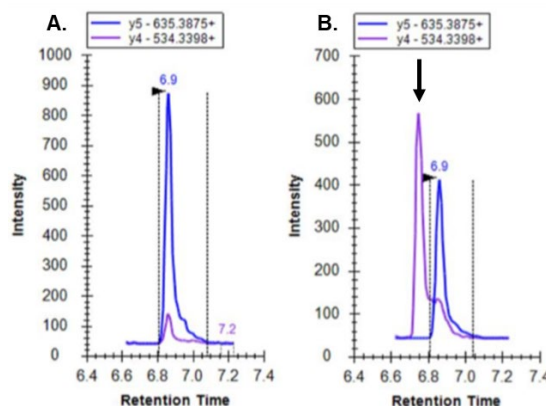


Figure 10: Prostatic acid phosphatase peptide FVTLFR peak area response in (A) neat seminal fluid and (B) seminal fluid and peripheral blood mixture. The m/z 534.3398⁺ transition (purple peak denoted with arrow) was removed and replaced.

4.1.7 Specificity Studies

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

[illegible]

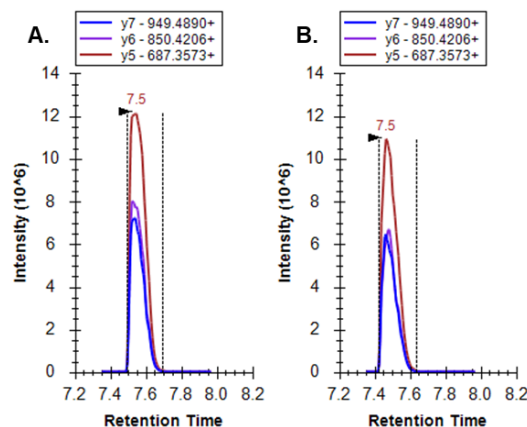


Figure 11: Hemoglobin peptide LLVVYPWTQR peak area response in (A) Rhesus monkey and (B) human peripheral blood.

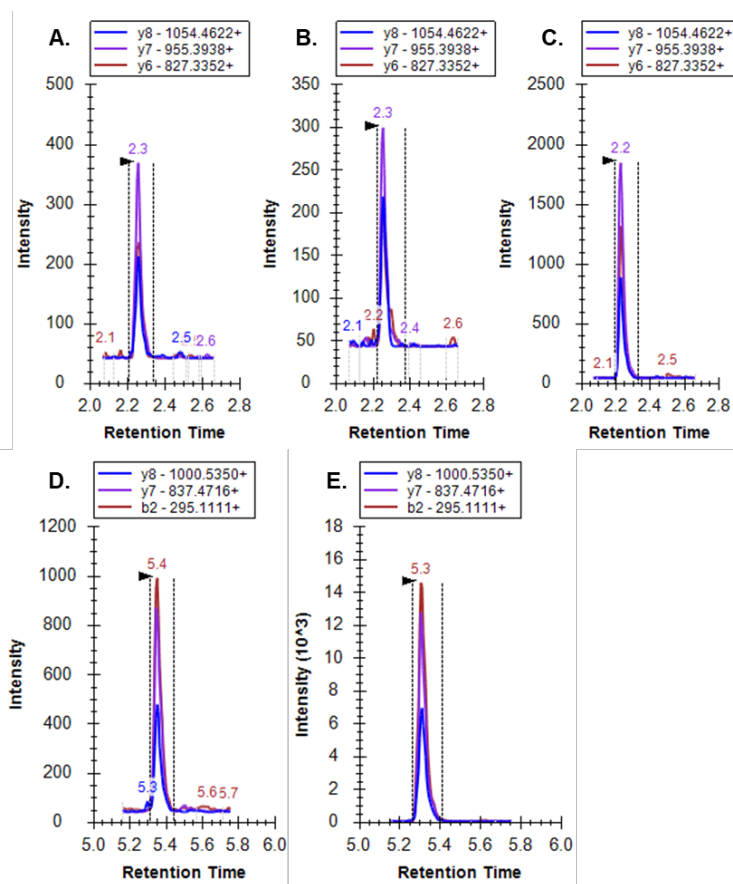


Figure 12: Peak area response of vaginal fluid markers in saliva samples. Ly6/PLAUR peptide GCVQDEFCTR peak area intensity in (A) bovine saliva, (B) Rhesus saliva, and (C) human vaginal fluid control samples. Neutrophil gelatinase peptide MYATIYELK peak area intensity in (D) Rhesus saliva and (E) human vaginal fluid control samples.

There were no instances of cross reactivity with the established assay for peripheral blood from chicken, coyote, or dog in addition to alpaca saliva (Figure 13). A more comprehensive assessment with the inclusion of additional target fluids from various

species could present further chromatographic interferences. Overall, the results of this experimentation stress the importance of clear interpretation guidelines, particularly when examining potential trace amounts of body fluid when only one biomarker is present.

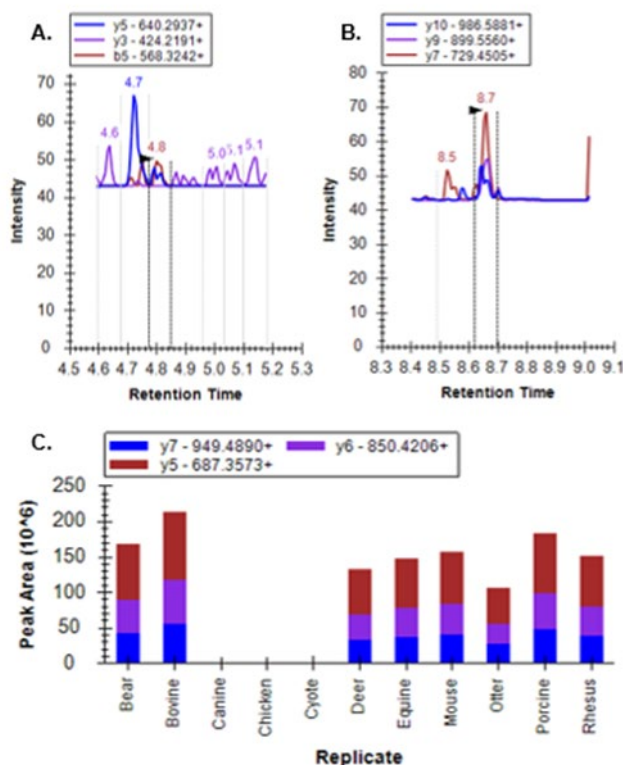


Figure 13: Peak area response of species specificity samples evaluated. (A) alpaca saliva sample protein cystatin peptide ALHFVISEYNK, (B) alpaca saliva sample protein amylase peptide LSGLLDLALGK, (C) hemoglobin peptide LLVVYPWTQR across all blood sample obtained.

4.1.8 Casework Samples

Laboratory simulated forensic casework type samples were evaluated to identify additional performance limitations, particularly with the analysis of trace level samples, and to aid in the generation of finalized Standard Operating Procedures and interpretation guidelines. Final SOP can be found in appendix B and sample preparation details in appendix C.

4.1.8.1 Substrates

Various substrate compositions were analyzed to ensure chemical products (i.e., indigo dye) or extraction efficiency off different materials (i.e., cotton versus leather)

would not interfere with the detection of protein biomarkers. Single source body fluids were evaluated on the following substrates: cotton, leather, denim, carpet, drywall, plastic bottle, aluminum bottle, and feminine hygiene menstrual pad. Results from this study are detailed in Table 22.

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

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

Similar vaginal fluid biomarker dropout was evident during menstrual blood analysis. Menstrual blood was applied to cotton, denim, and a feminine hygiene menstrual pad. Although all peripheral blood markers were positively detected, instances of vaginal fluid peptide dropout and complete protein dropout were observed. Across all substrates tested, three proteins exhibited complete dropout: suprabasin, involucrin, and Ly6/PLAUR. Furthermore, peptide dropout for proteins cornulin and periplakin was also observed, leaving proteins neutrophil gelatinase and small proline-rich protein 3 as the only vaginal fluid markers completely identified.

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

Table 22: Detection of body fluid markers in substrate samples. Proteins that were positively identified by all of their respective peptide markers are shown in dark green. Light green indicates where at least one peptide marker for the respective protein dropped out. Red indicates complete protein dropout. White indicates not tested.

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

4.1.8.2 Environmental Contaminants

A variety of environmental contaminants were selected and tested to evaluate potential concerns with enzymatic digestion, peptide cleanup, or to detect any instances of unanticipated interferences during instrumental analysis. Single source body fluids were tested with the following environmental contaminants: dirt, rust, 10% bleach solution,

chewing tobacco, cigarette butt, spermicide lubricated condom, and water-based personal lubricant. Results from this study are detailed in Table 23.

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

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

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

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

Table 23: Detection of body fluid markers in samples subjected to environmental contaminants. Proteins that were positively identified by all of their respective peptide markers are shown in dark green. Light green indicates where at least one peptide marker for the respective protein dropped out. Red indicates complete protein dropout. White indicates not tested.

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

4.1.8.3 Mixtures

The purpose of this study was to evaluate the performance of the optimized assay when analyzing mixed samples with a major and minor component. Five two-fluid mixtures were prepared, with pairings selected based on sample types routinely tested for in operational forensic laboratories. For example, a seminal fluid minor contributor in a vaginal fluid major contributor was prepared to simulate a sexual assault type sample. Results from this study are summarized in Table 24.

With the exception of urine, for which protein biomarkers are not included within the developed assay, all major and minor body fluid contributors were correctly identified. Beginning with the vaginal fluid minor contributor in a urine major contributor, high rates of vaginal fluid protein marker dropout were observed. Fluid identification was made from the presence of small proline rich protein 3 and cornulin. The urine matrix predictably diluted the vaginal fluid markers past the limit of detection of the assay. In addition, although the average urea composition (9.5 g/L) in urine is below the concentration incompatible with the BCA protein quantitation assay [159], the presence of urea and additional salts endogenous to urine may have negatively affected the ability to produce a reliable protein quantitation value. Therefore, if the total protein quantitation was underestimated, less than optimal protein amounts would have been utilized for testing.

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

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

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

The last two-component mixture analyzed was a saliva minor contributor in a menstrual blood major contributor. The saliva contributor was identified based on the sole presence of protein alpha amylase. Because this protein has demonstrated cross-expression in other body fluids and tissues, the presence of saliva in this particular sample cannot be reported as confirmatory. Furthermore, as observed with the seminal fluid in menstrual blood mixture previously described, there was consistent vaginal fluid protein biomarker dropout exhibited in this sample.

Table 24: Detection of body fluid markers in mixture samples. Proteins that were positively identified by all of their respective peptide markers are shown in dark green. Light green indicates where at least one peptide marker for the respective protein dropped out. Red indicates complete protein dropout. White indicates not tested.

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

4.1.8.4 Sexual Assault Samples

4.1.8.4.1 Simulated Sexual Assault Samples

Prepared samples were allowed to incubate at 37 °C for up to and including eleven days after preparation. At time zero, proteins semenogelin 1, semenogelin 2, and epididymal secretory protein were positively identified (Table 25). The complete panel of seminal fluid peptide markers was not detected in full during any timepoints evaluated during this study. Only epididymal secretory protein was consistently detected over the course of the eleven-day incubation study. Furthermore, a single peptide for both semenogelin 1 and 2 were consistently identified over the course of the study (Figure 14). Peptide dropout for proteins prostatic acid phosphatase and prostate specific antigen was observed at time zero, with complete protein dropout of prostate specific antigen observed for the remainder of the study. Prostatic acid phosphatase exhibited protein dropout after day seven.

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

Table 25: Detection of body fluid markers in simulated sexual assault samples. Proteins that were positively identified by all of their respective peptide markers are shown in dark green. Light green indicates where at least one peptide marker for the respective protein dropped out. White indicates complete protein dropout.

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

4.1.8.4.2 Mock Sexual Assault Kit Samples

4.2 Mock Sexual Assault Kit Samples

Mock sexual assault kit samples were prepared in duplicate using semen provided by a vasectomized and a non-vasectomized male individual (Table 26). For sexual assault swabs prepared with semen from a non-vasectomized donor, seminal fluid was positively identified through the detection of the following protein fingerprints. Beginning with vaginal swab samples, two occurrences of seminal fluid protein dropout were recorded for a single sample (SA01_01.2). Prostate specific antigen and epididymal secretory protein were not positively identified; however, prostatic acid phosphatase, semenogelin 1, and semenogelin 2 were completely characterized. In addition, given the sample type, vaginal fluid protein markers were successfully detected and identified for both samples being assessed. Similar results were recorded for the oral swab sample types. All seminal fluid biomarkers were detected, with the exception of epididymal secretory protein, which exhibited complete dropout. Furthermore, given the sample type, all saliva biomarkers were accurately detected with no protein dropout exhibited. Rectal swab samples were shown to exhibit protein inhibition, with occurrences of seminal fluid protein dropout for both sample collections. Prostate specific antigen and epididymal secretory protein were not detected, in addition to peptide dropout observed for prostatic acid phosphatase and semenogelin 2 (Figure 14).

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

acid phosphatase and semenogelin 2 were reported, with instances of semenogelin 1 peptide dropout for sample SA02_03.1 (Figure 15).

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

Table 26: Detection of body fluid markers in sexual assault kit samples. Proteins that were positively identified by all of their respective peptide markers are shown in dark green. Light green indicates where at least one peptide marker for the respective protein dropped out. White indicates complete protein dropout.

[illegible]

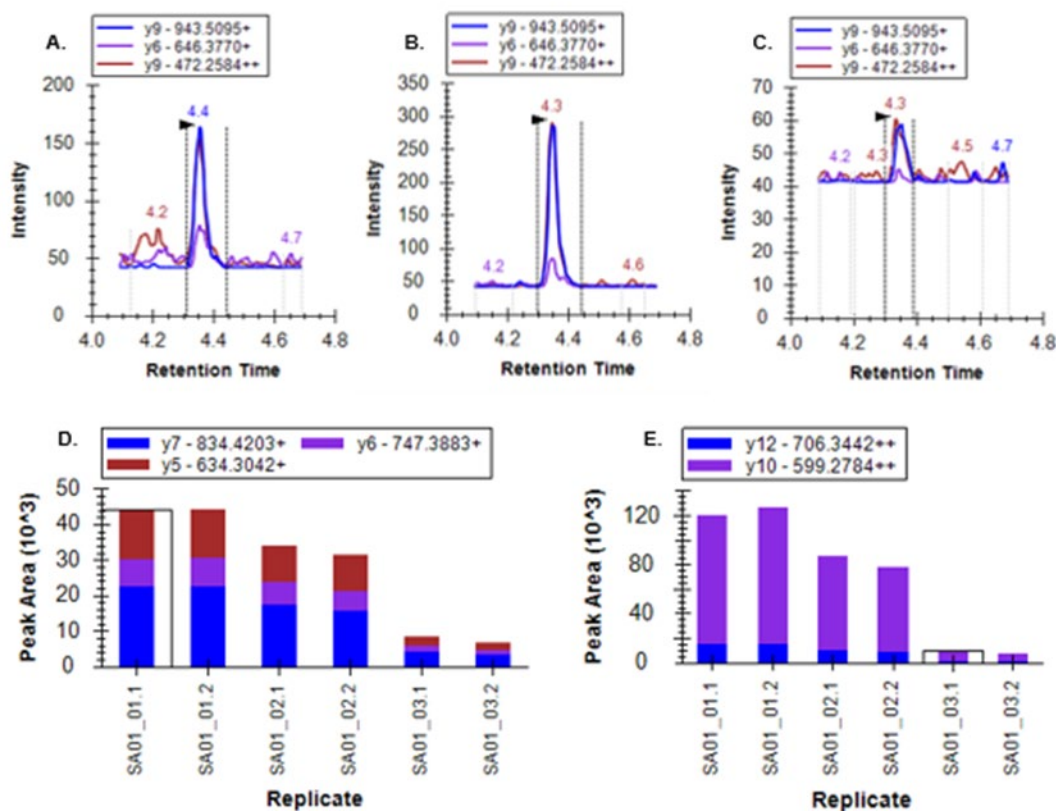


Figure 14: Peak area response of seminal fluid biomarkers in mock sexual assault kit samples containing semen from a non-vasectomized donor. Prostate specific antigen peptide LSEPAELTDAVK peak area intensity in (A) vaginal swab (B) oral swab (C) rectal swab. Semenogelin 2 peptide GSISIQTEEK peak area intensity (D) across vaginal, oral, and rectal swab sample replicates. Semenogelin 1 peptide QITIPSQEQEHSQK peak area intensity (E) across vaginal, oral, and rectal swab sample replicates.

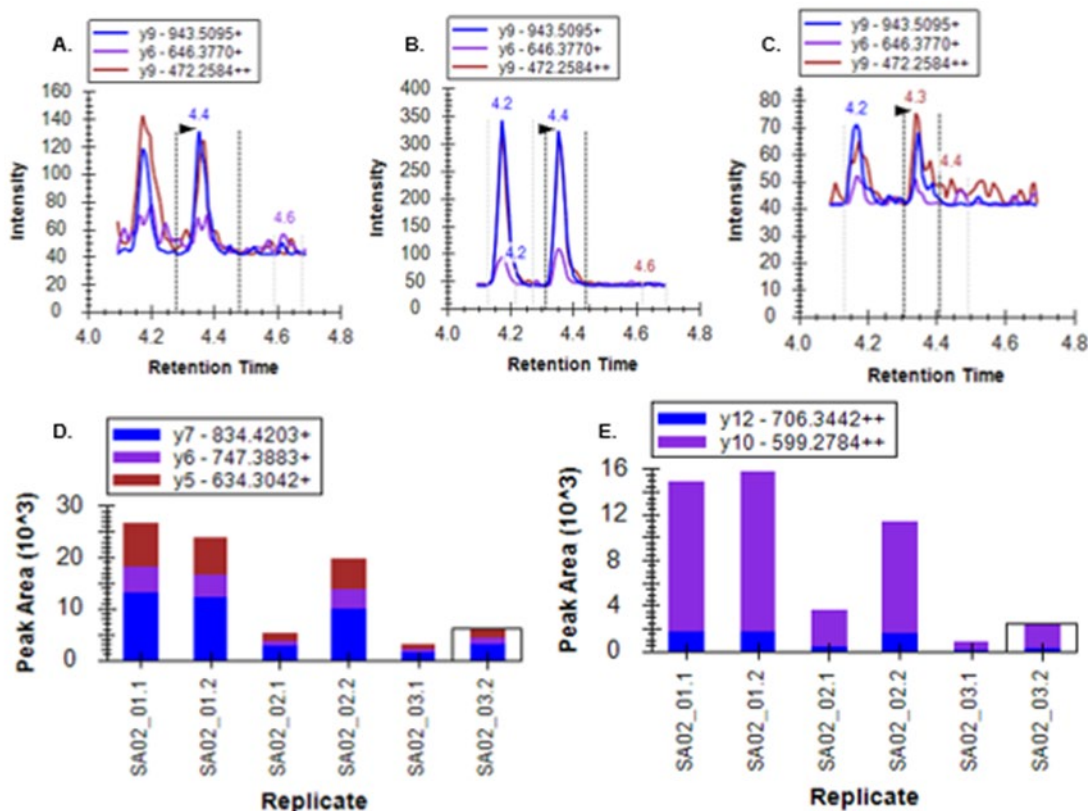


Figure 15: Peak area response of seminal fluid biomarkers in mock sexual assault kit samples containing semen from a vasectomized donor. Prostate specific antigen peptide LSEPAELTDAVK peak area intensity in (A) vaginal swab (B) oral swab (C) rectal swab. Semenogelin 2 peptide GSISIQTEEK peak area intensity (D) across vaginal, oral, and rectal swab sample replicates. Semenogelin 1 peptide QITIPSQEQEHSQK peak area intensity (E) across vaginal, oral, and rectal swab sample replicates.

4.1.8.4.3 Digital Swabs

Digital swab samples were collected on separate days for a total of two swab types, with each sample serving as a distinct sample collection. A summary of results is outlined in Table 27. Beginning with the oral penetration digital swabs, all saliva protein biomarkers were positively identified for both sample collections. The first sample evaluated was positive for alpha amylase and submaxillary gland androgen-regulated protein 3B, with peptide dropout observed for proteins statherin and cystatin SA. The second sample analyzed was positive for submaxillary gland androgen-regulated protein 3B and cystatin SA, with peptide dropout exhibited for alpha amylase and statherin. There were no instances of complete protein dropout observed for oral penetration digital swabs. Although inconsistencies were observed for alpha amylase detection between the

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

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

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

Table 27: Detection of body fluid markers in digital swab samples. Proteins that were positively identified by all of their respective peptide markers are shown in dark green. Light green indicates where at least one peptide marker for the respective protein dropped out. White indicates complete protein dropout.

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

4.1.8.5 Degradation Samples

Substrates were prepared to simulate authentic items of evidence that have been laundered. Upon analysis, both the stain region and the control (non-stain region) of the same substrate were processed concurrently. A complete summary of results is depicted in Table 28. Peripheral blood containing samples (cotton bath towel, denim) yielded positive identifications for all target peripheral blood biomarkers. For the towel sample, peptide dropout for apolipoprotein and serotransferrin was observed. The denim sample containing peripheral blood did not exhibit any instances of peptide or protein dropout. Both the towel and denim control regions were negative for all peripheral blood protein targets.

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

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

Table 28: Detection of body fluid markers in degraded samples. Proteins that were positively identified by all of their respective peptide markers are shown in dark green. Light green indicates where at least one peptide marker for the respective protein dropped out. White indicates complete protein dropout.

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

4.1.8.6 Aged Samples

Aged body fluid swabs were prepared to assess the degradation of target protein markers in addition to extraction efficiency when target body fluids were not stored under ideal conditions. Protein identification results for all aged samples are outlined in Table 29. Peripheral blood biomarkers were detected at all time points collected. No instances of protein degradation were observed, with all peak intensities remaining consistent over the course of the study. Similar results regarding peripheral blood markers in menstrual blood were obtained; however, vaginal fluid marker dropout was observed. Proteins Ly6/PLAUR, suprabasin, and involucrin were not detected during this study. All other vaginal fluid markers did exhibit stability for the time points collected, with no additional protein or peptide dropout exhibited.

Seminal fluid and saliva behaved similar to peripheral blood. All target protein markers were correctly identified during each time point collected, with no instances of

protein or peptide dropout over the course of the study. The amount of material present for vaginal fluid (i.e., 50 μ L vs. 150 μ L) effected the positive identification of specific markers, as originally hypothesized. Peptide dropout was observed for protein perioplakin during the study, with inconsistent peptide dropout exhibited by protein suprabasin. This suggests that the selected peptide marker AQSLQSAK for suprabasin does not exhibit the same high degree of robustness when compared with remaining vaginal fluid markers.

Table 29: Detection of body fluid markers in aged samples. Proteins that were positively identified by all of their respective peptide markers are shown in dark green. Light green indicates where at least one peptide marker for the respective protein dropped out. White indicates complete protein dropout.

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

4.1.8.7 Sensitivity Samples

Sensitivity samples were prepared by extracting a serial dilution of target body fluid from cotton swabs. These samples differ from those prepared under section 3.1.2.2 by diluting target body fluid and recovering from a substrate, in comparison with evaluating diluted target fluid directly in a test tube. A summary of results is detailed in Tables 30-32.

Beginning with peripheral blood detection, all protein markers were successfully identified for neat, 1:2, 1:10, and 1:100 dilution samples (Table 30). Hemopexin dropout was observed at the 1:1,000 dilution, with additional protein dropout of alpha-1 antitrypsin and serotransferrin peptide dropout at the 1:2,000 dilution. From this dilution, the only protein marker consistently detected was hemoglobin, with peptide or protein dropout reported for all other markers at the 1:5,000, 1:10,000 and 1:20,000 dilutions (Figure 16). No peripheral blood biomarkers were detected at the 40,000-fold dilution.

Similar results were exhibited by menstrual blood sensitivity analysis (Table 30). All peripheral blood markers were accurately detected through the 100-fold dilution, with peptide dropout for serotransferrin and apolipoprotein at the 1:1,000 dilution. Peripheral blood marker dropout of hemopexin and alpha-1 antitrypsin was observed at the 1:2,000 dilution, with all proteins except hemoglobin not detected at the 1:5,000 dilution. Hemoglobin was detected in all menstrual blood dilution samples analyzed. As hypothesized, vaginal fluid protein markers did not exhibit the same level of sensitivity as the peripheral blood markers in menstrual blood. Protein dropout for involucrin, Ly6/PLAUR, and suprabasin was observed for all samples evaluated. Furthermore, peptide dropout for cornulin and periplakin was exhibited for all samples. These results remained consistent up to and including the 100-fold dilution sample. Additional protein dropout of periplakin and neutrophil gelatinase was reported at the 1,000-fold dilution sample. Further dilutions exhibited inconsistent protein and peptide detection. Small proline rich protein 3 was detected at the 1:10,000 and 1:20,000 dilutions (Figure 16); however, exhibited peptide dropout at the 1:5,000 and 1:40,000 dilutions. As with other casework type samples analyzed, the endogenous low abundances of target vaginal fluid protein markers attributes to the lack of detection at lower dilution samples.

Robust detection of seminal fluid biomarkers over the course of the dilution study was observed (Table 30). All protein targets were identified in the neat, 1:2, and 1:10 dilutions, with two instances of peptide dropout for proteins prostatic acid phosphatase and prostate specific antigen at the 1:100 dilution. Prostate specific antigen and epididymal secretory protein were not detected at the 1:1,000 dilution, with additional prostatic acid phosphatase dropout at the 2,000-fold dilution. Although peptide dropout was exhibited, semenogelin 1 and 2 were positively identified at the 1:5,000, 1:10,000, and 1:20,000

dilution samples. Furthermore, semenogelin 1 peptide QITIPSQEQEHSQK was accurately detected at 40,000-fold dilution extract (Figure 16).

Saliva dilution samples, as hypothesized, did not demonstrate the same degree of sensitivity as semen and peripheral blood based on the lower endogenous levels of protein quantity (Table 31). All protein markers were positively identified at the neat, 1:2, and 1:10 dilution extracts, with complete dropout of cystatin SA at 1:100. From this point, inconsistencies in biomarker detection were observed. Beginning with protein alpha amylase, peptide dropout was exhibited at the 500-fold dilution but was completely detected at the 1,000-fold dilution sample (Figure 16). Similarly, submaxillary gland androgen-regulated protein 3B demonstrated protein dropout at 1:500, with a single peptide detected at 1:1,000. The remaining samples analyzed (1:2,000; 1:5,000; 1:10,000) were negative for all target protein markers consistent with saliva.

Lastly, vaginal fluid sensitivity was evaluated (Table 32). Three instances of peptide dropout were observed at the initial 10-fold dilution analyzed: periplakin, Ly6/PLAUR, and involucrin. After this dilution, almost complete protein dropout was exhibited at 1:100, with only two peptides being accurately detected for small proline rich protein 3 and periplakin. From this point, only a single small proline rich protein 3 peptide was detected for the remainder of the samples analyzed (Figure 16). It should be restated that the dilution evaluated was initially applied to a cotton swab and subsequently extracted in 1 mL of deionized water, further diluting any protein material that was originally present. The large extraction volume was necessary to produce enough material for additional studies conducted during subsequent aim of this project, as described in section 4. This decrease in recovery contributed to poor sensitivity in comparison with the sensitivity study conducted in section 3.1.2.2. However, in common forensic biology laboratory protocols, swabs are extracted in sufficient liquid to ensure saturation, further concentrating present targets prior to analysis.

Table 30: Detection of body fluid markers in casework sensitivity samples for peripheral blood, menstrual blood, and seminal fluid. Proteins that were positively identified by all of their respective peptide markers are shown in dark green. Light green indicates where at least one peptide marker for the respective protein dropped out. White indicates complete protein dropout.

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

Table 31: Detection of body fluid markers in casework sensitivity samples for saliva. Proteins that were positively identified by all of their respective peptide markers are shown in dark green. Light green indicates where at least one peptide marker for the respective protein dropped out. White indicates complete protein dropout.

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

Table 32: Detection of body fluid markers in casework sensitivity samples for vaginal fluid. Proteins that were positively identified by all of their respective peptide markers are shown in dark green. Light green indicates where at least one peptide marker for the respective protein dropped out. White indicates complete protein dropout.

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

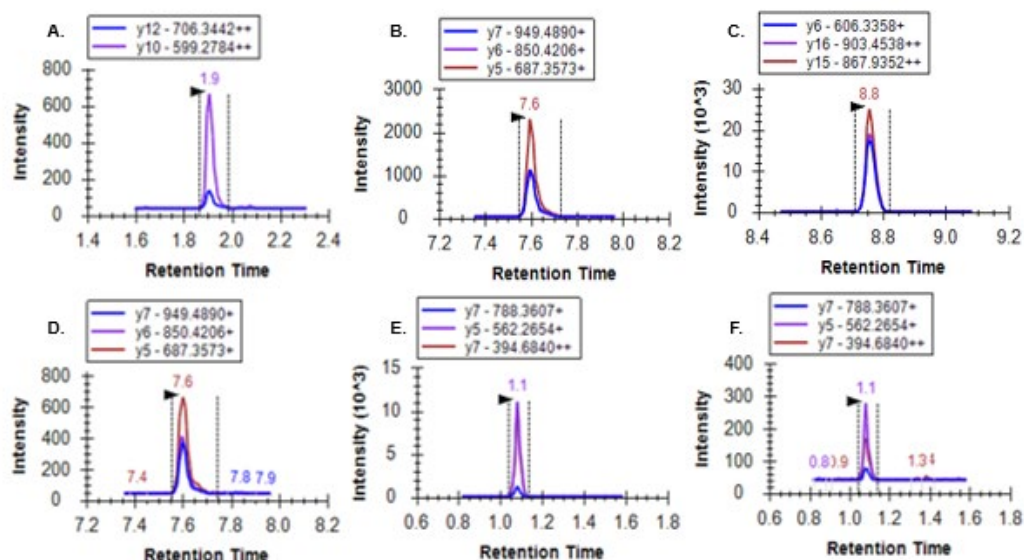


Figure 16: Peak area response of peptide biomarker at lowest detected sensitivity sample. (A) seminal fluid protein semenogelin 1 peptide QITPSQEQEHSQK at 40,000-fold dilution, (B) peripheral blood protein hemoglobin peptide LLVVPWTQR at 20,000-fold dilution, (C) saliva protein amylase peptide IAEYMNHLIDIGVAGFR at 1,000-fold dilution, (D) menstrual blood protein hemoglobin peptide LLVVPWTQR at 40,000-fold dilution, (E) menstrual blood protein small proline rich protein 3 peptide VPEPGCTK at 20,000-fold dilution, and (F) vaginal fluid protein small proline rich protein 3 peptide VPEPGCTK at 20,000-fold dilution.

4.1.9 Limit of Detection

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

A summary of results obtained are detailed in Tables 33 and 34. Overall, the LOD values for target peptide markers ranged from 1.0 to 25 femtomoles per individual peptide. Beginning with peripheral blood, the LOD for target peptide markers ranged from 2.5 fmol to 10 fmol per injection analyzed (Table 33). Hemoglobin peptides SAVTALWGK and LLVVPWTQR were found to have a higher LOD of 10 fmol due to observed peak area fluctuations of qualifier ions. Furthermore, this higher LOD is beneficial for analysis

because both hemoglobin peptides were found to have high instances of carryover between sample injections.

Seminal fluid peptide marker LOD values ranged from 1.0 fmol to 25 fmol (Table 33). Similar to hemoglobin, semenogelin 1 peptide DIFSTQDELLVYNK exhibited an LOD of 25 fmol, a higher value than those observed for remaining targets, and was found to have greater instances of carryover. On the contrary, LOD values for proteins prostatic acid phosphatase and prostate specific antigen were on the lower end of the range, with peptide FVTLVFR exhibiting an LOD of 1.0 fmol.

Derived LOD values for vaginal fluid ranged from 1.0 fmol to 25 fmol, with two instances of target isotopically-labeled peptide standard dropout (Table 34). Cornulin peptide AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR and Ly6/PLAUR peptide GLDHGLLAFIQLQCAQDR did not produce sufficient chromatographic peak shape or acceptable ion ratios at the 50 fmol fortification (Figure 17). Therefore, these peptides were not assigned a LOD and should be interpreted with caution. However, more robust protein biomarkers such as neutrophil gelatinase, exhibited acceptable LOD values at 5.0 fmol. Similar results were obtained with AQUA analysis in menstrual blood matrix, with LOD values ranging from 2.5 fmol to 25 fmol for both vaginal fluid and peripheral blood target markers (Table 3.30). In comparison with vaginal fluid matrix analysis, eight vaginal fluid targets produced comparable results in menstrual blood, including the dropped peptide biomarkers of cornulin and Ly6/PLAUR. However, five peptide targets were found to exhibit a greater LOD in menstrual blood than in vaginal fluid matrix. All peripheral blood target LOD values were comparable in menstrual blood, with the exception of hemopexin peptide NFPSPVDAAFR, exhibiting a greater LOD in peripheral blood than in menstrual blood matrix.

Isotopically-labeled peptide standards were not available for purchase at an acceptable purity for saliva proteins statherin and submaxillary gland androgen-regulated protein 3B. Peptide synthesis becomes exponentially more difficult as peptide length increases, with the peptide targets for these particular proteins outside of accurate synthesis range. Furthermore, peptides with a high content of certain amino acid residues (i.e., proline) add additional difficulties to the synthesis process. The remaining four peptide

targets (from proteins alpha amylase and cystatin SA) produced LOD values of 5 fmol or less, with LSGLLDLALGK peptide exhibiting an LOD of 2.5 fmol (Table 34).

Table 33: Limit of detection quantities of peripheral blood, saliva, and seminal fluid peptide biomarkers.

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

Table 34: Limit of detection quantities for vaginal fluid and menstrual blood peptide biomarkers. Not Detected (ND).

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

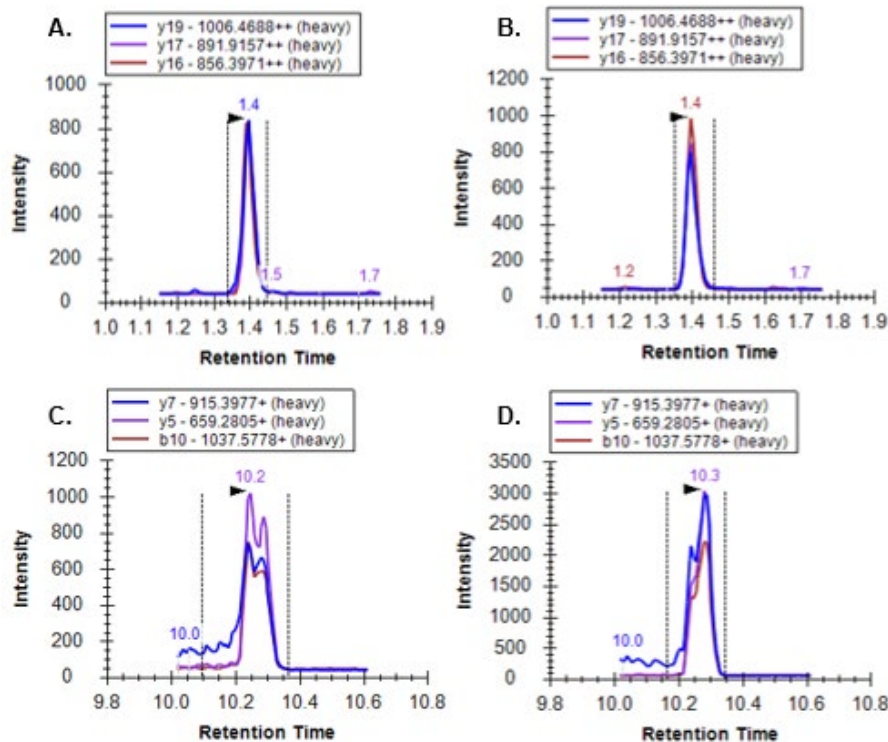


Figure 17: Peak area response of peptide biomarkers at 50 fmol limit of detection. (A) Menstrual blood matrix detection of cornulin peptide AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR, (B) vaginal fluid matrix detection of cornulin peptide AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR, (C) menstrual blood matrix detection of Ly6/PLAUR peptide GLDLHGLLAFIQLQCAQDR, and (D) vaginal fluid matrix detection of Ly6/PLAUR peptide GLDLHGLLAFIQLQCAQDR.

4.1.10 Ion Suppression

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

To assess peptide suppression or enhancement, the percent difference of peak area average between the neat standards and the AQUA fortified fluid matrix was calculated (Tables 35 and 36). Peptides that expressed a negative percent difference were marked as having ion suppression, indicating the observed peak area of the peptide in the digested

body fluid sample was less than that observed of the neat standard. Peptides that expressed a positive percent difference were interpreted as having ion enhancement, evident by a greater peak area response in the fortified digested body fluid than the neat standard. Substantial ion suppression was observed in 22% of peptide targets, with calculated percent difference greater than 30%. For example, the hemoglobin peptides SAVTALWGK and LLVVYPWTQR showed 55% and 94% suppression, respectively, when analyzed in neat peripheral blood (Figure 18). Similar suppression of hemoglobin peptides was also observed in menstrual blood. Substantial suppression of hemoglobin was expected, although not anticipated at such extreme rates, for both peripheral blood and menstrual blood matrices due to their endogenous complexity.

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

Table 35: Calculated percent difference of peripheral blood, seminal fluid, and saliva peptide biomarkers to assess ion suppression and enhancement.

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

Table 36: Calculated percent difference of vaginal fluid and menstrual blood peptide biomarkers to assess ion suppression and enhancement.

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

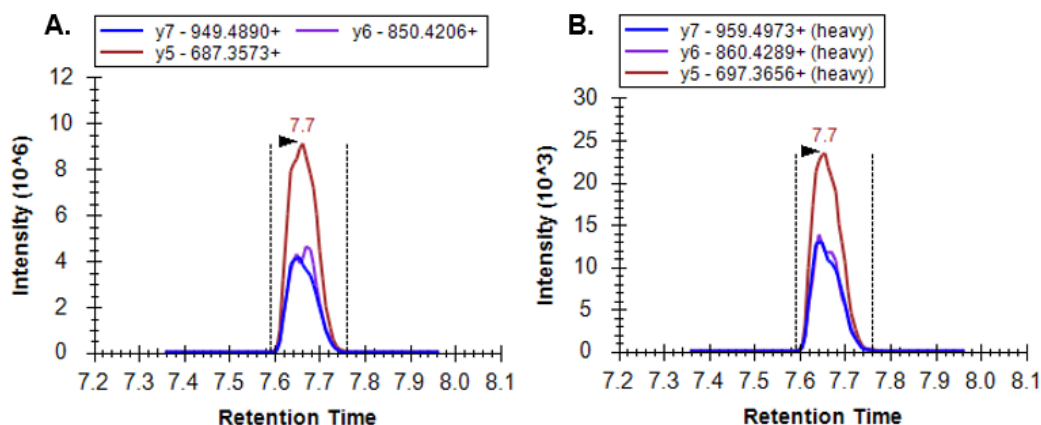


Figure 18: Peak area response of (A) endogenous hemoglobin peptide LLVYPWTQR and (B) AQUA hemoglobin peptide LLVYPWTQR.

4.1.11 Carryover Study

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

Preliminary carryover samples included 10 µg, 20 µg, and 30 µg of prepared material analyzed on column followed by a blank injection. Carryover in the subsequent blank was observed for two protein biomarkers. First, hemoglobin in peripheral blood and menstrual blood matrices was detected in blanks after all three loading amounts evaluated. Peptide LLVYPWTQR produced linear peak response in blank injections, with linearity of sample injections decreasing as the loading amount increased, indicating saturation of the mass spectrometer detector. Peptide SAVTALWGK was detected in blank injections after 20 µg and 30 µg sample amounts. Second, semenogelin 1 exhibited high levels of carryover in all subsequent blank injections (Figure 19). Peptide DIFSTQDELLVYNK peak area responses produced linear results in blank injection analysis, with peak areas greater than 7,400,000 in sample injections at 10 µg loading amounts. Similar to hemoglobin peptide LLVYPWTQR, semenogelin 1 peptide DIFSTQDELLVYNK is relatively hydrophobic, eluting toward the end of the analytical run time. With reverse-phase liquid

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

Because hemoglobin and semenogelin 1 carryover was observed at substantial peak area responses, samples were reinjected using the same loading amounts previously described followed by two blank injections. This was performed to evaluate the retention of the hydrophobic peptides. As expected, both hemoglobin and semenogelin 1 were detected in the second blank injection analyzed after a 10 µg injection (Figure 20).

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

Lower sample injection amounts were found to limit instances of carryover while maintaining accurate peak area responses for fluid identification. However, hemoglobin and semenogelin 1 carryover persisted, albeit at low-level abundance (Figure 21). Although semenogelin 1 exhibited carryover at the 2.5 µg injection amount, observed peak areas were sufficiently less than those observed from hemoglobin carryover at all loading amounts evaluated. Due to these effects, hemoglobin and semenogelin 1 targets must be called at a greater intensity, regardless of ion ratio and chromatographic acceptance at lower peak areas. Therefore, a 2.5 µg loading amount was selected as the optimal amount of protein on column during analysis. Although 2.5 µg of material still produced sufficient response for less abundant targets, specifically target vaginal fluid biomarkers, two instances of protein dropout, Ly6/PLAUR and epididymal secretory protein E1, were

observed at the 2.5 μ g injection. A complete overview of observed carryover is outlined in Table 37. The presence of carryover was addressed in drafted interpretation guidelines, with strict detail given on the acceptance of semenogelin 1 and hemoglobin peaks above a designated value in order to prevent the false identification of carryover peaks.

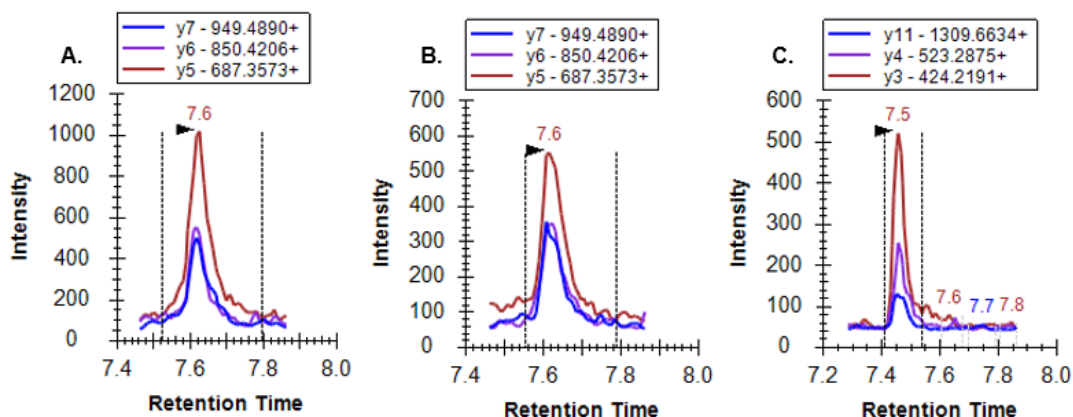


Figure 19: Peak area response of (A) hemoglobin peptide LLVYPWTQR in peripheral blood, (B) hemoglobin peptide LLVYPWTQR in menstrual blood, and (C) semenogelin 1 peptide DIFSTQDELLVYNK in seminal fluid in blank injections directly following a 10 μ g sample injection.

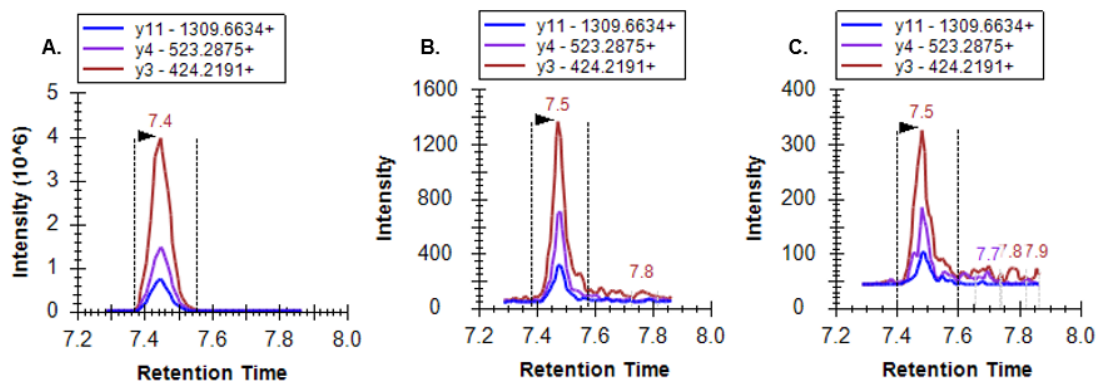


Figure 20: Peak area response of semenogelin 1 peptide DIFSTQDELLVYNK in (A) 10 μ g sample injection, (B) first subsequent blank injection, and (C) second subsequent blank injection.

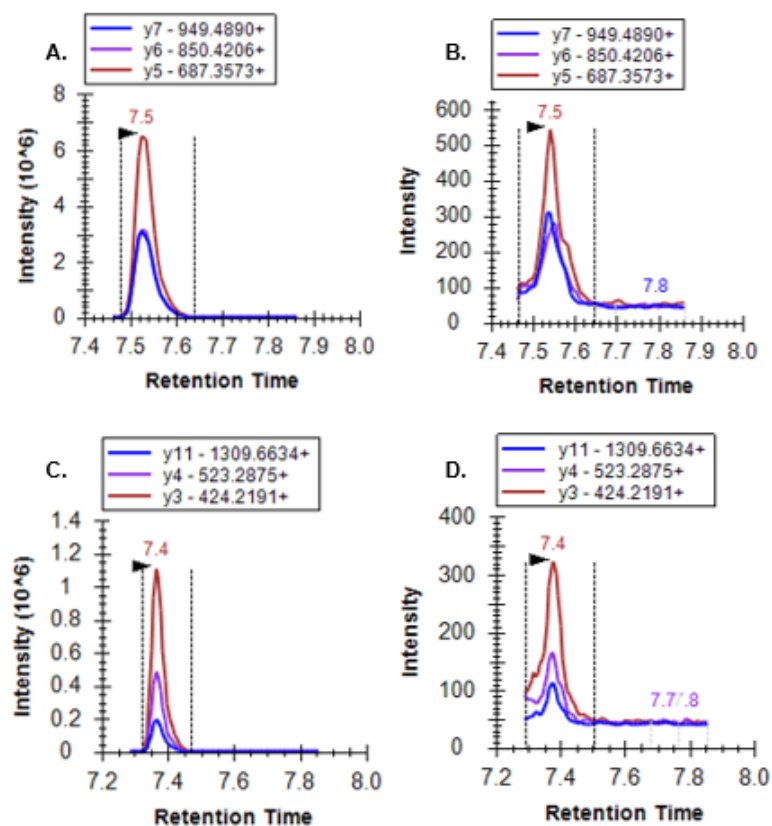


Figure 21: Peak area response of (A) hemoglobin peptide LLVVYPWTQR in 0.5 μ g peripheral blood, (B) hemoglobin peptide LLVVYPWTQR in subsequent blank injection, (C) semenogelin 1 peptide DIFSTQDELLVYNK in 2.5 μ g seminal fluid, and (D) semenogelin 1 peptide DIFSTQDELLVYNK in subsequent blank injection.

Table 37: Detection of body fluid markers during carryover assessment. Proteins that were positively identified by all of their respective peptide markers are shown in green. White indicates complete protein dropout.

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

4.1.12 Blind Sample Analysis

A series of 50 samples containing fiber-tipped swabs, swatches of cloth, and condoms were received from the grant agency for blind analysis. Utilizing the described LC-MS/MS method, body fluid identification assignments were made. In total, 41 of 50 blind samples (82%) analyzed were correctly identified when compared with true sample contents. The remaining 9 samples (18%) were misidentified, with 3 false positive

identifications and 5 false negative (i.e., non-detections observed) results recorded. A comprehensive summation of experimental identifications, true identifications, and sample preparation information is outlined in Appendix H. For example, a cotton swab sample (Sample #15) was identified as containing biomarkers consistent with seminal fluid and vaginal fluid; however, biomarkers consistent with saliva were not detected (Figure 22). This sample was characterized as a false negative, having contained a 3-part mixture (10 μ L semen and 1 μ L of saliva on a vaginal swab). On the contrary, a cotton swab (Sample #22) was misidentified as containing biomarkers consistent with vaginal fluid and characterized as a false positive identification (Figure 23).

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

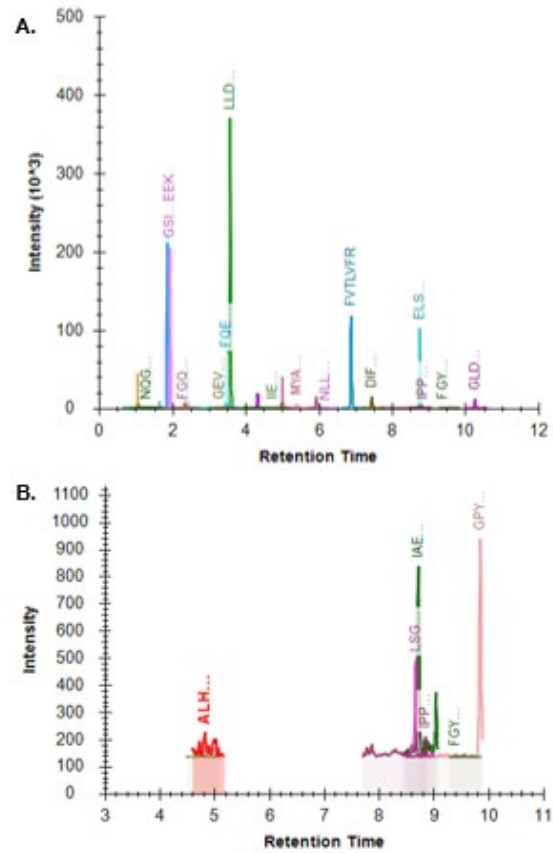


Figure 22: Peptide biomarker profile of Blind Sample #15. (A) Biomarkers consistent with seminal fluid and vaginal fluid and (B) lack of biomarkers consistent with saliva, prompting a false negative identification.

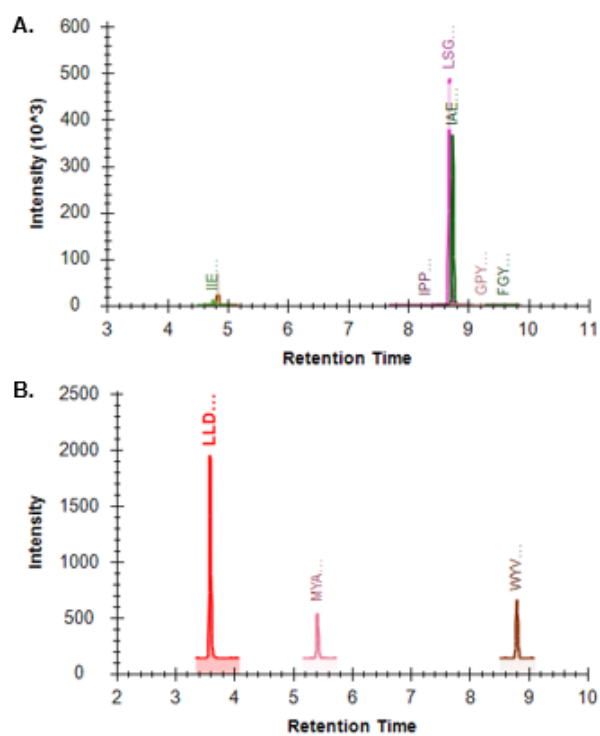


Figure 23: Peptide biomarker profile of Blind Sample #22. (A) Biomarkers consistent with saliva and (B) biomarkers consistent with vaginal fluid, prompting a false positive identification.

4.2 Comparison of a Validated LC-QQQ to Existing Forensic Casework Models

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

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

With the lack of advancements achieved within serological screening, a shift in existing casework models has been observed within the forensic biology community. Traditionally, evidence received would undergo serological screening, with results dictating the prioritization of samples for subsequent genetic testing. However, with the lack of reliable body fluid tests, serological screening as a gatekeeper for DNA analysis has been reconsidered. More recently, laboratories have opted for a “direct to DNA” approach, with small portions of evidence quickly extracted and quantitated for genetic material. The resulting quantity and quality of DNA determines the type of genetic testing

performed, with serological screening only conducted if needed. Although a practical alternative to the traditional workflow, there has been little research on the efficacy of a direct to DNA procedure, particularly with challenging sample types. By evaluating the use of the proteomic workflow within existing casework models, the use of serological screening as a gatekeeper for genetic testing has the capability of providing copious amounts of information vital to criminal investigations that would have otherwise been absent, in addition to reducing costs associated with advanced genetic analyses.

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

4.2.1 Substrate Samples

Substrate samples were designed to ensure chemical products or composition would not interfere with protein biomarker detection or recovery. Overall, the proteomic assay was successful in identifying the target body fluid of each substrate sample (Table 38), with three instances of vaginal fluid protein dropout observed for menstrual blood samples. Matched samples were compared with serological screening tests (Table 38). With the exception of two peripheral blood samples, all substrate samples were positive on traditional immunological and enzymatic based assays. Peripheral blood applied to carpet (sample ID SUB03) and to leather (sample ID SUB04) produced a negative result when evaluated with RSID™ Blood, with carpet exhibited in Figure 24A. In addition, when compared with a positive control test, a majority of peripheral blood and menstrual blood containing samples resulted in weak or very weak positive results. For example, menstrual blood on cotton resulted in a weak positive result on RSID™ Blood in comparison with ABACard® HemaTrace (Figure 24B). However, the proteomic assay was able to positively identify blood-containing samples by all targeted peptide biomarkers. Similarly, target proteins for semen and saliva samples were positively characterized by the proteomic assay, in addition to positive identification using traditional screening tests, with examples

highlighted in Figure 24C and Figure 24D, respectively. DNA analysis was not performed on this set of casework samples.

With the exception of the aforementioned negative peripheral blood samples on the single manufacturer test, the traditional assays and proteomic assay were comparable in identification of target body fluid. However, given the inherent nature of the LC-MS/MS technology, the proteomic assay fundamentally provided precise protein identification and additional information not capable of the lateral flow assays.

Table 38: Summary of comparative results for substrate samples. Positive identifications are represented by a green box. Negative identifications are represented by a red box. White boxes represent no testing was performed with that specific assay. Vaginal fluid samples were removed from the table as no additional testing or comparison was conducted.

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

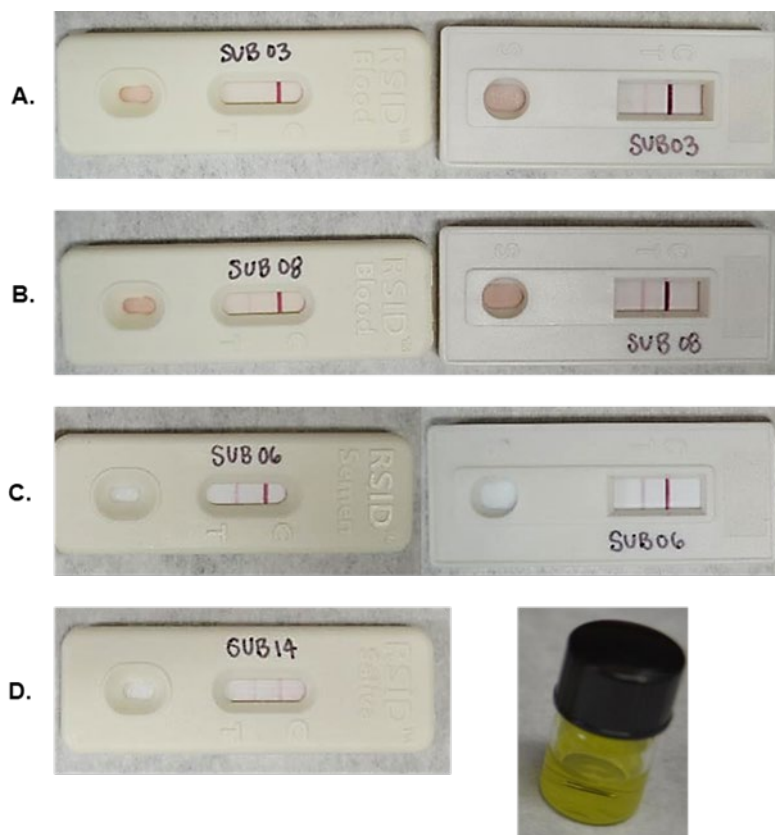


Figure 24: Selected serological screening test results on substrate samples. RSID™ tests are pictured on the left. ABAcard® tests and SALigAE® are pictured on the right. Within the lateral flow test membrane window, the test line is positioned on the left and the control line on the right. Results for (A) peripheral blood on carpet, (B) menstrual blood on cotton, (C) semen on cotton, and (D) saliva on plastic bottle. On lateral flow assays, a positive result is indicated by the presence of a red line at both the test and control zones. For SALigAE®, a positive result is indicated by a yellow colored reagent.

4.2.2 Environmental Contaminants

This subset of samples was prepared to assess the effects of environmental contaminants on sample preparation chemistries of the developed proteomic workflow. When evaluated with the proteomic assay, three samples exhibited protein and peptide dropout (Table 39). Menstrual blood samples (sample ID CON04 and CON05) illustrated vaginal fluid protein and peptide dropout. The third sample, vaginal fluid and water-based lubricant, exhibited complete protein dropout; however, this sample was unable to be directly compared with other serological screening tests as there are none commercially available. As observed with substrate samples, the traditional and proteomic assays produced comparable results in regard to target fluid identification. A summary of serological screening results is outlined in Table 39. Overall, samples subjected to chemical insult did not inhibit the ability to produce a positive result with traditional

serological assays. A very weak positive was observed for peripheral blood and 10% bleach (Figure 25A), which contradicts the positive protein identifications observed during proteomic analysis. It was originally hypothesized that the presence of personal lubricant would decrease advantageous protein binding events and result in an increase in negative reporting. However, immunological assays accurately characterized semen and menstrual blood samples containing personal lubricant (Figure 25B and 25C). There was no inhibition of salivary amylase, with both saliva case samples characterized as exhibited by saliva extracted from a cigarette filter (Figure 25D). DNA analysis was not performed on this set of casework samples. In summary, comparable results between the proteomic method and traditional methodologies were observed; however, the proteomic method provides a greater depth of coverage in specific biomarker identifications.

Table 39: Summary of comparative results for environmental contaminant samples. Positive identifications are represented by a green box. White boxes represent no testing was performed with that specific assay. Vaginal fluid samples were removed from the table as no additional testing or comparison was conducted.

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

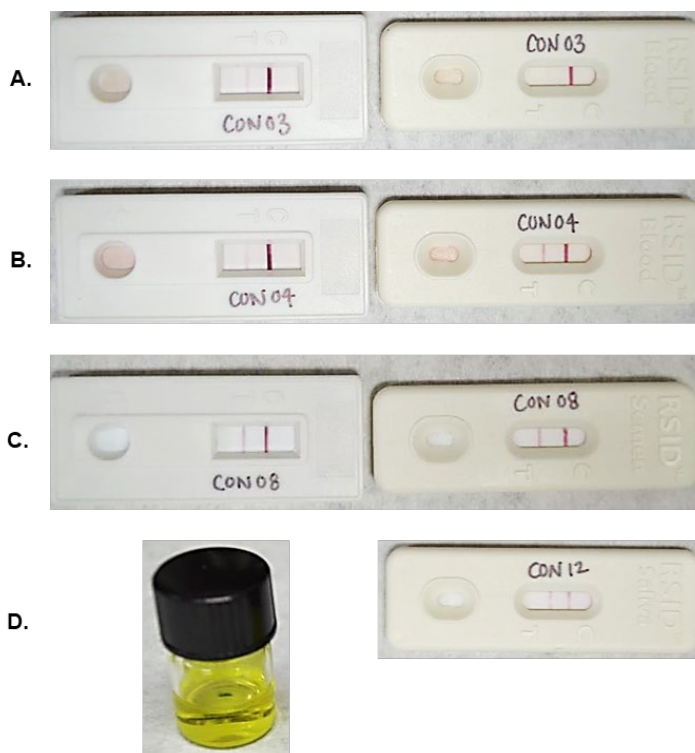


Figure 25: Selected serological screening test results for environmental contaminant samples. ABAcard® test and SALigAE® are pictured on the left. RSID™ test is pictured on the right. Within the membrane window, the test line is positioned on the left and the control line on the right. Results for (A) peripheral blood and 10% bleach, (B) menstrual blood and personal lubricant, (C) semen and personal lubricant, and (D) saliva on a cigarette. On lateral flow assays, a positive result is indicated by the presence of a red line at both the test and control zones. For SALigAE®, a positive result is indicated by a yellow colored reagent.

4.2.3 Mixture Samples

For this portion of the study, mixture samples evaluated are separate from those used during validation to determine transition interference. Casework-type mixture samples were designed to assess the capability of each testing strategy to detect a minor contributor when in the matrix of a major contributor, simulating commonly received sample types in a forensic biology laboratory. Of the five mixture samples assessed, the minor contributor was characterized when evaluated with the proteomic assay. When directly comparing a matched sample using traditional techniques, two instances of negative detection were observed (Table 40). With the detection of a minor saliva contributor, the enzymatic test SALigAE® was unable to characterize the presence of the target fluid in either a vaginal fluid or menstrual blood matrix; however, RSID™ Saliva exhibited a positive result for both MIX01 and MIX04 samples (Figure 26). It should be

noted that the inherent color of the MIX04 extract caused the SALIgAE® buffer to become discolored, potentially masking the visualization of a positive result. There were no reported discrepancies for the detection of seminal fluid with either antibody-based assay (Figure 27). Due to the lack of commercially available serological tests for vaginal fluid, a matched sample for MIX05, vaginal fluid in a urine major contributor, was not evaluated using antibody-based or enzymatic tests.

Allele calls produced by mixtures samples for short tandem repeat (STR) and Y chromosome STR (Y-STR) analyses are outlined in Tables 41 and 42, respectively, with alleles unique to the major contributor removed for ease of comparison. The allele calls presented are unique to the minor contributor or shared with the major contributor. For sample MIX01, a minor saliva contributor with a major vaginal fluid contributor, 2 out of 4 salivary protein biomarkers were detected with the proteomic assay; however, the DNA profile generated was consistent with the vaginal fluid major contributor. The DNA profile produced was single source, and even with allele sharing between the two contributors, the profile did not indicate instances of peak imbalance or YINDEL/DYS391 detection (Table 41). For samples MIX02 and MIX03, a minor semen contributor in a major vaginal fluid and menstrual blood contributor, respectively, were extracted using an organic differential protocol. The sperm fractions were analyzed for Y-STR's and produced full haplotypes (Table 42). Furthermore, all 5 target seminal fluid biomarkers were detected with the proteomic assay for these samples. For sample MIX04, a minor saliva contributor in a major menstrual blood contributor, no unique alleles consistent with the minor contributor were detected. And lastly, MIX05, a minor vaginal fluid contributor in a major urine contributor, produced a complete profile for the minor contributor, with 2 out of 7 vaginal fluid markers detected with the proteomic assay.

Table 40: Summary of comparative results for mixture samples. Positive identifications are represented by a green box. Negative results are represented by a red box. White boxes indicate no testing with that specific assay.

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

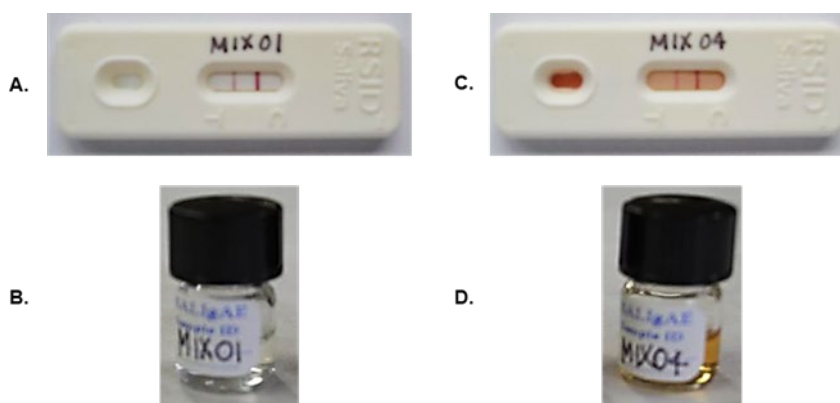


Figure 26: Serological screening test results for mixture samples containing saliva as a minor contributor. Within the membrane window, the test line is positioned on the left and the control line on the right. Results for saliva in a vaginal fluid major contributor using (A) RSID™ Saliva and (B) SALigAE®. Results for saliva in a menstrual blood major contributor using (C) RSID™ Saliva and (D) SALigAE®. On lateral flow assays, a positive result is indicated by the presence of a red line at both the test and control zones. For SALigAE®, a positive result is indicated by a yellow colored reagent.

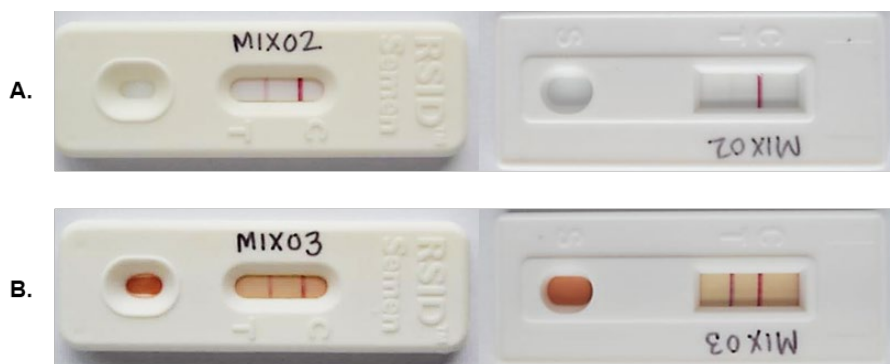


Figure 27: Serological screening test results for mixture samples containing semen as a minor contributor. RSID™ Semen test is pictured on the left. ABACard® p30 test is pictured on the right. Within the membrane window, the test line is positioned on the left and the control line on the right. Results for (A) semen in a vaginal fluid major contributor and (B) semen in a menstrual blood major contributor. A positive result is indicated by the presence of a red line at both the test and control zones.

Table 41: STR genetic results for mixture samples. Unique allele ratio for the minor contributor was calculated using reference profiles. For clarity, unique major contributor alleles have been removed from case samples. Alleles depicted represent shared and/or unique minor contributor alleles.

Mixtures		Semen, Saliva, Urine Reference	Vaginal Fluid Reference	Menstrual Blood Reference	MIX01 Saliva in Vaginal Fluid	MIX02 Semen in Vaginal Fluid	MIX03 Semen in Menstrual Blood	MIX04 Saliva in Menstrual Blood	MIX05 Vaginal Fluid in Urine
BLUE	D3S1358	15,17	16,17	16, 18	17	17	-	-	16
	VWA	17,18	15,18	16	18	18	-	-	15
	D16S539	11,12	9,12	13, 14	12	12	-	-	9
	CSF1PO	10,11	11,14	12, 13	11	11	-	-	14
	TPOX	8,11	8,11	9, 12	8,11	8,11	-	-	-
GREEN	YINDEL	2	-	-	-	-	2	-	-
	Amelogenin	XY	X	X	X	X	X	X	X
	D8S1179	10,13	12,13	13, 14	13	13	13	13	12
	D21S11	31.2,32.2	27,30	27, 30	-	-	-	-	27,30
	D18S51	11,15	10,14	12, 13	-	-	-	-	10,14
YELLOW	DYS391	12	-	-	-	-	-	-	-
	D2S441	11,14	11,12	10, 11	11	11	11	11	12
	D19S433	13,14	14,15	13	14	13,14	13	13	15
	THO1	6,7	9,9.3	6, 9.3	-	-	6	6	9,9.3
	FGA	20,24	20	20, 24	20	20	20,24	20,24	-
RED	D22S1045	15,16	11,15	15, 16	15	15	15,16	15,16	11
	D53818	11,12	11,12	12, 13	11,12	11,12	12	12	-
	D13S317	10,12	11,12	10, 11	12	12	10	10	11
	D73280	10,12	10,12	10, 11	10,12	10,12	10	10	-
	SE33	21,28.2	17,26.2	28.2*, 29.2	-	-	-	-	17,26.2
PURPLE	D10S1248	14,15	14,15	13, 15	14,15	14,15	15	15	-
	D1S1656	15	14,17	15, 16.3	-	-	15	15	14,17
	D12S391	15,16	18,20	15, 22	-	-	15	15	18,20
	D2S1338	17,24	17,19	19, 24	17	17	24	24	19
Minor Contributor Unique Allele Ratio		-	-	-	0/24	1/24	1/27	0/27	22/22

Table 42: Y-STR genetic results for mixture samples with semen as a minor contributor. Y-STR analysis was performed based on quantification values.

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

4.2.4 Sexual Assault Samples

4.2.4.1 Simulated Sexual Assault Samples

Simulated sexual assault samples were designed to imitate the environment of the vaginal cavity and its effects on the breakdown of semen. The ability to identify constituents of seminal fluid post-coitus, particularly after 48 hours, is advantageous to forensic practitioners. The poor sensitivity of currently available immunological screening assays has been reported in the literature for the detection of seminal fluid in an extended post-coital interval [50,162]. A sufficient difference in sensitivity between manufacturers was observed for this subset of samples (Table 43). Consistent with proteomic identification, RSID™ Semen positively characterized seminal fluid for all time points evaluated. However, ABACard® p30 failed to detect seminal fluid via prostate specific antigen after time point zero (Figure 28).

In total, samples from all time points assessed produced at least 20 of 24 (83%) unique male alleles, with four samples generating 100% unique male alleles (Table 44). Time points at days three, five, seven, and nine report complete detection of the 24 unique alleles from the male contributor, with day zero returning the smallest number of unique alleles, with a minimum of 20 alleles detected from the male contributor.

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

Table 43: Summary of comparative results for simulated sexual assault samples. Positive identifications are represented by a green box. Negative results are represented by a white box.

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

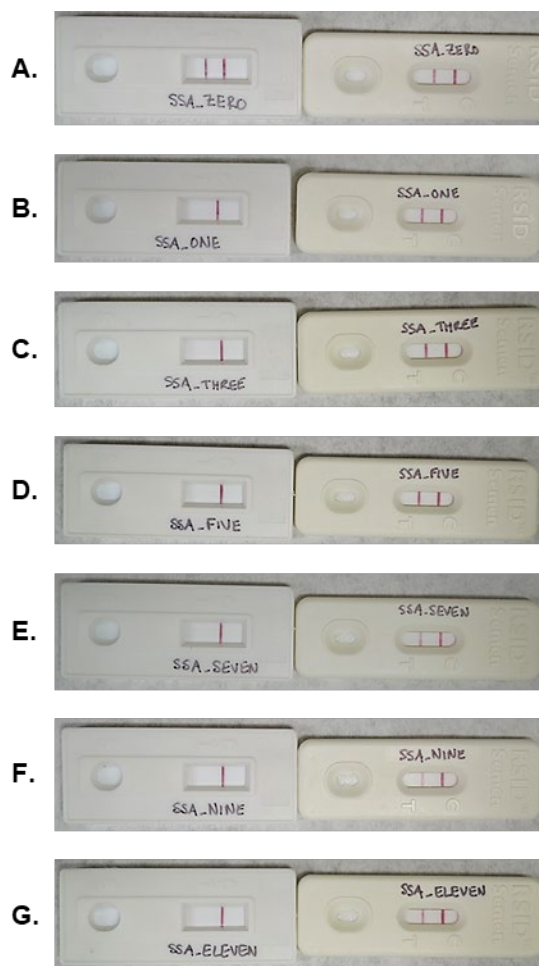


Figure 28: Immunochromatographic results for simulated sexual assault samples. ABAcard® p30 test is pictured on the left. RSID™ Semen test is pictured on the right. Within the membrane window, the test line is positioned on the left and the control line on the right. Results for (A) timepoint zero, (B) day one, (C) day three, (D) day five, (E) day seven, (F) day nine, and (G) day eleven. A positive result is indicated by the presence of a red line at both the test and control zones.

Table 44: STR genetic results for simulated sexual assault samples. The male reference sample depicts unique male alleles in bold.

		Male Reference	Female Reference	Day Zero	Day One	Day Three	Day Five	Day Seven	Day Nine	Day Eleven
BLUE	D3S1358	15,17	16,17	15,17	15,17	15,17	15,17	15,17	15,17	15,17
	VWA	17,18	15,18	17,18	17,18	17,18	17,18	17,18	17,18	17,18
	D16S539	11,12	9,12	11,12	12	11,12	11,12	11,12	11,12	11,12
	CSF1PO	10,11	11,14	10,11	10,11	10,11	10,11	10,11	10,11	10,11
	TPOX	8,11	8,11	8,11	8,11	8,11	8,11	8,11	8,11	8,11
GREEN	YINDEL	2	-	2	2	2	2	2	2	2
	Amelogenin	XY	X	X	XY	XY	XY	XY	XY	XY
	D8S1179	10,13	12,13	10,13	10,13	10,13	10,13	10,13	10,13	10,13
	D21S11	31,2,32,2	27,30	31,2,32,2	31,2,32,2	31,2,32,3	31,2,32,2	31,2,32,3	31,2,32,2	31,2,32,2
	D18S51	11,15	10,14	11,15	11,15	11,15	11,15	11,15	11,15	11,15
YELLOW	DYS391	12	-	-	12	12	12	12	12	12
	D2S441	11,14	11,12	11	11,14	11,14	11,14	11,14	11,14	11,14
	D19S433	13,14	14,15	13,14	13,14	13,14	13,14	13,14	13,14	13,14
	THO1	6,7	9,9,3	7	6,7	6,7	6,7	6,7	6,7	6,7
	FGA	20,24	20	20	20,24	20,24	20,24	20,24	20,24	20
RED	D22S1045	15,16	11,15	15,16	15,16	15,16	15,16	15,16	15,16	15,16
	D5S3818	11,12	11,12	11,12	11,12	11,12	11,12	11,12	11,12	11,12
	D13S317	10,12	11,12	10,12	10,12	10,12	10,12	10,12	10,12	10,12
	D7S830	10,12	10,12	10,12	10,12	10,12	10,12	10,12	10,12	10,12
	SE33	21,28,2	17,26,2	21,28,2	21,28,2	21,28,2	21,28,2	21,28,2	21,28,2	21,28,2
PURPLE	D10S1248	14,15	14,15	14,15	14,15	14,15	14,15	14,15	14,15	14,15
	D1S1656	15	14,17	15	15	15	15	15	15	-
	D12S391	15,16	18,20	15,16	15,16	15,16	15,16	15,16	15,16	15,16
	D2S1338	17,24	17,19	17,24	17,24	17,24	17,24	17,24	17,24	17,24
	Unique Male Allele Ratio	-	-	20/24	23/24	24/24	24/24	24/24	24/24	22/24

4.2.4.2 Mock Sexual Assault Samples

Both sets of laboratory-prepared sexual assault samples, one set containing semen from a non-vasectomized donor and the second set containing semen from a vasectomized donor, were reserved for analysis during this portion of the study (Table 45). When screening for seminal fluid using the proteomic assay, at least 3 of 5 target seminal fluid protein markers were detected across all swabs. Immunological lateral flow assays produced comparable results among the two sample sets evaluated. A positive result was observed for all vaginal swabs on RSID™ Semen and ABACard® p30, with oral swabs producing a positive result on ABACard® p30 only (Figure 29). However, all rectal swab samples exhibited a negative result when evaluated with a lateral flow assay.

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

samples containing semen from a vasectomized individual (Table 45, Appendix J). Nonetheless, mass spectrometry results show at least 3 seminal fluid biomarkers detected in each sample type of this group. Complete and partial DNA profiles were observed for samples containing semen from a non-vasectomized individual (Table 46). The variation in profile generated can be attributed to each sample having been prepared individually (i.e., not prep replicates), in order to best simulate sample-to-sample variability. For example, case sample SA01_02.1, an oral swab, generated 9 out of 24 unique male alleles; however, the second oral swab produced a full STR profile.

Table 45: Summary of comparative results for mock sexual assault samples containing semen from a non-vasectomized and a vasectomized donor. Positive identifications are represented by a green box. Negative results are represented by a white box.

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

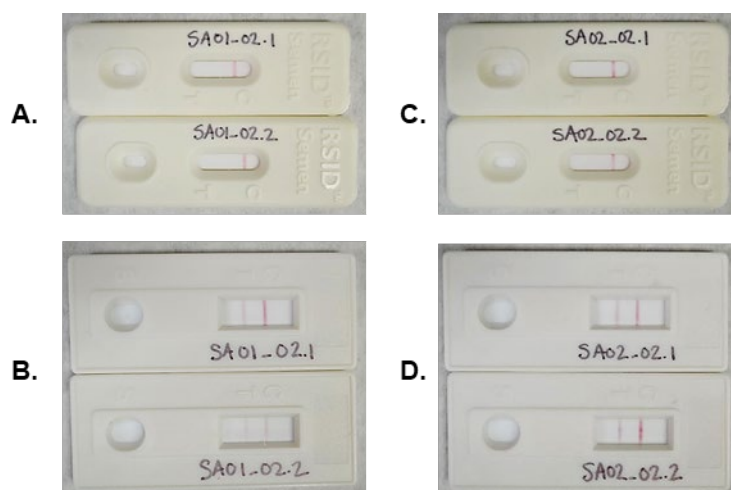


Figure 29: Immunochromatographic results for mock sexual assault samples. Within the membrane window, the test line is positioned on the left and the control line on the right. Results for (A) oral swab replicates using RSID™ Semen, (B) oral swab replicates using ABACard® p30, (C) oral swab replicates containing semen from a vasectomized donor using RSID™ Semen, and (D) oral swab replicates containing semen from a vasectomized donor using ABACard® p30. A positive result is indicated by the presence of a red line at both the test and control zones.

Table 46: STR genetic results for simulated sexual assault samples. The male reference sample depicts unique male alleles in bold.

		Male Reference	Female Reference	SA01_01.1	SA01_01.2	SA01_02.1	SA01_02.2	SA01_03.1	SA01_03.2
				Vaginal Swab	Vaginal Swab	Oral Swab	Oral Swab	Rectal Swab	Rectal Swab
BLUE	D3S1358	15,17	16,17	15,17	17	17	15,17	17	15,17
	VWA	17,18	15,18	17,18	-	-	17,18	17,18	17,18
	D16S539	11,12	9,12	11,12	11	11	11,12	11,12	11,12
	CSF1PO	10,11	11,14	10,11	-	-	10,11	10,11	10,11
	TPOX	8,11	8,11	8,11	-	8	8	8,11	8,11
GREEN	YINDEL	2	-	2	-	2	2	2	2
	Amelogenin	XY	X	XY	X	XY	XY	XY	XY
	D8S1179	10,13	12,13	10,13	10,13	13	10,13	10,13	10,13
	D21S11	31,2,32,2	27,30	31,2,32,2	-	-	31,2,32,2	31,2,32,2	31,2,32,2
	D18S51	11,15	10,14	11,15	-	11	11,15	11,15	11,15
YELLOW	DYS391	12	-	12	-	12	12	-	12
	D2S441	11,14	11,12	11,14	11	11	11,14	11,14	11,14
	D19S433	13,14	14,15	13,14	-	13,14	13,14	13,14	13,14
	TH01	6,7	9,9,3	6,7	-	6	6,7	6,7	6,7
	FGA	20,24	20	20,24	-	20	20,24	20,24	20,24
RED	D22S1045	15,16	11,15	15,16	-	-	15,16	15,16	15,16
	D5S818	11,12	11,12	11,12	11	12	11,12	11,12	11,12
	D13S317	10,12	11,12	10,12	-	-	10,12	10,12	10,12
	D7S830	10,12	10,12	10,12	-	12	10,12	10,12	10,12
	SE33	21,28,2	17,26,2	21,28,2	-	-	21,28,2	21,28,2	21,28,2
PURPLE	D10S1248	14,15	14,15	14,15	14,15	14	14,15	14,15	14,15
	D1S1656	15	14,17	15	15	15	15	15	15
	D12S391	15,16	18,20	15,16	15	16	15,16	15,16	15,16
	D2S1338	17,24	17,19	17,24	17	17,24	17,24	17,24	17,24
Unique Male Allele Ratio		-	-	24/24	4/24	9/24	24/24	22/24	24/24

4.2.4.3 Digital Swab Samples

Self-collected digital swab samples were obtained to mimic casework type samples as closely as possible. However, it should be noted that because the samples were self-collected, the likelihood of a mixed genetic profile was reduced as the body fluid and digit were of the same individual. Overall, serological tests produced comparable results with the proteomic assay and full genetic profiles were obtained from all samples (Table 47). Both oral cavity replicates produced a positive result from RSID™ Saliva and SALIgAE® tests (Figure 30A). When assessed for the presence of blood, menstrual blood was positively characterized by RSID™ Blood, with a negative result produced using ABACard® HemaTrace for a single replicate (Figure 30B). A complete DNA profile consistent with the female contributor was produced by all digital swab samples (Table 48).

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

Table 47: Summary of comparative results for digital swab samples. Positive identifications are represented by a green box. Negative results are represented by a red box. White boxes indicate no testing was performed.

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



Figure 30: Selected serological screening test results for digital swab samples. ABACard® test and SALigAE® are pictured on the left. RSID™ test is pictured on the right. Within the membrane window, the test line is positioned on the left and the control line on the right. Results for (A) digital swab saliva and (B) digital swab menstrual blood. On lateral flow assays, a positive result is indicated by the presence of a red line at both the test and control zones. For SALigAE®, a positive result is indicated by a yellow colored reagent.

Table 48: STR genetic results for digital swab samples.

		Female Reference	DS01.1	DS01.2	DS02.1	DS02.2	DS03.1	DS03.2
			Oral Penetration	Oral Penetration	Vaginal Penetration	Vaginal Penetration	Vaginal Penetration (Menstrual)	Vaginal Penetration (Menstrual)
BLUE	D3S1358	16,17	16,17	16,17	16,17	16,17	16,17	16,17
	VWA	15,18	15,18	15,18	15,18	15,18	15,18	15,18
	D16S539	9,12	9,12	9,12	9,12	9,12	9,12	9,12
	CSF1PO	11,14	11,14	11,14	11,14	11,14	11,14	11,14
	TPOX	8,11	8,11	8,11	8,11	8,11	8,11	8,11
GREEN	YINDEL	-	-	-	-	-	-	-
	Amelogenin	X	X	X	X	X	X	X
	D8S1179	12,13	12,13	12,13	12,13	12,13	12,13	12,13
	D21S11	27,30	27,30	27,30	27,30	27,30	27,30	27,30
	D18S51	10,14	10,14	10,14	10,14	10,14	10,14	10,14
YELLOW	DYS391	-	-	-	-	-	-	-
	D2S441	11,12	11,12	11,12	11,12	11,12	11,12	11,12
	D19S433	14,15	14,15	14,15	14,15	14,15	14,15	14,15
	THO1	9,9.3	9,9.3	9,9.3	9,9.3	9,9.3	9,9.3	9,9.3
RED	FGA	20	20	20	20	20	20	20
	D22S1045	11,15	11,15	11,15	11,15	11,15	11,15	11,15
	D5S3818	11,12	11,12	11,12	11,12	11,12	11,12	11,12
	D13S317	11,12	11,12	11,12	11,12	11,12	11,12	11,12
	D7S830	10,12	10,12	10,12	10,12	10,12	10,12	10,12
PURPLE	SE33	17,26.2	17,26.2	17,26.2	17,26.2	17,26.2	17,26.2	17,26.2
	D10S1248	14,15	14,15	14,15	14,15	14,15	14,15	14,15
	D1S1656	14,17	14,17	14,17	14,17	14,17	14,17	14,17
	D12S391	18,20	18,20	18,20	18,20	18,20	18,20	18,20
	D2S1338	17,19	17,19	17,19	17,19	17,19	17,19	17,19

4.2.5 Degradation Samples

Laundered substrates, composed of a set of substrates containing semen and a set of clothing containing peripheral blood, were carried over to this section of research. As noted in section 3.1.6.5, all samples and control regions were prepared, laundered, dried, and analyzed. Overall, the expected results were observed for the proteomic assay and

genetic analyses (Table 49). All samples produced a full genetic profile (Table 50). For the set of samples containing semen, the proteomic assay was capable of detecting protein signatures on both the bed sheet and underwear, with only a single protein biomarker (epididymal secretory protein) not detected on the bed sheet (Table 49). Conversely, immunological lateral flow assays failed to detect trace levels of seminal fluid (Figure 31A and Figure 31B). Both control regions exhibited negative results for both the proteomic assay and immunochromatographic assays. The second set of degraded samples, a cloth bath towel and a pair of denim jeans, targeted peripheral blood identification. Both samples produced comparable results. All protein biomarkers were detected with the proteomic assay and weak positive results were observed with lateral flow assays (Table 49, Figure 31C, Figure 31D). Interestingly, the extracted control samples from both the towel and the jeans produced a positive and weak positive result on RSID™ Blood, respectively. Using the LC-MS/MS method as a confirmation, these immunological assay results were not able to be verified, as this approach produced no identification of peripheral blood biomarkers. Furthermore, no genetic profile was produced from these control samples, even with manual analysis of electropherograms identifying no true alleles below the established analytical threshold (Appendix J).

Table 49: Summary of comparative results for degraded samples. Positive identifications are represented by a green box. Negative results are represented by a white box.

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

Table 50: STR genetic results for degraded samples.

		Semen on Bed Sheet	Semen on Underwear	Peripheral Blood on Towel	Peripheral Blood on Jeans
BLUE	D3S1358	15,17	15,17	15,17	15,17
	VWA	17,18	17,18	16,17	16,17
	D16S539	11,12	11,12	11,13	11,13
	CSF1PO	10,11	10,11	9,11	9,11
	TPOX	8,11	8,11	8,9	8,9
GREEN	YINDEL	2	2	2	2
	Amelogenin	XY	XY	XY	XY
	D8S1179	10,13	10,13	13	13
	D21S11	31.2,32.2	31.2,32.2	28,30	28,30
	D18S51	11,15	11,15	17	17
YELLOW	DYS391	12	12	11	11
	D2S441	11,14	11,14	11,14	11,14
	D19S433	13,14	13,14	14,15.2	14,15.2
	THO1	6,7	6,7	9	9
RED	FGA	20,24	20,24	18,22	18,22
	D22S1045	15,16	15,16	11,17	11,17
	D5S3818	11,12	11,12	12,13	12,13
	D13S317	10,12	10,12	11,12	11,12
	D7S3280	10,12	10,12	11	11
PURPLE	SE33	21,28.2	21,28.2	18,30.2	18,30.2
	D10S1248	14,15	14,15	12,14	12,14
	D1S1656	15	15	15,17	15,17
	D12S391	15,16	15,16	20,22	20,22
	D2S1338	17,24	17,24	19,20	19,20

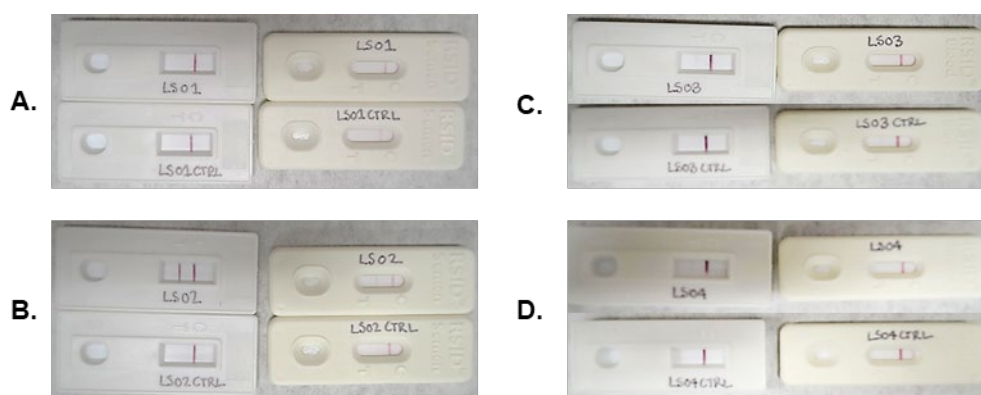


Figure 31 Immunochromatographic results for degraded samples. ABACard® tests are pictured on the left. RSID™ tests are pictured on the right. Question samples are oriented on the top of each frame. Control samples are oriented on the bottom of each frame. Within the membrane window, the test line is positioned on the left and the control line on the right. Results for (A) semen extracted from a bed sheet, (B) semen extracted from underwear, (C) blood extracted from a towel, and (D) blood extracted from denim jeans. A positive result is indicated by the presence of a red line at both the test and control zones.

4.2.6 Aged Samples

Swabs fortified with neat body fluid were kept at room temperature for time points zero, three, five, seven, and thirty-five days. Comparative results are outlined in Table 51 for this portion of the study. No instances of negative detection were recorded for evaluation using traditional screening techniques or the proteomic assay (Figure 4.9); however, weak and very weak positives were observed for ABACard® HemaTrace® for peripheral and menstrual blood samples. No change in detection was observed over the 35-day period. DNA analysis was not performed on this set of casework samples.

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

Table 51: Summary of comparative results for aged samples. Positive identifications are represented in green.

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

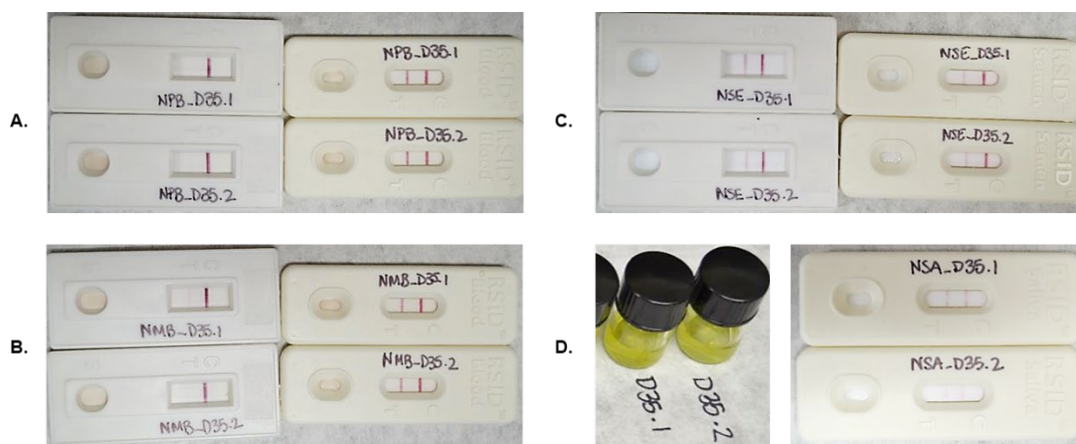


Figure 32: Serological screening test results for aged samples at Day 35. ABACard® test and SALigAE® are pictured on the left. RSID™ test is pictured on the right. Within the membrane window, the test line is positioned on the left and the control line on the right. Results for (A) peripheral blood, (B) menstrual blood, (C) semen, and (D) saliva. On lateral flow assays, a positive result is indicated by the presence of a red line at both the test and control zones. For SALigAE®, a positive result is indicated by a yellow colored reagent.

4.2.7 Sensitivity Samples

This particular cohort of sensitivity samples were designed differently than those used for analytical sensitivity determination of the LC-MS/MS assay. Diluted body fluid was spotted on cotton swabs prior to analysis, resulting in further dilution of target material during sample solubilization, representing an inherent limitation to the preparation workflow. A summary of proteomic, serological, and genetic testing results for each target fluid are outlined in Tables 52-56. The lowest diluted sample to still produce a positive result when screened with serological tests are pictured in Figure 33. Reviewing proteomic results, peripheral blood was characterized at the 20,000-fold dilution with the detection of a single hemoglobin peptide (Table 52). With processing a matched sample on commercially available lateral flow assays, the RSID™ Blood and ABACard® HemaTrace tests produced a positive result up to and including the 100-fold dilution. Conversely, STR analysis results were more consistent with the rate of peptide identification using the proteomic assay (Table 57). A complete STR profile was recovered through the 100-fold dilution, mirroring the complete characterization of peripheral blood peptide markers to the same dilution. The capability of STR profiling drastically decreased at a 5,000-fold and 10,000-fold dilutions, with samples producing a 7% and 5% STR profile, respectively. Remaining samples did not generate an STR profile; however, as previously stated,

hemoglobin was still detected at the 20,000-fold dilution with the proteomic assay. Overall, the proteomic assay was demonstrated to be 200 times more sensitivity than immunological assays for the detection of peripheral blood. In addition, the proteomic assay exceeded the sensitivity of routine STR typing chemistries.

Menstrual blood was characterized down to the 40,000-fold dilution sample on the proteomic method, with the detection of a single hemoglobin peptide, similar to peripheral blood described above (Table 53). But only 4 of 7 vaginal fluid markers were accurately characterized with the proteomic assay. Cornulin, a more specific vaginal fluid marker, was detected at the 100-fold dilution, with small proline rich protein 3 sporadically identified for the remainder of the dilution series. Interestingly, two instances of high dose hook effect were observed with immunochromatographic testing, with the neat and 2-fold dilution samples exhibiting a negative result on the antibody-based assays. In regard to identifying the blood component of menstrual blood, the proteomic assay outperformed the antibody-based tests by a factor of 400. Furthermore, with the absence of a vaginal fluid screening test, the proteomic assay inherently provided additional pertinent information down to the 100-fold dilution. A complete STR profile was obtained through the 100-fold dilution, with informative partial profiles produced at the 1,000- and 2,000-fold dilutions (Table 58). Sufficient allele and locus dropout were recorded for the remaining samples.

Seminal fluid was accurately identified at the lowest dilution on the proteomic assay, with detection of semenogelin peptide QITIPSQEQEHSQK (Table 54). However, in comparison, the lateral flow assays exhibited poor sensitivity. RSID™ Semen failed to produce a positive result after the 100-fold dilution (Figure 33C). Furthermore, ABACard® p30 demonstrated decreased sensitivity limits, reproducibly exhibiting a positive result only to the 10-fold dilution evaluated. In comparison, the proteomic assay demonstrated comparable results with STR and Y-STR genetic typing (Tables 59). With all protein biomarkers positively identified through the 100-fold dilution and a complete STR profile generated, seminal fluid identification using the validated assay strongly mirrors the abilities of routine genetic testing. As the number of peptides detected inherently decreases, so did the number of alleles characterized. In regard to genetic testing, samples beginning with the 10,000-fold dilution failed to produce a male quantitation value and were therefore amplified for Y-STR typing (Table 59). Few Y-STR alleles were characterized at the lower

end of the dilution series, with the 20,000-fold and 40,000-fold samples producing only a 7% and a 4% Y-STR profile, respectively. Overall, the proteomic assay demonstrated a sensitivity level 400 times more sensitivity than immunological tests but exhibited a similar sensitivity range of that of STR and Y-STR chemistries.

Diluted saliva was characterized reproducibly at the 1,000-fold dilution, with the detection of a single amylase peptide (Table 55). One antibody-based test and one enzymatic test were acquired for traditional serological testing for this portion of the study. Both RSID™ Saliva and SALIgAE® tests performed equally, with positive results observed up to and including the 10-fold dilution samples (Figure 33D). As previously described, the proteomic assay kept pace with the ability to generate an STR profile; however, the capabilities of genetic typing and reaching a larger sensitivity range outweigh those of the proteomic assay (Table 61). Identification of peptide markers began to decrease at the 100-fold dilution, where a full STR profile was still generated. Furthermore, out to the 2,000-fold dilution, a 70% complete STR profile was reported whereas the proteomic assay failed to detect saliva for the remainder of samples evaluated. To conclude, the proteomic assay demonstrated a sensitivity range 100 times more robust than that of the traditional screening assays. Conversely, routine genetic testing remained more sensitive than the validated assay.

Vaginal fluid was characterized by all target protein markers at the 10-fold dilution, with 2 of 7 markers detected at the 100-fold dilution and only a single protein (small proline rich protein 3) detected for the remaining samples (Table 56). In comparison, a full STR profile was produced down to the 500-fold dilution, with partial profiles observed for the remaining samples (Table 62). For the 100-fold dilution sample, no quantitation value was recorded and no DNA profile was produced. This result is inconsistent with the remaining data, and is therefore, being viewed as an outlier. However, no matched comparison could be generated given the lack of a commercially available assay for vaginal fluid screening.

Overall, the validated proteomic assay greatly outperformed existing serological screening tests. It should be noted that samples were not solubilized in kit-specific buffer provided by the commercial entities for immunochromatographic testing. Although solubilization in buffer is recommended by the manufacturers, concentrated samples did not seem to inhibit the functionality of the assays (with the exception of the high dose hook

effect for menstrual blood). Research has been conducted on the effect of pH and the ability to produce false positive and false negative results on the lateral flow assays. It was determined that extreme acidic or alkaline pH is not the sole contributor to false positive or false negative results. One study found that it is the presence of additional organic acids that causes disruption in the antibody-dye labeling mechanism [55].

Table 52: Summary of comparative results for peripheral blood sensitivity samples. Positive identifications are represented by a green box. Negative results are represented by a white box.

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

Table 53: Summary of comparative results for menstrual blood sensitivity samples. Positive identifications are represented by a green box. Negative results are represented by a white box.

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

Table 54: Summary of comparative results for seminal fluid sensitivity samples. Positive identifications are represented by a green box. Negative results are represented by a white box.

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

Table 55: Summary of comparative results for saliva sensitivity samples. Positive identifications are represented by a green box. Negative results are represented by a white box.

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

Table 56: Summary of comparative results for vaginal fluid sensitivity samples. Positive identifications are represented by a green box. Negative results are represented by a white box.

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

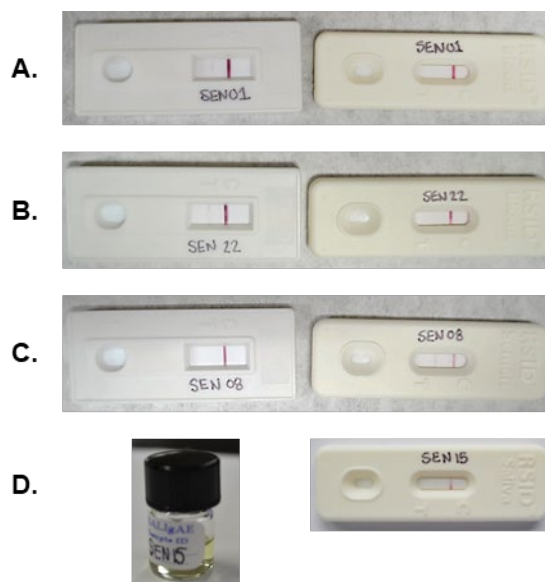


Figure 33: Serological screening test results for sensitivity samples. ABACard® test and SALigAE® are pictured on the left. RSID™ test is pictured on the right. Within the membrane window, the test line is positioned on the left and the control line on the right. Results for (A) 100-fold dilution of peripheral blood, (B) 100-fold dilution of menstrual blood, (C) 100-fold dilution of semen, and (D) 10-fold dilution of saliva. On lateral flow assays, a positive result is indicated by the presence of a red line at both the test and control zones. For SALigAE®, a positive result is indicated by a yellow colored reagent.

Table 57: STR genetic results for peripheral blood sensitivity samples. Complete locus identification represented by a green box. Partial locus identification represented by a grey box. Locus dropout represented by a white box.

Peripheral Blood		Neat	2	10	100	1,000	2,000	5,000	10,000	20,000	40,000
BLUE	D3S1358	15,17	15,17	15,17	15,17	17					
	VWA	18,21	18,21	18,21	18,21	21					
	D16S539	9,11	9,11	9,11	9,11	9,11					
	CSF1PO	12,13	12,13	12,13	12,13	12,13	13				
	TPOX	8,9	8,9	8,9	8,9	8,9	9				
GREEN	YINDEL	2	2	2	2	2					
	Amelogenin	XY	XY	XY	XY	XY	X				
	D8S1179	14,15	14,15	14,15	14,15	14,15	15				
	D21S11	28	28	28	28	28	28	28	28		
	D18S51	16,17	16,17	16,17	16,17	16,17	16				
YELLOW	DYS391	11	11	11	11	11	11				
	D2S441	11,12	11,12	11,12	11,12	11,12	11,12				
	D19S433	11,12	11,12	11,12	11,12	11	12		11		
	THO1	7	7	7	7	7	7				
	FGA	25,27	25,27	25,27	25,27	25,27	25				
RED	D22S1045	11,15	11,15	11,15	11,15	11,15	11				
	D53818	12	12	12	12	12		12			
	D13S317	12,13	12,13	12,13	12,13	12	12,13	13			
	D73280	10,12	10,12	10,12	10,12	10,12	10				
	SE33	21,26.2	21,26.2	21,26.2	21,26.2	21,26.2					
PURPLE	D10S1248	12,13	12,13	12,13	12,13	12,13	13				
	D1S1656	12,16.3	12,16.3	12,16.3	12,16.3	12,16.3					
	D12S391	17,20	17,20	17,20	17,20	17,20	20				
	D2S1338	23,26	23,26	23,26	23,26	23,26	23,26				
Profile Percentage		100	100	100	100	90.2	46.3	7.3	4.9	0	0

Table 58: STR genetic results for menstrual blood sensitivity samples. Complete locus identification represented by a green box. Partial locus identification represented by a grey box. Locus dropout represented by a white box. Menstrual blood for this subset of samples was provided by two donors (Reference #1 and Reference #2).

Menstrual Blood		Reference #1	Neat	2	10	Reference #2	100	1,000	2,000	5,000	10,000	20,000	40,000
BLUE	D3S1358	16, 18	16,18	16,18	16,18	16,17	16,17	16,17	17				
	VWA	16	16	16	16	15,18	15,18	15,18	15		18		
	D16S539	13, 14	13,14	13,14	13,14	9,12	9,12	9,12			9		
	CSF1PO	12, 13	12,13	12,13	12,13	11,14	11,14		11,14				
	TPOX	9, 12	9,12	9,12	9,12	8,11	8,11	8,11	8,11		11		8
GREEN	YINDEL	-	-	-	-	-	-	-	-	-	-	-	-
	Amelogenin	X	X	X	X	X	X	X	X	X		X	
	D8S1179	13, 14	13,14	13,14	13,14	12,13	12,13	12,13	12,13	12,13	12,13		
	D21S11	27, 30	27,30	27,30	27,30	27,30	27,30	27,30	27,30	27,30			30
	D18S51	12, 13	12,13	12,13	12,13	10,14	10,14	10,14	10,14				
YELLOW	DYS391	-	-	-	-	-	-	-	-	-	-	-	-
	D2S441	10, 11	10,11	10,11	10,11	11,12	11,12	11,12	11,12		11,12	11	
	D19S433	13	13	13	13	14,15	14,15	14,15	14,15		15		
	THO1	6, 9.3	6,9.3	6,9.3	6,9.3	9,9.3	9,9.3	9,9.3	9,9.3	9.3	9.3		
	FGA	20, 24	20,24	20,24	20,24	20	20	20	20		20	20	
RED	D22S1045	15, 16	15,16	15,16	15,16	11,15	11,15	11,15					
	D53818	12, 13	12,13	12,13	12,13	11,12	11,12	11,12	11,12				
	D13S317	10, 11	10,11	10,11	10,11	11,12	11,12		11,12		11,12	12	
	D73280	10, 11	10,11	10,11	10,11	10,12	10,12		10,12		10		
	SE33	29.2	29.2	29.2	29.2	17,26.2	17,26.2		17,26.2	17	17		
PURPLE	D10S1248	13, 15	13,15	13,15	13,15	14,15	14,15		14				
	D1S1656	15, 16.3	15,16.3	15,16.3	15,16.3	14,17	14,17	14,17	14,17				
	D12S391	15, 22	15,22	15,22	15,22	18,20	18,20	18,20	18,20				
	D2S1338	19, 24	19,24	19,24	19,24	17,19	17,19	17,19	17,19	19	17	17	
Profile Percentage		-	100	100	100	-	100	75.6	82.9	17.1	36.6	9.7	4.9

Table 59: STR genetic results for seminal fluid sensitivity samples. Complete locus identification represented by a green box. Partial locus identification represented by a grey box. Locus dropout represented by a white box.

	Semen	Reference	Neat	2	10	100	1,000	2,000	5,000
BLUE	D3S1358	15,17	15,17	15,17	15,17	15,17	15,17	15	15
	VWA	17,18	17,18	17,18	17,18	17,18	17,18	17,18	
	D16S539	11,12	11,12	11,12	11,12	11,12	11,12	12	
	CSF1PO	10,11	10,11	10,11	10,11	10,11	10,11	10,11	
	TPOX	8,11	8,11	8,11	8,11	8,11	8,11	11	
GREEN	YINDEL	2	2	2	2	2		2	2
	Amelogenin	XY	XY	XY	XY	XY	X	Y	
	D8S1179	10,13	10,13	10,13	10,13	10,13	10	10,13	10
	D21S11	31.2,32.2	31.2,32.2	31.2,32.2	31.2,32.2	31.2,32.2	31.2,32.2	32.2	31.2
	D18S51	11,15	11,15	11,15	11,15	11,15	11,15	11,15	15
YELLOW	DYS391	12	12	12	12	12	12	12	
	D2S441	11,14	11,14	11,14	11,14	11,14	11	11	11,14
	D19S433	13,14	13,14	13,14	13,14	13,14	13	13	
	THO1	6,7	6,7	6,7	6,7	6,7	7	6,7	
	FGA	20,24	20,24	20,24	20,24	20,24	20,24	20,24	24
RED	D22S1045	15,16	15,16	15,16	15,16	15,16	15,16	15,16	
	D53818	11,12	11,12	11,12	11,12	11,12	11,12	12	
	D13S317	10,12	10,12	10,12	10,12	10,12	10,12	10,12	12
	D73280	10,12	10,12	10,12	10,12	10,12	10,12	10,12	12
	SE33	21,28.2	21,28.2	21,28.2	21,28.2	21,28.2	21	21,28.2	
PURPLE	D10S1248	14,15	14,15	14,15	14,15	14,15	14,15	15	
	D1S1656	15	15	15	15	15	15	15	15
	D12S391	15,16	15,16	15,16	15,16	15,16	15,16	15,16	
	D2S1338	17,24	17,24	17,24	17,24	17,24	17		17
Profile Percentage		-	100	100	100	100	83.7	76.7	27.9

Table 60: Y-STR genetic results for seminal fluid sensitivity samples. Complete locus identification represented by a green box. Partial locus identification represented by a grey box. Locus dropout represented by a white box.

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

Table 61: STR genetic results for saliva sensitivity samples. Complete locus identification represented by a green box. Partial locus identification represented by a grey box. Locus dropout represented by a white box.

	Saliva	Reference	Neat	2	10	100	500	1,000	2,000	5,000	10,000
BLUE	D3S1358	15,17	15,17	15,17	15,17	15,17	15,17	15	15		
	VWA	17,18	17,18	17,18	17,18	17,18	17,18	17,18			
	D16S539	11,12	11,12	11,12	11,12	11,12	11,12	12			
	CSF1PO	10,11	10,11	10,11	10,11	10,11	10,11	10,11			
GREEN	TPOX	8,11	8,11	8,11	8,11	8,11	8,11		8		8
	YINDEL	2	2	2	2	2	2	2			
	Amelogenin	XY	XY	XY	XY	XY	XY	X	X		
	D8S1179	10,13	10,13	10,13	10,13	10,13	10,13	10,13	13		
YELLOW	D21S11	31.2,32.2	31.2,32.2	31.2,32.2	31.2,32.2	31.2,32.2	31.2,32.2	31.2,32.2	31.2,32.2		
	D18S51	11,15	11,15	11,15	11,15	11,15	11,15	11,15	11		
	DYS391	12	12	12	12	12	12	12	12		
	D2S441	11,14	11,14	11,14	11,14	11,14	11,14	11,14	11,14		11
RED	D19S433	13,14	13,14	13,14	13,14	13,14	13,14	13,14	13,14		
	THO1	6,7	6,7	6,7	6,7	6,7	6,7	6	6,7		
	FGA	20,24	20,24	20,24	20,24	20,24	20,24	20,24	20,24		
	D22S1045	15,16	15,16	15,16	15,16	15,16	15,16	15,16			
PURPLE	D5S3818	11,12	11,12	11,12	11,12	11,12	11,12	11,12	11,12		11
	D13S317	10,12	10,12	10,12	10,12	10,12	10,12	10,12	12		10
	D7S3280	10,12	10,12	10,12	10,12	10,12	10	10,12			10
	SE33	21,28.2	21,28.2	21,28.2	21,28.2	21,28.2	21,28.2	21,28.2	28.2		
PURPLE	D10S1248	14,15	14,15	14,15	14,15	14,15	14	14			15
	D1S1656	15	15	15	15	15	15	15	15		
	D12S391	15,16	15,16	15,16	15,16	15,16	15,16	15,16	16		
	D2S1338	17,24	17,24	17,24	17,24	17,24	17,24	17,24	17		
Profile Percentage		-	100	100	100	100	95.3	86	51.1	0	13.9

Table 62: STR genetic results for vaginal fluid sensitivity samples. Complete locus identification represented by a green box. Partial locus identification represented by a grey box. Locus dropout represented by a white box.

Vaginal Fluid	Reference	10	100	500	1,000	2,000	5,000	10,000
BLUE	D3S1358	16,17		16,17	16,17	16,17	17	
	VWA	15,18		15,18	15,18	15,18	15	
	D16S539	9,12		9,12	9,12	9,12		
	CSF1PO	11,14		11,14	11,14	11		
	TPOX	8,11		8,11	8,11	8,11	11	
GREEN	YINDEL	-	-	-	-	-	-	-
	Amelogenin	X	X	X	X	X	X	
	D8S1179	12,13		12,13	12,13	12,13	13	13
	D21S11	27,30		27,30	27,30		30	
	D18S51	10,14		10,14	10,14	10,14		
YELLOW	DYS391	-	-	-	-	-	-	-
	D2S441	11,12		11,12	11,12	11,12	11,12	
	D19S433	14,15		14,15	14,15	14,15	14,15	
	THO1	9,9.3		9,9.3	9,9.3	9	9.3	9
RED	FGA	20		20	20	20	20	20
	D22S1045	11,15		11,15	11	11,15	11	
	D53818	11,12		11,12	11,12	11,12		
	D13S317	11,12		11,12	12	11,12		
	D73280	10,12		10,12	10	12		
PURPLE	SE33	17,26.2		17,26.2	17	17,26.2		
	D10S1248	14,15		14,15	14,15	14,15		
	D1S1656	14,17		14,17	17	14,17		
	D12S391	18,20		18,20	18,20	18,20		
Profile Percentage	D2S1338	17,19		17,19	17,19	19		
		-	100	0	100	87.8	90.2	29.2
								7.3

4.3 Solid Phase Cleanup of Lubricant Contaminated Samples

In the previous sections, it was demonstrated that protein mass spectrometry techniques exhibit increased sensitivity and specificity to conduct accurate screening of evidentiary items for the presence of biological fluids. This approach has proven the LC-MS/MS analytical method to be robust and reliable in the detection of protein signatures, particularly for sexual assault type samples. With the developed workflow, it was demonstrated that protein components were able to be identified with a level of sensitivity comparable to that of STR/Y-STR profiling. The Uniform Crime Report states that in the year 2019, 17% of rape offenses were also linked with instances of sodomy and 6.5% with instances of sexual assault with a foreign object [163]. In the context of sexual assault sample screening, the identification of seminal fluid in addition to saliva and vaginal fluid play a significant role in sample processing and generation of critical sample information.

Of the laboratory-generated case samples utilized during validation and implementation assessments, a particular set of samples, those treated with personal lubricant as part of the environmental contaminant subset, were identified for additional analyses. In particular, peak area responses of vaginal fluid biomarkers were inhibited or complete protein dropout was observed with samples subjected to a personal lubricant.

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

4.3.1 Lubricants and Vaginal Fluid Biomarker Detection

4.3.1.1 Digest Efficiency

The total protein concentration of control and experimental samples was determined using the BCA assay. Quantitation was used to normalize the amount of total protein input into the enzymatic digestion procedure. Should the presence of personal lubricants negatively impact the ability to accurately determine the total protein quantity in a sample, the protein input for tryptic digestion would be incorrect. This would result in insufficient digestion or missed cleavages. No effect on digest efficiency was observed across and lubricant type or lubricant volume, resulting in no sufficient variation in protein concentration (Figure 34). A median protein concentration was measured at 266.42 $\mu\text{g/mL}$ and 272.18 $\mu\text{g/mL}$ for control and experimental samples, respectively.

Trypsin performance was monitored by assessing peak area ratios of intact internal positive control to digested internal positive control (IPC). Acceptance criteria outlined during validation studies were employed during this assessment to ascertain IPC performance. IPC ratios that fell within ratio bounds of 15.8 (upper bound) and 11.5 (lower bound) were considered acceptable and unaffected by experimental procedures. Digest performance was impacted by several personal lubricant types prior to implementing a supplemental pre-digestion cleanup (Figure 35). Greatest protease inhibition prior to supplemental cleanup was observed for Sliquid® H2O, Coconut Oil, and Swiss Navy® lubricants. For example, the average IPC ratio for 1 μ L coconut oil samples was 19.15, a ratio that falls outside the acceptable range. With the addition of the pre-digestion cleanup, this ratio fell to an acceptable 12.42. Interestingly, Sliquid® Silk demonstrated an inverse dose-response relationship, with 1 μ L volumes of personal lubricant above the established range, 5 μ L volumes within the established range, and 15 μ L volumes below the established range. Protease activity was not negatively affected by Astroglide®. Acceptable IPC ratios for all lubricants assessed were restored with the addition of the pre-digestion cleanup to the sample processing protocol.

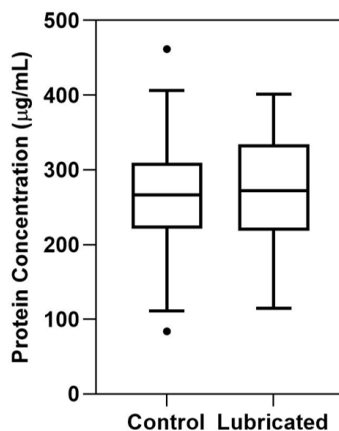


Figure 34: Quantitative results comparing total protein concentration of control and lubricated vaginal swab samples. Box = 25th and 75th percentiles; bars = Tukey fences. Outliers were calculated as any values outside ± 1.5 Inter Quartile Range (IQR).

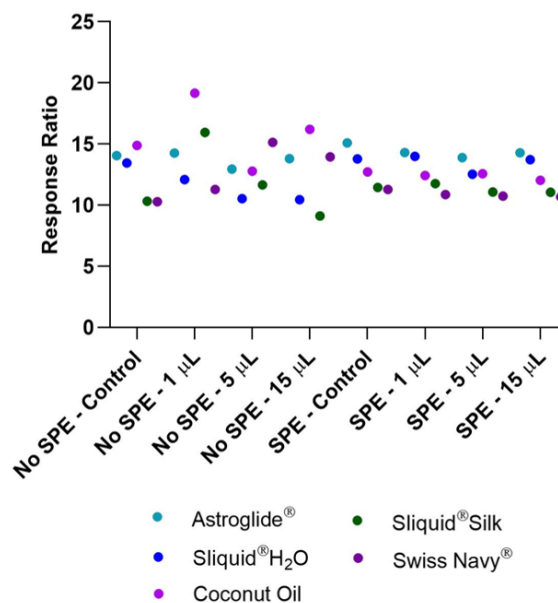


Figure 35: Internal positive control response ratio for control and lubricated samples which have or have not undergone an additional SPE purification during sample preparation. Response ratios were averaged across three replicates.

4.3.1.2 Biomarker Detection

The effect of personal lubricant on biomarker detection was assessed by calculating the percent difference of endogenous and internal standard peak area responses for all samples evaluated. Similar to the results observed for digestion efficiency, biomarker detection was negatively affected by the presence of personal lubricant, particularly prior to the addition of the pre-digestion cleanup. Overall, impact of personal lubricant was sufficiently reduced at all lubricant volumes with pre-digestion cleanup (Figure 36).

The greatest deleterious effects were observed for Sliquid® H₂O, Coconut Oil, and Sliquid® Silk lubricants. Sliquid® H₂O, the water-based lubricant without glycerin, exhibited a dose-response relationship for both endogenous and internal standard biomarkers. When fortified with lubricant, a median percent difference for endogenous vaginal fluid biomarkers of 0.31%, -11.28%, and -37.54% were observed at the 1 µL, 5 µL, and 15 µL lubricant volumes, respectively. The greater percent difference at the 15 µL volume was mitigated with a pre-digestion cleanup, resulting in a percent difference of -14.28% (Figure 36B). A similar dose-response relationship was exhibited by samples

treated with Sliquid® Silk (Figure 36D). Samples treated with Coconut Oil exhibited negative impact on biomarker detection regardless of lubricant volume. A percent difference of -25.04%, -22.15%, and -25.03% were observed for 1 µL, 5 µL, and 15 µL volumes, respectively. However, a wide range in response ratios was demonstrated. The lower whisker extends to -80.94% and the median lies at the top of the inter quartile range (IQR). Internal standard responses exhibited a negative percent difference, but not to the same extent as the endogenous biomarkers (Figure 36C).

To further illustrate the negative effects of certain lubricants on specific endogenous biomarkers, Figure 37 depicts the response of neutrophil gelatinase peptide WYVVGLAGNAILR when subjected to each lubricant evaluated. This biomarker was selected given the specificity of neutrophil gelatinase in vaginal fluid and the hydrophobic nature of the WYVVGLAGNAILR peptide (retention time of 8.7 minutes). The response of this specific peptide was unaffected by Astroglide® and Sliquid® Silk; however, sufficient decrease in response was exhibited with Sliquid® H2O, Coconut Oil, and Swiss Navy®. With the addition of a pre-digestion cleanup, inconsistencies were not entirely eliminated, but minimized in most cases. For example, Figure 38 illustrates the increase in peak area response of endogenous neutrophil gelatinase peptide WYVVGLAGNAILR with the addition of the pre-digestion cleanup.

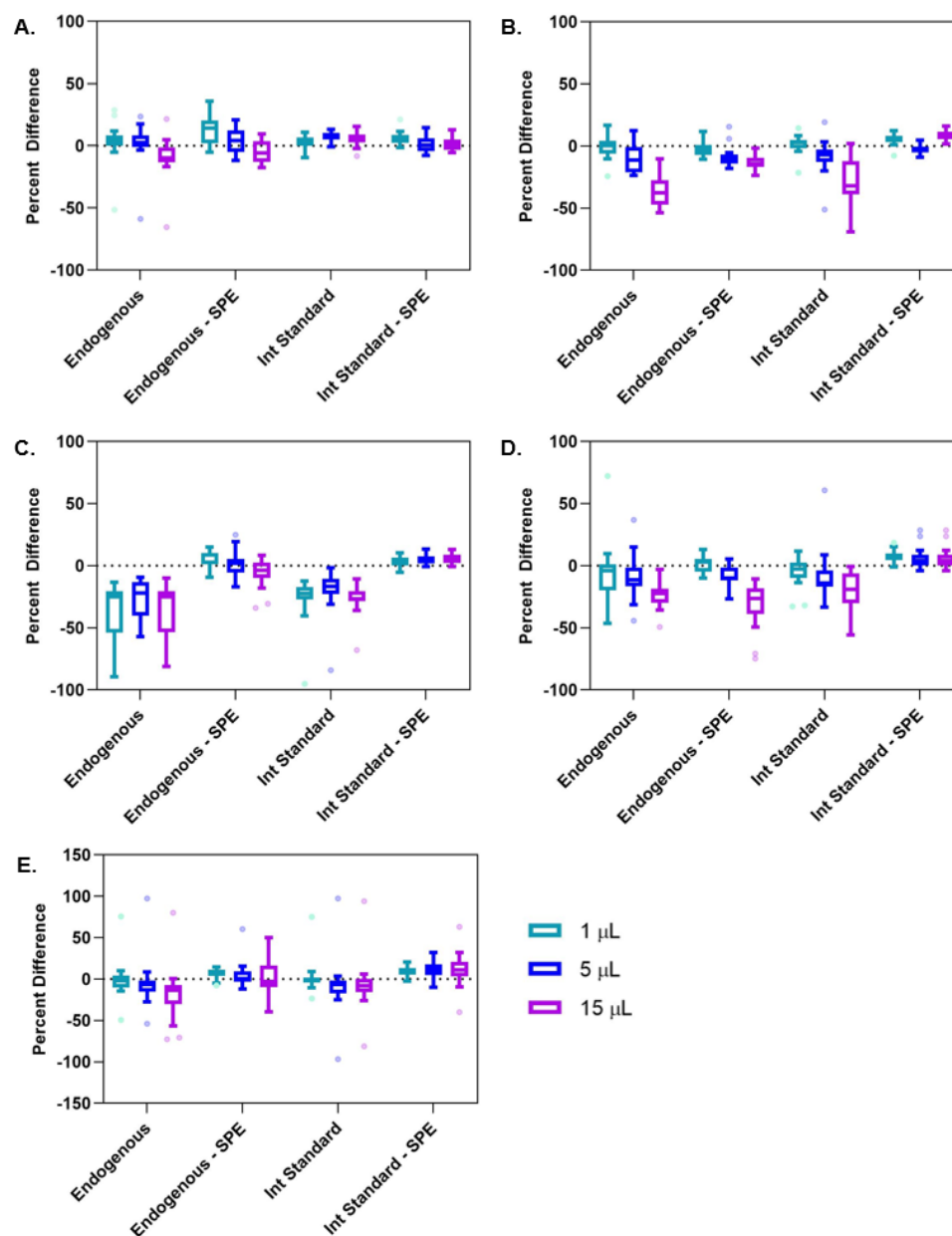


Figure 36: Effect of (A) Astroglide®, (B) Sliquid® H2O, (C) Coconut Oil, (D) Sliquid® Silk, and (E) Swiss Navy® lubricants measured as percent difference on peak area response of endogenous vaginal fluid proteins and isotopically-labeled internal standards both with and without pre-digestion SPE cleanup. Box = 25th and 75th percentiles; bars = Tukey fences. Outliers were calculated as any values outside ± 1.5 IQR.

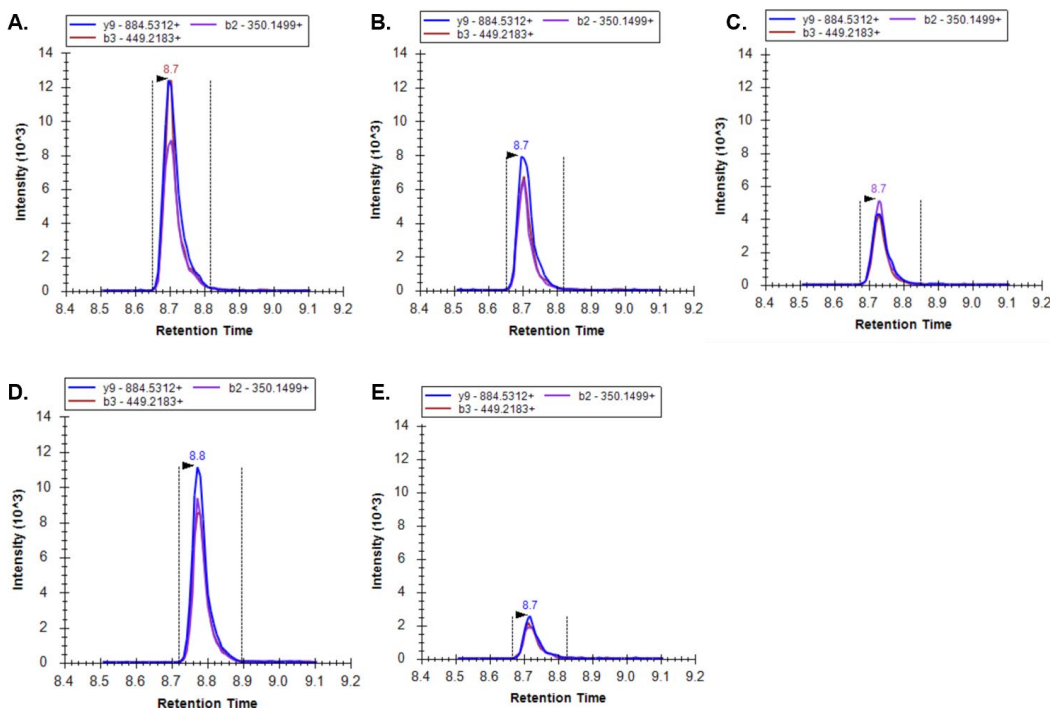


Figure 37: Chromatographic peak response for endogenous neutrophil gelatinase peptide WYVVGLAGNAILR when subjected to 15 μ L of (A) Astroglide®, (B) Sliquid® H2O, (C) Coconut Oil, (D) Sliquid® Silk, and (E) Swiss Navy®

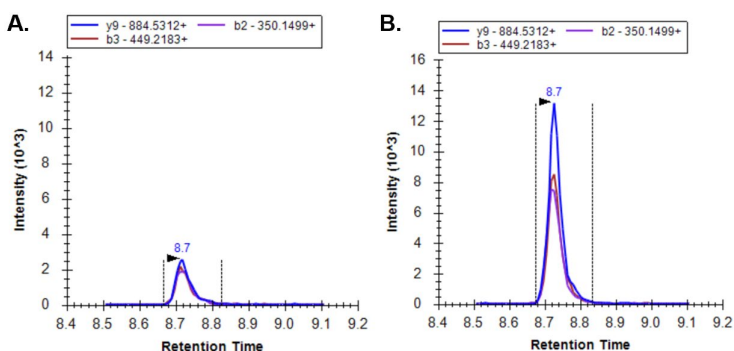


Figure 38: Chromatographic peak response for endogenous neutrophil gelatinase peptide WYVVGLAGNAILR (A) without pre-digestion cleanup and (B) with pre-digestion cleanup, when subjected to 15 μ L of Swiss Navy®.

4.3.2 Lubricants and the Detection of Semen and Saliva Biomarkers

The overall effect of personal lubricant was further assessed with mock sexual assault samples. Generally, when sexual assault evidence is received in a forensic laboratory, it will be screened for the presence of seminal fluid and/or saliva. Therefore, in addition to the effect of personal lubricant on the detection of vaginal fluid, it was vital to establish any deleterious effects that may inhibit the identification of seminal fluid and/or

saliva. To simulate sexual assault samples, pooled vaginal extract was fortified with seminal fluid, saliva, and a mixture of both target fluids in addition to increasing amounts of personal lubricant. All lubricant classes and selected volumes were evaluated in triplicate.

In summary, all seminal fluid and saliva protein biomarkers were detected at all lubricant volumes and for all lubricant classes evaluated, in both the prepared single source and mixture samples. For prepared samples containing saliva, marginal suppression or enhancement effects were observed for samples fortified with Astroglide®, Sliquid® H2O, and Coconut Oil. In comparison with the detection of vaginal fluid markers, sufficient losses in peptide response were observed for samples fortified with Sliquid® Silk, even with the pre-digest cleanup (Figure 39A). A median saliva biomarker signal decrease of -37.0%, -54.7%, and -93.3% at the 1 μ L, 5 μ L, and 15 μ L volumes was observed with the Sliquid® Silk lubricant. Furthermore, at the 15 μ L volume, sufficient enhancement effects were observed for Swiss Navy®, with a median signal increase of 120.5%. These observed effects were consistent with recorded peak area responses of the internal standards of single source saliva samples (Figure 39B). The greatest suppression effects were illustrated by endogenous seminal fluid protein biomarkers, with consistent suppression noted for Astroglide®, Sliquid® H2O, Coconut Oil, and Sliquid® Silk. A median signal decrease of -23.3% and -85.7% were observed at the 5 μ L and 15 μ L Sliquid® Silk volumes, respectively. These two samples exhibit a large IQR. However, small instances of enhancement were recorded for the 15 μ L Sliquid® Silk and 1 μ L Swiss Navy® lubricants, with signal increases of 4.9% and 4.8%, respectively (Figure 39C). Conversely, minimal suppression and enhancement effects were observed for seminal fluid internal standard responses (Figure 39D).

Interestingly, the severe instances of peak response suppression for Sliquid® Silk were not as evident in samples containing a mixture of seminal fluid and saliva (Figure 40A and 40C) Although an average peptide response loss of -49.7% for saliva and -26.4% for seminal fluid were observed at the 15 μ L volume of Sliquid® Silk, peak response enhancement for Coconut Oil was illustrated (Figure 40A and 40C). Internal standard response demonstrated limited suppression for both target fluids, with enhancement observed for Coconut Oil and Sliquid® Silk lubricants (Figure 40B and 40D). For example,

decrease in peak area response for semenogelin 2 and alpha amylase is depicted in Figure 41. Endogenous peak area response (red peaks) demonstrates a linear decrease while the internal standard response (blue peaks) remains relatively unchanged across lubricant volumes.

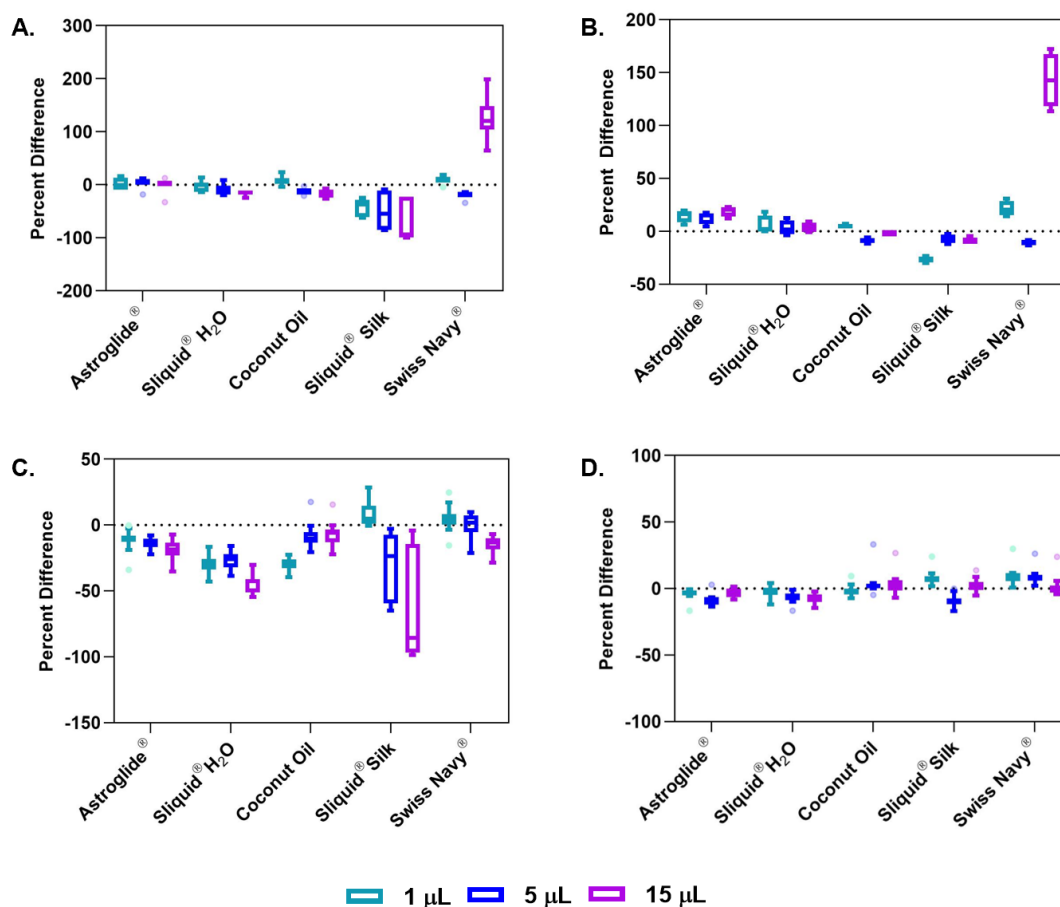


Figure 39: Effects of lubricant on (A) endogenous saliva biomarkers, (B) internal standard saliva biomarkers, (C) endogenous seminal fluid biomarkers, and (D) internal standard seminal fluid biomarkers. Plotted is the percent difference of average signal intensity for control versus experimental samples for all target biomarkers. Box = 25th and 75th percentiles; bars = Tukey fences. Outliers were calculated as any values outside ± 1.5 IQR.

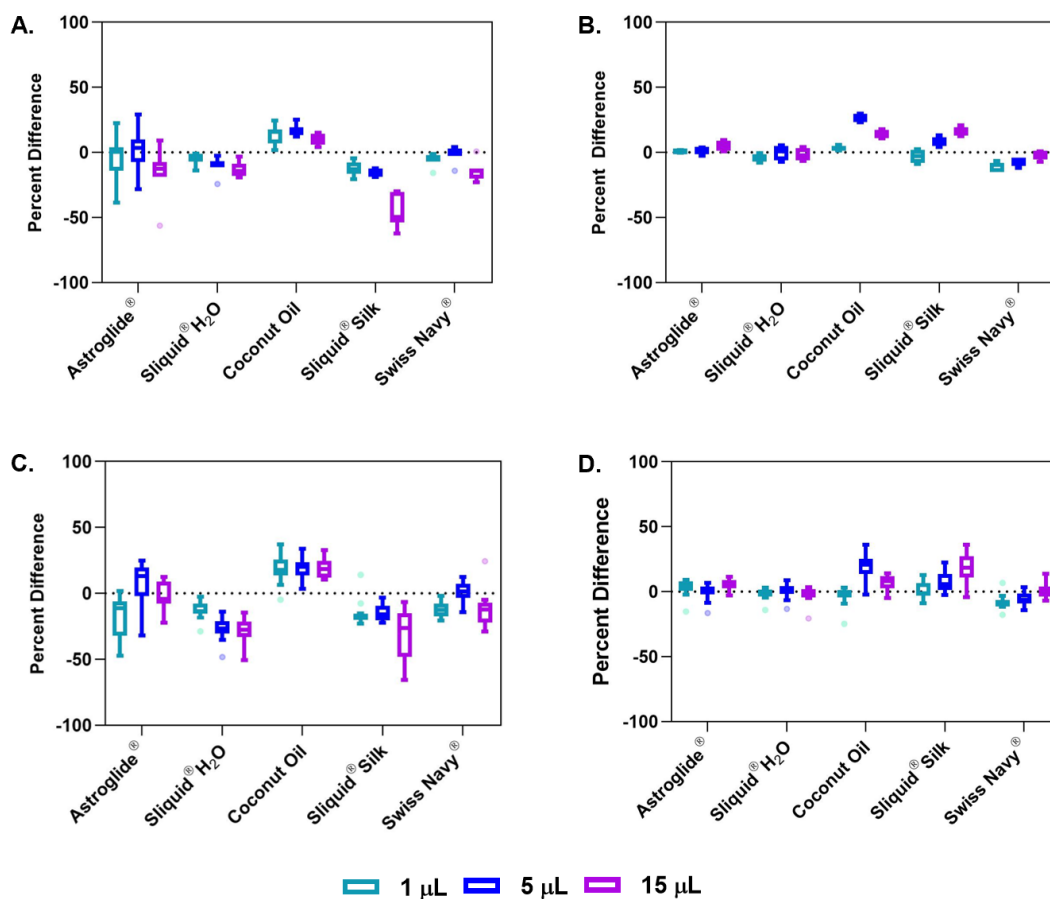


Figure 40: Effects of lubricant on (A) endogenous saliva biomarkers, (B) internal standard saliva biomarkers, (C) endogenous seminal fluid biomarkers, and (D) internal standard seminal fluid biomarkers in mixture samples. Plotted is the percent difference of average signal intensity for control versus experimental samples for all target biomarkers. Box = 25th and 75th percentiles; bars = Tukey fences. Outliers were calculated as any values outside ± 1.5 IQR.

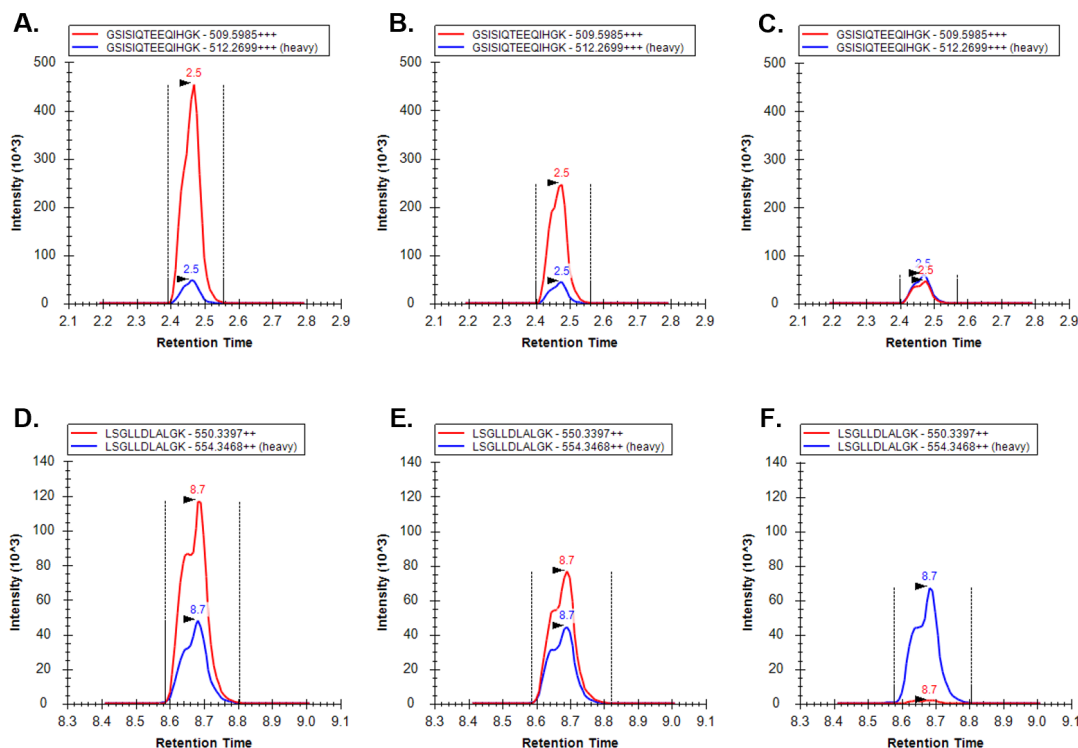


Figure 41: Semenogelin 2 peptide GSISIQTEEQIHGK peak area response in mixture samples containing (A) 1 μ L, (B) 5 μ L, and (C) 15 μ L volumes of Sliquid® Silk lubricant. Alpha amylase peptide LSGLLDLALGK peak area response in mixture samples containing (D) 1 μ L, (E) 5 μ L, and (F) 15 μ L volumes of Sliquid® Silk lubricant. Red peaks represent endogenous peptide peak area response. Blue peaks represent internal standard peak area response.

4.3.3 Vaginal Fluid Biomarker Detection from Condoms

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

To further assess this hypothesis, lubricated condoms both with and without spermicidal additives were evaluated. The external surface of each condom was swabbed with a vaginal swab to capture the proprietary lubricant and/or spermicide additive at concentrations commonly encountered in forensic casework. Unlubricated condoms

(Trojan™ ENZ™) served as the condom control, in addition to pooled, untreated vaginal extract serving as the negative control. In addition to the unlubricated condom type, SKYN® Original, SKYN® Extra Lube, and Durex® Performax® condoms were selected for this portion of the study. After initial observations made during sample handling, the Durex® Performax® condoms contained the greatest amount of surface lubricant and, although marketed as unlubricated, the Trojan™ ENZ™ condoms did contain a minimal amount of lubricant on the external surface.

Overall, target peptide response of vaginal fluid markers was greater across all condom types in comparison to negative control samples (Figure 42). It should be noted that inconsistency in sample data was observed, as depicted by the percent difference range of endogenous targets and large IQR of internal standard response. For endogenous biomarkers, the calculated percent differences of peak area response were evenly distributed between the first and third quartiles. However, outliers greater than 1.5 IQR were observed for Durex® Performax®, SKYN® Original, and SKYN® Extra Lube condom samples. A median 50% gain in peptide response was exhibited by vaginal fluid markers recovered from swabs subjected to the surfaces of SKYN® Extra Lube and Trojan™ ENZ™ condoms (Figure 42A). Overall, peak response of isotopically-labeled internal standard was enhanced in comparison with control samples (Figure 42B). The internal standard response demonstrated greater agreement among sample replicates than endogenous biomarkers responses, evident by the absence of outliers and a more consistent IQR. Chromatographic endogenous and internal standard peak area responses across condom replicate samples are depicted in Figure 43.

An active ingredient in Durex® Performax® condoms that may attribute to the larger IQR and presence of outlier data points is benzocaine. Benzocaine is a common ingredient in over-the-counter topical pain ointments, such as those used for oral and otic pain. The mechanism of action of benzocaine that results in pain relief has also been leveraged for use in contraceptives. Benzocaine acts by inhibiting voltage-dependent sodium channels, preventing an action potential from propagating down a neuron membrane. When applied to condoms, benzocaine can prevent premature ejaculation by decreasing sensitivity of male sex organs. Given the compound's structure, it is hydrophobic in nature and readily soluble in alcohol-based solvents. Therefore, benzocaine

has great affinity for the C18 sorbent material of solid phase extraction cartridges and for the acetonitrile-based elution solvent.

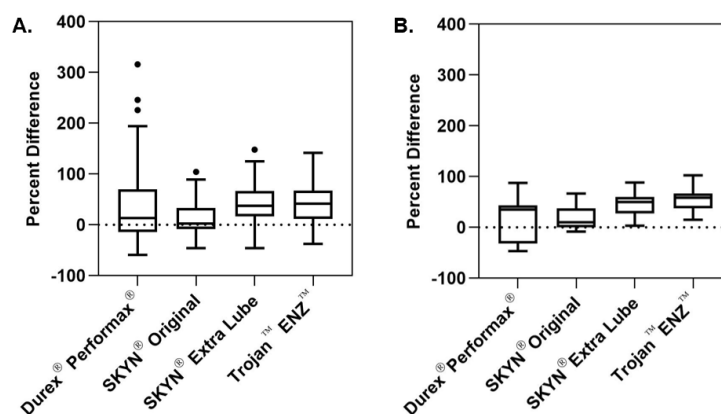


Figure 42: Effects of condom lubricant on (A) endogenous vaginal fluid biomarkers and (B) internal standard vaginal fluid biomarkers. Plotted is the percent difference of average signal intensity for control versus experimental samples for all target biomarkers. Box = 25th and 75th percentiles; bars = Tukey fences. Outliers were calculated as any values outside ± 1.5 IQR.

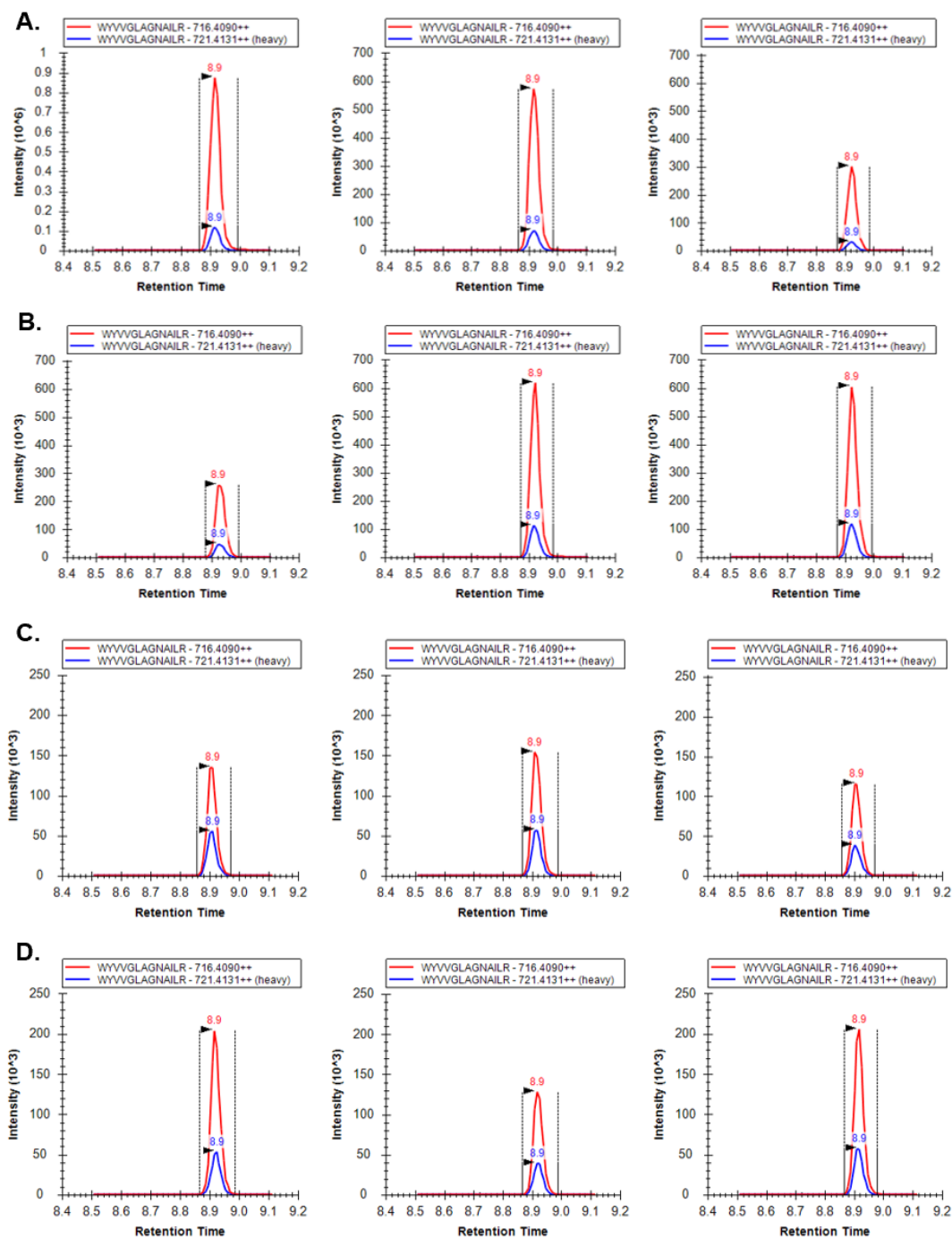


Figure 43: Neutrophil gelatinase peptide WYVVLGNAILR peak area response for (A) Durex® Performax®, (B) SKYN® Original, (C) SKYN® Extra Lube, and (D) Trojan™ ENZ™ condom triplicate samples. Red peaks represent endogenous peptide peak area response. Blue peaks represent internal standard peak area response.

4.4 Body fluid Identification by Protease-Free High Resolution Mass Spectrometry

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

A multiplex assay using high-specificity protein biomarker panels for the identification of five forensically-relevant body fluids (i.e., peripheral blood, saliva, seminal fluid, vaginal/menstrual fluids) was developed and validated, as described previously. This approach has proven to be broadly robust and reliable, particularly for the processing of sexual assault-type samples. With this workflow, the protein components, and by association the body fluid source, of stains can be detected with a level of sensitivity comparable to that of DNA profiling. The assay has been rigorously validated in accordance with the Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology [167] and the FBI's Quality Assurance Standards (QAS) [168] to establish the reliability and limitations of the methodology. Studies on assay performance with casework-type samples have shown that

the method is fully compatible with downstream genetic testing and that assay sensitivity greatly exceeds that of traditional lateral flow assays, particularly with trace level samples. Additional studies, discussed previously, that targeted the potential impact of substances commonly encountered in sexual assault-type evidence (e.g., personal lubricants and spermicidal compounds) provided additional insight on how best to maximize the successful detection of body fluids in casework.

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

4.4.1 Biomarker Selection and Population Study

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

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

In summary, purchased semen samples from healthy male donors displayed an average donor age of 31.6 years, with an age range of 18 to 54 years. A majority (80%) of semen samples were collected from Caucasian donors, with 3 donors of African American descent, 3 donors of Hispanic descent, 2 donors of Asian descent, and 2 donors from Asian/Caucasian descent. Semen purchased from vasectomized male donors displayed an average donor age of 51.6 years, with an age range of 39 to 61 years. All vasectomized samples were donated by individuals of Caucasian descent. Overall, this approach consistently identified SEMG1, SEMG2, KLK3, and PPAP across the population. Summary search results for non-vasectomized and vasectomized sample sets can be found in Tables 63 and 64, respectively, with complete detailed search results outlined in Appendix K. Only a single instance of protein dropout was observed within the sample population (sample ID 31978-07), with KLK3 not detected in this particular sample. Otherwise, all non-vasectomized and vasectomized samples were positive for the four target protein biomarkers stated previous. There was little difference between the two sample populations. A slightly greater average percent coverage was exhibited by the non-vasectomized population for biomarkers KLK3 and PPAP, displaying a 15.18% and 15.48% increase, respectively. There was no change in percent coverage observed for SEMG2 and a minute 2.48% increase in coverage for vasectomized samples for SEMG1. A similar trend was illustrated for the average number of unique peptides identified by the bioinformatics software. Overall, the developed sample preparation protocol was able to characterize semen-specific protein biomarkers in both non-vasectomized and vasectomized samples. These results were used to identify surrogate peptides that are consistently identified across all individuals, as described in the next section.

Purchased saliva samples from healthy donors displayed an average donor age of 40.38 years, an age range of 19 to 70 years, with 48% female and 52% male donors represented. Racial decent was not provided for this sample set. Similar to the semen samples, consistency in protein coverage was observed with multiple protein biomarkers being confidently identified in the saliva sample population. Summary search results can be found in Table 65 with complete detailed search results outlined in Appendix L. Several proteins were identified across all samples (e.g., STAT, SMR3B, and HIS1). Other targets, such as HIS3 and CST1 were identified in 64% and 68% of samples, respectively. While more protein loss was observed with the saliva population in comparison with the semen population, it is likely that this is a function of the data dependent acquisition approach utilized. A targeted method would likely detect even the proteins which were not identified in each sample. This is due to certain salivary biomarkers being of lower abundance in relation to the limited number of precursor ions which the instrument can measure in parallel.

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

A total of 32 individuals were retained for the vaginal fluid population assessment. In summary, purchased vaginal swab samples (25 samples) from healthy donors displayed an average donor age of 39.16 years, with an age range of 23 to 61 years. All vaginal swab samples were collected from Caucasian donors. No identifying information was recorded for IRB collected samples (7 samples). Summary search results can be found in Table 66, with complete detailed search results outlined in Appendix M. Overall, no candidate protein biomarkers were consistently detected in all samples of this population; however,

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

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

Table 63: Descriptive statistics of non-vasectomized semen population.

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

Table 64: Descriptive statistics of vasectomized semen population.

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

Table 65: Descriptive statistics of saliva population.

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

Table 66: Descriptive statistics of vaginal swab population.

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

4.4.2 Target Peptide Biomarker Selection

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

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

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

Using the criteria stated above, a preliminary list of targets included 77 semen peptides, 92 saliva peptides, and 30 vaginal fluid peptides, for which reference material was purchased and evaluated. The salivary protein HIS3 was removed from the peptide list due to low quality spectra and sample occurrence. All other listed protein biomarkers were represented in the peptide list. Furthermore, prolactin inducible protein (PIP) found in seminal fluid was re-introduced. Although not originally described during the population assessment, this protein exhibited higher quality spectra with a range of sample occurrence that prompted additional interest in this particular biomarker. In total, 38 seminal fluid peptides, 44 saliva peptides, and 12 vaginal fluid peptides were selected for the final inclusion library (Tables 67-69).

Table 67: Peptide markers for seminal fluid. Prostate specific antigen (KLK3), prolactin inducible protein (PIP), prostatic acid phosphatase (PPAP), semenogelin 1 (SEMG1), and semenogelin 2 (SEMG2). RT = retention time, AA = amino acid.

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

Table 68: Peptide markers for saliva. Histatin-1 (HIS1), basic salivary proline-rich protein 1 (PRP_1), basic salivary proline-rich protein 2 (PRP_2), basic salivary proline-rich protein 4 (PRP_4), salivary acidic proline-rich protein 1/2

(PRPC), submaxillary androgen-regulated protein 3B (SMR3B), and statherin (STAT). RT = retention time, AA = amino acid.

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

Table 69: Peptide markers for vaginal fluid. Cornulin (CRNN), involucrin (IVL), periplakin (PPL), and suprabasin (SBSN). RT = retention time, AA = amino acid.

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

4.4.3 Performance Assessment

4.4.3.1 Sensitivity Samples

Sensitivity replicates were prepared by diluting target body fluid with deionized water and applying the dilution to a cotton swab. Therefore, the dilution factor was inherently diluted a second time during the solubilization step of the developed workflow. Data for seminal fluid, saliva, and vaginal fluid dilutions are outlined in Tables 70-72. Proteins were positively identified by the presence of at least one peptide target from the inclusion list. Seminal fluid was reproducibly identified at the lowest dilution factor (10,000-fold) by both SEMG1 and SEMG2. However, the remaining seminal fluid proteins showed poor sensitivity, with little to no identifications at the 100-fold dilution. Salivary protein biomarkers demonstrated robust sensitivity, with a majority of proteins detected at the 100-fold dilution. Basic salivary proline-rich protein 1, salivary acidic proline-rich protein 1/2, and submaxillary gland androgen-regulated protein 3B were detected in at least one replicate at the lowest dilution factor. Vaginal fluid sensitivity was contingent on cornulin identification, which was positive through the 100-fold dilution. The remaining protein biomarkers demonstrated poor sensitivity. Full details for these and all remaining casework samples can be found in appendix N.

Table 70: Sensitivity sample results for seminal fluid. Prostate specific antigen (KLK3), prolactin inducible protein (PIP), prostatic acid phosphatase (PPAP), semenogelin 1 (SEMG1), and semenogelin 2 (SEMG2). (+) indicates at least one peptide detected for the designated protein. (-) indicates a negative result.

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

Table 71: Sensitivity sample results for saliva. (+) indicates at least one peptide detected for the designated protein. (-) indicates a negative result. Histatin-1 (HIS1), basic salivary proline-rich protein 1 (PRP_1), basic salivary proline-rich protein 2 (PRP_2), basic salivary proline-rich protein 4 (PRP_4), salivary acidic proline-rich protein 1/2 (PRPC), submaxillary androgen-regulated protein 3B (SRM3B), and statherin (STAT).

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

Table 72: Sensitivity sample results for vaginal fluid. (+) indicates at least one peptide detected for the designated protein. (-) indicates a negative result. Cornulin (CRNN), involucrin (IVL), periplakin (PPL), and suprabasin (SBSN).

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

4.4.3.2 Aged Samples

The effect of biomarker recovery and characterization from aged biological material was assessed using a two-pronged approach. Each target fluid was evaluated as a single source in addition to a vaginal swab matrix fortified with either semen or saliva. Samples were kept at room temperature for 2, 3, 7, and 30 days, with detailed results outlined in Table 73. Seminal fluid as a single source was positively characterized at every timepoint; however, PPAP exhibited complete dropout. Consistency between replicates was observed, with a single replicate exhibiting a positive KLK3 result. Similar results were obtained for seminal fluid recovered from a vaginal swab matrix. However, complete dropout of PIP was recorded. It is hypothesized that a protease may be present in vaginal

fluid that may degrade PIP past the point of characterization. A comparable observation was made for saliva and saliva recovered from a vaginal swab matrix. Although consistency in replicate characterization was recorded for saliva on its own, complete dropout of PRP_2, histatin-1, and statherin was exhibited when saliva was recovered from a vaginal swab. And lastly, as seen with sensitivity samples, vaginal fluid characterization relied on cornulin detection, which exhibited consistency with each replicate at all timepoints.

Table 73: Aged sample results. (+) indicates at least one peptide detected for the designated protein for each replicate. (-) indicates a negative result. NT = not tested. Prostate specific antigen (KLK3), prolactin inducible protein (PIP), prostatic acid phosphatase (PPAP), semenogelin 1 (SEMG1), and semenogelin 2 (SEMG2), histatin-1 (HIS1), basic salivary proline-rich protein 1 (PRP_1), basic salivary proline-rich protein 2 (PRP_2), basic salivary proline-rich protein 4 (PRP_4), salivary acidic proline-rich protein 1/2 (PRPC), submaxillary androgen-regulated protein 3B (SRM3B), and statherin (STAT), cornulin (CRNN), involucrin (IVL), periplakin (PPL), and suprabasin (SBSN).

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

4.4.3.3 Sexual Assault Samples

Laboratory prepared sexual assault kit samples were generated to simulate authentic samples, with results outlined in Table 74. Of surprise was the ability of the developed method to positively characterize both seminal fluid and saliva when recovered from a rectal swab. Given the endogenous bacterial presence common with this sample type, the observation of reproducible peptide identifications demonstrates the robustness of a peptidomic strategy. Where previous studies have indicated the presence of fecal matter to inhibit the processivity of trypsin, the removal of proteolytic cleavage sites has

eliminated this issue. For example, during validation of the bottom up LC-MS/MS method, KLK3 was not detected on rectal swab samples, whereas this protein was consistently detected on replicates with the peptidomic technique. Furthermore, the recovery of saliva from rectal swabs was included in this portion of research. The presence of fecal matter exhibited no deleterious effects in the ability to identify saliva. For example, salivary protein SMR3B demonstrated excellent ion coverage and spectral quality (Figure 44).

Table 74: Sexual assault kit sample results. (+) indicates at least one peptide detected for the designated protein for each replicate. (-) indicates a negative result. NT = not tested. Prostate specific antigen (KLK3), prolactin inducible protein (PIP), prostatic acid phosphatase (PPAP), semenogelin 1 (SEMG1), and semenogelin 2 (SEMG2), histatin-1 (HIS1), basic salivary proline-rich protein 1 (PRP_1), basic salivary proline-rich protein 2 (PRP_2), basic salivary proline-rich protein 4 (PRP_4), salivary acidic proline-rich protein 1/2 (PRPC), submaxillary androgen-regulated protein 3B (SRM3B), and statherin (STAT), cornulin (CRNN), involucrin (IVL), periplakin (PPL), and suprabasin (SBSN).

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

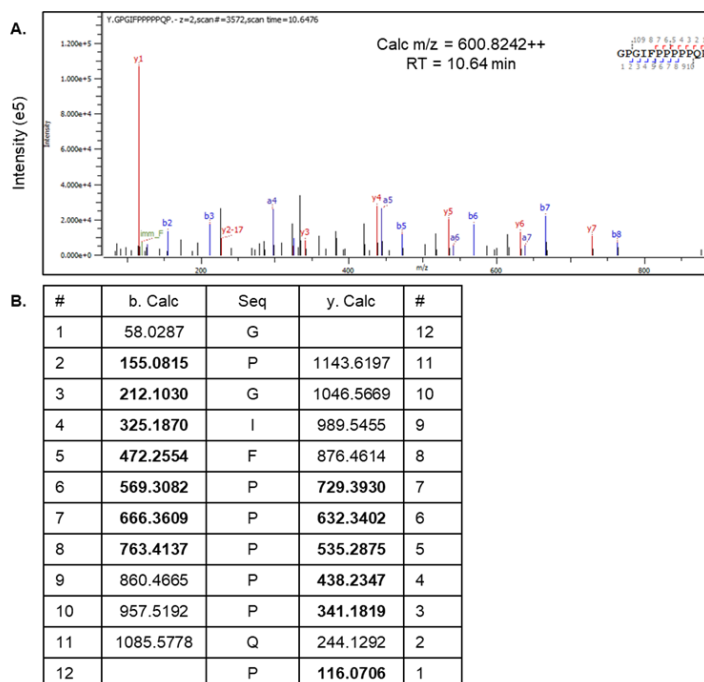


Figure 44: (A) Mass spectrum of saliva recovered from a rectal swab replicate. Depicted is peptide GPGIFPPPPQP of protein submaxillary androgen-regulated protein 3B (SRM3B). **(B)** y and b ions characterized for the peptide sequence are shown in bold type.

4.4.3.4 Mixture Samples

Volume mixtures of seminal fluid and saliva were applied to vaginal swabs to assess ion suppression, particularly for a minor saliva contributor in a major seminal fluid contributor. Vaginal swabs were selected as the matrix in order to simulate sexual assault type samples more closely. In summary, all target fluids were positively identified (Table 75). Seminal fluid is protein rich, and despite the greater volumes, did not inhibit the ability to positively characterize saliva or vaginal fluid peptide targets. Saliva was consistently identified by the presence of PRPC, even when the amount of semen was greater (MIX03) or equivalent to (MIX04) the saliva contribution. As seen with previous performance samples, complete dropout of statherin was exhibited when in the presence of vaginal fluid.

Table 75: Mixture sample results. (+) indicates at least one peptide detected for the designated protein for each replicate. (-) indicates a negative result. Prostate specific antigen (KLK3), prolactin inducible protein (PIP), prostatic acid phosphatase (PPAP), semenogelin 1 (SEMG1), and semenogelin 2 (SEMG2), histatin-1 (HIS1), basic salivary proline-rich protein 1 (PRP_1), basic salivary proline-rich protein 2 (PRP_2), basic salivary proline-rich protein 4 (PRP_4), salivary acidic proline-rich protein 1/2 (PRPC), submaxillary androgen-regulated protein 3B (SRM3B), and statherin (STAT), cornulin (CRNN), involucrin (IVL), periplakin (PPL), and suprabasin (SBSN).

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

4.4.3.5 Contaminant Samples

The recovery of target biomarkers when subjected to chemical insult and environmental contaminants was of interest to this research in order to more closely simulate authentic forensic evidence received in operational laboratories (Table 76). Based on data reported previously, personal lubricants, in addition to bleach and dish soap, were identified as contaminants that have the potential to prevent the identification of seminal fluid. SEMG1 and SEMG2 were positively identified when in the presence of all contaminants. Uniform protein dropout PIP and PPAP was recorded. Overall, the presence of contaminants did not impede the ability to correctly characterize seminal fluid, particularly when in the presence of 10% bleach (Figure 45). SEMG2 peptide NVVDVREE exhibited a clean and clear spectrum, with characteristic b, y, and y++ ions detected.

Saliva characterization in the presence of chemical insults was not as robust as observed with seminal fluid biomarkers. Negative results for all peptide targets were

recorded for saliva treated with 10% bleach. For the remaining samples, salivary acidic proline-rich protein 1/2 and SMR3B were identified by the presence of at least one target peptide. And lastly, vaginal fluid was identified from a menstrual swab based on the positive characterization of cornulin in all three replicates. Detergents and formulations containing halogen substances, such as bleach, present additional challenges with mass spectrometric analysis. Halogens, such as chlorine, have a characteristic isotope distribution on a mass spectra. This distribution is straightforward with small molecule analysis; however, with larger peptide masses, the chlorine isotope distribution can impact the quality of a mass spectrum.

Table 76: Contaminant samples results. (+) indicates at least one peptide detected for the designated protein for each replicate. (-) indicates a negative result. NT = not tested. Prostate specific antigen (KLK3), prolactin inducible protein (PIP), prostatic acid phosphatase (PPAP), semenogelin 1 (SEMG1), and semenogelin 2 (SEMG2), histatin-1 (HIS1), basic salivary proline-rich protein 1 (PRP_1), basic salivary proline-rich protein 2 (PRP_2), basic salivary proline-rich protein 4 (PRP_4), salivary acidic proline-rich protein 1/2 (PRPC), submaxillary androgen-regulated protein 3B (SRM3B), and statherin (STAT), cornulin (CRNN), involucrin (IVL), periplakin (PPL), and suprabasin (SBSN).

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

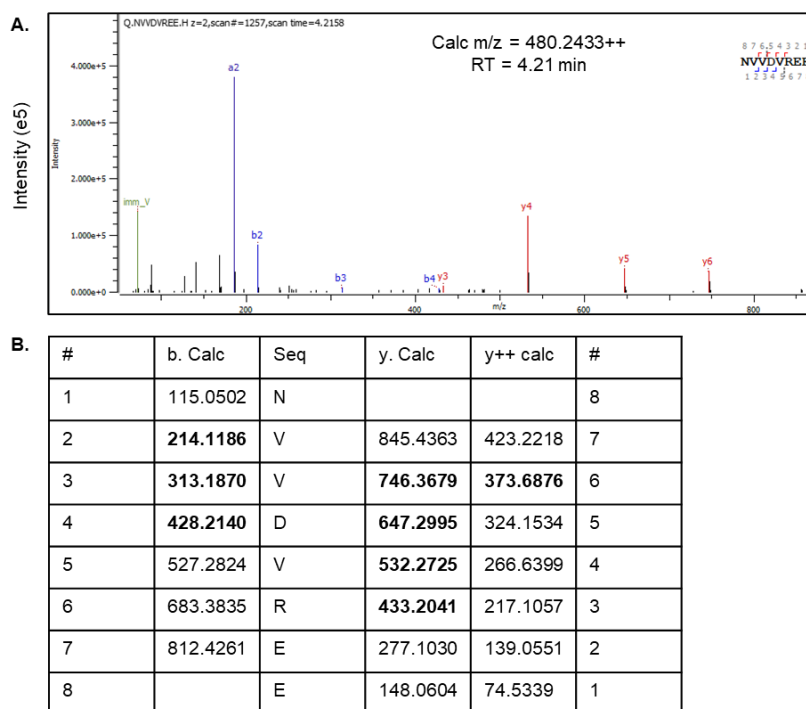


Figure 45: (A) Mass spectrum of seminal fluid treated with 10% bleach. Depicted is peptide NVVDVREE of protein semenogelin 2 (SEMG2). (B) y and b ions characterized for the peptide sequence are shown in bold type.

4.4.3.6 Substrate Samples

In conjunction with contaminants, the ability to positively characterize biological fluids recovered from various substrates was important to consider. Four fabric types were selected for this portion of the study: 100% cotton, 100% polyester, polyester blend (60% polyester, 40% cotton), and denim. Each fabric had small quantities of biological fluid applied, with the 100% cotton underwear worn by a female subject prior to fluid application. Detailed results are outlined in Table 77. As with previous data sets, SEMG1 and SEMG2 outperformed the remaining seminal fluid protein targets. However, all protein targets were characterized when recovered from denim. It is hypothesized that, given the texture of denim fabric, the applied semen was retained over a smaller surface area and easily released back into solution once solubilized. In addition to these samples, semen stains were excised and recovered from bed sheets to assess biomarker loss during laundering. Seminal fluid was positively characterized on both bed sheets evaluated by the presence of SEMG1 and SEMG2, in addition to a single KLK3 peptide identification on the polyester blend bed sheet (Figure 46). Semenogelin 2 peptide NVVDVREE

demonstrated great intensity from laundered items in comparison with the 10% bleach solution, further highlighting the lower sensitivity limits of the developed methodology.

Saliva protein characterization was substrate dependent. As with seminal fluid, recovery of saliva from denim was nearly complete, with protein dropout of basic salivary proline-rich protein 4 exhibited. However, both statherin and SMR3B, confirmatory saliva biomarkers, were identified. Additional protein dropout and greater inconsistency between replicates were reported for polyester blend and 100% polyester (Table 77).

Table 77: Substrate samples results. (+) indicates at least one peptide detected for the designated protein for each replicate. (-) indicates a negative result. NT = not tested. Prostate specific antigen (KLK3), prolactin inducible protein (PIP), prostatic acid phosphatase (PPAP), semenogelin 1 (SEMG1), and semenogelin 2 (SEMG2), histatin-1 (HIS1), basic salivary proline-rich protein 1 (PRP_1), basic salivary proline-rich protein 2 (PRP_2), basic salivary proline-rich protein 4 (PRP_4), salivary acidic proline-rich protein 1/2 (PRPC), submaxillary androgen-regulated protein 3B (SRM3B), and statherin (STAT).

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

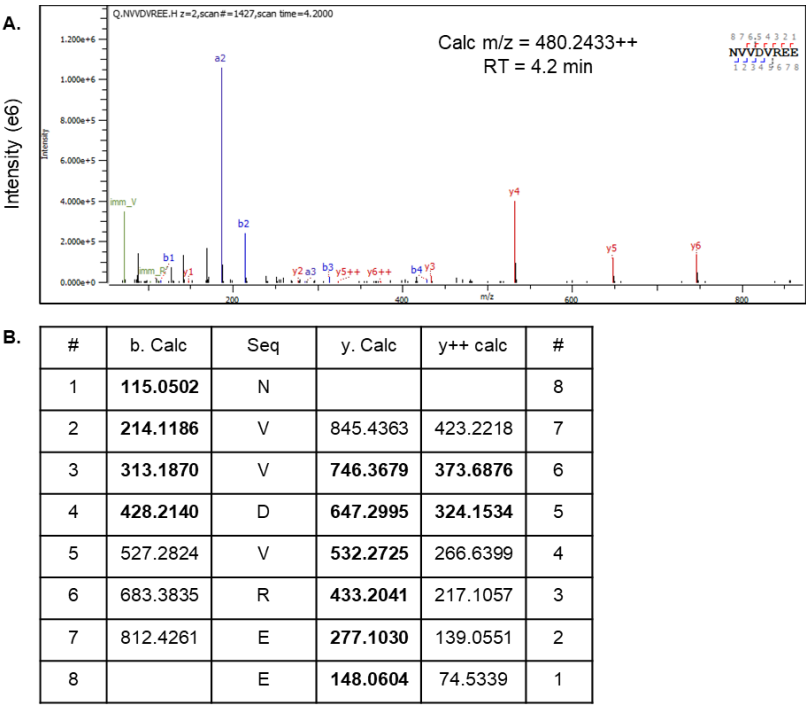


Figure 46: (A) Mass spectrum of seminal fluid recovered from laundered polyester blend bed sheet. Depicted is peptide NVVDVREE of protein semenogelin 2 (SEMG2). **(B)** y and b ions characterized for the peptide sequence are shown in bold type.

4.5 Saliva Identification by Intact High Resolution Mass Spectrometry

Previous sections have shown that protein mass spectrometry is a sensitive and reliable approach for body fluid identification. Moreover, section 4.4, which focused on a protease-free approach, has shown that traditional sample preparation strategies (denaturation, enzymatic digestion, SPE cleanup) can be omitted to increase the throughput of body fluid identification by mass spectrometry. In this section, one-step solid phase extraction as well as anti-protein immunoaffinity purification coupled to intact mass analysis.

The objective of this phase of research was to develop an intact workflow for the identification of human Statherin and assess two separate sample preparation approaches. This was carried out by two specific aims. These included intact statherin confirmation in a small-scale population assessment and a performance assessment. This intact workflow streamlined saliva identification.

4.5.1 Population Assessment

Two extraction methods were developed to assess the recovery and purification of intact Statherin. This included a “routine” SPE-based extraction as well as an immunopurification based assay. Both extraction methods were performed on the AssayMap Bravo. From previous biomarker verification research, it was determined that statherin was consistently detected in 90% of the sample population. Similar results were observed during the present study. Of 20 individuals assessed, statherin was positively identified in 18 samples by the immunopurification extraction and 17 samples by the C18 extraction (Table 78).

Table 78: Population assessment of 20 individuals for the presence of statherin. Y = statherin detected. N = statherin not present. Reinject = samples will be re-analyzed on the Q Exactive.

Sample ID	IP	C18
1	N	N
2	Y	Y
3	Y	Y
4	Y	Y
5	Y	Y
6	Y	Y
7	Y	Y

8	Y	Y
9	Y	Y
10	Y	Y
11	Y	Y
12	Y	Y
13	Y	Y
14	Y	Y
15	Y	Y
16	N	N
17	Y	Y
18	Y	Y
19	Y	Y
20	Y	N

4.5.2 Performance Assessment

Casework-type samples were prepared to assess the performance of the assay and have been divided into the following categories: aged, sensitivity, mixture, substrates, contaminants, and miscellaneous. All sample types were analyzed in duplicate. A complete list of samples and results is outlined in Table 79. Processed samples were analyzed both with the developed immunoprecipitation protocol in addition to a previously developed C18 solid phase microextraction on the AssayMAP Bravo liquid handling platform for comparison purposes. In summary, the immunoprecipitation protocol was successful with identifying statherin, with only one sample type resulting in a negative detection. Statherin in saliva samples treated with a 10% bleach solution failed to be detected. Overall, the immunoprecipitation protocol outperformed the C18 microextraction procedure, particularly in regard to the analysis of mixture samples. Statherin was unable to be identified in all C18 microextraction preparations of mixtures including saliva applied to a menstrual swab and is likely due to the inherent competition on the stationary phase on the sorbent bed.

Sensitivity casework samples were positively identified for statherin regardless of sample preparation technique. However, although the C18 microextraction produced greater signal intensity for concentrated samples, the immunoprecipitation protocol outperformed at the greatest diluted sample (Figure 47). Finally, statherin has been reported

to be detectable in nasal swabs. This was shown to be the case here as well with both extraction procedures successfully identifying statherin from two nasal swabs.

Table 79: Casework-type samples for statherin identification. Y = statherin identified. N = statherin not identified. All substrate control samples were negative of statherin.

Category	Sample Information	Immunoprecipitation		C18 Extraction	
		Replicate 1	Replicate 2	Replicate 1	Replicate 2
Aged Swabs	30 days + 10 ul saliva	Y	Y	Y	Y
	7 days + 10 ul saliva	Y	Y	Y	Y
	3 days + 10 ul saliva	Y	Y	Y	Y
	2 days + 10 ul saliva	Y	Y	Y	Y
Sensitivity	10 ul saliva	Y	Y	Y	Y
	1 ul saliva	Y	Y	Y	Y
	0.5 ul saliva	Y	Y	Y	Y
	0.1 ul saliva	Y	Y	Y	Y
Substrates	jeans + 10 ul saliva	Y	Y	Y	Y
	100% cotton bedsheet + 10 ul saliva	Y	Y	Y	Y
	aluminum can	Y	Y	Y	Y
	cigarette butt	Y	Y	Y	Y
	100% synthetic fabric + 10 ul saliva	Y	Y	Y	Y
Insults	10 ul lubricant + 10 ul saliva	Y	Y	Y	Y
	25 ul lubricant + 10 ul saliva	Y	Y	Y	Y
	25 ul dish soap solution + 10 ul saliva	Y	Y	N	N
	25 ul 10% bleach + 10 ul saliva	N	N	N	N
Mixtures	10 ul blood + 10 ul saliva	Y	Y	N	N
	50 ul blood + 10 ul saliva	Y	Y	N	N
	10 ul semen + 10 ul saliva	Y	Y	N	N
	50 ul semen + 10 ul saliva	Y	Y	N	N
Misc.	skin swab/breast swab + 10 ul saliva	Y	Y	Y	Y
	menstrual swab + 10 ul saliva	Y	Y	N	N
	envelope	Y	Y	Y	Y
	vaginal swab + 10 ul saliva	Y	Y	Y	Y
	Nasal swab	Y	Y	Y	Y
Controls	cotton sheet control	N	N	N	N
	denim control	N	N	N	N
	polyester sheet control	N	N	N	N

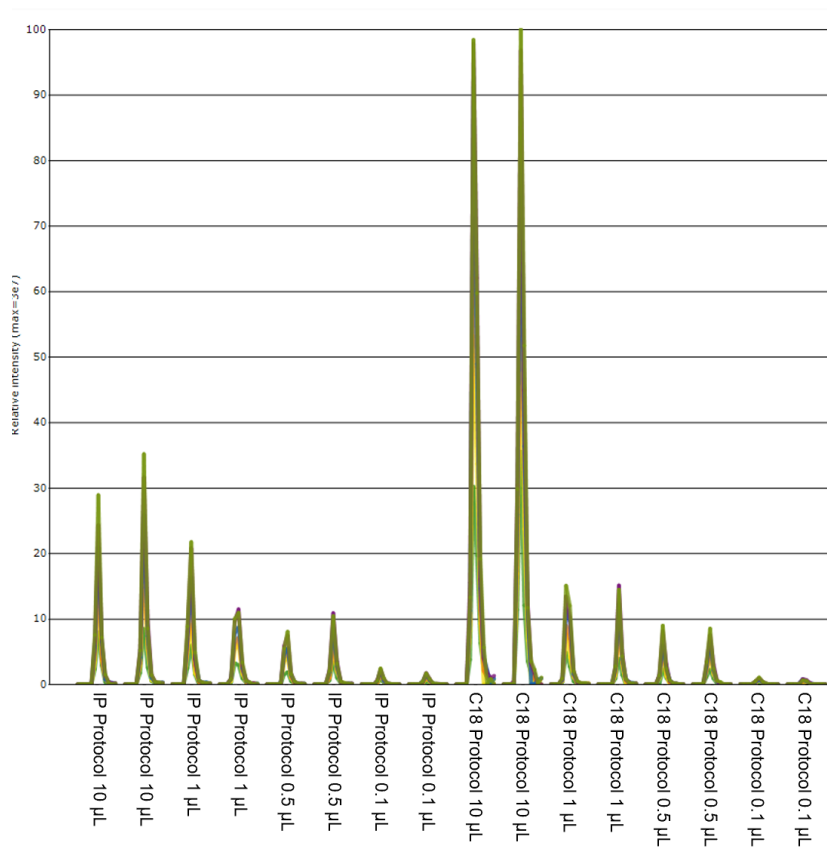


Figure 47: Statherin sensitivity ion distribution for immunoprecipitation (IP) and C18 microextraction procedures. Sensitivity samples represent 10, 1.0, 0.5, and 0.1 µL of saliva.

5 Conclusions

The objective of this project was to develop and rigorously validate a high-specificity forensic serology assay. The assay employed sample preparation robotics and ultra-performance liquid chromatography coupled to triple quadrupole mass spectrometry. Following the successful validation of the assay, additional applications were explored including the development of a robust pre-processing protocol to remove the deleterious effect of personal lubricants, a middle-down peptidomic assay to increase sample throughput, and finally the development and testing of a top-down protocol to identify saliva.

With the completion of the developmental validation in section 4.1, the robustness and reliability of the automated QQQ multiplex assay described was exhaustively demonstrated. Validation studies included repeatability and reproducibility, sensitivity, stability, freeze thaw stability, autosampler stability, mixture studies, specificity studies, various casework samples, the limit of detection, ion suppression, carryover, and blind sample analysis.

In addition, the inherent limitations observed through the analysis of both routine and challenging forensic type evidence have elucidated a defined understanding of presumptive and confirmatory biomarkers utilized for identification. Overall, the culmination of results detailed within section 4.1 are the final product of a fully validated analytical method for the serological analysis of peripheral blood, seminal fluid, saliva, and vaginal/menstrual fluids. With achieving sufficient sensitivity coverage over several orders of magnitude, in addition to demonstrating the specificity of fluid-specific biomarkers, the use of protein mass spectrometry as an advanced serological technique offers significant advantages over currently existing catalytic and immunological assays routinely employed in operational laboratories. The deliverable to the forensic community following the developmental validation results detailed in this section include not only a robust analytical multiplex assay, but comprehensive interpretation guidelines (Appendix I). The purpose of the drafted interpretation guidelines is to serve as a resource for consistent and reliable identification of the body fluids assessed between forensic analysts and laboratories.

Section 4.2 extensively compared the performance of the automated LC-QQQ assay to traditional serological testing strategies. The data demonstrated that the validated

proteomic assay enables significant gains in sensitivity over currently utilized antibody- and enzyme-based screening techniques. Although various types of samples were deployed for this portion of the assessment, perhaps the greatest discrepancies between the traditional and proteomic analyses were observed with the sensitivity samples. The proteomic assay greatly outperformed traditional tests and provided detailed information on the proteins/peptides identified. This information can present other avenues for interpretation and subsequent testing to a forensic analyst. Still, one particular area of advancement worth highlighting is with the analysis of seminal fluid from a vasectomized individual. When considering the scenario of a suspected rape, the ability to accurately characterize semenogelin off a vaginal swab that will test negative for sperm cells has the potential to provide critical information to an investigation. This data further supports the necessity for confirmatory seminal fluid identification, given that not all male perpetrators may contain sperm cells that could be visualized using routine microscopic techniques.

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

With the completion of section 4.3, which assessed the inhibitory effects of personal lubricants on fluid identification by mass spectrometry, it was demonstrated that the deleterious effects of personal lubricants, specifically oil and silicone-based formulas, can be mitigated through the addition of a second solid phase extraction cleanup. When implemented prior to digestion, this cleanup eliminated interferents that would otherwise negatively affect trypsin processivity and mass spectrometry detection. Reverse phase solid phase extraction was used for additional sample cleanup. Reverse phase employs a polar mobile phase and a nonpolar sorbent stationary phase. Given that serological samples are generally maintained in water or saline solution, reverse phase extraction was a clear solution to mitigating the effects of hydrophobic compounds common in lubricant formulas.

Astroglide[®] contains the compound (1-(3-chloroallyl)-3,5,7-triaza-1-azoniaadamantane chloride), referred to as polyquaternium-15. Although water soluble, this compound has surfactant properties. Surfactants, even at low concentrations, can severely suppress ionization during mass spectrometry analysis. Swiss Navy[®], the silicone lubricant selected for this study, contains the compound dimethicone that has surfactant properties as well. Dimethicone is a hydrophobic compound, as is Vitamin E acetate (α -tocopheryl acetate) and cyclopentasiloxane. These three compounds would be irreversibly retained during reverse phase solid phase extraction. In order to selectively remove retained proteins from interferents, a relatively polar elution solvent was necessary. In comparison with other organic solvents such as ethyl acetate or acetone, acetonitrile is a polar solvent. Therefore, protein material would have greater affinity for the elution solvent than severely hydrophobic interferents, such as dimethicone, which would stay retained to the sorbent material. In summary, it was demonstrated that the analysis of sexual assault samples subjected to personal lubricants was achievable with modified sample preparation protocols.

Section 4.4 aimed to improve sample throughput by developing a protease-free approach for fluid identification. In summary, the use of a peptidomic approach for seminal fluid, saliva, and vaginal fluid identification revealed that multiple non-tryptic peptide

fragments are produced through endogenous cleavage and can be analyzed via high resolution mass spectrometry. The protein biomarkers identified with this technique were comparable to those detected with the validated bottom-up LC-MS/MS assay. Therefore, the ability to prepare biological stains for analysis with an expedited, simple extraction prior to chromatographic separation eliminated the need for lengthy enzymatic digestion incubation periods. The results presented provide support for further development of peptidomic applications in the field of forensic serology. From a forensic biology standpoint, it is recommended that reporting of results be based on the identification of inclusion peptides acquired during targeted data dependent analysis. Although additional peptide fragments are identified by the proteomic software, the peptides included in the library have undergone quality control assessment during verification using purified reference material. Significant proteins include semenogelin 1 and 2 for seminal fluid identification in addition to statherin and submaxillary gland androgen-regulated protein 3B for saliva identification.

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

Finally, in section 4.5, direct detection of saliva proteins was enabled through immunopurification and intact mass analysis. This was carried out by identifying a polyclonal anti-statherin antibody and developing an antibody enrichment protocol using the AssayMap bravo. This was coupled to intact mass analysis on the Q-Exactive HRMS. In this study, throughput gains were further achieved compared to the peptidomic approach. Statherin extraction was carried out in approximately 2.5 hours and analyzed in only 10 minutes by mass spectrometry. This strategy proved to be sensitive with sub microliters of saliva readily identified through this approach. In addition, complex two component mixtures could be readily identified.

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

In toto, the specific objectives outlined for completion of this research were successfully evaluated. Proteomic techniques in the form of bottom up, peptidomic, and top down methodologies were demonstrated to surpass the sensitivity and specificity capable of traditional serological screening techniques. The transferring of a multiplex mass spectrometry-based assay from a nanoflow workflow designed previously to high performance LC-MS/MS analytical system sufficiently increased sample throughput. In addition, an automated sample processing procedure mitigated sources of human error and contributed to increased sample throughput without a loss in peptide intensity. The multiplex LC-MS/MS assay underwent rigorous validation and comparison, illustrating its robust performance and compatibilities with existing forensic biology workflows. It has been demonstrated that the ability to produce serological information at sensitivity levels consistent with STR/Y-STR typing is achievable with protein mass spectrometry technology. Body fluid identification of samples subjected to personal lubricants was

achieved through specific sample preparation procedures and detection of surrogate peptides using the validated LC-MS/MS assay. an expedited proteomic analysis of body fluids consistent with sexual assault evidence was developed using peptidomic techniques. The use of proteomics and advanced analytical instrumentation is a viable solution to meeting the sensitivity and specificity demands of the forensic biology community for the confirmation of biological origin, therefore contributing to the identification and individualization of evidentiary material. And lastly, an intact methodology was developed and assessed to identify saliva in forensic samples.

6 Recommendations for Further Applications

The identification of biological stains has posed significant challenges since the onset and acceleration of DNA individualization. Currently, practitioners have no means of reliably confirming the presence of biological stains, especially the differentiation of saliva and vaginal fluid. The presumptive results obtained through serological screening prevent definitive statements regarding the origin of a biological stain by an expert witness during courtroom testimony. While implementing a new technology such as protein mass spectrometry comes with significant onboarding actions, such as new analyst training and purchasing of instrumentation, the cost-to-benefit ratio of investing in this next generation technique is substantial.

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

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

evidence submitted in sexual assault cases involving vaginal rape with a foreign object would greatly benefit from such an assay.

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

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

7 References

- [1] E. Locard, *L'enquête criminelle et les méthodes scientifiques*, E. Flammarion, Paris, 1920.
- [2] Federal Bureau of Investigation, CODIS-NDIS Statistics, (2021).
<https://www.fbi.gov/services/laboratory/biometric-analysis/codis/ndis-statistics> (accessed May 12, 2021).
- [3] S. Stanton, D. Smith, D. Kasler, M. Sullivan, 'Day of reckoning' — Joseph DeAngelo admits guilt in Golden State Killer murders, rapes, *The Modesto Bee*. (2020).
<https://www.modbee.com/news/california/article243868517.html>.
- [4] Man convicted in 1987 killings of Canadian couple gets life, *Associated Press*. (2019).
<https://apnews.com/article/3ac9a37c2203445ea0c5cf44f371bec0>.
- [5] R.A.H. van Oorschot, D.L. McColl, J.E. Alderton, M.L. Harvey, R.J. Mitchell, B. Szkuta, Activities between activities of focus-Relevant when assessing DNA transfer probabilities, *Forensic Science International: Genetics Supplement Series*. 5 (2015) e75–e77.
<https://doi.org/10.1016/j.fsigss.2015.09.031>.
- [6] B. Martin, R. Blackie, D. Taylor, A. Linacre, DNA profiles generated from a range of touched sample types, *Forensic Science International: Genetics*. 36 (2018) 13–19.
<https://doi.org/10.1016/j.fsigen.2018.06.002>.
- [7] G.E. Meakin, E. v. Butcher, R.A.H. van Oorschot, R.M. Morgan, Trace DNA evidence dynamics: An investigation into the deposition and persistence of directly- and indirectly-transferred DNA on regularly-used knives, *Forensic Science International: Genetics*. 29 (2017) 38–47.
<https://doi.org/10.1016/j.fsigen.2017.03.016>.
- [8] B. Szkuta, K.N. Ballantyne, B. Kokshoorn, R.A.H. van Oorschot, Transfer and persistence of non-self DNA on hands over time: Using empirical data to evaluate DNA evidence given activity level propositions, *Forensic Science International: Genetics*. 33 (2018) 84–97.
<https://doi.org/10.1016/j.fsigen.2017.11.017>.
- [9] P. Ramos, O. Handt, D. Taylor, Investigating the position and level of DNA transfer to undergarments during digital sexual assault, *Forensic Science International: Genetics*. 47 (2020) 102316. <https://doi.org/10.1016/j.fsigen.2020.102316>.
- [10] R.A.H. van Oorschot, B. Szkuta, G.E. Meakin, B. Kokshoorn, M. Goray, DNA transfer in forensic science: A review, *Forensic Science International: Genetics*. 38 (2019) 140–166.
<https://doi.org/10.1016/j.fsigen.2018.10.014>.
- [11] P. Gill, J. Whitaker, C. Flaxman, N. Brown, J. Buckleton, An investigation of the rigor of interpretation rules for STRs derived from less than 100 pg of DNA, *Forensic Science International*. 112 (2000) 17–40. [https://doi.org/10.1016/S0379-0738\(00\)00158-4](https://doi.org/10.1016/S0379-0738(00)00158-4).
- [12] M.D. Coble, J.A. Bright, Probabilistic genotyping software: An overview, *Forensic Science International: Genetics*. 38 (2019) 219–224. <https://doi.org/10.1016/j.fsigen.2018.11.009>.
- [13] J.G. Cino, Deploying the Secret Police: the Use of Algorithms in the Criminal Justice System, *Georgia State University Law Review*. 34 (2018).
- [14] P.W. Nutter, Machine Learning Evidence: Admissibility and Weight, *University of Pennsylvania Journal of Constitutional Law*. 21 (2019). <https://edition.cnn.com/2018/03/22/us/>.
- [15] R. Li, *Forensic Biology*, 2nd ed., Taylor & Francis Group, LLC, Boca Raton, FL, 2015.

- [16] V. Barrera, B. Fliss, S. Panzer, S.A. Bolliger, Gunshot residue on dark materials: a comparison between infrared photography and the use of an alternative light source, *International Journal of Legal Medicine*. 133 (2019) 1115–1120. <https://doi.org/10.1007/s00414-018-1965-7>.
- [17] K.G. Wiggins, Forensic Textile Fiber Examination Across the USA and Europe, *Journal of Forensic Sciences*. 46 (2001) 15150J. <https://doi.org/10.1520/jfs15150j>.
- [18] A. Zamir, B. Springer, B. Glattstein, Fingerprints and DNA: STR Typing of DNA Extracted from Adhesive Tape after Processing for Fingerprints, *Journal of Forensic Sciences*. 45 (2000) 14749J. <https://doi.org/10.1520/jfs14749j>.
- [19] R.E. Gaennsslen, F.R. Camp, Sourcebook in forensic serology, immunology and biochemistry, *Forensic Science International*. 14 (1979) 147–148. [https://doi.org/10.1016/0379-0738\(79\)90239-1](https://doi.org/10.1016/0379-0738(79)90239-1).
- [20] M.N. Hochmeister, B. Budowle, R. Sparkes, O. Rudin, C. Gehrig, M. Thali, L. Schmidt, A. Cordier, R. Dirnhofer, Validation studies of an immunochromatographic 1-step test for the forensic identification of human blood., *Journal of Forensic Sciences*. 44 (1999) 597–602.
- [21] J. Glaister, The Kastle-Meyer Test for the Detection of Blood: Considered from the Medico-Legal Aspect, *BMJ*. 1 (1926) 650–652. <https://doi.org/10.1136/bmj.1.3406.650>.
- [22] J.L. Webb, J.I. Creamer, T.I. Quickenden, A comparison of the presumptive luminol test for blood with four non-chemiluminescent forensic techniques, *Luminescence*. 21 (2006) 214–220. <https://doi.org/10.1002/bio.908>.
- [23] J.I. Creamer, T.I. Quickenden, M. v. Apanah, K.A. Kerr, P. Robertson, A comprehensive experimental study of industrial, domestic and environmental interferences with the forensic luminol test for blood, *Luminescence*. 18 (2003) 193–198. <https://doi.org/10.1002/bio.723>.
- [24] A.M. Gross, K.A. Harris, G.L. Kaldun, The effect of luminol on presumptive tests and DNA analysis using the polymerase chain reaction, *J Forensic Sci*. 44 (1999) 837–840. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10432617.
- [25] D.J.A. Kerr, V.H. Mason, The Haemochromogen Crystal Test for Blood, *BMJ*. 1 (1926) 134–136. <https://doi.org/10.1136/bmj.1.3395.134>.
- [26] N. Pernick, PAS (Periodic Acid-Schiff, *PathologyOutlines.Com*. (2018). <http://www.pathologyoutlines.com/topic/stainspas.html>.
- [27] B. Randall, Glycogenated squamous epithelial cells as a marker of foreign body penetration in sexual assault., *Journal of Forensic Sciences*. 33 (1988) 511–514.
- [28] R. Hausmann, B. Schellmann, Forensic value of the Lugol's staining method: Further studies on glycogenated epithelium in the male urinary tract, *International Journal of Legal Medicine*. 107 (1994) 147–151. <https://doi.org/10.1007/BF01225602>.
- [29] R. Hausmann, C. Pregler, B. Schellmann, The value of the Lugol's iodine staining technique for the identification of vaginal epithelial cells, *International Journal of Legal Medicine*. 106 (1994) 298–301. <https://doi.org/10.1007/BF01224775>.
- [30] K. Mirakovitz, *Forensic Science: The Basics*, 2nd ed., CRC Press, 2016.
- [31] E.T. Dane, D.L. Herman, Haematoxylin-phyloxine-alcian blue-orange G differential staining of prekeratin, keratin and mucin, *Stain Technology*. 38 (1963) 97–101.

- [32] C.E. v French, C.G. Jensen, S.K. Vintiner, D.A. Elliot, S.R. McGlashan, A novel histological technique for distinguishing between epithelial cells in forensic casework, *Forensic Science International*. 178 (2008) 1–6. <https://doi.org/10.1016/j.forsciint.2008.01.010>.
- [33] J.-P. Allery, N. Telmon, R. Mieusset, A. Blanc, D. Rouge, Cytological detection of spermatozoa: comparison of three staining methods, *Journal of Forensic Sciences*. 46 (2001) 349–351. <https://doi.org/10.1520/JFS14970J>.
- [34] C.G. Westring, M. Wiuf, S.J. Nielsen, J.C. Fogleman, J.B. Old, C. Lenz, K.A. Reich, N. Morling, SPERM HY-LITER for the identification of spermatozoa from sexual assault evidence, *Forensic Science International: Genetics*. 12 (2014) 161–167. <https://doi.org/10.1016/j.fsigen.2014.06.003>.
- [35] K.W.P. Miller, J. Old, B.R. Fischer, B. Schweers, S. Stipinaite, K. Reich, Developmental validation of the SPERM HY-LITER™ Kit for the identification of human spermatozoa in forensic samples, *Journal of Forensic Sciences*. 56 (2011) 853–865. <https://doi.org/10.1111/j.1556-4029.2011.01796.x>.
- [36] P.S. Raju, N.K. Iyengar, Acid Phosphatase Reaction as a Specific Test for the Identification of Seminal Stains, *The Journal of Criminal Law, Criminology, and Police Science*. 55 (1964) 522. <https://doi.org/10.2307/1140908>.
- [37] P.J. Ablett, The Identification of the Precise Conditions for Seminal Acid Phosphatase (SAP) and Vaginal Acid Phosphatase (VAP) Separation by Isoelectric Focusing Patterns, *Journal of the Forensic Science Society*. 23 (1983) 254–256. [https://doi.org/10.1016/S0015-7368\(83\)72254-1](https://doi.org/10.1016/S0015-7368(83)72254-1).
- [38] A.F. Schiff, Reliability of the Acid Phosphatase Test for the Identification of Seminal Fluid, *Journal of Forensic Sciences*. 23 (1978) 10745J. <https://doi.org/10.1520/JFS10745J>.
- [39] B.C.M. Pang, B.K.K. Cheung, Applicability of two commercially available kits for forensic identification of saliva stains, *Journal of Forensic Sciences*. 53 (2008) 1117–1122. <https://doi.org/10.1111/j.1556-4029.2008.00814.x>.
- [40] J.R. Myers, W.K. Adkins, Comparison of Modern Techniques for Saliva Screening, n.d.
- [41] B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts, P. Walter, eds., *Molecular Biology of the Cell*, 5th ed., Garland Science, Taylor Francis Group, LLC, New York, New York, 2008.
- [42] K. Reich, Forensic test for human semen, US 2007/0196871A1, 2007.
- [43] K. Reich, Forensic test for human saliva, US 2007/0092977A1, 2007.
- [44] K. Reich, Forensic test for human blood, US 2008/0171399A1, 2008.
- [45] P.S. Bagaria, Test device for detecting semen and method of use, 6,686,167B2, 2004.
- [46] P.S. Bagaria, Test device for detecting human blood and method of use, 7,067,264B2, 2006.
- [47] Seratec®, SERATEC® HemDirect Hemoglobin Assay A visual one-step immunoassay for the rapid identification of human blood in forensic samples by the determination of hemoglobin, Göttingen, Germany, 2009.
- [48] Seratec®, SERATEC® AmylaseTest: An Overview for Users, Göttingen, Germany, 2017. https://www.seratec.com/docs/AmylaseTest_05_2017.pdf.
- [49] Seratec®, Seratec® PSA Semiquant In-vitro diagnostic test for professional forensic use for the detection of seminal fluid by the semi-quantitative determination of PSA (Prostate-specific antigen), Göttingen, Germany, 2011.

- [50] J. Old, B. Schweers, P.W. Boonlayangoor, K. Reich, Developmental Validation Studies of RSID™ - Semen A Lateral Flow Immunochromatographic Strip test for the Forensic Detection of Seminal Fluid, 2010.
- [51] J. Old, B. Schweers, P.W. Boonlayangoor, K. Reich, Developmental Validation Studies of RSID-Saliva Lateral Flow Immunochromatographic Strip test for the forensic detection of Saliva, (2006).
- [52] H.E. McKiernan, Targeted-Ion Mass Spectrometry for the Identification of Forensically Relevant Biological Fluids and Samples from Sexual Assault Evidence, 2019.
- [53] S.E. Bitner, False Positives Observed on the Seratec PSA SemiQuant Cassette Test with Condom Lubricants, *Journal of Forensic Sciences*. 57 (2012) 1545–1548. <https://doi.org/10.1111/j.1556-4029.2012.02141.x>.
- [54] M.C. Snead, A.P. Kourtis, C.M. Black, C.K. Mauck, T.M. Brown, A. Penman-Aguilar, J.H. Melendez, M.F. Gallo, D.J. Jamieson, M. Macaluso, Effect of topical vaginal products on the detection of prostate-specific antigen, a biomarker of semen exposure, using ABACards, *Contraception*. 88 (2013) 382–386. <https://doi.org/10.1016/j.contraception.2012.10.034>.
- [55] M.M. Foley, C.O. Brown, C.G. Westring, P.B. Danielson, H.E. McKiernan, Effects of organic acids and common household products on the occurrence of false positive test results using immunochromatographic assays, *Forensic Science International*. 308 (2020). <https://doi.org/10.1016/j.forsciint.2020.110165>.
- [56] A. Vidaki, M. Kayser, Recent progress, methods and perspectives in forensic epigenetics, *Forensic Science International: Genetics*. 37 (2018) 180–195. <https://doi.org/10.1016/j.fsigen.2018.08.008>.
- [57] D.M. Jeziorska, R.J.S. Murray, M. de Gobbi, R. Gaentzsch, D. Garrick, H. Ayyub, T. Chen, E. Li, J. Telenius, M. Lynch, B. Graham, A.J.H. Smith, J.N. Lund, J.R. Hughes, D.R. Higgs, C. Tufarelli, DNA methylation of intragenic CpG islands depends on their transcriptional activity during differentiation and disease, *Proceedings of the National Academy of Sciences of the United States of America*. 114 (2017) E7526–E7535. <https://doi.org/10.1073/pnas.1703087114>.
- [58] T. Madi, K. Balamurugan, R. Bombardi, G. Duncan, B. Mccord, The determination of tissue-specific DNA methylation patterns in forensic biofluids using bisulfite modification and pyrosequencing, *Electrophoresis*. 33 (2012) 1736–1745. <https://doi.org/10.1002/elps.201100711>.
- [59] D. Frumkin, A. Wasserstrom, B. Budowle, A. Davidson, DNA methylation-based forensic tissue identification, *Forensic Science International: Genetics*. 5 (2011) 517–524. <https://doi.org/10.1016/j.fsigen.2010.12.001>.
- [60] P. Nyrén, B. Pettersson, M. Uhlén, Solid phase DNA minisequencing by an enzymatic luminometric inorganic pyrophosphate detection assay, *Analytical Biochemistry*. 208 (1993) 171–175. <https://doi.org/10.1006/abio.1993.1024>.
- [61] J. Antunes, D.S.B.S. Silva, K. Balamurugan, G. Duncan, C.S. Alho, B. Mccord, High-resolution melt analysis of DNA methylation to discriminate semen in biological stains, *Analytical Biochemistry*. 494 (2016) 40–45. <https://doi.org/10.1016/j.ab.2015.10.002>.

- [62] S. Forat, B. Huettel, R. Reinhardt, R. Fimmers, G. Haidl, D. Denschlag, K. Olek, Methylation markers for the identification of body fluids and tissues from forensic trace evidence, *PLoS ONE*. 11 (2016) 1–19. <https://doi.org/10.1371/journal.pone.0147973>.
- [63] Y.C. Lin, L.C. Tsai, J.C.I. Lee, C.W. Su, J.T.C. Tzen, A. Linacre, H.M. Hsieh, Novel identification of biofluids using a multiplex methylation sensitive restriction enzyme-PCR system, *Forensic Science International: Genetics*. 25 (2016) 157–165. <https://doi.org/10.1016/j.fsigen.2016.08.011>.
- [64] Q.T. Gauthier, S. Cho, J.H. Carmel, B.R. McCord, Development of a body fluid identification multiplex via DNA methylation analysis, *Electrophoresis*. 40 (2019) 2565–2574. <https://doi.org/10.1002/elps.201900118>.
- [65] A. Vidaki, D. Ballard, A. Aliferi, T.H. Miller, L.P. Barron, D. Syndercombe Court, DNA methylation-based forensic age prediction using artificial neural networks and next generation sequencing, *Forensic Science International: Genetics*. 28 (2017) 225–236. <https://doi.org/10.1016/j.fsigen.2017.02.009>.
- [66] H. Holtkötter, K. Schwender, P. Wiegand, H. Pfeiffer, M. Vennemann, Marker evaluation for differentiation of blood and menstrual fluid by methylation-sensitive snapshot analysis, *International Journal of Legal Medicine*. 132 (2018) 387–395. <https://doi.org/10.1007/s00414-018-1770-3>.
- [67] S. Silva, J. Antunes, K. Balamurugan, G. Duncan, C.S. Alho, B. Mccord, Evaluation of DNA methylation markers and their potential to predict human aging, *Electrophoresis*. 36 (2015) 1775–1780. <https://doi.org/10.1002/elps.201500137>.
- [68] J. Juusola, J. Ballantyne, Messenger RNA profiling: A prototype method to supplant conventional methods for body fluid identification, *Forensic Science International*. 135 (2003) 85–96. [https://doi.org/10.1016/S0379-0738\(03\)00197-X](https://doi.org/10.1016/S0379-0738(03)00197-X).
- [69] J. Juusola, J. Ballantyne, Multiplex mRNA profiling for the identification of body fluids, *Forensic Science International*. 152 (2005) 1–12. <https://doi.org/10.1016/j.forsciint.2005.02.020>.
- [70] J. Juusola, J. Ballantyne, mRNA profiling for body fluid identification by multiplex quantitative RT-PCR, *Journal of Forensic Sciences*. 52 (2007) 1252–1262. <https://doi.org/10.1111/j.1556-4029.2007.00550.x>.
- [71] C. Haas, E. Hanson, M.J. Anjos, W. Bar, R. Banemann, A. Berti, E. Borges, C. Bouakaze, A. Carracedo, M. Carvalho, V. Castella, A. Choma, G. de Cock, M. Dotsch, P. Hoff-Olsen, P. Johansen, F. Kohlmeier, P.A. Lindenbergh, B. Ludes, O. Maronas, D. Moore, M.L. Morerod, N. Morling, H. Niederstätter, F. Noel, W. Parson, G. Patel, C. Popielarz, E. Salata, P.M. Schneider, T. Sijen, B. Sviezena, M. Turanska, L. Zatkalikovi, J. Ballantyne, RNA/DNA co-analysis from blood stains - Results of a second collaborative EDNAP exercise, *Forensic Science International: Genetics*. 6 (2012) 70–80. <https://doi.org/10.1016/j.fsigen.2011.02.004>.
- [72] C. Haas, E. Hanson, M.J. Anjos, R. Banemann, A. Berti, E. Borges, A. Carracedo, M. Carvalho, C. Courts, G. de Cock, M. Dötsch, S. Flynn, I. Gomes, C. Hollard, B. Hjort, P. Hoff-Olsen, K. Hríbková, A. Lindenbergh, B. Ludes, O. Maroñas, N. McCallum, D. Moore, N. Morling, H. Niederstätter, F. Noel, W. Parson, C. Popielarz, C. Rapone, A.D. Roeder, Y. Ruiz, E. Sauer, P.M. Schneider, T. Sijen, D.S. Court, B. Sviežená, M. Turanská, A. Vidaki, L. Zatkáliková, J. Ballantyne, RNA/DNA co-analysis from human saliva and semen stains-Results of a third collaborative EDNAP exercise,

- Forensic Science International: Genetics. 7 (2013) 230–239.
<https://doi.org/10.1016/j.fsigen.2012.10.011>.
- [73] C. Haas, E. Hanson, M.J. Anjos, K.N. Ballantyne, R. Banemann, B. Bhoelai, E. Borges, M. Carvalho, C. Courts, G. de Cock, K. Drobic, M. Dötsch, R. Fleming, C. Franchi, I. Gomes, G. Hadzic, S.A. Harbison, J. Hartevelde, B. Hjort, C. Hollard, P. Hoff-Olsen, C. Hüls, C. Keyser, O. Maroñas, N. McCallum, D. Moore, N. Morling, H. Niederstätter, F. Noël, W. Parson, C. Phillips, C. Popielarz, A.D. Roeder, L. Salvaderi, E. Sauer, P.M. Schneider, G. Shanthan, D.S. Court, M. Turanská, R.A.H. van Oorschot, M. Vennemann, A. Vidaki, L. Zatkáliková, J. Ballantyne, RNA/DNA co-analysis from human menstrual blood and vaginal secretion stains: Results of a fourth and fifth collaborative EDNAP exercise, *Forensic Science International: Genetics*. 8 (2014) 203–212.
<https://doi.org/10.1016/j.fsigen.2013.09.009>.
- [74] P.P. Albani, J. Patel, R.I. Fleming, Detection of five specified menstrual fluid mRNA markers over the uterine cycle, *Forensic Science International: Genetics*. 49 (2020) 102359.
<https://doi.org/10.1016/j.fsigen.2020.102359>.
- [75] C. Haas, E. Hanson, R. Banemann, A.M. Bento, A. Berti, Á. Carracedo, C. Courts, G. de Cock, K. Drobic, R. Fleming, C. Franchi, I. Gomes, G. Hadzic, S.A. Harbison, B. Hjort, C. Hollard, P. Hoff-Olsen, C. Keyser, A. Kondili, T. Sijen, D.S. Court, M. Turanská, M. van den Berge, M. Vennemann, A. Vidaki, L. Zatkáliková, J. Ballantyne, RNA/DNA co-analysis from human skin and contact traces – results of a sixth collaborative EDNAP exercise, *Forensic Science International: Genetics*. 16 (2015) 139–147. <https://doi.org/10.1016/j.fsigen.2015.01.002>.
- [76] C. Nussbaumer, E. Gharehbaghi-Schnell, I. Korschneck, Messenger RNA profiling: A novel method for body fluid identification by Real-Time PCR, *Forensic Science International*. 157 (2006) 181–186. <https://doi.org/10.1016/j.forsciint.2005.10.009>.
- [77] C. Haas, B. Klessner, C. Maake, W. Bär, A. Kratzer, mRNA profiling for body fluid identification by reverse transcription endpoint PCR and realtime PCR, *Forensic Science International: Genetics*. 3 (2009) 80–88. <https://doi.org/10.1016/j.fsigen.2008.11.003>.
- [78] E. Hanson, J. Ballantyne, Circulating microRNA for the identification of forensically relevant body fluids, *Methods in Molecular Biology*. 1024 (2013) 221–234.
- [79] P. Danaher, R.L. White, E.K. Hanson, J. Ballantyne, Facile semi-automated forensic body fluid identification by multiplex solution hybridization of NanoString barcode probes to specific mRNA targets, *Forensic Science International: Genetics*. 14 (2015) 18–30.
<https://doi.org/10.1016/j.fsigen.2014.09.005>.
- [80] S. Ingold, G. Dørum, E. Hanson, A. Berti, W. Branicki, Body fluid identification using a targeted mRNA massively parallel sequencing approach – results of a EUROFORGEN/EDNAP collaborative exercise, *Forensic Science International: Genetics*. 34 (2018) 105–115.
<https://doi.org/10.1016/j.fsigen.2018.01.002>.
- [81] S. Ingold, G. Dørum, E. Hanson, J. Ballantyne, C. Haas, Assigning forensic body fluids to donors in mixed body fluids by targeted RNA/DNA deep sequencing of coding region SNPs, *International Journal of Legal Medicine*. (2020) 473–485. <https://doi.org/10.1007/s00414-020-02252-w>.
- [82] B. Liu, Q. Yang, H. Meng, C. Shao, J. Jiang, H. Xu, K. Sun, Y. Zhou, Y. Yao, Z. Zhou, H. Li, Y. Shen, Z. Zhao, Q. Tang, J. Xie, Development of a multiplex system for the identification of forensically

- relevant body fluids, *Forensic Science International: Genetics*. 47 (2020).
<https://doi.org/10.1016/j.fsigen.2020.102312>.
- [83] M. Setzer, J. Juusola, J. Ballantyne, Recovery and stability of RNA in vaginal swabs and blood, semen, and saliva stains, *Journal of Forensic Sciences*. 53 (2008) 296–305.
<https://doi.org/10.1111/j.1556-4029.2007.00652.x>.
- [84] D. Zubakov, E. Hanekamp, M. Kokshoorn, W. van IJcken, M. Kayser, Stable RNA markers for identification of blood and saliva stains revealed from whole genome expression analysis of time-wise degraded samples, *International Journal of Legal Medicine*. 122 (2008) 135–142.
<https://doi.org/10.1007/s00414-007-0182-6>.
- [85] S.S. Silva, C. Lopes, A.L. Teixeira, M.J.C. de Sousa, R. Medeiros, Forensic miRNA: Potential biomarker for body fluids?, *Forensic Science International: Genetics*. 14 (2015) 1–10.
<https://doi.org/10.1016/j.fsigen.2014.09.002>.
- [86] E. Hanson, H. Lubenow, J. Ballantyne, Identification of forensically relevant body fluids using a panel of differentially expressed microRNAs, *Forensic Science International: Genetics Supplement Series*. 2 (2009) 503–504. <https://doi.org/10.1016/j.fsigs.2009.08.184>.
- [87] D. Zubakov, A.W.M. Boersma, Y. Choi, P.F. van Kuijk, E.A.C. Wiemer, M. Kayser, MicroRNA markers for forensic body fluid identification obtained from microarray screening and quantitative RT-PCR confirmation, *International Journal of Legal Medicine*. 124 (2010) 217–226.
<https://doi.org/10.1007/s00414-009-0402-3>.
- [88] C. Courts, B. Madea, Specific micro-RNA signatures for the detection of saliva and blood in forensic body-fluid identification, *Journal of Forensic Sciences*. 56 (2011) 1464–1470.
<https://doi.org/10.1111/j.1556-4029.2011.01894.x>.
- [89] E.K. Hanson, M. Mirza, K. Rekab, J. Ballantyne, The identification of menstrual blood in forensic samples by logistic regression modeling of miRNA expression, *Electrophoresis*. 35 (2014) 3087–3095. <https://doi.org/10.1002/elps.201400171>.
- [90] J.L. Park, S.M. Park, O.H. Kwon, H. chul Lee, J. young Kim, H.H. Seok, W.S. Lee, S.H. Lee, Y.S. Kim, K.M. Woo, S.Y. Kim, Microarray screening and qRT-PCR evaluation of microRNA markers for forensic body fluid identification, *Electrophoresis*. 35 (2014) 3062–3068.
<https://doi.org/10.1002/elps.201400075>.
- [91] E. Sauer, A.K. Reinke, C. Courts, Differentiation of five body fluids from forensic samples by expression analysis of four microRNAs using quantitative PCR, *Forensic Science International: Genetics*. 22 (2016) 89–99. <https://doi.org/10.1016/j.fsigen.2016.01.018>.
- [92] S. Seashols-Williams, C. Lewis, C. Calloway, N. Peace, A. Harrison, C. Hayes-Nash, S. Fleming, Q. Wu, Z.E. Zehner, High-throughput miRNA sequencing and identification of biomarkers for forensically relevant biological fluids, *Electrophoresis*. 37 (2016) 2780–2788.
<https://doi.org/10.1002/elps.201600258>.
- [93] M. Sirker, R. Fimmers, P.M. Schneider, I. Gomes, Evaluating the forensic application of 19 target microRNAs as biomarkers in body fluid and tissue identification, *Forensic Science International: Genetics*. 27 (2017) 41–49. <https://doi.org/10.1016/j.fsigen.2016.11.012>.
- [94] C. Mayes, S. Seashols-Williams, S. Hughes-Stamm, A capillary electrophoresis method for identifying forensically relevant body fluids using miRNAs, *Legal Medicine*. 30 (2018) 1–4.
<https://doi.org/10.1016/j.legalmed.2017.10.013>.

- [95] L.J. Saidi, L.H. Müller, B. Madea, M. Grabmüller, Specific miRNA profiling from DNA eluates for body fluid identification, *Forensic Science International: Genetics Supplement Series*. 7 (2019) 692–694. <https://doi.org/10.1016/j.fsigss.2019.10.141>.
- [96] S. Cassol, M.J. Gill, R. Pilon, M. Cormier, R.F. Voigt, B. Willoughby, J. Forbes, Quantification of human immunodeficiency virus type 1 RNA from dried plasma spots collected on filter paper, *Journal of Clinical Microbiology*. 35 (1997) 2795–2801. <https://doi.org/10.1128/jcm.35.11.2795-2801.1997>.
- [97] R.T. Marquez, S. Bandyopadhyay, E.B. Wendlandt, K. Keck, B.A. Hoffer, M.S. Icardi, R.N. Christensen, W.N. Schmidt, A.P. McCaffrey, Correlation between microRNA expression levels and clinical parameters associated with chronic hepatitis C viral infection in humans, *Laboratory Investigation*. 90 (2010) 1727–1736. <https://doi.org/10.1038/labinvest.2010.126>.
- [98] J.G. Grasselli, M.K. Snavely, B.J. Bulkin, Applications of Raman spectroscopy, *Physics Reports*. 65 (1980) 231–344. [https://doi.org/10.1016/0370-1573\(80\)90065-4](https://doi.org/10.1016/0370-1573(80)90065-4).
- [99] C.M. Hodges, J. Akhavan, The use of Fourier Transform Raman spectroscopy in the forensic identification of illicit drugs and explosives, *Spectrochimica Acta Part A: Molecular Spectroscopy*. 46 (1990) 303–307. [https://doi.org/10.1016/0584-8539\(90\)80098-J](https://doi.org/10.1016/0584-8539(90)80098-J).
- [100] M.J. West, M.J. Went, Detection of drugs of abuse by Raman spectroscopy, *Drug Testing and Analysis*. 3 (2011) 532–538. <https://doi.org/10.1002/dta.217>.
- [101] L. Burgio, R.J.H. Clark, R.R. Hark, Raman microscopy and x-ray fluorescence analysis of pigments on medieval and Renaissance Italian manuscript cuttings, *Proceedings of the National Academy of Sciences of the United States of America*. 107 (2010) 5726–5731. <https://doi.org/10.1073/pnas.0914797107>.
- [102] E.M. Suzuki, M. Carrabba, In Situ Identification and Analysis of Automotive Paint Pigments Using Line Segment Excitation Raman Spectroscopy: I. Inorganic Topcoat Pigments, *Journal of Forensic Sciences*. 46 (2001) 15099J. <https://doi.org/10.1520/jfs15099j>.
- [103] S. Botti, L. Cantarini, A. Palucci, Surface-enhanced Raman spectroscopy for trace-level detection of explosives, *Journal of Raman Spectroscopy*. 41 (2010) 866–869. <https://doi.org/10.1002/jrs.2649>.
- [104] P. Buzzini, G. Massonnet, The analysis of colored acrylic, cotton, and wool textile fibers using micro-raman spectroscopy. Part 2: Comparison with the traditional methods of fiber examination, *Journal of Forensic Sciences*. 60 (2015) 712–720. <https://doi.org/10.1111/1556-4029.12654>.
- [105] K. Virkler, I.K. Lednev, Raman spectroscopy offers great potential for the nondestructive confirmatory identification of body fluids, *Forensic Science International*. 181 (2008) 1–5. <https://doi.org/10.1016/j.forsciint.2008.08.004>.
- [106] V. Sikirzhyski, A. Sikirzhyskaya, I.K. Lednev, Multidimensional Raman spectroscopic signatures as a tool for forensic identification of body fluid traces: A review, *Applied Spectroscopy*. 65 (2011) 1223–1232. <https://doi.org/10.1366/11-06455>.
- [107] S. Boyd, M.F. Bertino, S.J. Seashols, Raman spectroscopy of blood samples for forensic applications, *Forensic Science International*. 208 (2011) 124–128. <https://doi.org/10.1016/j.forsciint.2010.11.012>.

- [108] T. Schlagetter, B.W. Kammrath, C.L. Glynn, The Use of Raman Spectroscopy for the Identification of Forensically Relevant Body Fluid Stains, *Spectroscopy*. 32 (2017) 19–24.
- [109] J. Fujihara, Y. Fujita, T. Yamamoto, N. Nishimoto, K. Kimura-Kataoka, S. Kurata, Y. Takinami, T. Yasuda, H. Takeshita, Blood identification and discrimination between human and nonhuman blood using portable Raman spectroscopy, *International Journal of Legal Medicine*. 131 (2017) 319–322. <https://doi.org/10.1007/s00414-016-1396-2>.
- [110] G. McLaughlin, K.C. Doty, I.K. Lednev, Raman spectroscopy of blood for species identification, *Analytical Chemistry*. 86 (2014) 11628–11633. <https://doi.org/10.1021/ac5026368>.
- [111] K.C. Doty, I.K. Lednev, Differentiation of human blood from animal blood using Raman spectroscopy: A survey of forensically relevant species, *Forensic Science International*. 282 (2018) 204–210. <https://doi.org/10.1016/j.forsciint.2017.11.033>.
- [112] Y. Zou, P. Xia, F. Yang, F. Cao, K. Ma, Z. Mi, X. Huang, N. Cai, B. Jiang, X. Zhao, W. Liu, X. Chen, Y.J. Kim, S. Hahn, G. Yoon, K.D. Wael, L. Lepot, F. Gason, B. Gilbert, K. Virkler, I.K. Lednev, D. Rohleder, G. Kocherscheidt, K. Gerber, W. Kiefer, W. Kohler, J. Mocks, W. Petrich, F. SaverioRomolo, P. Margot, E. Widjaja, E.M. Ali, H.G. Edwards, I.J. Scowen, C. Hodges, J. Akhavan, J. Thomas, P. Buzzini, G. Massonnet, B. Reedy, C. Roux, D. Broughton, C. Rodger, T. Coyle, N. Anwar, V. Sikirzhytski, A. Sikirzhytskaya, I.K. Lednev, V. Sikirzhytski, K. Virkler, I.K. Lednev, K. Virkler, I.K. Lednev, S. Boyda, M.F. Bertino, S.J. Seashols, H. Sato, H. Chiba, H. Tashiro, Y. Ozaki, T.G. Spiro, T.C. Strekas, A. Sikirzhytskaya, V. Sikirzhytski, I.K. Lednev, M. Pecul, A. Rizzo, J. Leszczynski, S. Choi, T.G. Spiro, K.C. Langry, K.M. Smith, D.L. Budd, G.N. la Mar, M. Abe, T. Kitagawa, Y. Kyogoku, C.R. Johnson, M. Ludwig, S.A. Asher, M.A. Raso, M. v. Garcia, J. Morcillo, A.I. Ivanov, R.G. Zhabankov, E.A. Korolenko, E. v. Korolik, L.A. Meleshchenko, M. Marchewka, H. Ratajczak, J. Liang, Y. Cheng, H. Han, K.L. Aubrey, G.J.T. Jr, A. Eapen, I.H. Joe, V. Fawcett, D.A. Long, A. Bertoluzza, C. Fagnano, P. Finelli, M.A. Morelli, R. Simoni, R. Tosi, M. Ludwig, S.A. Asher, J. Twardowski, I. Nowak, D.J. Stufkens, T.L. Snoeck, S. Mohan, N. Sundaraganesan, A.J.D. Moreno, P.T.C. Freire, I. Guedes, F.E.A. Melo, J.M. Filho, J.A. Sanjurjo, Whole blood and semen identification using mid-infrared and Raman spectrum analysis for forensic applications, *Anal. Methods*. 8 (2016) 3763–3767. <https://doi.org/10.1039/C5AY03337C>.
- [113] C.K. Muro, K.C. Doty, L. Fernandes, I.K. Lednev, Forensic body fluid identification and differentiation by Raman spectroscopy, *Forensic Chemistry*. 1 (2016) 31–38. <https://doi.org/10.1016/j.forc.2016.06.003>.
- [114] I. Feine, R. Gafny, I. Pinkas, Combination of prostate-specific antigen detection and micro-Raman spectroscopy for confirmatory semen detection, *Forensic Science International*. 270 (2017) 241–247. <https://doi.org/10.1016/j.forsciint.2016.10.012>.
- [115] S.S. Tobe, N. Watson, N.N. Daéid, Evaluation of six presumptive tests for blood, their specificity, sensitivity, and effect on high molecular-weight DNA, *Journal of Forensic Sciences*. 52 (2007) 102–109. <https://doi.org/10.1111/j.1556-4029.2006.00324.x>.
- [116] U S Supreme Court, DAUBERT et ux., individually and as guardians ad litem for DAUBERT, et al. v. MERRELL DOW PHARMACEUTICALS, INC., (1992) 585–589.
- [117] K. Varmuza, ed., *Chemometrics in Practical Applications*, InTechOpen, 2012. <https://doi.org/10.5772/1150>.

- [118] G. McLaughlin, M.A. Fikiet, M. Ando, H. o. Hamaguchi, I.K. Lednev, Universal detection of body fluid traces in situ with Raman hyperspectroscopy for forensic purposes: Evaluation of a new detection algorithm (HAMAND) using semen samples, *Journal of Raman Spectroscopy*. 50 (2019) 1147–1153. <https://doi.org/10.1002/jrs.5621>.
- [119] A. Sikirzhyskaya, V. Sikirzhyski, I.K. Lednev, Raman spectroscopic signature of vaginal fluid and its potential application in forensic body fluid identification, *Forensic Science International*. 216 (2012) 44–48. <https://doi.org/10.1016/j.forsciint.2011.08.015>.
- [120] K. Virkler, I.K. Lednev, Forensic body fluid identification: The Raman spectroscopic signature of saliva, *Analyst*. 135 (2010) 512–517. <https://doi.org/10.1039/b919393f>.
- [121] V. Sikirzhyski, A. Sikirzhyskaya, I.K. Lednev, Multidimensional Raman spectroscopic signature of sweat and its potential application to forensic body fluid identification, *Analytica Chimica Acta*. 718 (2012) 78–83. <https://doi.org/10.1016/j.aca.2011.12.059>.
- [122] E. Mistek, L. Halámková, I.K. Lednev, Phenotype profiling for forensic purposes: Nondestructive potentially on scene attenuated total reflection Fourier transform-infrared (ATR FT-IR) spectroscopy of bloodstains, *Forensic Chemistry*. 16 (2019) 100176. <https://doi.org/10.1016/j.forc.2019.100176>.
- [123] K. Suhre, M.I. McCarthy, J.M. Schwenk, Genetics meets proteomics: perspectives for large population-based studies, *Nature Reviews Genetics*. (2020). <https://doi.org/10.1038/s41576-020-0268-2>.
- [124] A. Bodzon-Kulakowska, A. Bierczynska-Krzsik, T. Dylag, A. Drabik, P. Suder, M. Noga, J. Jarzebinska, J. Silberring, Methods for samples preparation in proteomic research, *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*. 849 (2007) 1–31. <https://doi.org/10.1016/j.jchromb.2006.10.040>.
- [125] A.P. Bruins, Mechanistic aspects of electrospray ionization, *Journal of Chromatography A*. 794 (1998) 345–357. [https://doi.org/10.1016/S0021-9673\(97\)01110-2](https://doi.org/10.1016/S0021-9673(97)01110-2).
- [126] D. Lin, D.L. Tabb, J.R. Yates, Large-scale protein identification using mass spectrometry, *Biochimica et Biophysica Acta - Proteins and Proteomics*. 1646 (2003) 1–10. [https://doi.org/10.1016/S1570-9639\(02\)00546-0](https://doi.org/10.1016/S1570-9639(02)00546-0).
- [127] R.A. Zubarev, A. Makarov, Orbitrap mass spectrometry, *Analytical Chemistry*. 85 (2013) 5288–5296. <https://doi.org/10.1021/ac4001223>.
- [128] K. Scheffler, R. Viner, E. Damoc, High resolution top-down experimental strategies on the Orbitrap platform, *Journal of Proteomics*. 175 (2018) 42–55. <https://doi.org/10.1016/j.jprot.2017.03.028>.
- [129] Y. Zhang, B.R. Fonslow, B. Shan, M.C. Baek, J.R. Yates, Protein analysis by shotgun/bottom-up proteomics, *Chemical Reviews*. 113 (2013) 2343–2394. <https://doi.org/10.1021/cr3003533>.
- [130] K.M. Legg, R. Powell, N. Reisdorph, R. Reisdorph, P.B. Danielson, Discovery of highly specific protein markers for the identification of biological stains, *Electrophoresis*. 35 (2014) 3069–3078. <https://doi.org/10.1002/elps.201400125>.
- [131] P. Picotti, R. Aebersold, Selected reaction monitoring-based proteomics: Workflows, potential, pitfalls and future directions, *Nature Methods*. 9 (2012) 555–566. <https://doi.org/10.1038/nmeth.2015>.

- [132] A. Doerr, DIA mass spectrometry, *Nature Methods*. 12 (2014) 35.
<https://doi.org/10.1038/nmeth.3234>.
- [133] A. Thompson, J. Schäfer, K. Kuhn, S. Kienle, J. Schwarz, G. Schmidt, T. Neumann, C. Hamon, Tandem mass tags: A novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS, *Analytical Chemistry*. 75 (2003) 1895–1904.
<https://doi.org/10.1021/ac0262560>.
- [134] D.M. Desiderio, M. Kai, Preparation of stable isotope-incorporated peptide internal standards for field desorption mass spectrometry quantification of peptides in biologic tissue, *Biological Mass Spectrometry*. 10 (1983) 471–479. <https://doi.org/10.1002/bms.1200100806>.
- [135] D.C. Liebler, L.J. Zimmerman, Targeted quantitation of proteins by mass spectrometry, *Biochemistry*. 52 (2013) 3797–3806. <https://doi.org/10.1021/bi400110b>.
- [136] A.D. Catherman, O.S. Skinner, N.L. Kelleher, Top Down proteomics: Facts and perspectives, *Biochemical and Biophysical Research Communications*. 445 (2014) 683–693.
<https://doi.org/10.1016/j.bbrc.2014.02.041>.
- [137] O.S. Skinner, N.A. Haverland, L. Fornelli, R.D. Melani, L.H.F. do Vale, H.S. Seckler, P.F. Doubleday, L.F. Schachner, K. Srzentić, N.L. Kelleher, P.D. Compton, Top-down characterization of endogenous protein complexes with native proteomics, *Nature Chemical Biology*. 14 (2018) 36–41. <https://doi.org/10.1038/nchembio.2515>.
- [138] A. Cristobal, F. Marino, H. Post, H.W.P. van den Toorn, S. Mohammed, A.J.R. Heck, Toward an Optimized Workflow for Middle-Down Proteomics, *Analytical Chemistry*. 89 (2017) 3318–3325.
<https://doi.org/10.1021/acs.analchem.6b03756>.
- [139] V.H. Wysocki, K.A. Resing, Q. Zhang, G. Cheng, Mass spectrometry of peptides and proteins, *Methods*. 35 (2005) 211–222. <https://doi.org/10.1016/j.ymeth.2004.08.013>.
- [140] F. Boulund, R. Karlsson, L. Gonzales-Siles, A. Johnning, N. Karami, O. AL-Bayati, C. Ahrén, E.R.B. Moore, E. Kristiansson, Typing and characterization of bacteria using bottom-up tandem mass spectrometry proteomics, *Molecular and Cellular Proteomics*. 16 (2017) 1052–1063.
<https://doi.org/10.1074/mcp.M116.061721>.
- [141] R. Karlsson, M. Davidson, L. Svensson-Stadler, A. Karlsson, K. Olesen, E. Carlsohn, E.R.B. Moore, Strain-level typing and identification of bacteria using mass spectrometry-based proteomics, *Journal of Proteome Research*. 11 (2012) 2710–2720. <https://doi.org/10.1021/pr2010633>.
- [142] G.J. Parker, T. Leppert, D.S. Anex, J.K. Hilmer, N. Matsunami, L. Baird, J. Stevens, K. Parsawar, B.P. Durbin-Johnson, D.M. Rocke, C. Nelson, D.J. Fairbanks, A.S. Wilson, R.H. Rice, S.R. Woodward, B. Bothner, B.R. Hart, M. Leppert, Demonstration of protein-based human identification using the hair shaft proteome, *PLoS ONE*. 11 (2016) 1–26.
<https://doi.org/10.1371/journal.pone.0160653>.
- [143] D.M. Schieltz, S.C. McGrath, L.G. McWilliams, J. Rees, M.D. Bowen, J.J. Kools, L.A. Dauphin, E. Gomez-Saladin, B.N. Newton, H.L. Stang, M.J. Vick, J. Thomas, J.L. Pirkle, J.R. Barr, Analysis of active ricin and castor bean proteins in a ricin preparation, castor bean extract, and surface swabs from a public health investigation, *Forensic Science International*. 209 (2011) 70–79.
<https://doi.org/10.1016/j.forsciint.2010.12.013>.
- [144] M. Dupré, B. Gilquin, F. Fenaille, C. Feraudet-Tarisse, J. Dano, M. Ferro, S. Simon, C. Junot, V. Brun, F. Becher, Multiplex Quantification of Protein Toxins in Human Biofluids and Food

- Matrices Using Immunoextraction and High-Resolution Targeted Mass Spectrometry, *Analytical Chemistry*. 87 (2015) 8473–8480. <https://doi.org/10.1021/acs.analchem.5b01900>.
- [145] H. Yang, B. Zhou, H. Deng, M. Prinz, D. Siegel, Body fluid identification by mass spectrometry, *International Journal of Legal Medicine*. 127 (2013) 1065–1077. <https://doi.org/10.1007/s00414-013-0848-1>.
- [146] K. van Steendam, M. de Ceuleneer, M. Dhaenens, D. van Hoofstat, D. Deforce, Mass spectrometry-based proteomics as a tool to identify biological matrices in forensic science, *International Journal of Legal Medicine*. 127 (2013) 287–298. <https://doi.org/10.1007/s00414-012-0747-x>.
- [147] K.M. Legg, R. Powell, N. Reisdorph, R. Reisdorph, P.B. Danielson, Verification of protein biomarker specificity for the identification of biological stains by quadrupole time-of-flight mass spectrometry, *Electrophoresis*. 38 (2017) 833–845. <https://doi.org/10.1002/elps.201600352>.
- [148] A. Illiano, V. Arpino, G. Pinto, A. Berti, V. Verdoliva, G. Peluso, P. Pucci, A. Amoresano, Multiple Reaction Monitoring Tandem Mass Spectrometry Approach for the Identification of Biological Fluids at Crime Scene Investigations, *Analytical Chemistry*. 90 (2018) 5627–5636. <https://doi.org/10.1021/acs.analchem.7b04742>.
- [149] S. Jackson, B.S. Frey, M.N. Bates, D.J. Swiner, A.K. Badu-Tawiah, Direct differentiation of whole blood for forensic serology analysis by thread spray mass spectrometry, *The Analyst*. 145 (2020) 5615–5623. <https://doi.org/10.1039/d0an00857e>.
- [150] T. Browne, M. Concheiro-Guisan, M. Prinz, Semi quantitative detection of signature peptides in body fluids by liquid chromatography tandem mass spectrometry (LC–MS/MS), *Forensic Science International: Genetics Supplement Series*. (2019). <https://doi.org/10.1016/j.fsigss.2019.09.080>.
- [151] S. Kranes, S.A. Sterling, K. Mason, D. Anex, B. Hart, G. Parker, M. Prinz, Simultaneous DNA and protein extraction using trypsin, *Forensic Science International: Genetics Supplement Series*. 6 (2017) e203–e204. <https://doi.org/10.1016/j.fsigss.2017.09.081>.
- [152] K.E. Mason, D. Anex, T. Grey, B. Hart, G. Parker, Protein-based forensic identification using genetically variant peptides in human bone, *Forensic Science International*. 288 (2018) 89–96. <https://doi.org/10.1016/j.forsciint.2018.04.016>.
- [153] Z.C. Goecker, M.R. Salemi, N. Karim, B.S. Phinney, R.H. Rice, G.J. Parker, Optimal processing for proteomic genotyping of single human hairs, *Forensic Science International: Genetics*. 47 (2020) 102314. <https://doi.org/10.1016/j.fsigen.2020.102314>.
- [154] J.F. Kellie, J.C. Tran, W. Jian, B. Jones, J.T. Mehl, Y. Ge, J. Henion, K.P. Bateman, Intact Protein Mass Spectrometry for Therapeutic Protein Quantitation, Pharmacokinetics, and Biotransformation in Preclinical and Clinical Studies: An Industry Perspective, *Journal of the American Society for Mass Spectrometry*. (2020). <https://doi.org/10.1021/jasms.0c00270>.
- [155] C.M. Stoscheck, Quantitation of protein, *Methods in Enzymology*. 182 (1990) 50–68. [https://doi.org/10.1016/0076-6879\(90\)82008-P](https://doi.org/10.1016/0076-6879(90)82008-P).
- [156] I. Fridolin, M. Magnusson, L.G. Lindberg, On-line monitoring of solutes in dialysate using absorption of ultraviolet radiation: Technique description, *International Journal of Artificial Organs*. 25 (2002) 748–761. <https://doi.org/10.1177/039139880202500802>.
- [157] H. Edelhoch, Spectroscopic Determination of Tryptophan and Tyrosine in Proteins, *Biochemistry*. 6 (1967) 1948–1954. <https://doi.org/10.1021/bi00859a010>.

- [158] J.D. Russell, Z. Van, D. Heuvel, M. Bovee, S. Murphy, Automation for LC/MS Sample Preparation: High Throughput In-Solution Digestion and Peptide Cleanup Enabled by the Agilent AssayMAP Bravo Platform Application Note, 2016.
- [159] Thermo Scientific, User Guide: Pierce BCA Protein Assay Kit, 2011.
<https://doi.org/10.1016/j.ijproman.2010.02.012>.
- [160] H. Mkaouar, N. Akermi, V. Mariaule, S. Boudebouze, N. Gaci, F. Szukala, N. Pons, J. Marquez, A. Gargouri, E. Maguin, M. Rhimi, Siropins, novel serine protease inhibitors from gut microbiota acting on human proteases involved in inflammatory bowel diseases, *Microbial Cell Factories*. 15 (2016). <https://doi.org/10.1186/s12934-016-0596-2>.
- [161] Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology, *Journal of Analytical Toxicology*. 37 (2013) 452–474.
<https://doi.org/10.1093/jat/bkt054>.
- [162] E.S. Boward, S.L. Wilson, A comparison of ABACard p30 and RSID-Semen test kits for forensic semen identification, *Journal of Forensic and Legal Medicine*. 20 (2013) 1126–1130.
<https://doi.org/10.1016/j.jflm.2013.09.007>.
- [163] F.B. of Investigation, Crime Data Explorer, (n.d.). <https://crime-data-explorer.fr.cloud.gov/explorer/national/united-states/crime> (accessed May 12, 2021).
- [164] S. Sinozich, L. Langton, Rape and Sexual Assault Victimization Among College-Age Females, 1995-2013, 2014.
- [165] P. Tjaden, N. Thoennes, K. v Rhodes, D. Houry, C. Cerulli, H. Straus, N.J. Kaslow, L.-A. McNutt, R.J. Reid, A.E. Bonomi, F.P. Rivara, M.L. Anderson, P. a Fishman, D.S. Carrell, R.S. Thompson, A.L. Coker, K.E. Davis, I. Arias, S. Desai, M. Sanderson, H.M. Brandt, P.H. Smith, H. Cho, B. Callie, M. Rennison, D. Ph, S. Welchans, Full Report of the Prevalence, Incidence, and Consequences of Violence Against Women, *American Journal of Preventive Medicine*. 23 (2000) 260–268.
<https://doi.org/10.1177/0886260512436391>.
- [166] G. Menschaert, T.T.M. Vandekerckhove, G. Baggerman, L. Schoofs, W. Luyten, W. van Criekeing, Peptidomics coming of age: A review of contributions from a bioinformatics angle, *Journal of Proteome Research*. 9 (2010) 2051–2061. <https://doi.org/10.1021/pr900929m>.
- [167] S. Scientific Working Group for Forensic Toxicology, Standard Practices for Method Validation in Forensic Toxicology, (2013) 1–52. <https://doi.org/10.1093/jat/bkt054>.
- [168] F.B. of Investigation, Quality Assurance Standards (QAS) for DNA Databasing Laboratories, (2020). http://www.fbi.gov/about-us/lab/codis/qas_databasalabs.
- [169] C.O. Brown, B.L. Robbins, H.E. McKiernan, P.B. Danielson, K.M. Legg, Direct seminal fluid identification by protease-free high-resolution mass spectrometry, *Journal of Forensic Sciences*. 66 (2021) 1017–1023. <https://doi.org/10.1111/1556-4029.14646>.
- [170] H. Koistinen, R. Koistinen, a Dell, H.R. Morris, R.L. Easton, M.S. Patankar, S. Oehninger, G.F. Clark, M. Seppälä, Glycodelin from seminal plasma is a differentially glycosylated form of contraceptive glycodelin-A., *Molecular Human Reproduction*. 2 (1996) 759–65.
<http://www.ncbi.nlm.nih.gov/pubmed/9239694>.
- [171] M. Uhlén, L. Fagerberg, B.M. Hallström, C. Lindskog, P. Oksvold, A. Mardinoglu, Å. Sivertsson, C. Kampf, E. Sjöstedt, A. Asplund, I.M. Olsson, K. Edlund, E. Lundberg, S. Navani, C.A.K. Szigartyo, J. Odeberg, D. Djureinovic, J.O. Takanen, S. Hober, T. Alm, P.H. Edqvist, H. Berling, H. Tegel, J.

- Mulder, J. Rockberg, P. Nilsson, J.M. Schwenk, M. Hamsten, K. von Feilitzen, M. Forsberg, L. Persson, F. Johansson, M. Zwahlen, G. von Heijne, J. Nielsen, F. Pontén, Tissue-based map of the human proteome, *Science*. 347 (2015). <https://doi.org/10.1126/science.1260419>.
- [172] P. Stanley, N. Taniguchi, M. Aebi, *Essentials of Glycobiology Chapter 9 N-Glycans*, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2017.
- [173] K. Lapid, N. Sharon, Meet the multifunctional and sexy glycoforms of glycodelin, *Glycobiology*. 16 (2006) 39–45. <https://doi.org/10.1093/glycob/cwj059>.
- [174] T. Borja, N. Karim, Z. Goecker, M. Salemi, B. Phinney, M. Naeem, R. Rice, G. Parker, Proteomic genotyping of fingerprint donors with genetically variant peptides, *Forensic Science International: Genetics*. 42 (2019) 21–30. <https://doi.org/10.1016/j.fsigen.2019.05.005>.
- [175] L.A. Catlin, R.M. Chou, Z.C. Goecker, L.A. Mullins, D. Silva, R.R. Spurbeck, G.J. Parker, Demonstration of a mitochondrial DNA-compatible work flow for genetically variant peptide identification from human hair samples, *Forensic Science International: Genetics*. 43 (2019). <https://doi.org/10.1016/j.fsigen.2019.102148>.

APPENDIX A: FINAL BIOMARKER LIST AND ASSOCIATED COLLISION ENERGIES

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

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

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

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

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

BODY FLUID IDENTIFICATION BY LC-MS/MS

TABLE OF CONTENTS

Materials and Reagents

Controls

Body Fluid Extraction and Quantitation

Protein Sample Digestion

Peptide Sample Cleanup

Analysis by Mass Spectrometry

Appendix I: AssayMAP Bravo Startup/Shutdown

Appendix II: Human Serum Albumin Acquisition Parameters

Appendix III: Body Fluid Identification Acquisition Parameters

MATERIALS AND REAGENTS

Equipment

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

General Plastics and Labware

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

AssayMAP and Automation Labware

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

Chemicals

1. 100 mM Tris-HCl (Thermo #15568-025)
 - a. Dilute 1M Tris-HCl 1:10 in LCMS grade water
2. 8 M Urea (Sigma BioUltra #51456)
 - a. 3.123 grams in 4 mL 100 mM Tris-HCl
3. 1 M Tris(2-carboxyethyl)phosphine hydrochloride, TCEP (Thermo #20490)
 - a. 0.286 grams in 1 mL LCMS grade water. 35 μ L aliquots stored at -80 °C.
4. 100 mM Iodoacetamide, IAA (Sigma BioUltra #11149)
 - a. 0.036 grams in 2.0 mL LCMS grade water

General Solvents

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

AssayMAP Solvents

1. Priming Solvent: 50% Acetonitrile in water with 0.5% TFA
 - a. 500 mL acetonitrile, 500 mL water, 5 mL TFA
2. Equilibration and Wash Solvent: Water with 0.5% TFA

- a. 1,000 mL water, 5 mL TFA
3. Elution Solvent: 70% Acetonitrile in water with 0.1% FA
 - a. 700 mL acetonitrile, 300 mL water, 1 mL formic acid
4. Cartridge Wash (60% Methanol)
 - a. 600 mL Methanol, 400 mL water

Mass Spectrometry Solvents

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

Enzymes:

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

Myelin Internal Control Reagents:

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

System Suitability:

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

CONTROLS

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

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

BODY FLUID EXTRACTION AND QUANTITATION

Initial preparations:

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

Extraction protocols:

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

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

Quantitation protocols:

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

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

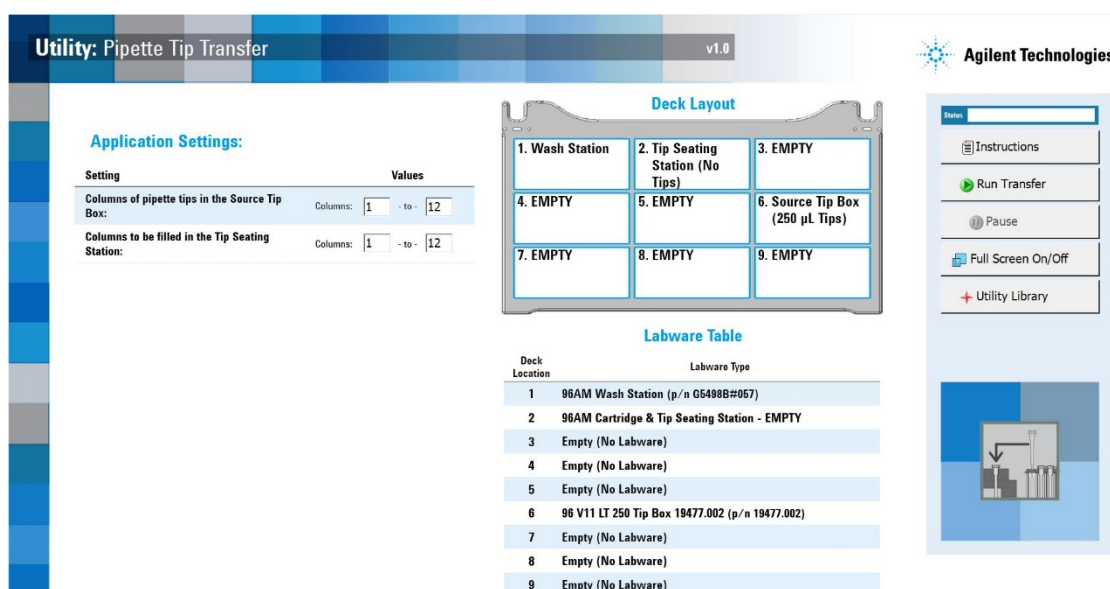
2. For sample preparation, dilute sample supernatant in 100mM Tris-HCl at the discretion of the analyst
 - a. Blood and Semen Samples 1:100 dilution
 - b. Saliva and Vaginal Fluid 1:50 dilution
 - c. Dependent on the concentration and physical appearance of the extract
3. Prepare BCA Protein Assay working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

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

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

PROTEIN SAMPLE DIGESTION

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



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

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

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

In-Solution Digestion: Single Plate v1.0 Agilent Technologies

A. Select Method

Browse for a Method:

Method Loaded: Load Values

Method Status:

B. Input Sample Settings

Setting	Value
Sample Plate Labware	96 Greiner 650207_U-Bottom, White PolyPro
Sample Plate Lidded	☒
Starting Sample Volume [μL]	0
Number of Full Columns of Samples	6

C. Input Addition Step Settings

Addition Number	Addition Name	Reagent Deck Location	Addition Volume [μL]	Mixing Cycles	Incubation Time (min)	Incubation Temp (°C)	Pause After Addition	Labware Selection	Plate Lidded	Use Tips for Addition	Number of Wash Cycles
1	Denaturation	5	55	15	45	25	☐	96 Greiner 650207_U-Bottom, White PolyPro	☐	☒	3
2	Alkylation	6	6	15	30	25	☐	96 Greiner 650207_U-Bottom, White PolyPro	☒	☒	3
3	Diluent	8	170	15	0	25	☐	96 Greiner 650207_U-Bottom, White PolyPro	☐	☒	3
4	Protease 1	9	10	15	0	25	☐	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro	☐	☒	3

D. Update Deck Layout

Update Deck Layout

<Pos 1 Wash Station>
AM Wash Station

<Pos 2 Tip Seating Station>
96AM Cartridge and Tip Seating Station with 6 Columns of Tips

<Pos 3 Lid Hotel 2>
Empty Location. Reserved for Lids

<Pos 4 Sample Plate>
96 Greiner 650207_U-Bottom, White PolyPro
Lid Required

<Pos 5 Denaturation>
96 Greiner 650207_U-Bottom, White PolyPro
Tip Lid Needed

<Pos 6 Alkylation>
96 Greiner 650207_U-Bottom, White PolyPro
Lid Required

<Pos 7 Lid Hotel 1>
Empty Location. Reserved for Lids
No Lid Needed

<Pos 8 Diluent>
96 Greiner 650207_U-Bottom, White PolyPro
No Lid Needed

<Pos 9 Protease 1>
96 Eppendorf 30129300, PCR, Full Skirt, PolyPro
No Lid Needed

Status

Cumulative Sample Volume [μL]: N/A

Instructions

Run Digestion

Pause

Restore Defaults

Full Screen On/Off

App Library

Save Method

Name:

Overwrite if Name Exists: ☐

Save

10. Run the digestion application.
11. With the completion of the digestion application, remove the sample plate and seal with an Eppendorf storage film. Place the sample plate in an incubator set at 37 °C for 14-16 hours.
12. Run AssayMap shutdown procedure found in Appendix I.

PEPTIDE SAMPLE CLEANUP

Sample Acidification:

1. Run AssayMap startup procedure found in Appendix I.
2. Allow the sample plate to come to room temperature. Remove Myelin Isotopically Labelled Peptide Stock Solution from freezer.
3. Remove storage film. With a repeater pipette, add 10 μL of Myelin Isotopically Labelled Peptide stock solution at 1 $\mu\text{g/mL}$ for a 10 ng spike per sample.
4. Open the Pipette Tip Transfer Utility Application in the VWorks Software Utility Library. Arrange the pipette tip rack according to the deck layout diagram displayed. Input the number of columns to transfer according to the number of samples being processed. A full row of tips should be transferred, regardless of whether the sample plate contains a complete row of samples to be analyzed. Run the Pipette Tip Transfer Application. Remove the pipette tip rack from the instrument.

Utility: Pipette Tip Transfer v1.0 Agilent Technologies

Application Settings:

Setting	Values
Columns of pipette tips in the Source Tip Box:	Columns: 1 - to - 12
Columns to be filled in the Tip Seating Station:	Columns: 1 - to - 12

Deck Layout

1. Wash Station	2. Tip Seating Station (No Tips)	3. EMPTY
4. EMPTY	5. EMPTY	6. Source Tip Box (250 μL Tips)
7. EMPTY	8. EMPTY	9. EMPTY

Labware Table

Deck Location	Labware Type
1	96AM Wash Station (p/n G5408B#067)
2	96AM Cartridge & Tip Seating Station - EMPTY
3	Empty (No Labware)
4	Empty (No Labware)
5	Empty (No Labware)
6	96 V11 LT 250 Tip Box 19477.002 (p/n 19477.002)
7	Empty (No Labware)
8	Empty (No Labware)
9	Empty (No Labware)

State:

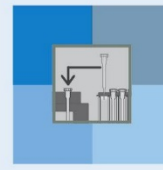
Instructions

Run Transfer

Pause

Full Screen On/Off

Utility Library



5. Open the Reagent Transfer Utility application in the VWorks Software Utility library.
6. Fill the appropriate number of channels of a 12 Column Reservoir Plate with 25% TFA solution. Move the plate to the source location of the deck layout displayed.
7. Place the sample plate containing digested material to the destination location of the deck layout displayed.

8. In the Reagent Transfer Utility application, select the number of columns to be transferred, check the box indicating the use of pipette tips to transfer, and input 10 μL of 25% TFA to be transferred to each well.

Utility: Reagent Transfer v1.0 Agilent Technologies

Application Settings

General Settings	Value
Use Pipette Tips for Transfer:	☒
Number of Columns of Samples to be Transferred:	6
Initial Syringe/Tip Wash Cycles:	0
Final Syringe/Tip Wash Cycles:	0

Source Plate Settings	Value
Initial Well Volume:	900 μL
Volume to Transfer:	10 μL

Destination Plate Settings	Value
Initial Well Volume:	251 μL
Mix Cycles after Transfer:	15 μL
Blowout Volume:	10 μL

Deck Layout

1. Wash Station	2. Tip Seating Station (with 250 μL Tips)	3. EMPTY
4. EMPTY	5. EMPTY	6. EMPTY
7. Source Plate	8. Destination Plate	9. EMPTY

Labware Table

Deck Location	Labware Type
1	96AM Wash Station (p/n G5498B#057)
2	96AM Cartridge & Tip Seating Station (with 250 μL Tips)
3	Empty (No Labware)
4	Empty (No Labware)
5	Empty (No Labware)
6	Empty (No Labware)
7	12 Column, Low Profile Reservoir, Natural PP
8	96 Greiner 650207_U-Bottom, White PolyPro
9	Empty (No Labware)

On the right side of the interface, there is a 'Status' section with buttons for 'Instructions', 'Run Transfer', 'Pause', 'Full Screen On/Off', and 'Utility Library'. Below these buttons is a small diagram of the labware setup.

9. Run the Reagent Transfer application.
10. Discard the pipette tips.
11. Discard the 25% TFA solution into solvent waste.

Cartridge Transfer:

1. Open the Cartridge Transfer Utility application in VWorks Software Utility library.
2. Arrange the cartridge rack according to the deck layout diagram displayed.
3. Input the number of columns to transfer according to the number of samples being processed.

Note that full columns should be transferred.

Utility: CARTRIDGE TRANSFER v1.0

A. Application Settings

First Column in Source Cartridge Rack: 1

Number of Columns to Transfer: 6

First Column in Destination Cartridge Rack: 1

Reverse Process: ☐

B. Deck Layout

1. Wash Station	2. Destination: Seating Station	3. Empty
4. Empty	5. Empty	6. Source: Cartridge Rack
7. Empty	8. Empty	9. Empty

C. Labware Table

Deck Location	Labware Type
1	96AM Wash Station
2	96AM Cartridge & Tip Seating Station
3	none
4	none
5	none
6	96AM Cartridge Rack and Receiver Plate
7	none
8	none
9	none

Agilent AssayMAP

Instructions

Run Cartridge Transfer

Pause

Save Settings

Restore Defaults

Utility Library

Agilent Technologies

4. Run the Cartridge Transfer application.
5. Remove the cartridge rack from the instrument.

Peptide Cleanup Protocol:

1. Open the Peptide Cleanup application in VWorks Software application library.
2. Prepare the Prime solution. Fill the corresponding number of channels to capacity of a labeled 12 Column Reservoir Plate to the number of columns being processed. Move the plate position 5 on the AssayMAP deck.
3. Prepare the Equilibration solution. Fill the corresponding number of channels to capacity of a labeled 12 Column Reservoir Plate to the number of columns being processed. Move the plate to position 6 on the AssayMAP deck.
4. Prepare the Elution solution. Fill the corresponding number of channels to capacity of a labeled 12 Column Reservoir Plate to the number of columns being processed. Move the plate to position 8 on the AssayMAP deck.
5. Place the 96 well plate containing digested and acidified sample in the corresponding position to the displayed deck layout.
6. Place a 12 Column Reservoir plate in the Organic Waste/position 3 on the deck.
7. Place a 96 well PCR plate in the Eluate Collection/position 9 on the deck.
8. Input the appropriate number of columns being processed in the software.

9. Verify the following parameters in the software:

Cleanup Step	Addition Volume	Flow Rate	# Wash Cycles
Initial Syringe Wash	---	---	4 wash cycles
Prime	100 μ L 50% ACN 0.5% TFA	300 μ L/min	3 wash cycles
Equilibrate	50 μ L of H ₂ O 0.5% TFA	10 μ L/min	3 wash cycles
Sample Load	220 μ L	15 μ L/min	3 wash cycles
Cup Wash	25 μ L	---	1 wash cycle
Internal Cartridge Wash	50 μ L	10 μ L/min	3 wash cycles
Stringent Syringe Wash	50 μ L	---	1 wash cycle
Elute	20 μ L 70% ACN 0.1% FA	5 μ L/min	3 wash cycles
Final Syringe Wash	---	---	3 wash cycles

Peptide Cleanup: Using AssayMAP v2.0

Application Settings

Number of Full Columns of Cartridges: 6

Step	Conduct Step?	Volume (μ L)	Flow Rate (μ L/min)	Wash Cycles
Initial Syringe Wash	☒			4
Prime	☒	100	300	3
Equilibrate	☒	50	10	3
Load Sample	☒	220	5	3
Collect Flow Through	☐			
Cup Wash	☒	25		1
Internal Cartridge Wash	☒	50	10	3
Collect Flow Through	☐			
Stringent Syringe Wash	☒	50		1
Elute	☒	25	5	1
Eluate Discard	☐	0		
Add to Flow Through	☐			
Existing Collection Volume		0		
Final Syringe Wash	☒			3

Deck Layout

1. Wash Station	2. Cartridges	3. Organic Waste
4. Sample	5. Priming & Syringe Wash Buffer	6. Elution Buffer
7. Flow Through Collection	8. Equilibration & Cartridge Wash Buffer	9. Eluate Collection

Labware Table

Deck Location	Labware Type
1	96AM Wash Station
2	96AM Cartridge & Tip Seating Station
3	12 Column, Low Profile Reservoir, Natural PP
4	96 Greiner 650207_U-Bottom, White PolyPro
5	12 Column, Low Profile Reservoir, Natural PP
6	12 Column, Low Profile Reservoir, Natural PP
7	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro
8	12 Column, Low Profile Reservoir, Natural PP
9	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro

Agilent Technologies

Status: Initializing Peptide Cleanup...

Run Peptide Cleanup

Pause

Save Settings

Restore Defaults

App Library

10. Run the Peptide Cleanup application.

11. Upon completion, remove the eluate collection plate from the instrument.

12. Dry down in the SpeedVac concentrator at 45 °C until lyophilized to dryness.

ANALYSIS BY MASS SPECTROMETRY

Initial Preparations:

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

Utility: Pipette Tip Transfer v1.0

Agilent Technologies

Application Settings:

Setting	Values
Columns of pipette tips in the Source Tip Box:	Columns: 1 - to - 12
Columns to be filled in the Tip Seating Station:	Columns: 1 - to - 12

Deck Layout

1. Wash Station	2. Tip Seating Station (No Tips)	3. EMPTY
4. EMPTY	5. EMPTY	6. Source Tip Box (250 µL Tips)
7. EMPTY	8. EMPTY	9. EMPTY

Labware Table

Deck Location	Labware Type
1	96AM Wash Station (p/n G5498B#067)
2	96AM Cartridge & Tip Seating Station - EMPTY
3	Empty (No Labware)
4	Empty (No Labware)
5	Empty (No Labware)
6	96 V11 LT 250 Tip Box 19477.002 (p/n 19477.002)
7	Empty (No Labware)
8	Empty (No Labware)
9	Empty (No Labware)

Buttons: Instructions, Run Transfer, Pause, Full Screen On/Off, Utility Library

3. Open the Reagent Transfer Utility application in the VWorks Software Utility library.
4. Fill the appropriate number of channels of a 12 Column Reservoir Plate with 2% acetonitrile with 0.1% formic acid in LCMS grade water.
5. Move the plate to the source location of the deck layout displayed. Place the sample plate containing lyophilized, purified sample to the destination location of the deck layout displayed.

Utility: Reagent Transfer v1.0 **Agilent Technologies**

Application Settings

General Settings	Value
Use Pipette Tips for Transfer:	☒
Number of Columns of Samples to be Transferred:	6
Initial Syringe/Tip Wash Cycles:	0
Final Syringe/Tip Wash Cycles:	0

Source Plate Settings	Value
Initial Well Volume:	900 μL
Volume to Transfer:	10 μL

Destination Plate Settings	Value
Initial Well Volume:	251 μL
Mix Cycles after Transfer:	15 μL
Blowout Volume:	10 μL

Deck Layout

1. Wash Station	2. Tip Seating Station (with 250 μL Tips)	3. EMPTY
4. EMPTY	5. EMPTY	6. EMPTY
7. Source Plate	8. Destination Plate	9. EMPTY

Labware Table

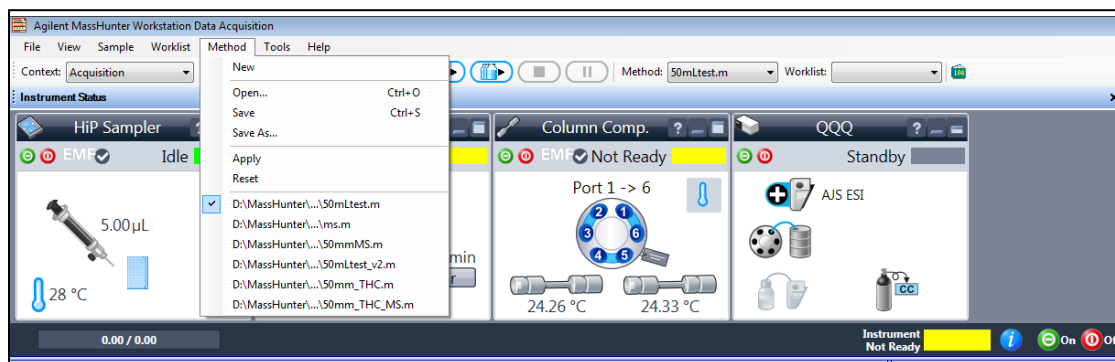
Deck Location	Labware Type
1	96AM Wash Station (p/n G5498B#057)
2	96AM Cartridge & Tip Seating Station (with 250 μL Tips)
3	Empty (No Labware)
4	Empty (No Labware)
5	Empty (No Labware)
6	Empty (No Labware)
7	12 Column, Low Profile Reservoir, Natural PP
8	96 Greiner 650207_U-Bottom, White PolyPro
9	Empty (No Labware)

Utility Library

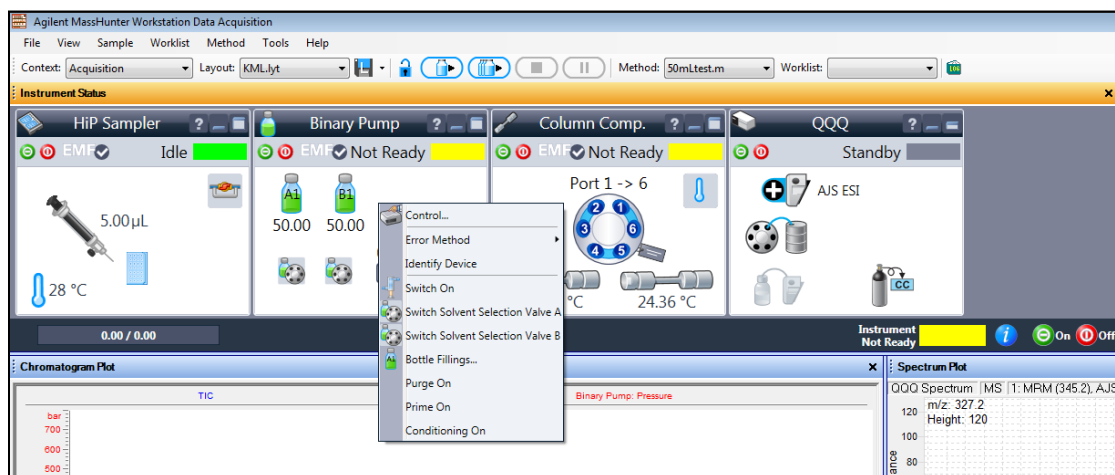
- In the Reagent Transfer Utility application, select the number of columns to be transferred, check the box indicating the use of pipette tips to transfer, and input 100 μL of 2% ACN 0.1% FA to be transferred to each well.
- Run the Reagent Transfer application.
- Discard the pipette tips.
- Discard the 2% ACN 0.1% FA solution into solvent waste.

Sample Plate Analysis:

- Ensure all solvents are not expired.
- Verify the Agilent Jet Stream source as been cleaned during the week of analysis or as necessary. For example, deterioration or material on the spray shield.
- Verify the 6495 MS has been fully tuned within 1 month and a check tune has been performed during the week of analysis. If necessary, tune the instrument.
- Open MassHunter Acquisition Software.
- Under the Method Tab, open and load the HSA method.

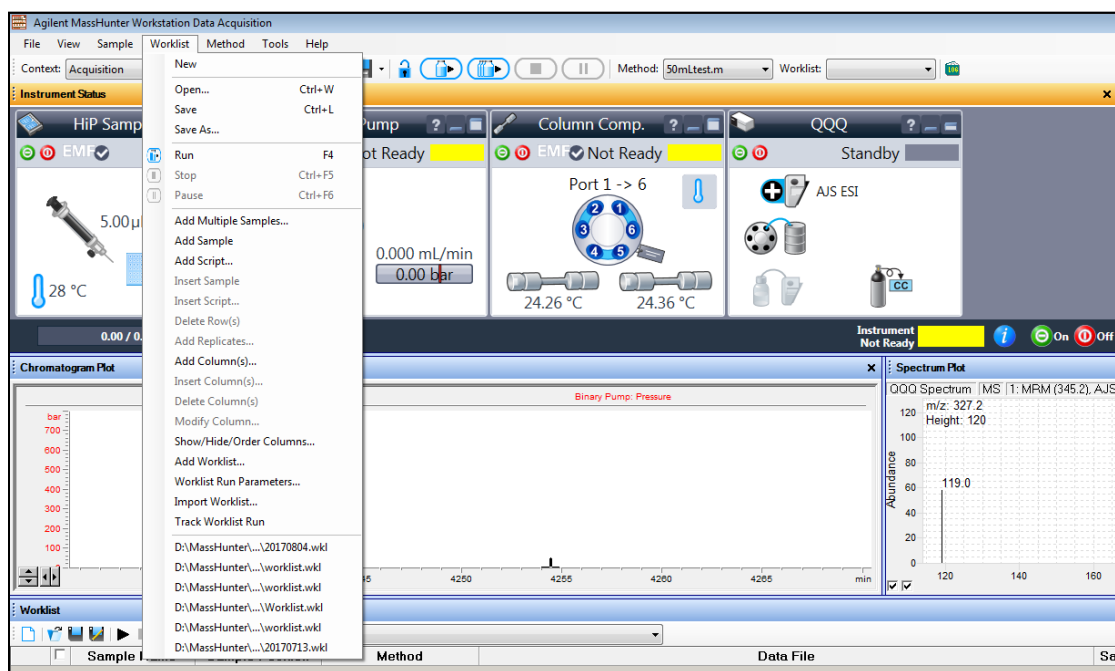


6. If fresh solvents were prepared, update the bottle fillings by right clicking the Binary Pump Module.

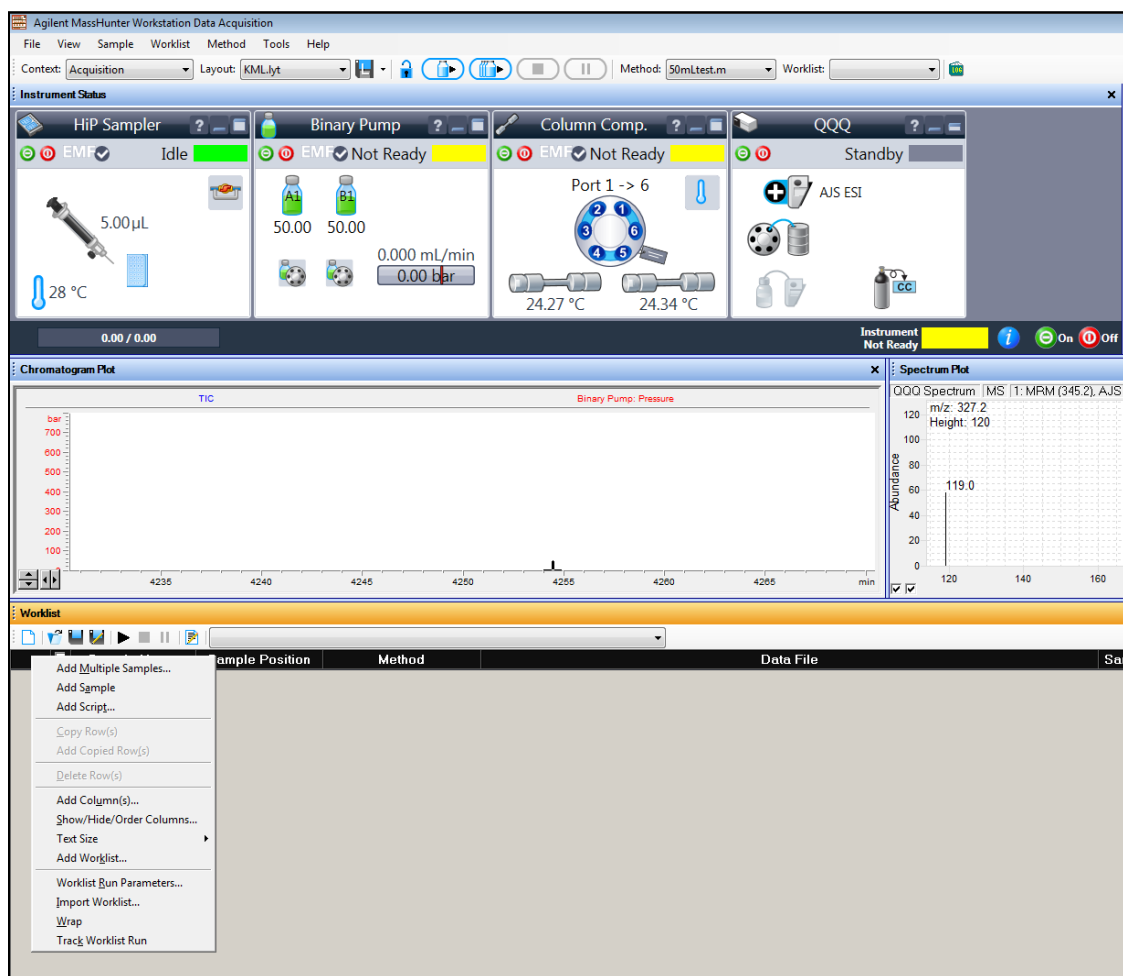


7. Purge the A and B Mobile Phase pump lines by right clicking the Binary Pump Module. Purge at 50:50 mobile phase A:B at 4 mL/min for 4 minutes.
8. Ensure the correct analytical column is connected in the proper position on the heat block. Agilent AdvancedBio Peptide Map column, 3x100mm, 2.7 µM, #655950-302.
9. Turn on the instrument by clicking the green 'On' button in the lower right corner of the Module bar. Allow the instrument to idly pump for approximately five minutes. Monitor the pressure listed on the Binary Pump Module. Pressures > 350 bar may indicate the column requires maintenance or replacement.
10. Create a new run folder on the computer hard drive, labeling appropriately according to laboratory protocol.

11. In MassHunter Acquisition Software, under the Worklist Tab, modify the Worklist Run Parameters. Input the new run folder and ensure the proper method and run folder is selected.

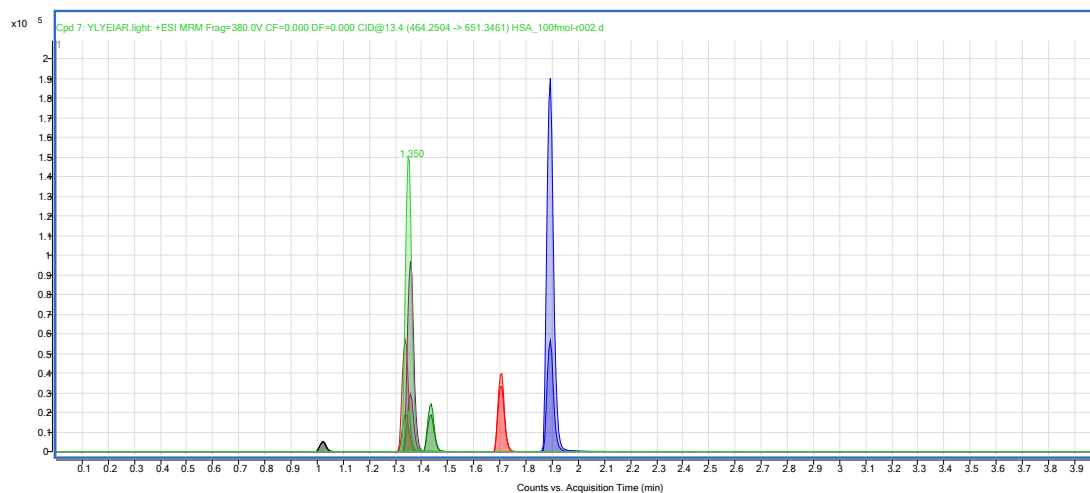


12. Click the Worklist tab in the bottom left corner of the screen. Begin a new worklist by right clicking the space and selecting Add Sample. Alternatively, open a previously made worklist by clicking the Worklist Tab at the top of the screen and opening the desired worklist. Label the samples appropriately and insert blanks where necessary. Always run HSA System Suitability samples in triplicate at the beginning of the worklist, to ensure proper instrument performance. Always include a shutdown script at the end of the worklist, communicating to the instrument to go into standby once the run is completed.



13. Once the worklist is prepared, place samples into the autosampler according to the plate/vial position.
14. Select all the samples in the worklist by checking the square box at the top left of the worklist.
Hit the triangle "Play" button to start the worklist.
15. Review the results for the HSA System Suitability samples in the Qualitative Analysis Software.
16. Verify that all six peptides are present in the system suitability injections. Verify retention times, relative intensities, as well as peak shape for the peptides. If a problem is observed, troubleshoot the issue before running the full batch.
17. Refer to the table and representative chromatogram below:

Peptide Sequence	Retention Time (min.)	Intensity (cts.)
AAFTEC[+57.0]C[+57.0]QAADK	1	5.5×10^3
AVMDDFAAFVEK	1.7	4.0×10^4
RPC[+57.0]FSALEVDETYVPK	1.4	2.5×10^4
HPYFYAPELLFFAK	1.8	2.0×10^5
LVNEVTEFAK	1.3	1.0×10^5
KVPQVSTPTLVEVSR	1.3	5.5×10^4
YLYEIAR	1.3	1.5×10^5



APPENDIX I ASSAYMAP BRAVO STARTUP AND SHUTDOWN

Startup

1. Turn on the AssayMAP Bravo automation system. Ensure the wash station carboy contains a sufficient amount of water and the waste carboy is empty. Open the Startup Utility Application in the VWorks Software. Run the application and follow the prompts on the computer.

AssayMAP Startup & Shutdown: Using AssayMAP

v2.0

Startup Procedure

The purpose of this procedure is to initialize the system, safely discard liquid introduced during the shutdown procedure, and prime the wash station tubing.

Startup Options	Value
Number of Syringe Wash Cycles	3
Wash Station Prime Duration	10 sec

Deck Layout

1. Wash Station	2. Cartridge Seating Station	3. Empty
4. Empty	5. Empty	6. Empty
7. Syringe Storage Liquid (optional)	8. Empty	9. Empty

Shutdown Procedure Description

This procedure is designed to introduce water into the AssayMAP syringes to protect their plunger seals from drying during extended periods of inactivity. Water is aspirated from the 96AM Wash Station at deck location 1. Running the Shutdown Procedure is recommended for AM Bravo idle periods of 1 hr - 1 week.

Shutdown Options	Value
Number of Syringe Wash Cycles	3
Syringe Storage Liquid Source	96AM Wash Station

Labware Table

Deck Location	Labware Type
1	96AM Wash Station (p/n G5498B#057)
2	96AM Cartridge and Tip Seating Station (p/n)
3	Empty (No Labware)
4	Empty (No Labware)
5	Empty (No Labware)
6	Empty (No Labware)
7	12 Column, Low Profile Reservoir, Natural PP
8	Empty (No Labware)
9	Empty (No Labware)

Status

Instructions

Run Startup

Run Shutdown

Pause

Full Screen on/off

Utility Library

Shutdown

1. Open the Startup & Shutdown Utility Application in the VWorks Software. Run the application and follow the prompts on the computer.

AssayMAP Startup & Shutdown: Using AssayMAP

v2.0

Startup Procedure

The purpose of this procedure is to initialize the system, safely discard liquid introduced during the shutdown procedure, and prime the wash station tubing.

Startup Options	Value
Number of Syringe Wash Cycles	3
Wash Station Prime Duration	10 sec

Deck Layout

1. Wash Station	2. Cartridge Seating Station	3. Empty
4. Empty	5. Empty	6. Empty
7. Syringe Storage Liquid (optional)	8. Empty	9. Empty

Shutdown Procedure Description

This procedure is designed to introduce water into the AssayMAP syringes to protect their plunger seals from drying during extended periods of inactivity. Water is aspirated from the 96AM Wash Station at deck location 1. Running the Shutdown Procedure is recommended for AM Bravo idle periods of 1 hr - 1 week.

Shutdown Options	Value
Number of Syringe Wash Cycles	3
Syringe Storage Liquid Source	96AM Wash Station

Labware Table

Deck Location	Labware Type
1	96AM Wash Station (p/n G5498B#057)
2	96AM Cartridge and Tip Seating Station (p/n)
3	Empty (No Labware)
4	Empty (No Labware)
5	Empty (No Labware)
6	Empty (No Labware)
7	12 Column, Low Profile Reservoir, Natural PP
8	Empty (No Labware)
9	Empty (No Labware)

Status

Instructions

Run Startup

Run Shutdown

Pause

Full Screen on/off

Utility Library

APPENDIX II HUMAN SERUM ALBUMIN ACQUISITION PARAMETERS

Instrument Parameters

- Instrumentation: Agilent 6495 Tandem Mass Spectrometer with an Agilent 1290 LC system.
 - Instrument Mode: MRM
- Agilent AdvancedBio Peptide Map column, 3x100mm, 2.7 μ M
 - Mobile Phases:
 - A: Water with 0.2% Formic Acid
 - B: Acetonitrile with 0.2% Formic Acid
 - Needle Wash: 1:1:1:1 Methanol:Acetonitrile:Isopropanol:Water
 - Injection Volume: 2.5 -10 μ L
- System Suitability: Agilent Human Serum Albumin peptide mix with 100 fmol on column.
- The ions monitored are listed below.

Compound Group	Compound Name	Precursor Ion	Product Ion
sp P02768 ALBU_HUMAN	AAFTEC[+57.0]C[+57.0]QAADK.light	686.3	981.4
sp P02768 ALBU_HUMAN	AAFTEC[+57.0]C[+57.0]QAADK.light	686.3	852.3
sp P02768 ALBU_HUMAN	AVMDDFAAFVEK.light	671.8	1,172.5
sp P02768 ALBU_HUMAN	AVMDDFAAFVEK.light	671.8	1,041.5
sp P02768 ALBU_HUMAN	RPC[+57.0]FSALEVDETYVPK.light	637.6	961.5
sp P02768 ALBU_HUMAN	RPC[+57.0]FSALEVDETYVPK.light	637.6	851.4
sp P02768 ALBU_HUMAN	HPYFYAPELLFFAK.light	581.6	779.4
sp P02768 ALBU_HUMAN	HPYFYAPELLFFAK.light	581.6	482.8
sp P02768 ALBU_HUMAN	LVNEVTEFAK.light	575.3	937.5
sp P02768 ALBU_HUMAN	LVNEVTEFAK.light	575.3	595.3
sp P02768 ALBU_HUMAN	KVPQVSTPTLVEVSR.light	547.3	702.4
sp P02768 ALBU_HUMAN	KVPQVSTPTLVEVSR.light	547.3	589.3
sp P02768 ALBU_HUMAN	YLYEIAR.light	464.3	651.3
sp P02768 ALBU_HUMAN	YLYEIAR.light	464.3	488.3

- The acquisition parameters are as follows:

Time Segment 1

Scan Segments

Cpd Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Dwell	Frag (V)	CE (V)	Cell Acc (V)	Polarity
AAFTEC [+57.0]C [+57.0] QAADK.lig ht	No	686.28701 9	Unit/Enh (6490)	981.37644 2	Unit/Enh (6490)	20	380	24	4	Positive
AAFTEC [+57.0]C [+57.0] QAADK.lig ht	No	686.28701 9	Unit/Enh (6490)	852.33384 9	Unit/Enh (6490)	20	380	26	4	Positive
AVMDDFA AFVEK.lig ht	No	671.82102	Unit/Enh (6490)	1172.5292 37	Unit/Enh (6490)	20	380	21.8	4	Positive
AVMDDFA AFVEK.lig ht	No	671.82102	Unit/Enh (6490)	1041.4887 52	Unit/Enh (6490)	20	380	23.8	4	Positive
RPC [+57.0] FSALEVD ETYVPK.ii ght	No	637.64874 3	Unit/Enh (6490)	961.45601 3	Unit/Enh (6490)	20	380	18.2	4	Positive
RPC [+57.0] FSALEVD ETYVPK.ii ght	No	637.64874 3	Unit/Enh (6490)	851.41452 5	Unit/Enh (6490)	20	380	18.2	4	Positive
HPYFYAP ELLFFAK.ii ght	No	581.63621 4	Unit/Enh (6490)	779.35113 7	Unit/Enh (6490)	20	380	12.1	4	Positive
HPYFYAP ELLFFAK.ii ght	No	581.63621 4	Unit/Enh (6490)	482.77875 3	Unit/Enh (6490)	20	380	12.1	4	Positive
LVNEVTEF AK.light	No	575.31114 6	Unit/Enh (6490)	937.46253 8	Unit/Enh (6490)	20	380	18.8	4	Positive
LVNEVTEF AK.light	No	575.31114 6	Unit/Enh (6490)	595.30860 3	Unit/Enh (6490)	20	380	18.8	4	Positive
KVPQVST PTLVEVS R.light	No	547.31743 3	Unit/Enh (6490)	702.41446 5	Unit/Enh (6490)	20	380	18.9	4	Positive
KVPQVST PTLVEVS R.light	No	547.31743 3	Unit/Enh (6490)	589.33040 1	Unit/Enh (6490)	20	380	18.9	4	Positive
YLVEIAR.ii ght	No	464.25036	Unit/Enh (6490)	651.34605 1	Unit/Enh (6490)	20	380	13.4	4	Positive
YLVEIAR.ii ght	No	464.25036	Unit/Enh (6490)	488.28272 3	Unit/Enh (6490)	20	380	15.4	4	Positive

Scan Parameters

Data Stg	Threshold
Centroid	0

Source Parameters

Parameter	Value (+)	Value (-)
Gas Temp (°C)	200	200
Gas Flow (l/min)	13	13
Nebulizer (psi)	35	35
SheathGasHeater	200	200
SheathGasFlow	12	12
Capillary (V)	3500	3000
VCharging	300	500

Ion Funnel Parameters

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

Chromatograms

Chrom Type	Label	Offset	Y-Range
TIC		0	10000000

Instrument Curves

Actual

Acquisition Method Info

Method Name HSA.m
 Method Path D:\MassHunter\Methods\Proteomics_DOD\3mm_Final\HSA.m
 Method Description 1 min source with trap column, HSA
 Device List
 HIP Sampler
 Binary Pump
 Column Comp.
 QQQ

MS QQQ Mass Spectrometer

Ion Source AJS ESI
 Stop Mode No Limit/As Pump
 Time Filter On
 Tune File atunes.TUNE.XML
 Stop Time (min) 1
 Time Filter Width (min) 0.03

Time Segments	Index	Start Time (min)	Scan Type	Ion Mode	Div Valve	Delta EMV	Store
	1	0	MRM	ESI+Agilent Jet Stream	To MS	200	Yes

Name: **HIP Sampler** Model: **G4226A**

Auxiliary

Draw Speed 100.0 µL/min
 Eject Speed 100.0 µL/min
 Draw Position Offset 0.0 mm
 Wait Time After Drawing 2.0 s
 Sample Flush Out Factor 5.0
 Vial/Well bottom sensing No

Injection

Injection Mode Injection with needle wash
 Injection Volume 1.00 µL
 Needle Wash
 Needle Wash Location Flush Port
 Wash Time 3.0 s

High throughput

Automatic Delay Volume Reduction No
 Overlapped Injection
 Enable Overlapped Injection No

Valve Switching

Valve Movements 0
 Valve Switch Time 1
 Switch Time 1 Enabled No
 Valve Switch Time 2
 Switch Time 2 Enabled No
 Valve Switch Time 3
 Switch Time 3 Enabled No
 Valve Switch Time 4
 Switch Time 4 Enabled No

Stop Time

Stop Time Mode As pump/No limit

Post Time

Post Time Mode Off

Name: Binary Pump**Model:** G4220A

Flow 1.000 mL/min
 Use Solvent Types Yes
 Stroke Mode Synchronized
 Low Pressure Limit 0.00 bar
 High Pressure Limit 500.00 bar
 Max. Flow Ramp Up 100.000 mL/min²
 Max. Flow Ramp Down 100.000 mL/min²
 Expected Mixer No check

Stroke A

Automatic Stroke Calculation A Yes

Stop Time

Stoptime Mode Time set
 Stoptime 4.00 min

Post Time

Posttime Mode Time set
 Posttime 3.00 min

Solvent Composition

	Channel	Ch. 1 Solv.	Name 1	Ch2 Solv.	Name 2	Selected	Used	Percent
1	A	100.0 % Water V.03		100.0 % Water V.03		Ch. 1	Yes	95.00 %
2	B	100.0 % Acetonitrile V.03		100.0 % Acetonitrile V.03		Ch. 1	Yes	5.00 %

Timetable

	Time	A	B	Flow	Pressure
1	2.40 min	40.00 %	60.00 %	--- mL/min	--- bar
2	3.10 min	20.00 %	80.00 %	--- mL/min	--- bar
3	3.80 min	20.00 %	80.00 %	--- mL/min	--- bar
4	3.90 min	95.00 %	5.00 %	--- mL/min	--- bar
5	4.00 min	95.00 %	5.00 %	--- mL/min	--- bar

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

Valve Position Port 1 -> 6
 Ready when front door open Yes
Left Temperature Control
 Temperature Control Mode Temperature Set
 Temperature 45.00 °C
 Enable Analysis Left Temperature
 Enable Analysis Left Temperature On Yes
 Enable Analysis Left Temperature Value 0.8 °C

Right Temperature Control

Right temperature Control Mode Not Controlled
 Enable Analysis Right Temperature
 Enable Analysis Right Temperature On Yes
 Enable Analysis Right Temperature Value 0.8 °C

Stop Time

Stoptime Mode As pump/injector

Post Time

Posttime Mode Off

APPENDIX III BODY FLUID IDENTIFICATION ACQUISITION PARAMETERS

Instrument Parameters:

- Instrumentation: Agilent 6495 Tandem Mass Spectrometer with an Agilent 1290 LC system.
 - Instrument Mode: MRM
- Agilent AdvancedBio Peptide Map column, 3x100mm, 2.7 μ M
 - Mobile Phases:
 - A: Water with 0.2% Formic Acid
 - B: Acetonitrile with 0.2% Formic Acid
 - Needle Wash: 1:1:1:1 Methanol:Acetonitrile:Isopropanol:Water
 - Injection Volume: 2.5 -10 μ L
- System Suitability: Agilent Human Serum Albumin peptide mix with 100 fmol on column.
- The ions monitored are listed below and with representative chromatograms for each protein target following.

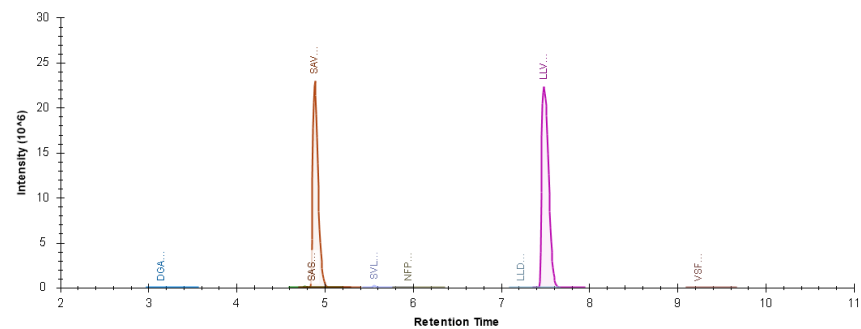
Compound Group	Compound Name	Precursor Ion	Product Ion
sp O95274 LYPD3_HUMAN	GLDLHGLLAFIQLQQC[+57.0]AQDR.light	766.1	649.3
sp O95274 LYPD3_HUMAN	GLDLHGLLAFIQLQQC[+57.0]AQDR.light	766.1	905.4
sp O95274 LYPD3_HUMAN	GLDLHGLLAFIQLQQC[+57.0]AQDR.light	766.1	1037.6
sp P02808 STAT_HUMAN	FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF.light	1215.2	1074.5
sp P02808 STAT_HUMAN	FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF.light	1215.2	1229.6
sp P02808 STAT_HUMAN	FGYGYGPYQPVPEQPLYPQPYQPQYQQYTF.light	1215.2	1687.8
sp P02647 APOA1_HUMAN	VSFLSALEEYTK.light	693.9	600.8
sp P02647 APOA1_HUMAN	VSFLSALEEYTK.light	693.9	940.5
sp P02647 APOA1_HUMAN	VSFLSALEEYTK.light	693.9	1053.5
sp P02814 SMR3B_HUMAN	GPYPGGLAPPQFPGFVPPPPPPYGPGR.light	1034.5	850.4
sp P02814 SMR3B_HUMAN	GPYPGGLAPPQFPGFVPPPPPPYGPGR.light	1034.5	1172.6
sp P02814 SMR3B_HUMAN	GPYPGGLAPPQFPGFVPPPPPPYGPGR.light	1034.5	1228.6
sp P80188 NGAL_HUMAN	WYVVGLAGNAILR.light	716.4	350.1
sp P80188 NGAL_HUMAN	WYVVGLAGNAILR.light	716.4	449.2
sp P80188 NGAL_HUMAN	WYVVGLAGNAILR.light	716.4	884.5
sp P15309 PPAP_HUMAN	ELSELSLLSLYGIHK.light	568.0	730.4
sp P15309 PPAP_HUMAN	ELSELSLLSLYGIHK.light	568.0	817.5
sp P15309 PPAP_HUMAN	ELSELSLLSLYGIHK.light	568.0	930.5
sp P04745 AMY1_HUMAN	IAEYMNHLIDIGVAGFR.light	640.3	606.3
sp P04745 AMY1_HUMAN	IAEYMNHLIDIGVAGFR.light	640.3	867.9
sp P04745 AMY1_HUMAN	IAEYMNHLIDIGVAGFR.light	640.3	903.5
sp P04745 AMY1_HUMAN	LSGLLDLALGK.light	550.3	729.5
sp P04745 AMY1_HUMAN	LSGLLDLALGK.light	550.3	899.6
sp P04745 AMY1_HUMAN	LSGLLDLALGK.light	550.3	986.6
sp P02814 SMR3B_HUMAN	IPPPPPAPYGPGFPPPPPPQP.light	710.7	535.3
sp P02814 SMR3B_HUMAN	IPPPPPAPYGPGFPPPPPPQP.light	710.7	729.4
sp P02814 SMR3B_HUMAN	IPPPPPAPYGPGFPPPPPPQP.light	710.7	1141.6
sp P68871 HBB_HUMAN	LLVVYPWTQR.light	637.9	687.4
sp P68871 HBB_HUMAN	LLVVYPWTQR.light	637.9	850.4
sp P68871 HBB_HUMAN	LLVVYPWTQR.light	637.9	949.5
sp P04279 SEMG1_HUMAN	DIFSTQDELLVYNK.light	842.9	424.2
sp P04279 SEMG1_HUMAN	DIFSTQDELLVYNK.light	842.9	523.3
sp P04279 SEMG1_HUMAN	DIFSTQDELLVYNK.light	842.9	1309.7
sp P02647 APOA1_HUMAN	LLDNWDSVTSTFSK.light	806.9	569.3
sp P02647 APOA1_HUMAN	LLDNWDSVTSTFSK.light	806.9	670.3
sp P02647 APOA1_HUMAN	LLDNWDSVTSTFSK.light	806.9	971.5
sp P15309 PPAP_HUMAN	FVTLVFR.light	441.3	247.1
sp P15309 PPAP_HUMAN	FVTLVFR.light	441.3	421.3
sp P15309 PPAP_HUMAN	FVTLVFR.light	441.3	635.4
sp O60437 PEPL_HUMAN	NLLDEIASR.light	515.8	228.1

sp O60437 PEPL_HUMAN	NLLDEIASR.light	515.8	690.3
sp O60437 PEPL_HUMAN	NLLDEIASR.light	515.8	803.4
sp P02790 HEMO_HUMAN	NFPSPVDAAFR.light	610.8	480.3
sp P02790 HEMO_HUMAN	NFPSPVDAAFR.light	610.8	775.4
sp P02790 HEMO_HUMAN	NFPSPVDAAFR.light	610.8	959.5
sp P07288 KLK3_HUMAN	FLRPGDDSSHDLMLLR.light	468.7	536.3
sp P07288 KLK3_HUMAN	FLRPGDDSSHDLMLLR.light	468.7	575.6
sp P07288 KLK3_HUMAN	FLRPGDDSSHDLMLLR.light	468.7	760.4
sp P01009 A1AT_HUMAN	SVLGQLGITK.light	508.3	531.4
sp P01009 A1AT_HUMAN	SVLGQLGITK.light	508.3	716.4
sp P01009 A1AT_HUMAN	SVLGQLGITK.light	508.3	829.5
sp P02687 MBP_BOVIN	DTGILDSLGR.light	523.8	660.4
sp P02687 MBP_BOVIN	DTGILDSLGR.light	523.8	830.5
sp P02687 MBP_BOVIN	DTGILDSLGR.heavy	528.8	670.4
sp P02687 MBP_BOVIN	DTGILDSLGR.heavy	528.8	840.5
sp P80188 NGAL_HUMAN	MYATIELK.light	566.3	295.1
sp P80188 NGAL_HUMAN	MYATIELK.light	566.3	837.5
sp P80188 NGAL_HUMAN	MYATIELK.light	566.3	1000.5
sp P68871 HBB_HUMAN	SAVTALWGK.light	466.8	574.3
sp P68871 HBB_HUMAN	SAVTALWGK.light	466.8	675.4
sp P68871 HBB_HUMAN	SAVTALWGK.light	466.8	774.5
sp Q02383 SEMG2_HUMAN	DVSQSSISFQIEK.light	734.4	751.4
sp Q02383 SEMG2_HUMAN	DVSQSSISFQIEK.light	734.4	951.5
sp Q02383 SEMG2_HUMAN	DVSQSSISFQIEK.light	734.4	1038.5
sp P02787 TRFE_HUMAN	SASDLTWDNLK.light	625.3	675.3
sp P02787 TRFE_HUMAN	SASDLTWDNLK.light	625.3	776.4
sp P02787 TRFE_HUMAN	SASDLTWDNLK.light	625.3	1091.5
sp P01009 A1AT_HUMAN	LSITGTYDLK.light	555.8	696.4
sp P01009 A1AT_HUMAN	LSITGTYDLK.light	555.8	797.4
sp P01009 A1AT_HUMAN	LSITGTYDLK.light	555.8	910.5
sp P09228 CYTT_HUMAN	ALHFVISEYNK.light	440.9	424.2
sp P09228 CYTT_HUMAN	ALHFVISEYNK.light	440.9	568.3
sp P09228 CYTT_HUMAN	ALHFVISEYNK.light	440.9	640.3
sp P09228 CYTT_HUMAN	IIEGGIYDADLNDER.light	846.9	947.4
sp P09228 CYTT_HUMAN	IIEGGIYDADLNDER.light	846.9	1110.5
sp P09228 CYTT_HUMAN	IIEGGIYDADLNDER.light	846.9	1466.6
sp P07288 KLK3_HUMAN	LSEPAELTDAVK.light	636.8	472.3
sp P07288 KLK3_HUMAN	LSEPAELTDAVK.light	636.8	646.4
sp P07288 KLK3_HUMAN	LSEPAELTDAVK.light	636.8	943.5
sp P07476 INVO_HUMAN	QEAQLELPEQQVGQPK.light	608.0	505.8
sp P07476 INVO_HUMAN	QEAQLELPEQQVGQPK.light	608.0	699.3

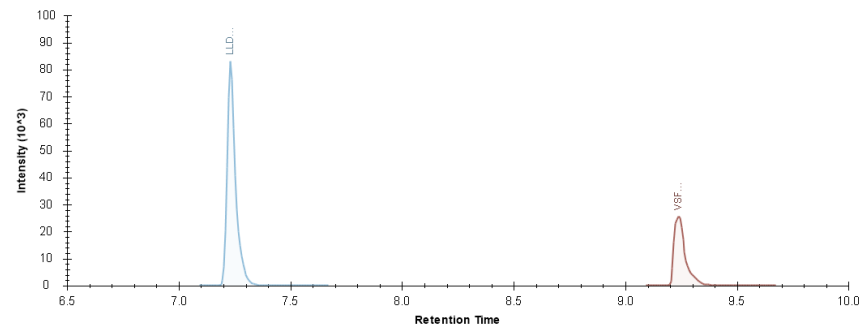
sp P07476 INVO_HUMAN	QEAQLELPEQQVGQPK.light	608.0	812.4
sp Q9UBG3 CRNN_HUMAN	LLDEDHTGTVEFK.light	501.9	294.2
sp Q9UBG3 CRNN_HUMAN	LLDEDHTGTVEFK.light	501.9	639.3
sp Q9UBG3 CRNN_HUMAN	LLDEDHTGTVEFK.light	501.9	695.8
sp P15309 PPAP_HUMAN	FQELESETLK.light	612.3	276.1
sp P15309 PPAP_HUMAN	FQELESETLK.light	612.3	819.4
sp P15309 PPAP_HUMAN	FQELESETLK.light	612.3	948.5
sp Q9UBG3 CRNN_HUMAN	ISPQIQLSGQTEQTQK.light	893.5	793.4
sp Q9UBG3 CRNN_HUMAN	ISPQIQLSGQTEQTQK.light	893.5	1006.5
sp Q9UBG3 CRNN_HUMAN	ISPQIQLSGQTEQTQK.light	893.5	1247.6
sp P07476 INVO_HUMAN	GEVLLPVEHQQQK.light	502.3	553.8
sp P07476 INVO_HUMAN	GEVLLPVEHQQQK.light	502.3	610.3
sp P07476 INVO_HUMAN	GEVLLPVEHQQQK.light	502.3	659.9
sp P02787 TRFE_HUMAN	DGAGDVAFVK.light	489.7	563.4
sp P02787 TRFE_HUMAN	DGAGDVAFVK.light	489.7	678.4
sp P02787 TRFE_HUMAN	DGAGDVAFVK.light	489.7	735.4
sp Q6UWP8 SBSN_HUMAN	ALDGINSGITHAGR.light	461.2	541.8
sp Q6UWP8 SBSN_HUMAN	ALDGINSGITHAGR.light	461.2	599.3
sp Q02383 SEMG2_HUMAN	GSISIQTEEQIHGK.light	509.6	461.6
sp Q02383 SEMG2_HUMAN	GSISIQTEEQIHGK.light	509.6	635.3
sp Q02383 SEMG2_HUMAN	GSISIQTEEQIHGK.light	509.6	691.9
sp P61916 NPC2_HUMAN	DC[+57.0]GSVDGVIK.light	525.3	276.1
sp P61916 NPC2_HUMAN	DC[+57.0]GSVDGVIK.light	525.3	630.4
sp P61916 NPC2_HUMAN	DC[+57.0]GSVDGVIK.light	525.3	774.4
sp Q6UWP8 SBSN_HUMAN	FGQGVHHGLSEGWK.light	513.6	603.8
sp Q6UWP8 SBSN_HUMAN	FGQGVHHGLSEGWK.light	513.6	696.3
sp Q95274 LYPD3_HUMAN	GC[+57.0]VQDEFC[+57.0]TR.light	636.3	827.3
sp Q95274 LYPD3_HUMAN	GC[+57.0]VQDEFC[+57.0]TR.light	636.3	955.4
sp Q95274 LYPD3_HUMAN	GC[+57.0]VQDEFC[+57.0]TR.light	636.3	1054.5
sp Q9UBC9 SPRR3_HUMAN	VPVPGYTK.light	430.7	381.2
sp Q9UBC9 SPRR3_HUMAN	VPVPGYTK.light	430.7	565.3
sp Q9UBC9 SPRR3_HUMAN	VPVPGYTK.light	430.7	664.4
sp Q02383 SEMG2_HUMAN	GSISIQTEEK.light	546.3	634.3
sp Q02383 SEMG2_HUMAN	GSISIQTEEK.light	546.3	747.4
sp Q02383 SEMG2_HUMAN	GSISIQTEEK.light	546.3	834.4
sp P04279 SEMG1_HUMAN	QITIPSQEQEHSQK.light	551.6	599.3
sp P04279 SEMG1_HUMAN	QITIPSQEQEHSQK.light	551.6	706.3
sp P61916 NPC2_HUMAN	SGINC[+57.0]PIQK.light	508.8	485.3
sp P61916 NPC2_HUMAN	SGINC[+57.0]PIQK.light	508.8	645.3
sp P61916 NPC2_HUMAN	SGINC[+57.0]PIQK.light	508.8	759.4
sp P07476 INVO_HUMAN	HLVQQEQGLEQKER.light	574.6	560.3

sp P07476 INVO_HUMAN	HLVQQEGQLEQQER.light	574.6	689.3
sp P07476 INVO_HUMAN	HLVQQEGQLEQQER.light	574.6	920.5
sp Q9UBG3 CRNN_HUMAN	AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR.light	839.9	851.4
sp Q9UBG3 CRNN_HUMAN	AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR.light	839.9	886.9
sp Q9UBG3 CRNN_HUMAN	AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR.light	839.9	1001.5
sp O60437 PEPL_HUMAN	NQGPQESVVR.light	557.3	243.1
sp O60437 PEPL_HUMAN	NQGPQESVVR.light	557.3	814.4
sp O60437 PEPL_HUMAN	NQGPQESVVR.light	557.3	871.5
sp Q9UBC9 SPRR3_HUMAN	VPEPGC[+57.0]TK.light	444.2	394.7
sp Q9UBC9 SPRR3_HUMAN	VPEPGC[+57.0]TK.light	444.2	562.3
sp Q9UBC9 SPRR3_HUMAN	VPEPGC[+57.0]TK.light	444.2	788.4
sp O60437 PEPL_HUMAN	AQSLQSAK.light	416.7	200.1
sp O60437 PEPL_HUMAN	AQSLQSAK.light	416.7	546.3
sp O60437 PEPL_HUMAN	AQSLQSAK.light	416.7	633.4

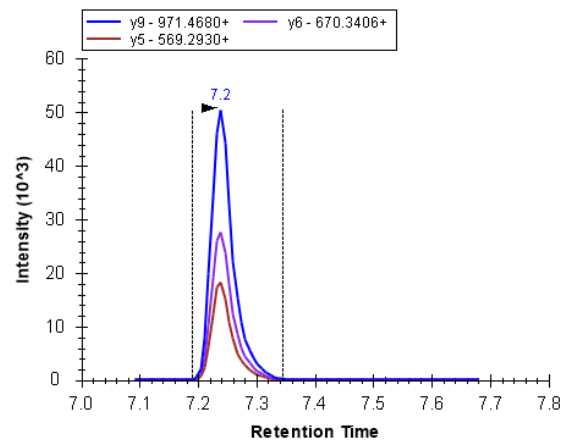
Peripheral Blood:



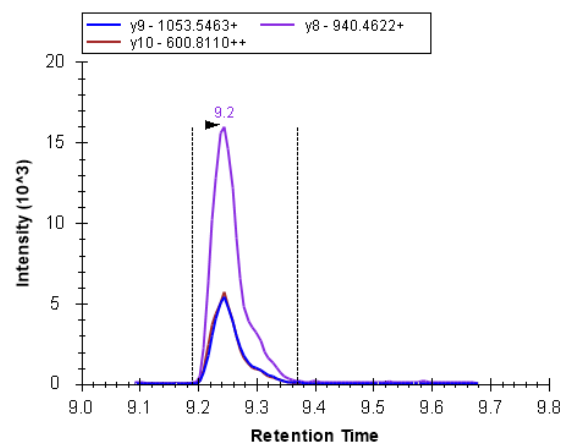
Apolipoprotein P02647|APOA1



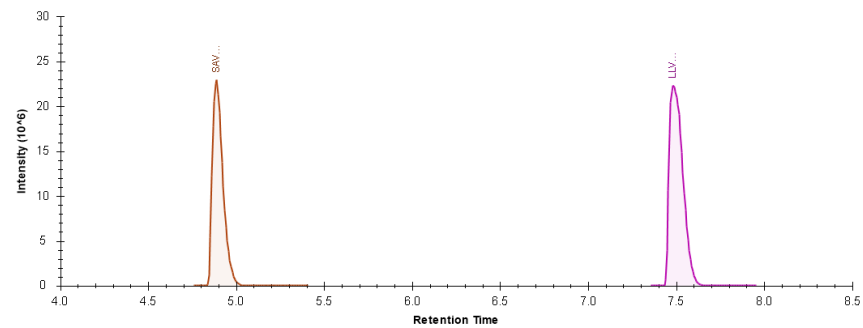
K.LLDNWDSVTSTFSK.L [69, 82]



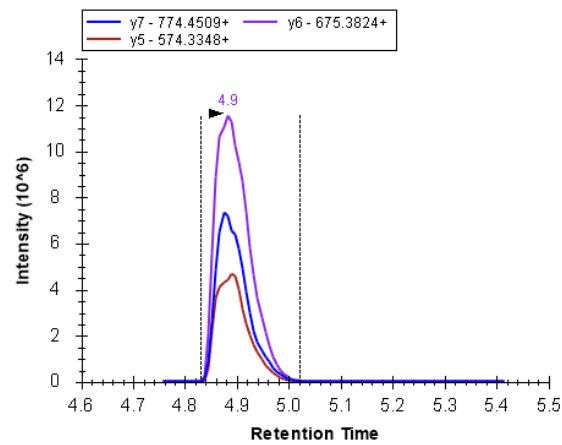
K.VSFLSALEEYTK.K [250, 261]



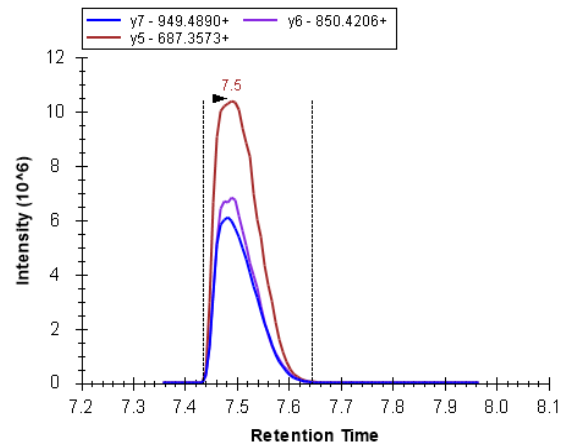
Hemoglobin Beta P68871|HBB

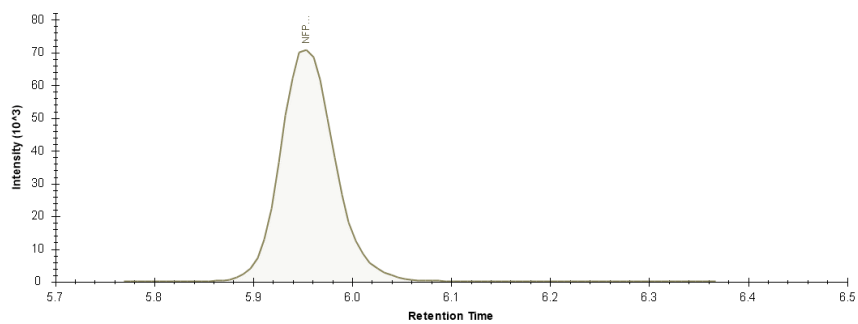
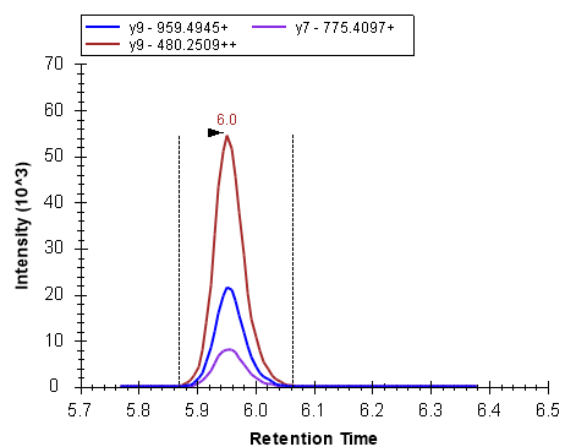


SAVTALWGK

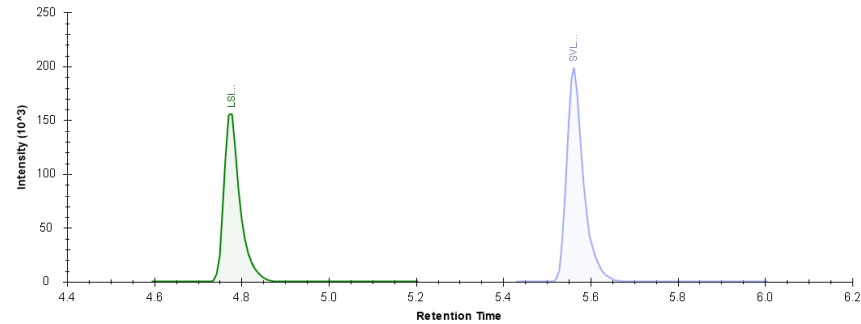


LLVVYPWTQR

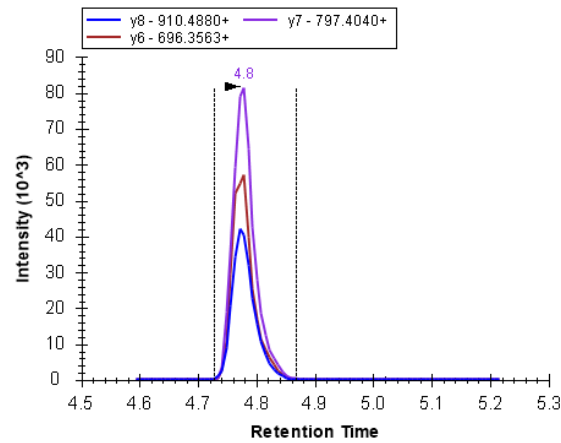


Hemopexin P02790|HEMO**K.NFPSPVDAAFR.Q [91, 101]**

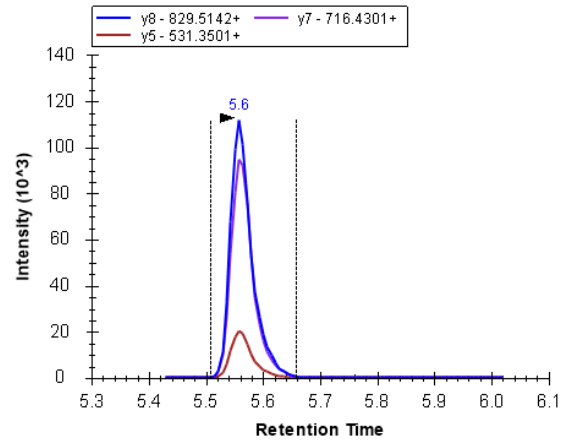
Alpha-1 Antitrypsin P01009|A1AT



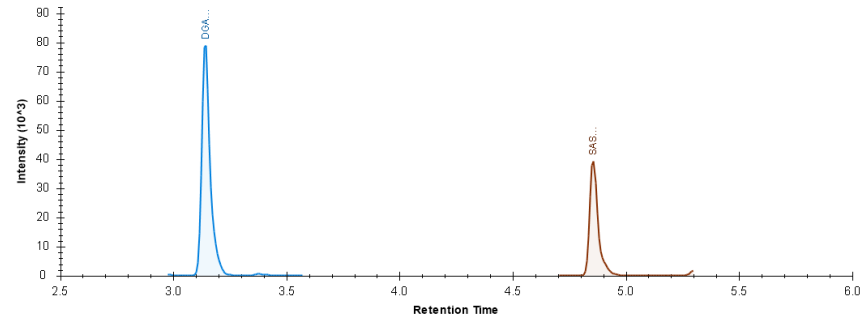
K.LSITGTYDLK.S [314, 323]



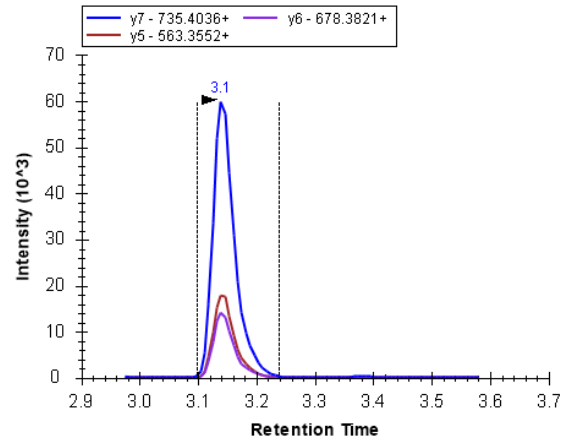
K.SVLGQLGITK.V [324, 333]



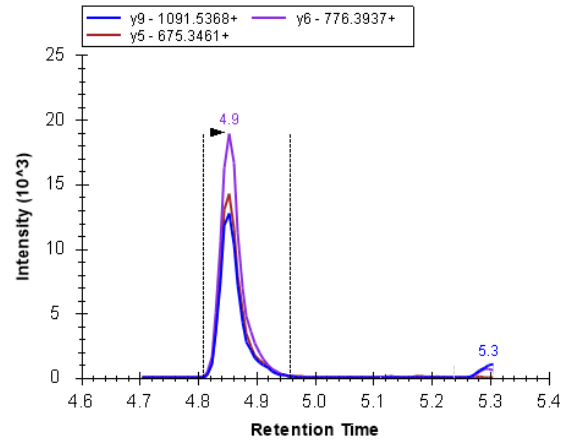
Serotransferrin P02787|TRFE



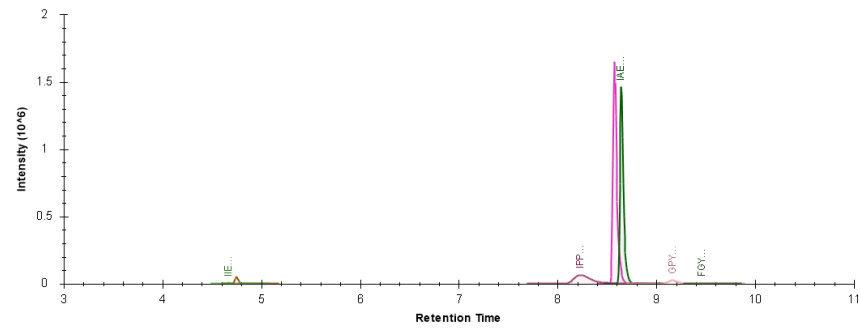
K.DGAGDVAFVK.H [215, 224]



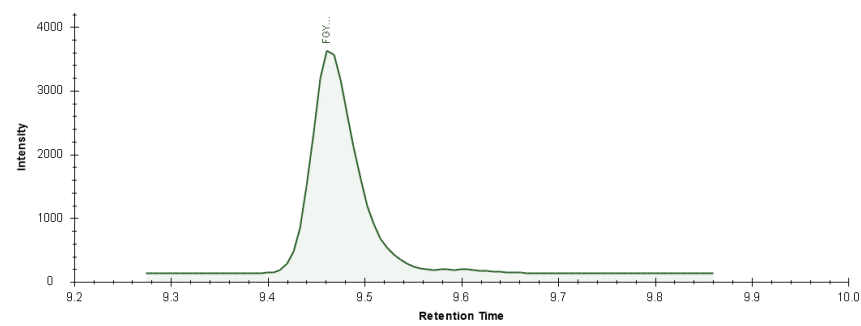
K.SASDLTWDNLK.G [453, 463]



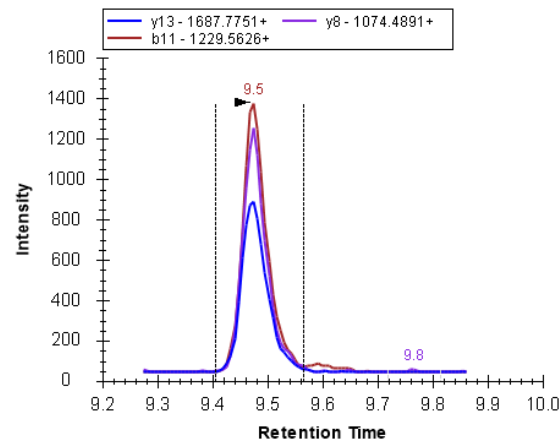
Saliva



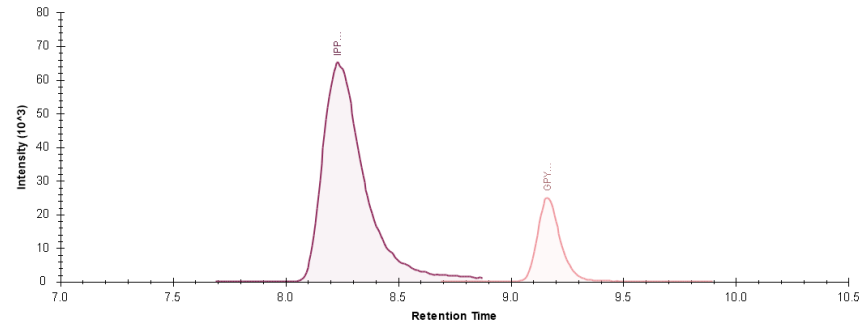
Statherin P02808 |STAT



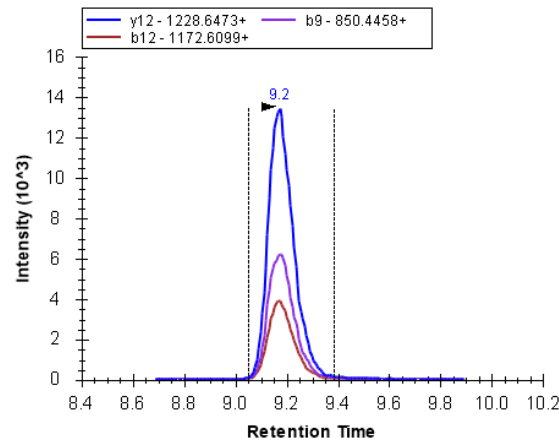
R.FGYGYGPYQPVPEQPLYPQYPYQPQYQQYTF - [32, 61]



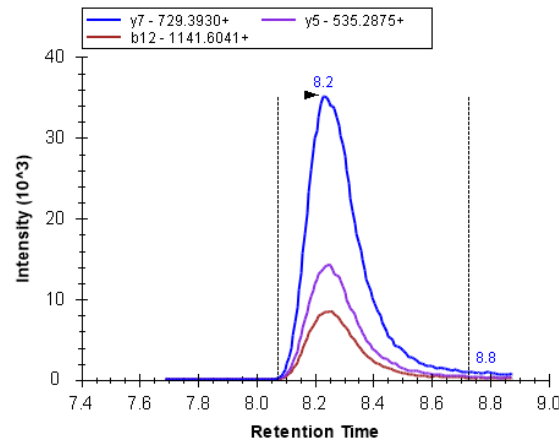
Submaxillary Gland Androgen-Regulated Protein 3B P02814|SMR3B

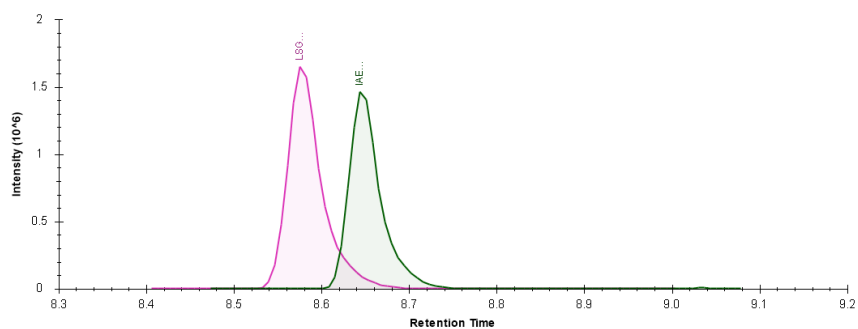
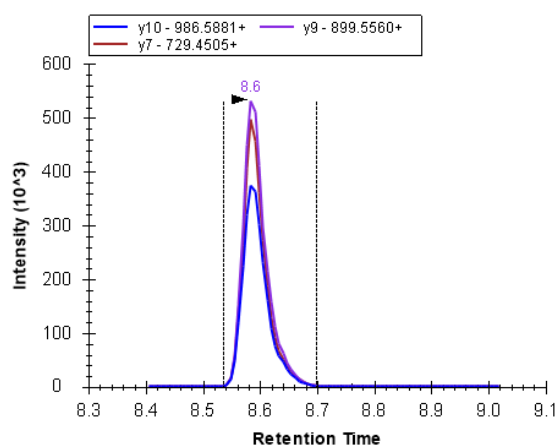
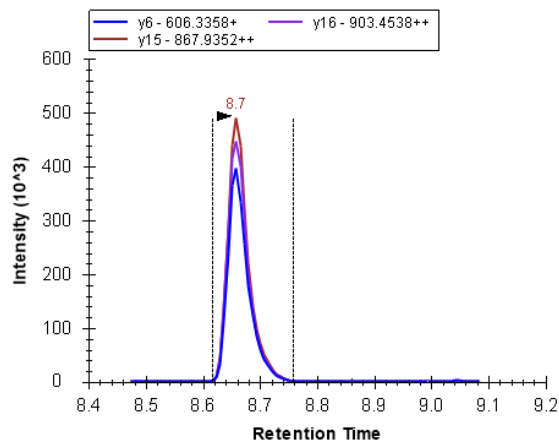


R.GPYPPGPLAPPQPFPGPGFVPPPPPPYGPGR.I [27, 57]

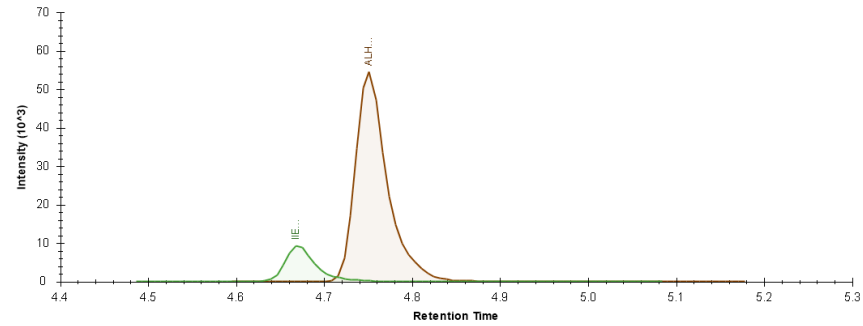


R.IPPPPPAPYGP GIFPPPPQP - [58, 78]

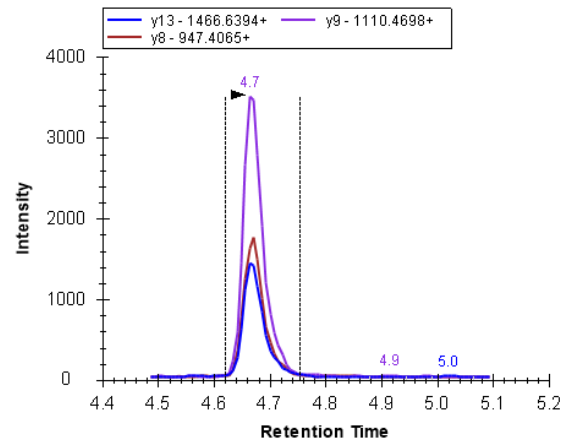


Alpha Amylase-1 P04745|AMY1**R.LSGLLDLALGK.D [176, 186]****K.IAEYMNHLIDIGVAGFR.I [193, 209]**

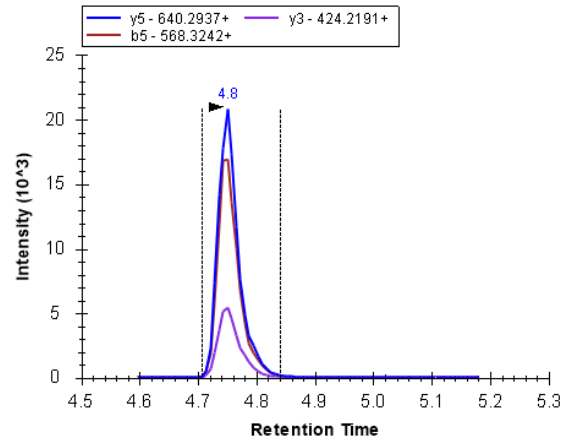
Cystatin SA P09228|CYTT



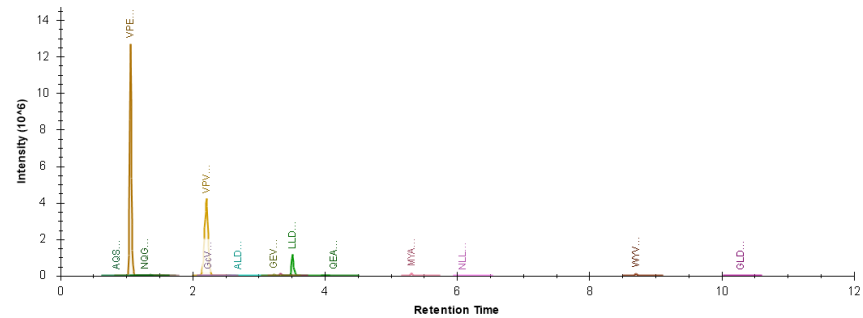
R.IIEGGIYDADLNDER.V [28, 42]



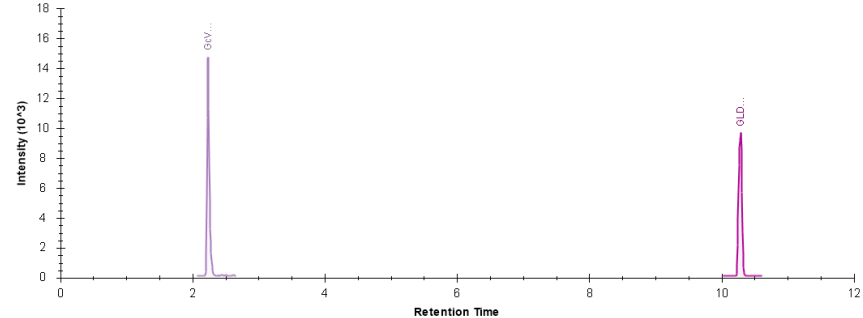
R.ALHFVISEYNK.A [46, 56]



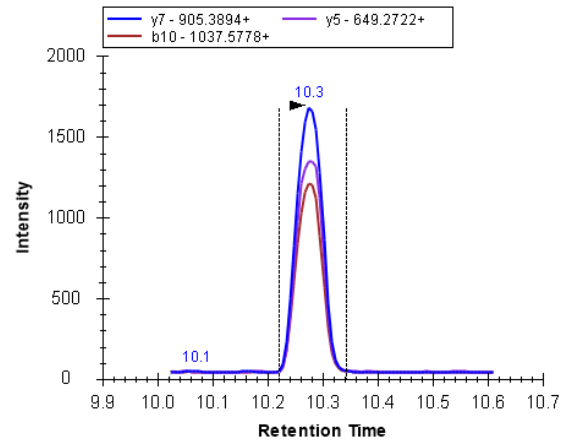
Vaginal Fluid



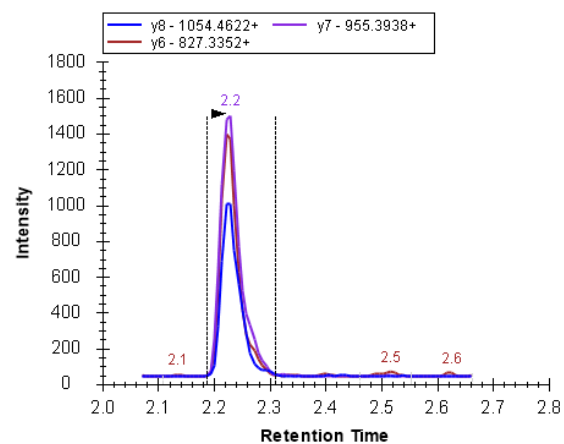
Ly6/PLAUR O95274|LYPD3



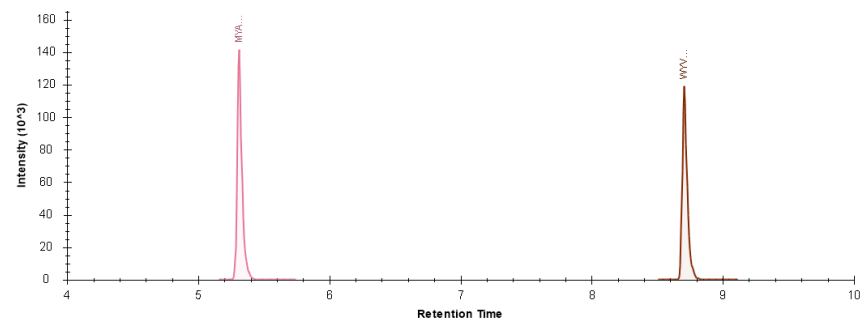
R.GLDLHGLLAFIQLQQCAQDR.C [92, 111]



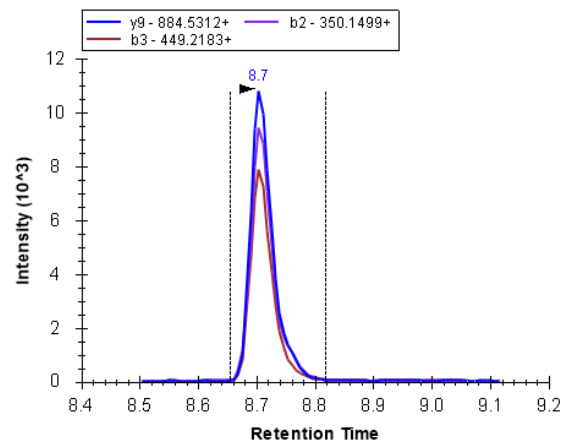
R.GCVQDEFCTR.D [191, 200]



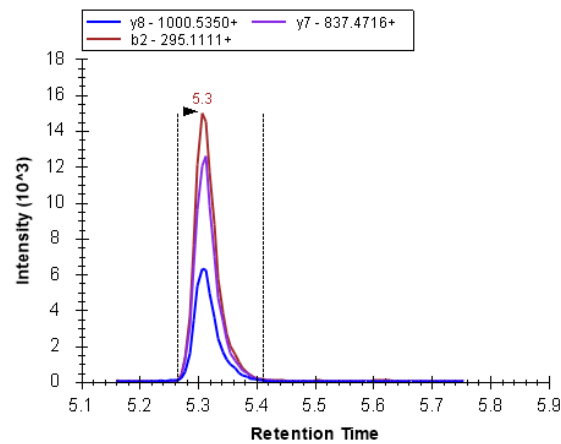
Neutrophil Gelatinase P80188|NGAL

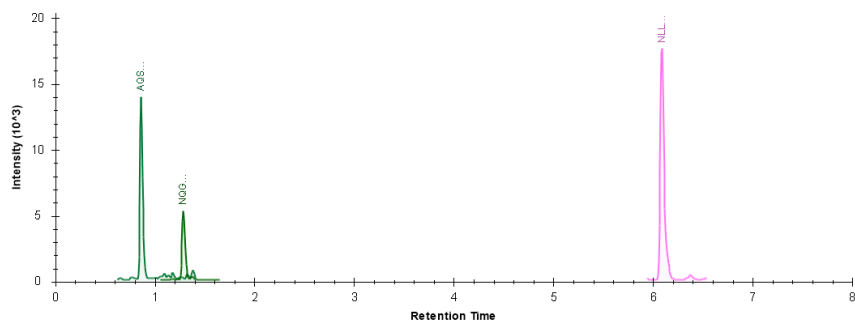
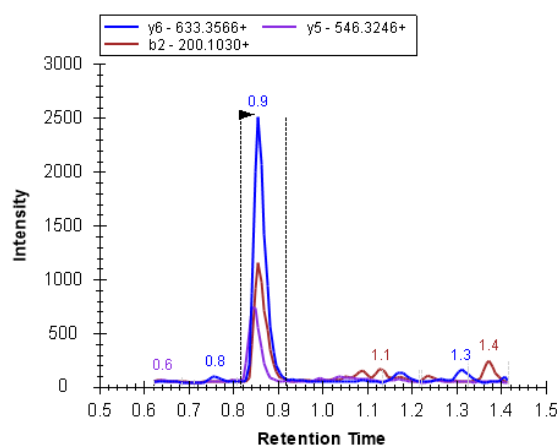
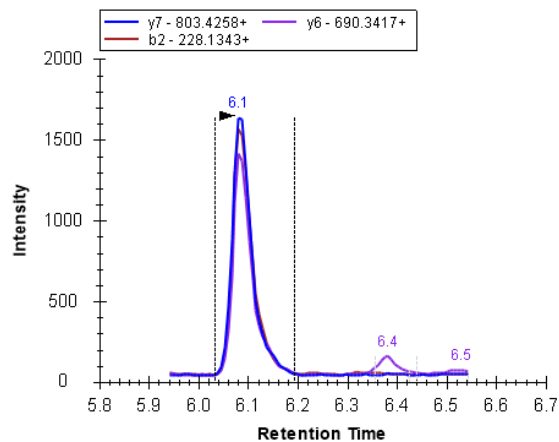


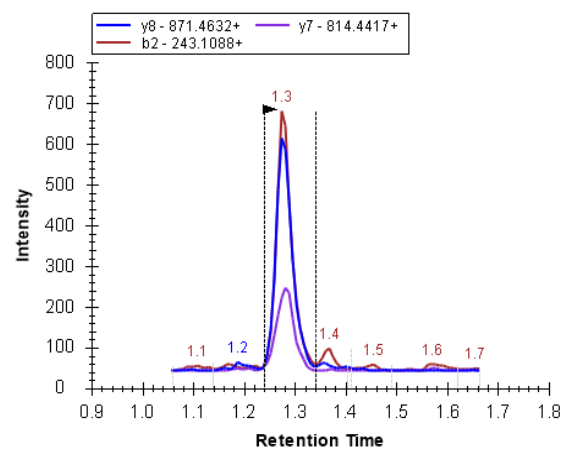
K.WYVVGLAGNAILR.E [50, 62]



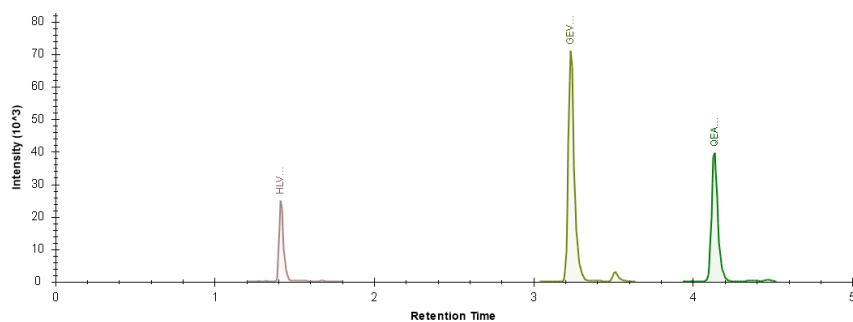
K.MYATIELK.E [70, 78]



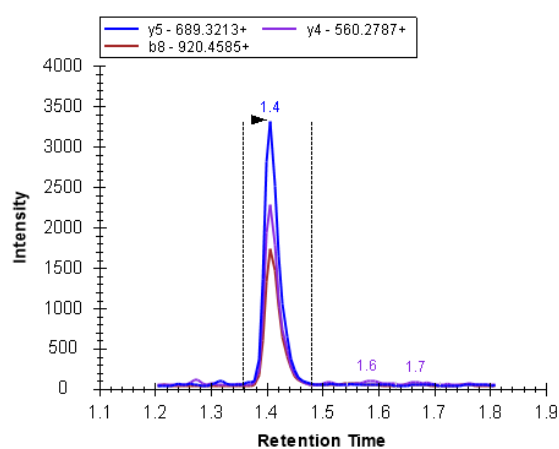
Periplakin O60437|PEPL**R.AQSLQSAK.A [721, 728]****K.NLLDEIASR.E [771, 779]****R.NQGPQESVVR.K [924, 933]**



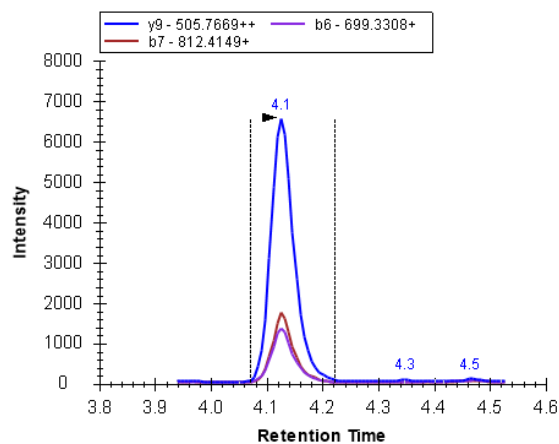
Involucrin P07476|INVO



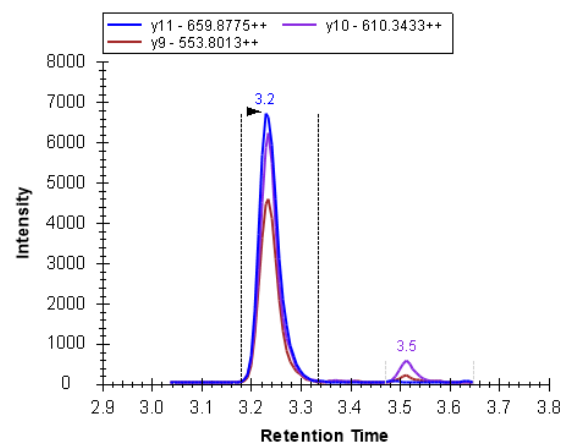
K.HLVQQEGQLEQQR.Q [404, 417]



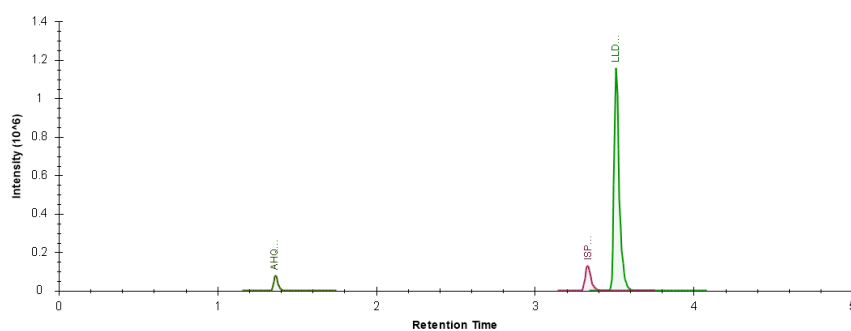
K.QEAQLELPEQQVGQPK.H [485, 500]



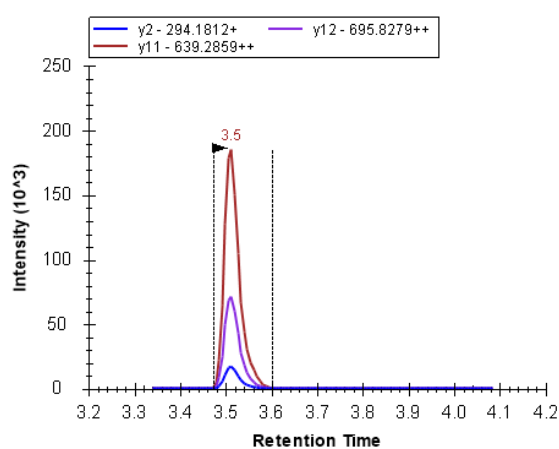
K.GEVLLPVEHQQK.Q [562, 574]



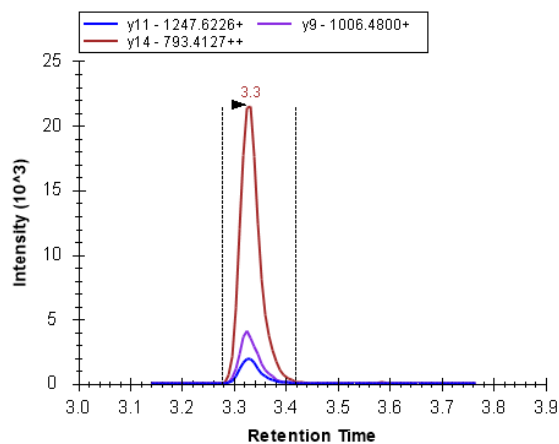
Cornulin Q9UBG3|CRNN



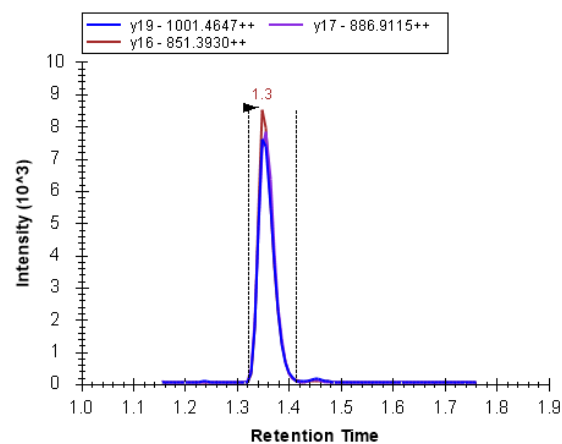
R.LLDEDHTGTVEFK.E [59, 71]

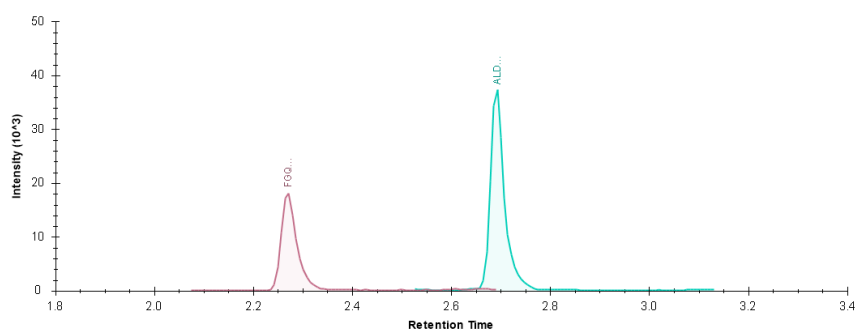
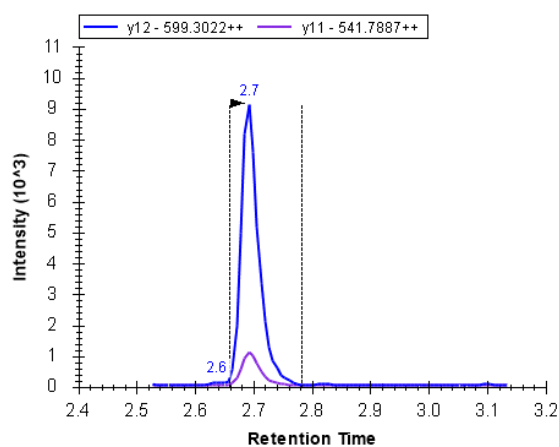
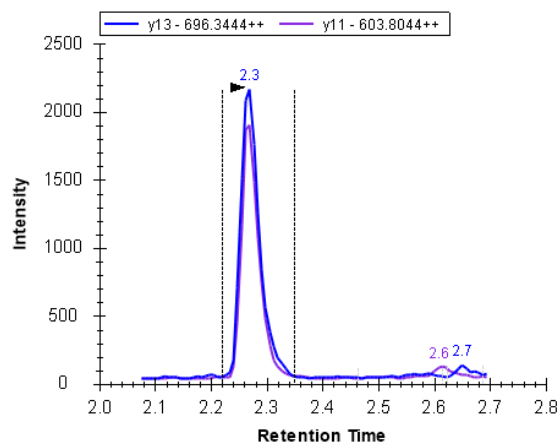


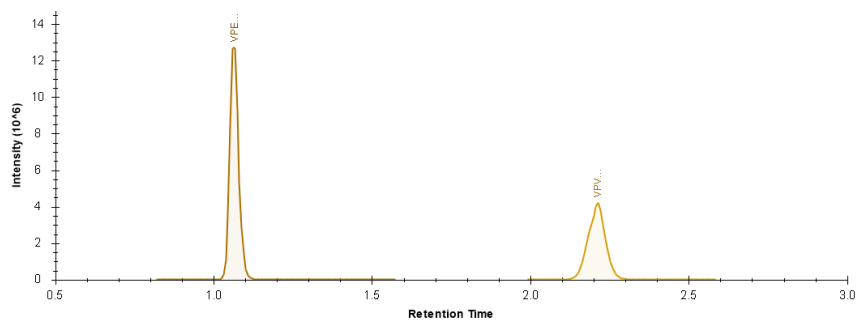
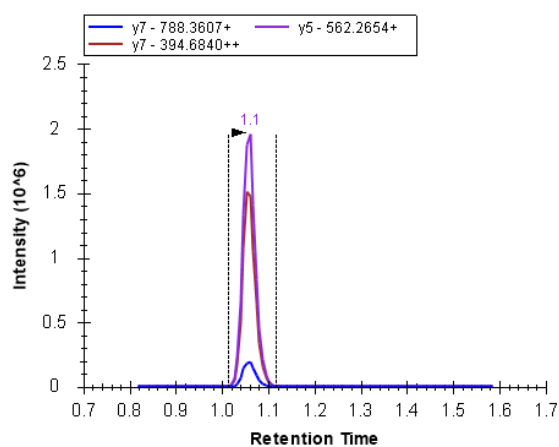
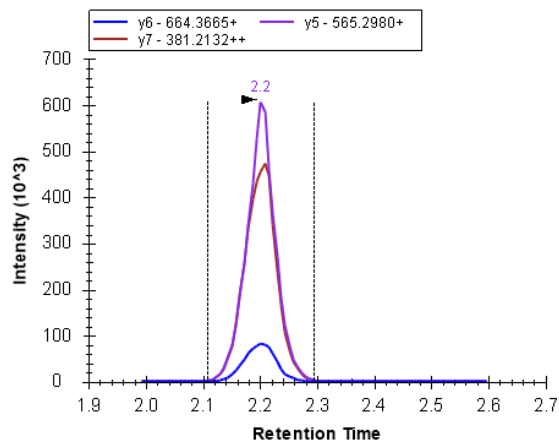
R.ISPQIQLSGQTEQTQK.A [178, 193]



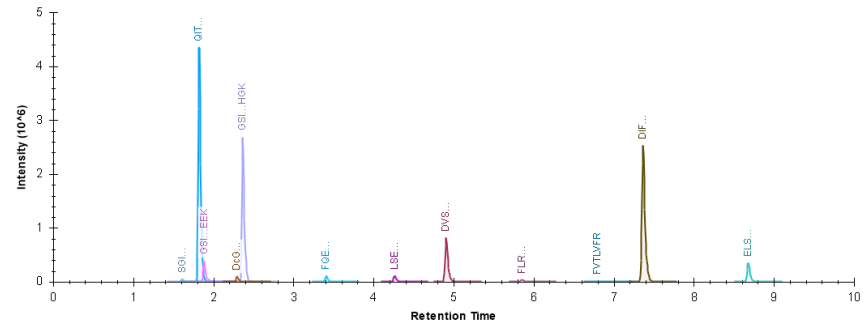
R.AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR.T [219, 251]



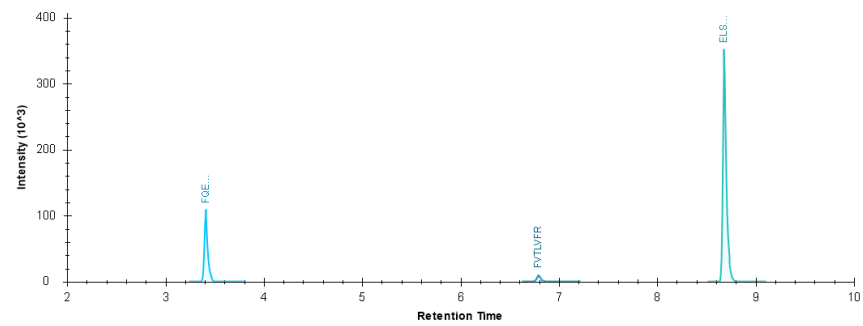
Suprabasin Q6UWP8|SBSN**K.ALDGINSGITHAGR.E [49, 62]****R.FGQGVHHGLSEGWK.E [331, 344]**

Small Proline Rich Protein 3 Q9UBC9|SPRR3**K.VPEPGCTK.V [60, 67]****K.VPVPGYTK.L [140, 147]**

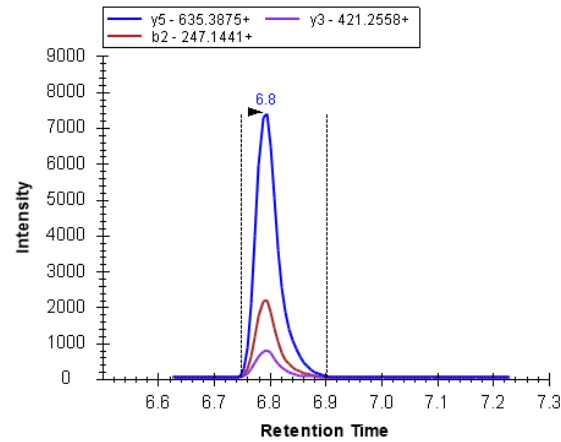
Seminal Fluid



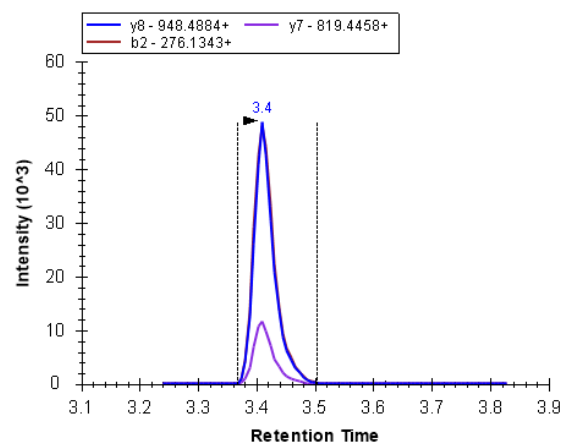
Prostatic Acid Phosphatase P15309|PPAP



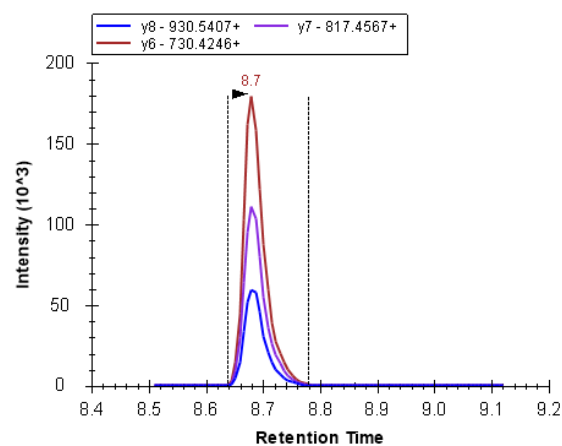
K.FVTLVFR.H [36, 42]

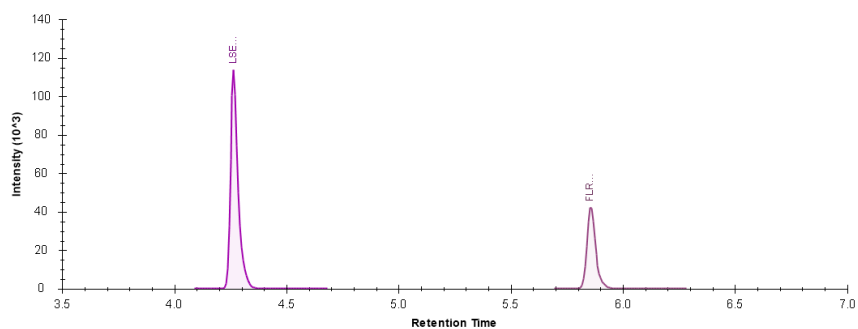
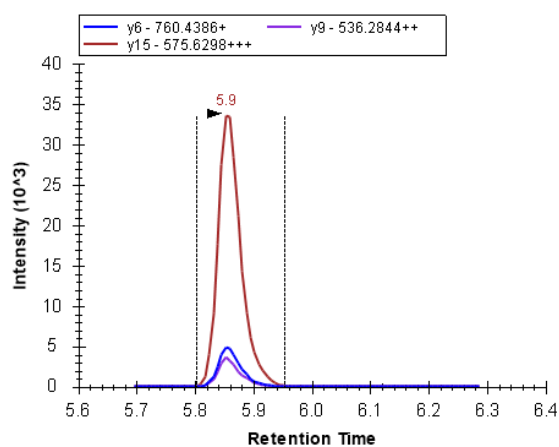
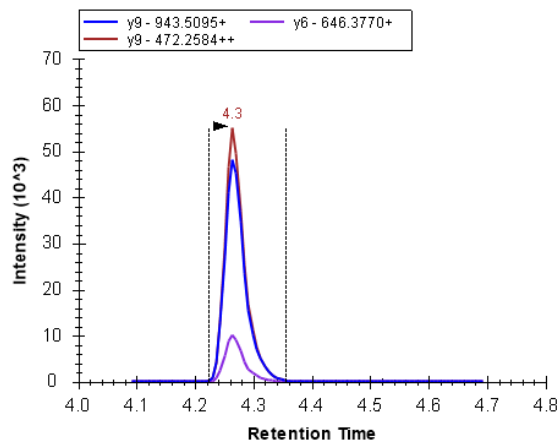


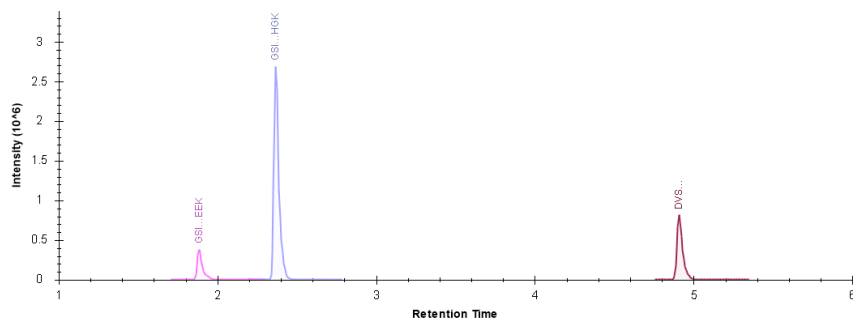
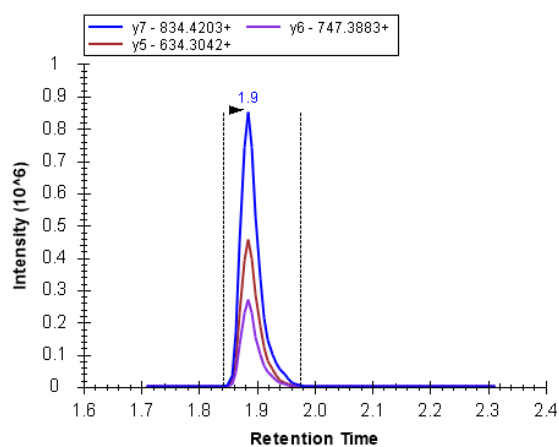
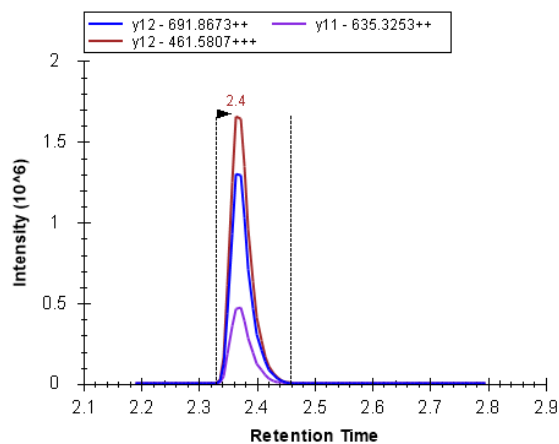
R.FQELESETLK.S [163, 172]

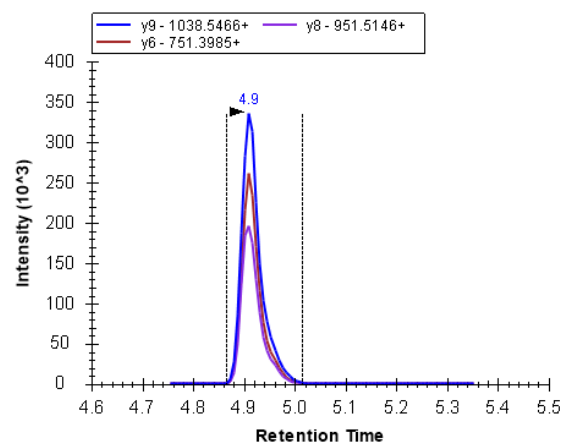


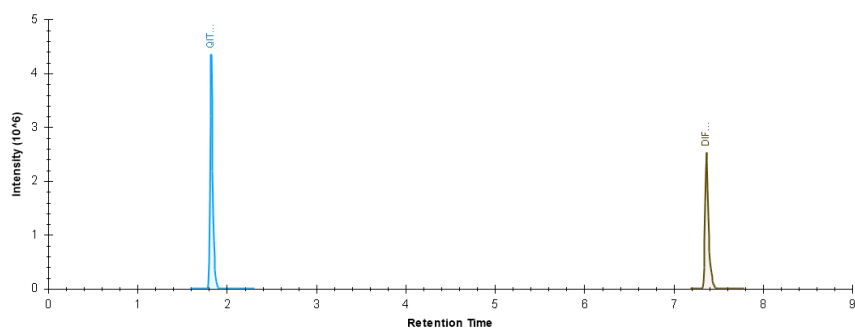
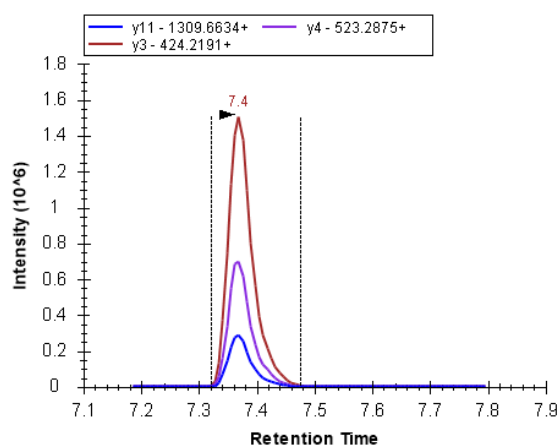
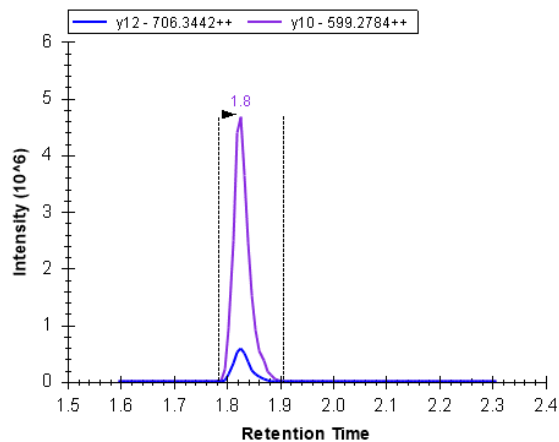
R.ELSELSLLSLYGIHK.Q [236, 250]



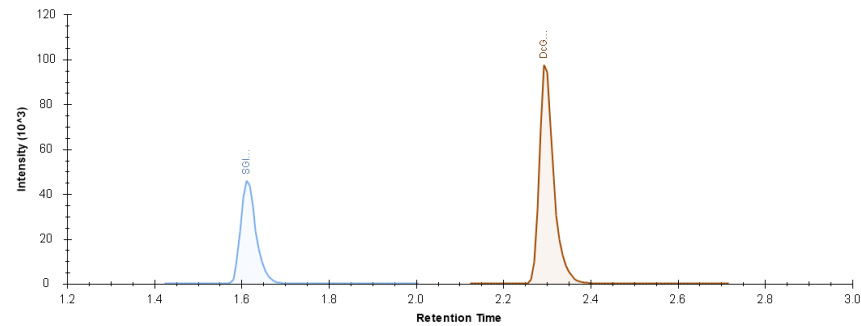
Prostate Specific Antigen P07288|KLK3**R.FLRPGDDSSHDLMLLR.L [109, 124]****R.LSEPAELTDAVK.V [125, 136]**

Semenogelin 2 Q02383|SEMG2**K.GSISIQTEEK.I [311, 320]****K.GSISIQTEEQIHGK.S [371, 384]****K.DVSQSSISFQIEK.L [487, 499]**

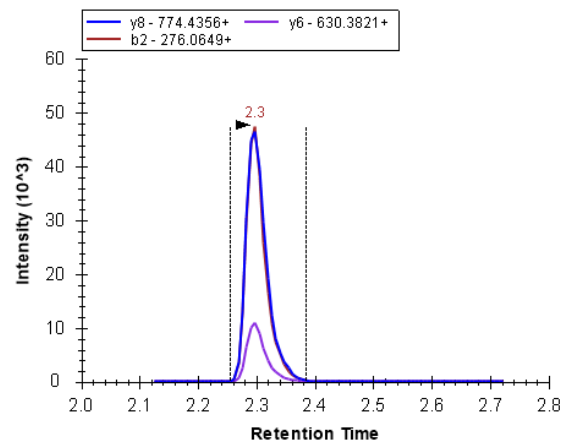


Semenogelin 1 P04279|SEMG1**K.DIFSTQDELLVYNK.N [251, 264]****K.QITIPSQEQEHSQK.A [328, 341]**

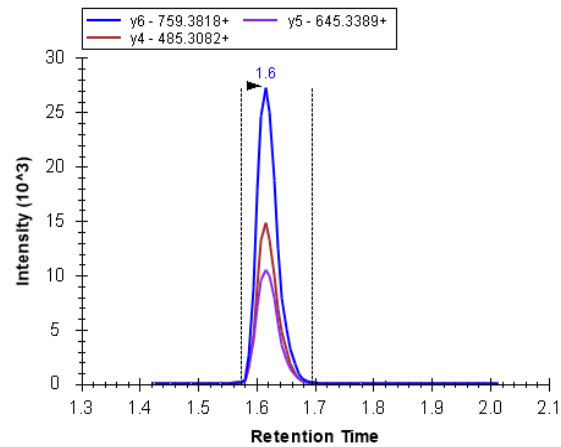
Epididymal Secretory Protein P61916|NPC2



K.DCGSVDGVIK.E [25, 34]



K.SGINCP^uPIQK.D [94, 102]



- The acquisition parameters are as follows:

Acquisition Method Info

Method Name 11min_dMRM_FINAL.m
 Method Path D:\MassHunter\methods\Proteomics_DOD\3mm_Final\11min_dMRM_FINAL.m
 Method Description 11 min 3x100
 Device List
 HIP Sampler
 Binary Pump
 Column Comp.
 QQQ

MS QQQ Mass Spectrometer

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

Time Segments

Index	Start Time (min)	Scan Type	Ion Mode	Div Valve	Delta EMV	Store	Cycle Time (ms)	Triggered?	MRM Repeats
1	0	DynamicMRM	ESI+Agilent Jet Stream	To MS	200	Yes	400	No	3

Time Segment 1

Scan Segments

Cpd Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Frag (V)	CE (V)	Cell Acc (V)	Ret Time (min)	Ret Window	Polarity
AHQGTGET VTGSGTQ TQAGATQ TVEQDSS HQTGR.lig ht	No	839.8888	Unit/Enh (6490)	1001.4646 63	Unit/Enh (6490)	380	28	4	1.46	0.6	Positive
AHQGTGET VTGSGTQ TQAGATQ TVEQDSS HQTGR.lig ht	No	839.8888	Unit/Enh (6490)	886.91153 5	Unit/Enh (6490)	380	22	4	1.46	0.6	Positive
AHQGTGET VTGSGTQ TQAGATQ TVEQDSS HQTGR.lig ht	No	839.8888	Unit/Enh (6490)	851.39297 8	Unit/Enh (6490)	380	25.4	4	1.46	0.6	Positive
ALDGIN GITHAGR.I light	No	461.24426 8	Unit/Enh (6490)	599.30217 5	Unit/Enh (6490)	380	11.8	4	2.83	0.6	Positive
ALDGIN GITHAGR.I light	No	461.24426 8	Unit/Enh (6490)	541.78870 4	Unit/Enh (6490)	380	15	4	2.83	0.6	Positive
ALHFVISE YNK.light	No	440.90307 2	Unit/Enh (6490)	640.29368 1	Unit/Enh (6490)	380	8	4	4.89	0.6	Positive
ALHFVISE YNK.light	No	440.90307 2	Unit/Enh (6490)	568.32419 4	Unit/Enh (6490)	380	14	4	4.89	0.6	Positive
ALHFVISE YNK.light	No	440.90307 2	Unit/Enh (6490)	424.21906 (6490)	Unit/Enh (6490)	380	14	4	4.89	0.6	Positive
AQSLQSA K.light	No	416.72979 2	Unit/Enh (6490)	633.35661 6	Unit/Enh (6490)	380	11	4	0.92	0.6	Positive
AQSLQSA K.light	No	416.72979 2	Unit/Enh (6490)	546.32458 8	Unit/Enh (6490)	380	17	4	0.92	0.6	Positive
AQSLQSA K.light	No	416.72979 2	Unit/Enh (6490)	200.10296 7	Unit/Enh (6490)	380	13.9	4	0.92	0.6	Positive
DC[+57.0] GSVDGVI K.light	No	525.25023 1	Unit/Enh (6490)	774.43559 5	Unit/Enh (6490)	380	14	4	2.43	0.6	Positive
DC[+57.0] GSVDGVI K.light	No	525.25023 1	Unit/Enh (6490)	630.38210 2	Unit/Enh (6490)	380	14	4	2.43	0.6	Positive
DC[+57.0] GSVDGVI K.light	No	525.25023 1	Unit/Enh (6490)	276.06486 8	Unit/Enh (6490)	380	17.3	4	2.43	0.6	Positive
DGAGDVA FVK.light	No	489.74818 1	Unit/Enh (6490)	735.40356 6	Unit/Enh (6490)	380	16.2	4	3.28	0.6	Positive
DGAGDVA FVK.light	No	489.74818 1	Unit/Enh (6490)	678.38210 2	Unit/Enh (6490)	380	16.2	4	3.28	0.6	Positive

Cpd Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Frag (V)	CE (V)	Cell Acc (V)	Ret Time (min)	Ret Window	Polarity
DGAGDVA FVK.light	No	489.74818 1 (6490)	Unit/Enh (6490)	563.35515 9 (6490)	Unit/Enh (6490)	380	19	4	3.28	0.6	Positive
DIFSTQDE LLVYNK.lig ht	No	842.92506 (6490)	Unit/Enh (6490)	1309.6634 22 (6490)	Unit/Enh (6490)	380	27.1	4	7.5	0.6	Positive
DIFSTQDE LLVYNK.lig ht	No	842.92506 (6490)	Unit/Enh (6490)	523.26747 4 (6490)	Unit/Enh (6490)	380	24	4	7.5	0.6	Positive
DIFSTQDE LLVYNK.lig ht	No	842.92506 (6490)	Unit/Enh (6490)	424.21906 (6490)	Unit/Enh (6490)	380	24	4	7.5	0.6	Positive
DTGILDLSL GR.heavy	Yes	528.78160 5 (6490)	Unit/Enh (6490)	840.48131 2 (6490)	Unit/Enh (6490)	380	17.2	4	5.5	0.6	Positive
DTGILDLSL GR.heavy	Yes	528.78160 5 (6490)	Unit/Enh (6490)	670.37578 4 (6490)	Unit/Enh (6490)	380	17.2	4	5.5	0.6	Positive
DTGILDLSL GR.light	No	523.77747 (6490)	Unit/Enh (6490)	830.47304 3 (6490)	Unit/Enh (6490)	380	17.2	4	5.5	0.6	Positive
DTGILDLSL GR.light	No	523.77747 (6490)	Unit/Enh (6490)	660.36751 5 (6490)	Unit/Enh (6490)	380	17.2	4	5.5	0.6	Positive
DVSQSSI SFQIEK.lig ht	No	734.36992 (6490)	Unit/Enh (6490)	1038.5466 02 (6490)	Unit/Enh (6490)	380	23.8	4	5.06	0.6	Positive
DVSQSSI SFQIEK.lig ht	No	734.36992 (6490)	Unit/Enh (6490)	951.51457 3 (6490)	Unit/Enh (6490)	380	27	4	5.06	0.6	Positive
DVSQSSI SFQIEK.lig ht	No	734.36992 (6490)	Unit/Enh (6490)	751.39848 1 (6490)	Unit/Enh (6490)	380	21	4	5.06	0.6	Positive
ELSELSSL SLYGIHK.ii ght	No	567.98557 2 (6490)	Unit/Enh (6490)	930.54072 8 (6490)	Unit/Enh (6490)	380	19	4	8.81	0.6	Positive
ELSELSSL SLYGIHK.ii ght	No	567.98557 2 (6490)	Unit/Enh (6490)	817.45666 4 (6490)	Unit/Enh (6490)	380	19	4	8.81	0.6	Positive
ELSELSSL SLYGIHK.ii ght	No	567.98557 2 (6490)	Unit/Enh (6490)	730.42463 6 (6490)	Unit/Enh (6490)	380	19	4	8.81	0.6	Positive
FQGGVHH GLSEGWK .light	No	513.58814 6 (6490)	Unit/Enh (6490)	696.34437 4 (6490)	Unit/Enh (6490)	380	13.7	4	2.4	0.6	Positive
FQGGVHH GLSEGWK .light	No	513.58814 6 (6490)	Unit/Enh (6490)	603.80435 4 (6490)	Unit/Enh (6490)	380	17	4	2.4	0.6	Positive
FGYGYGP YQVPPEQ PLYPQPY QPQYQQ YTF.light	No	1215.2330 17 (6490)	Unit/Enh (6490)	1687.7750 98 (6490)	Unit/Enh (6490)	380	31	4	9.57	0.6	Positive
FGYGYGP YQVPPEQ PLYPQPY QPQYQQ YTF.light	No	1215.2330 17 (6490)	Unit/Enh (6490)	1229.5625 86 (6490)	Unit/Enh (6490)	380	33	4	9.57	0.6	Positive
FGYGYGP YQVPPEQ PLYPQPY QPQYQQ YTF.light	No	1215.2330 17 (6490)	Unit/Enh (6490)	1074.4890 87 (6490)	Unit/Enh (6490)	380	33	4	9.57	0.6	Positive
FLRPGDD SSHDLML LR.light	No	468.74126 8 (6490)	Unit/Enh (6490)	760.43857 2 (6490)	Unit/Enh (6490)	380	15	4	5.99	0.6	Positive
FLRPGDD SSHDLML LR.light	No	468.74126 8 (6490)	Unit/Enh (6490)	575.62979 4 (6490)	Unit/Enh (6490)	380	18	4	5.99	0.6	Positive
FLRPGDD SSHDLML LR.light	No	468.74126 8 (6490)	Unit/Enh (6490)	536.28440 8 (6490)	Unit/Enh (6490)	380	15	4	5.99	0.6	Positive
FQELESE TLK.light	No	612.31134 3 (6490)	Unit/Enh (6490)	948.48841 8 (6490)	Unit/Enh (6490)	380	20	4	3.54	0.6	Positive
FQELESE TLK.light	No	612.31134 3 (6490)	Unit/Enh (6490)	819.44582 5 (6490)	Unit/Enh (6490)	380	23	4	3.54	0.6	Positive
FQELESE TLK.light	No	612.31134 3 (6490)	Unit/Enh (6490)	276.13426 7 (6490)	Unit/Enh (6490)	380	20	4	3.54	0.6	Positive
FVTLVFR.J ight	No	441.26581 3 (6490)	Unit/Enh (6490)	635.38752 2 (6490)	Unit/Enh (6490)	380	14.7	4	6.93	0.6	Positive
FVTLVFR.J ight	No	441.26581 3 (6490)	Unit/Enh (6490)	421.25578 (6490)	Unit/Enh (6490)	380	14.7	4	6.93	0.6	Positive
FVTLVFR.J ight	No	441.26581 3 (6490)	Unit/Enh (6490)	247.1441 (6490)	Unit/Enh (6490)	380	14.7	4	6.93	0.6	Positive
GC[+57.0] VQDEFC [+57.0] TR.light	No	636.26080 4 (6490)	Unit/Enh (6490)	1054.4622 2 (6490)	Unit/Enh (6490)	380	24	4	2.37	0.6	Positive

Cpd Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Frag (V)	CE (V)	Cell Acc (V)	Ret Time (min)	Ret Window	Polarity
GC(+57.0) VQDEFC [+57.0] TR.light	No	636.26080	Unit/Enh 4 (6490)	955.39380	Unit/Enh 6 (6490)	380	20.7	4	2.37	0.6	Positive
GC(+57.0) VQDEFC [+57.0] TR.light	No	636.26080	Unit/Enh 4 (6490)	827.33522	Unit/Enh 9 (6490)	380	24	4	2.37	0.6	Positive
GEVLLPV EHQQQK.I light	No	502.27545	Unit/Enh 7 (6490)	659.87751	Unit/Enh 9 (6490)	380	10	4	3.35	0.6	Positive
GEVLLPV EHQQQK.I light	No	502.27545	Unit/Enh 7 (6490)	610.34331	Unit/Enh 2 (6490)	380	13.3	4	3.35	0.6	Positive
GEVLLPV EHQQQK.I light	No	502.27545	Unit/Enh 7 (6490)	553.80128	Unit/Enh (6490)	380	13.3	4	3.35	0.6	Positive
GLDLHGL LAFIQLQQ C(+57.0)	No	766.06707	Unit/Enh 1 (6490)	1037.5778	Unit/Enh 42 (6490)	380	20	4	10.32	0.6	Positive
AQDR.light GLDLHGL LAFIQLQQ C(+57.0)	No	766.06707	Unit/Enh 1 (6490)	905.38939	Unit/Enh (6490)	380	22.8	4	10.32	0.6	Positive
AQDR.light GLDLHGL LAFIQLQQ C(+57.0)	No	766.06707	Unit/Enh 1 (6490)	649.27223	Unit/Enh 5 (6490)	380	26	4	10.32	0.6	Positive
AQDR.light GPYPGP LAPPQPF GPGFVPP PPPPYV PGR.light	No	1034.5393	Unit/Enh 88 (6490)	1228.6473	Unit/Enh 19 (6490)	380	29	4	9.3	1.2	Positive
GPYPGP LAPPQPF GPGFVPP PPPPYV PGR.light	No	1034.5393	Unit/Enh 88 (6490)	1172.6098	Unit/Enh 71 (6490)	380	29	4	9.3	1.2	Positive
GPYPGP LAPPQPF GPGFVPP PPPPYV PGR.light	No	1034.5393	Unit/Enh 88 (6490)	850.44576	Unit/Enh 5 (6490)	380	29	4	9.3	1.2	Positive
GSISIQTE EK.light	No	546.28258	Unit/Enh 5 (6490)	834.42033	Unit/Enh 9 (6490)	380	15	4	2.01	0.6	Positive
GSISIQTE EK.light	No	546.28258	Unit/Enh 5 (6490)	747.38831	Unit/Enh (6490)	380	21	4	2.01	0.6	Positive
GSISIQTE EK.light	No	546.28258	Unit/Enh 5 (6490)	634.30424	Unit/Enh 6 (6490)	380	14	4	2.01	0.6	Positive
GSISIQTE EQIHGK.I ght	No	509.59848	Unit/Enh 8 (6490)	691.86734	Unit/Enh 8 (6490)	380	8	4	2.5	0.6	Positive
GSISIQTE EQIHGK.I ght	No	509.59848	Unit/Enh 8 (6490)	635.32531	Unit/Enh 6 (6490)	380	20	4	2.5	0.6	Positive
GSISIQTE EQIHGK.I ght	No	509.59848	Unit/Enh 8 (6490)	461.58065	Unit/Enh 7 (6490)	380	11	4	2.5	0.6	Positive
HLVQQEG QLEQQER .light	No	574.62369	Unit/Enh 6 (6490)	920.45845	Unit/Enh 5 (6490)	380	13	4	1.51	0.6	Positive
HLVQQEG QLEQQER .light	No	574.62369	Unit/Enh 6 (6490)	689.32129	Unit/Enh 3 (6490)	380	15.9	4	1.51	0.6	Positive
HLVQQEG QLEQQER .light	No	574.62369	Unit/Enh 6 (6490)	560.2787	Unit/Enh (6490)	380	13	4	1.51	0.6	Positive
IAEYMNH LIDIGVAG FR.light	No	640.33297	Unit/Enh 7 (6490)	903.45379	Unit/Enh 6 (6490)	380	21	4	8.78	0.6	Positive
IAEYMNH LIDIGVAG FR.light	No	640.33297	Unit/Enh 7 (6490)	867.93523	Unit/Enh 9 (6490)	380	21	4	8.78	0.6	Positive
IAEYMNH LIDIGVAG FR.light	No	640.33297	Unit/Enh 7 (6490)	606.33582	Unit/Enh 1 (6490)	380	18.3	4	8.78	0.6	Positive
IEGGIYD ADLNDER. light	No	846.90739	Unit/Enh 8 (6490)	1466.6393	Unit/Enh 93 (6490)	380	30	4	4.8	0.6	Positive
IEGGIYD ADLNDER. light	No	846.90739	Unit/Enh 8 (6490)	1110.4698	Unit/Enh 08 (6490)	380	30	4	4.8	0.6	Positive

Cpd Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Frag (V)	CE (V)	Cell Acc (V)	Ret Time (min)	Ret Window	Polarity
IIEGGIYD ADLNDER. light	No	846.90739	Unit/Enh 8 (6490)	947.40647	Unit/Enh 9 (6490)	380	30	4	4.8	0.6	Positive
IPPPPPAP YGPQIFP PPPPQP.II ght	No	710.71893	Unit/Enh 7 (6490)	1141.6040	Unit/Enh 57 (6490)	380	24	4	8.29	1.2	Positive
IPPPPPAP YGPQIFP PPPPQP.II ght	No	710.71893	Unit/Enh 7 (6490)	729.39300	Unit/Enh 1 (6490)	380	15	4	8.29	1.2	Positive
IPPPPPAP YGPQIFP PPPPQP.II ght	No	710.71893	Unit/Enh 7 (6490)	535.28747	Unit/Enh 4 (6490)	380	20.8	4	8.29	1.2	Positive
ISPOIQLS GQTEQTO K.light	No	893.47069	Unit/Enh 7 (6490)	1247.6226	Unit/Enh 2 (6490)	380	32	4	3.46	0.6	Positive
ISPOIQLS GQTEQTO K.light	No	893.47069	Unit/Enh 7 (6490)	1006.4799	Unit/Enh 79 (6490)	380	32	4	3.46	0.6	Positive
ISPOIQLS GQTEQTO K.light	No	893.47069	Unit/Enh 7 (6490)	793.41265	Unit/Enh 1 (6490)	380	32	4	3.46	0.6	Positive
LLDEDHT GTVEFK.II ght	No	501.91570	Unit/Enh 6 (6490)	695.82788	Unit/Enh 8 (6490)	380	16	4	3.65	0.6	Positive
LLDEDHT GTVEFK.II ght	No	501.91570	Unit/Enh 6 (6490)	639.28585	Unit/Enh 6 (6490)	380	16	4	3.65	0.6	Positive
LLDEDHT GTVEFK.II ght	No	501.91570	Unit/Enh 6 (6490)	294.18121	Unit/Enh 8 (6490)	380	13.3	4	3.65	0.6	Positive
LLDNWDS VTSTFSK.I light	No	806.89630	Unit/Enh 2 (6490)	971.46801	Unit/Enh 7 (6490)	380	29	4	7.39	0.6	Positive
LLDNWDS VTSTFSK.I light	No	806.89630	Unit/Enh 2 (6490)	670.34063	Unit/Enh 2 (6490)	380	26	4	7.39	0.6	Positive
LLDNWDS VTSTFSK.I light	No	806.89630	Unit/Enh 2 (6490)	569.29295	Unit/Enh 3 (6490)	380	26	4	7.39	0.6	Positive
LLVVYPW TQR.light	No	637.86642	Unit/Enh 3 (6490)	949.48902	Unit/Enh 7 (6490)	380	18	4	7.66	0.6	Positive
LLVVYPW TQR.light	No	637.86642	Unit/Enh 3 (6490)	850.42061	Unit/Enh 3 (6490)	380	24	4	7.66	0.6	Positive
LLVVYPW TQR.light	No	637.86642	Unit/Enh 3 (6490)	687.35728	Unit/Enh 5 (6490)	380	18	4	7.66	0.6	Positive
LSEPAELT DAVK.light	No	636.83772	Unit/Enh 5 (6490)	943.50948	Unit/Enh 8 (6490)	380	20.7	4	4.4	0.6	Positive
LSEPAELT DAVK.light	No	636.83772	Unit/Enh 5 (6490)	646.37701	Unit/Enh 7 (6490)	380	24	4	4.4	0.6	Positive
LSEPAELT DAVK.light	No	636.83772	Unit/Enh 5 (6490)	472.25838	Unit/Enh 2 (6490)	380	20.7	4	4.4	0.6	Positive
LSGLDLA LGK.light	No	550.33970	Unit/Enh 6 (6490)	986.58807	Unit/Enh 3 (6490)	380	18.1	4	8.71	0.6	Positive
LSGLDLA LGK.light	No	550.33970	Unit/Enh 6 (6490)	899.55604	Unit/Enh 4 (6490)	380	15	4	8.71	0.6	Positive
LSGLDLA LGK.light	No	550.33970	Unit/Enh 6 (6490)	729.45051	Unit/Enh 6 (6490)	380	15	4	8.71	0.6	Positive
LSITGTYD LK.light	No	555.80569	Unit/Enh 6 (6490)	910.48802	Unit/Enh 4 (6490)	380	15	4	4.91	0.6	Positive
LSITGTYD LK.light	No	555.80569	Unit/Enh 6 (6490)	797.40396	Unit/Enh (6490)	380	18.2	4	4.91	0.6	Positive
LSITGTYD LK.light	No	555.80569	Unit/Enh 6 (6490)	696.35628	Unit/Enh 2 (6490)	380	15	4	4.91	0.6	Positive
MYATIVEL K.light	No	566.29136	Unit/Enh 8 (6490)	1000.5349	Unit/Enh 74 (6490)	380	18.6	4	5.46	0.6	Positive
MYATIVEL K.light	No	566.29136	Unit/Enh 8 (6490)	837.47164	Unit/Enh 6 (6490)	380	22	4	5.46	0.6	Positive
MYATIVEL K.light	No	566.29136	Unit/Enh 8 (6490)	295.11108	Unit/Enh 9 (6490)	380	16	4	5.46	0.6	Positive
NFPSPVD AAFR.light	No	610.80656	Unit/Enh 2 (6490)	959.49450	Unit/Enh 6 (6490)	380	23	4	6.08	0.6	Positive
NFPSPVD AAFR.light	No	610.80656	Unit/Enh 2 (6490)	775.40971	Unit/Enh 4 (6490)	380	26	4	6.08	0.6	Positive
NFPSPVD AAFR.light	No	610.80656	Unit/Enh 2 (6490)	480.25089	Unit/Enh 1 (6490)	380	17	4	6.08	0.6	Positive
NLLDEIAS R.light	No	515.78001	Unit/Enh 3 (6490)	803.42575	Unit/Enh 8 (6490)	380	17	4	6.25	0.6	Positive
NLLDEIAS R.light	No	515.78001	Unit/Enh 3 (6490)	690.34169	Unit/Enh 4 (6490)	380	17	4	6.25	0.6	Positive
NLLDEIAS R.light	No	515.78001	Unit/Enh 3 (6490)	228.13426	Unit/Enh 7 (6490)	380	14	4	6.25	0.6	Positive

Cpd Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Frag (V)	CE (V)	Cell Acc (V)	Ret Time (min)	Ret Window	Polarity
NOGPOES VVR.light	No	557.28599 4 (6490)	Unit/Enh	871.46320 6 (6490)	Unit/Enh	380	24	4	1.36	0.6	Positive
NOGPOES VVR.light	No	557.28599 4 (6490)	Unit/Enh	814.44174 3 (6490)	Unit/Enh	380	18.3	4	1.36	0.6	Positive
NOGPOES VVR.light	No	557.28599 4 (6490)	Unit/Enh	243.10878 1 (6490)	Unit/Enh	380	18.3	4	1.36	0.6	Positive
QEAQLEL PEQQVG QPK.light	No	607.98289 4 (6490)	Unit/Enh	812.41485 9 (6490)	Unit/Enh	380	14	4	4.24	0.6	Positive
QEAQLEL PEQQVG QPK.light	No	607.98289 (6490)	Unit/Enh	699.33079 5 (6490)	Unit/Enh	380	17.1	4	4.24	0.6	Positive
QEAQLEL PEQQVG QPK.light	No	607.98289 (6490)	Unit/Enh	505.76690 5 (6490)	Unit/Enh	380	14	4	4.24	0.6	Positive
QITIPSQE QEHSQK.II ght	No	551.61279 7 (6490)	Unit/Enh	706.34423 7 (6490)	Unit/Enh	380	12	4	1.9	0.6	Positive
QITIPSQE QEHSQK.II ght	No	551.61279 7 (6490)	Unit/Enh	599.27836 6 (6490)	Unit/Enh	380	18	4	1.9	0.6	Positive
SASDLTW DNLK.light	No	625.30659 2 (6490)	Unit/Enh	1091.5367 65 (6490)	Unit/Enh	380	20.4	4	5.02	0.6	Positive
SASDLTW DNLK.light	No	625.30659 2 (6490)	Unit/Enh	776.39373 (6490)	Unit/Enh	380	17	4	5.02	0.6	Positive
SASDLTW DNLK.light	No	625.30659 2 (6490)	Unit/Enh	675.34605 (6490)	Unit/Enh	380	20.4	4	5.02	0.6	Positive
SAVTALW GK.light	No	466.76363 4 (6490)	Unit/Enh	774.45085 1 (6490)	Unit/Enh	380	13	4	5.08	0.6	Positive
SAVTALW GK.light	No	466.76363 4 (6490)	Unit/Enh	675.38243 7 (6490)	Unit/Enh	380	15.5	4	5.08	0.6	Positive
SAVTALW GK.light	No	466.76363 4 (6490)	Unit/Enh	574.33475 8 (6490)	Unit/Enh	380	19	4	5.08	0.6	Positive
SGINC [+57.0] PIQK.light	No	508.76330 9 (6490)	Unit/Enh	759.38178 5 (6490)	Unit/Enh	380	16.8	4	1.72	0.6	Positive
SGINC [+57.0] PIQK.light	No	508.76330 9 (6490)	Unit/Enh	645.33885 8 (6490)	Unit/Enh	380	16.8	4	1.72	0.6	Positive
SGINC [+57.0] PIQK.light	No	508.76330 9 (6490)	Unit/Enh	485.30820 9 (6490)	Unit/Enh	380	16.8	4	1.72	0.6	Positive
SVLGQLGI TK.light	No	508.31094 9 (6490)	Unit/Enh	829.51417 9 (6490)	Unit/Enh	380	20	4	5.72	0.6	Positive
SVLGQLGI TK.light	No	508.31094 9 (6490)	Unit/Enh	716.43011 5 (6490)	Unit/Enh	380	14	4	5.72	0.6	Positive
SVLGQLGI TK.light	No	508.31094 9 (6490)	Unit/Enh	531.35007 4 (6490)	Unit/Enh	380	16.8	4	5.72	0.6	Positive
VPEPGC [+57.0] TK.light	No	444.21820 3 (6490)	Unit/Enh	788.36071 5 (6490)	Unit/Enh	380	18	4	1.13	0.6	Positive
VPEPGC [+57.0] TK.light	No	444.21820 3 (6490)	Unit/Enh	562.26535 8 (6490)	Unit/Enh	380	18	4	1.13	0.6	Positive
VPEPGC [+57.0] TK.light	No	444.21820 3 (6490)	Unit/Enh	394.68399 6 (6490)	Unit/Enh	380	18	4	1.13	0.6	Positive
VPVPGYT K.light	No	430.74745 3 (6490)	Unit/Enh	664.36645 2 (6490)	Unit/Enh	380	17	4	2.3	0.6	Positive
VPVPGYT K.light	No	430.74745 3 (6490)	Unit/Enh	565.29803 8 (6490)	Unit/Enh	380	14.4	4	2.3	0.6	Positive
VPVPGYT K.light	No	430.74745 3 (6490)	Unit/Enh	381.21324 6 (6490)	Unit/Enh	380	14.4	4	2.3	0.6	Positive
VSFLSALE EYTK.light	No	693.8612 (6490)	Unit/Enh	1053.5462 67 (6490)	Unit/Enh	380	22.5	4	9.39	0.6	Positive
VSFLSALE EYTK.light	No	693.8612 (6490)	Unit/Enh	940.46220 3 (6490)	Unit/Enh	380	22.5	4	9.39	0.6	Positive
VSFLSALE EYTK.light	No	693.8612 (6490)	Unit/Enh	600.81097 9 (6490)	Unit/Enh	380	20	4	9.39	0.6	Positive
WYVVGLA GNAILR.II ght	No	716.40898 6 (6490)	Unit/Enh	884.53122 6 (6490)	Unit/Enh	380	23.2	4	8.82	0.6	Positive
WYVVGLA GNAILR.II ght	No	716.40898 6 (6490)	Unit/Enh	449.21833 2 (6490)	Unit/Enh	380	23.2	4	8.82	0.6	Positive
WYVVGLA GNAILR.II ght	No	716.40898 6 (6490)	Unit/Enh	350.14991 8 (6490)	Unit/Enh	380	20	4	8.82	0.6	Positive

Scan Parameters

Data Stg
Centroid

Threshold
0

Source Parameters

Parameter	Value (+)	Value (-)
Gas Temp (°C)	200	200
Gas Flow (l/min)	13	13
Nebulizer (psi)	35	35
SheathGasHeater	200	200
SheathGasFlow	12	12
Capillary (V)	3500	3000
VCharging	300	500

Ion Funnel Parameters

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

Chromatograms

Chrom Type	Label	Offset	Y-Range
TIC	TIC	0	10000000

Instrument Curves

Actual

Name: HiP Sampler

Model: G4226A

Auxiliary

Draw Speed	100.0 µL/min
Eject Speed	100.0 µL/min
Draw Position Offset	-3.0 mm
Wait Time After Drawing	0.0 s
Sample Flush Out Factor	5.0
Vial/Well bottom sensing	No

Injection

Injection Mode	Injection with needle wash
Injection Volume	20.00 µL
Needle Wash	
Needle Wash Location	Flush Port
Wash Time	15.0 s

High throughput

Automatic Delay Volume Reduction	No
Overlapped Injection	
Enable Overlapped Injection	No

Valve Switching

Valve Movements	0
Valve Switch Time 1	
Switch Time 1 Enabled	No
Valve Switch Time 2	
Switch Time 2 Enabled	No
Valve Switch Time 3	
Switch Time 3 Enabled	No
Valve Switch Time 4	
Switch Time 4 Enabled	No

Stop Time

Stoptime Mode	As pump/No limit

Post Time

Posttime Mode	Off

Name: Binary Pump**Model:** G4220A

Flow 1.000 mL/min
 Use Solvent Types Yes
 Stroke Mode Synchronized
 Low Pressure Limit 0.00 bar
 High Pressure Limit 550.00 bar
 Max. Flow Ramp Up 100.000 mL/min²
 Max. Flow Ramp Down 100.000 mL/min²
 Expected Mixer No check

Stroke A

Automatic Stroke Calculation A Yes

Stop Time

Stoptime Mode Time set
 Stoptime 11.00 min

Post Time

Posttime Mode Time set
 Posttime 3.00 min

Solvent Composition

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

Timetable

	Time	A	B	Flow	Pressure
1	0.50 min	90.00 %	10.00 %	--- mL/min	--- bar
2	9.70 min	70.00 %	30.00 %	--- mL/min	--- bar
3	9.80 min	10.00 %	90.00 %	--- mL/min	--- bar
4	10.80 min	10.00 %	90.00 %	--- mL/min	--- bar
5	10.90 min	95.00 %	5.00 %	--- mL/min	--- bar
6	11.00 min	95.00 %	5.00 %	--- mL/min	--- bar

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

Valve Position Port 1 -> 6
 Ready when front door open Yes

Left Temperature Control

Temperature Control Mode Temperature Set
 Temperature 45.00 °C

Enable Analysis Left Temperature

Enable Analysis Left Temperature On Yes
 Enable Analysis Left Temperature Value 0.8 °C

Right Temperature Control

Right temperature Control Mode Not Controlled

Enable Analysis Right Temperature

Enable Analysis Right Temperature On Yes
 Enable Analysis Right Temperature Value 0.8 °C

Stop Time

Stoptime Mode As pump/injector

Post Time

Posttime Mode Off

APPENDIX C: SAMPLE PREPARATION LIST FOR CASEWORK

Descriptor	Identifier	Samples	Preparation
Aged	NPB_D#	Peripheral Blood	Neat fluid (single source) pipetted onto cotton swab. Incubated at room temperature for Day 0, 1, 3, 7, and 35 days in duplicate. Swab cut and placed in freezer at designated time, analyzed in a single batch.
	NMB_D#	Menstrual Blood	
	NSE_D#	Seminal Fluid	
	NVF50_D#	Vaginal Fluid	
	NSA_D#	Saliva	
Simulated Sexual Assault Incubation	SSA_ZERO	Day Zero	10 ul neat semen (single source) in 1 mL vaginal fluid (single source) incubated at 37 for the listed days. Tubes frozen at designated time, analyzed in a single batch.
	SSA_ONE	Day One	
	SSA_THREE	Day Three	
	SSA_FIVE	Day Five	
	SSA_SEVEN	Day Seven	
	SSA_NINE	Day Nine	
	SSA_ELEVEN	Day Eleven	
Substrates	SUB01.1 ; SUB01.2	Peripheral Blood on Cotton	50 ul neat peripheral blood on cutting of cotton T-shirt, dried at RT overnight
	SUB02.1 ; SUB02.2	Peripheral Blood on Denim	50 ul neat peripheral blood on cutting of denim, dried at RT overnight
	SUB03.1 ; SUB03.2	Peripheral Blood on Carpet	50 ul neat peripheral blood on cutting of carpet, dried at RT overnight
	SUB04.1 ; SUB04.2	Peripheral Blood on Leather	50 ul neat peripheral blood on cutting of leather, dried at RT overnight
	SUB05.1 ; SUB05.2	Peripheral Blood on Drywall	50 ul neat peripheral blood on cutting of drywall, cutting taken for analysis
	SUB06.1 ; SUB06.2	Semen on Cotton	50 ul neat semen on cutting of cotton T-shirt, dried at RT overnight
	SUB07.1 ; SUB07.2	Semen on Leather	50 ul neat semen on cutting of leather, dried at RT overnight, cutting taken for analysis
	SUB08.1 ; SUB08.2	Menstrual Blood on Cotton	50 ul neat menstrual blood on cutting of cotton T-shirt, dried at RT overnight
	SUB09.1 ; SUB09.2	Menstrual Blood on Denim	50 ul neat menstrual blood on cutting of denim, dried at RT overnight
	SUB10.1 ; SUB10.2	Menstrual Blood on Pad	50 ul neat menstrual blood on pad, dried at RT overnight, cutting taken for analysis
	SUB11.1 ; SUB11.2	Vaginal Fluid on Cotton	50 ul neat vaginal fluid on cutting of cotton T-shirt, dried at RT overnight
	SUB12.1 ; SUB12.2	Vaginal Fluid on Demin	50 ul neat vaginal fluid on cutting of denim, dried at RT overnight
	SUB13.1 ; SUB13.2	Vaginal Fluid on Leather	50 ul neat vaginal fluid on cutting of leather, dried at RT overnight
	SUB14.1 ; SUB14.2	Saliva on Plastic Bottle	50 ul on bottle, dried at RT overnight
	SUB15.1 ; SUB15.2	Saliva on Aluminum Can	50 ul on bottle, dried at RT overnight

Descriptor	Identifier	Samples	Preparation
Environmental Contaminants	CON01.1 ; CON01.2	Peripheral Blood & Dirt	50 ul neat peripheral blood & 50 ul dirt slurry on 1/2 swab, dried at RT overnight
	CON02.1 ; CON02.2	Peripheral Blood & Rust	50 ul neat peripheral blood & 50 ul rust slurry on 1/2 swab, dried at RT overnight
	CON03.1 ; CON03.2	Peripheral Blood & 10% Bleach	50 ul neat peripheral blood & 50 ul 10% bleach on 1/2 swab, dried at RT overnight
	CON04.1 ; CON04.2	Menstrual Blood & Lube	50 ul neat menstrual blood & 50 ul lube on 1/2 swab, dried at RT overnight
	CON05.1 ; CON05.2	Menstrual Blood & Spermicide Condom	50 ul neat menstrual blood on cutting of condom, dried at RT overnight
	CON06.1 ; CON06.2	Vaginal Fluid & Spermicide Condom	50 ul neat vaginal fluid on condom cutting of , dried at RT overnight
	CON07.1 ; CON07.2	Vaginal Fluid & Lube	50 ul neat vaginal fluid & 50 ul lube on 1/2 swab, dried at RT overnight
	CON08.1 ; CON08.2	Semen & Lube	50 ul neat semen & 50 ul lube on 1/2 swab, dried at RT overnight
	CON09.1 ; CON09.2	Semen & Spermicide Condom	50 ul neat semen on condom cutting of , dried at RT overnight
	CON10.1 ; CON10.2	Semen & 10% Bleach	50 ul neat semen & 50 ul 10% bleach on 1/2 swab, dried at RT overnight
	CON11.1 ; CON11.2	Saliva & Tobacco	50 ul saliva/tobacco slurry on 1/2 swab, dried at RT overnight
	CON12.1 ; CON12.2	Saliva & Cigarette	50 ul saliva on cutting of filter, dried at RT overnight
Mixtures	MIX01	Saliva (minor) & Vaginal Fluid (major)	10 ul minor fluid mixed with 500 ul major fluid, 100 ul spotted on full swab. Dried at RT overnight, resuspended in 1 ml deionized water. Immunochromatographic comparison. Single Source.
	MIX02	Semen (minor) & Vaginal Fluid (major)	
	MIX03	Semen (minor) & Menstrual Blood (major)	
	MIX04	Saliva (minor) & Menstrual Blood (major)	
	MIX05	Vaginal Fluid (minor) & Urine (major)	

Descriptor	Identifier	Samples	Preparation
Dilutions on Swabs	SEN01	Peripheral Blood 1:100	Dilutions prepared in deionized water, 150 ul spotted on full swabs. Dried at RT overnight, resuspended in 1 ml deionized water. Immunochromatographic. Single Source. Injected 20 uL on column.
	SEN02	Peripheral Blood 1:1,000	
	SEN03	Peripheral Blood 1:2,000	
	SEN04	Peripheral Blood 1:5,000	
	SEN05	Peripheral Blood 1:10,000	
	SEN06	Peripheral Blood 1:20,000	
	SEN07	Peripheral Blood 1:40,000	
	SEN08	Semen 1:100	
	SEN09	Semen 1:1,000	
	SEN10	Semen 1:2,000	
	SEN11	Semen 1:5,000	
	SEN12	Semen 1:10,000	
	SEN13	Semen 1:20,000	
	SEN14	Semen 1:40,000	
	SEN15	Saliva 1:10	
	SEN16	Saliva 1:100	
	SEN17	Saliva 1:500	
	SEN18	Saliva 1:1,000	
	SEN19	Saliva 1:2,000	
	SEN20	Saliva 1:5,000	
	SEN21	Saliva 1:10,000	
	SEN22	Menstrual Blood 1:100	
	SEN23	Menstrual Blood 1:1,000	
	SEN24	Menstrual Blood 1:2,000	
	SEN25	Menstrual Blood 1:5,000	
	SEN26	Menstrual Blood 1:10,000	
	SEN27	Menstrual Blood 1:20,000	
	SEN28	Menstrual Blood 1:40,000	
	SEN29	Vaginal Fluid 1:10	
	SEN30	Vaginal Fluid 1:100	
	SEN31	Vaginal Fluid 1:500	
	SEN32	Vaginal Fluid 1:1,000	
	SEN33	Vaginal Fluid 1:2,000	
	SEN34	Vaginal Fluid 1:5,000	
	SEN35	Vaginal Fluid 1:10,000	
Authentic	Sexual Assault Kit		Swabs taken from a single female individual in duplicate immediately before preparation. 10 ul of 1:100 semen dilution spotted on swabs, dried at RT overnight. Full swab resuspended in 1 mL deionized water. Immunochromatographic comparison. Single source male and female donors.
	SA01_01.1; SA01_01.2	Vaginal Swab	
	SA01_02.1; SA01_02.2	Oral Swab	
	SA01_03.1; SA01_03.2	Rectal Swab	
	Sexual Assault Kit-Vasectomized Suspect		
	SA02_01.1; SA02_01.2	Vaginal Swab	
	SA02_02.1; SA02_02.2	Oral Swab	
	SA02_03.1; SA02_03.2	Rectal Swab	
	Laundered Samples		1 mL of fluid 'squirted' on substrate, outlined with marker. Substrates washed separately (by fluid) on regular cycle with detergent and dried completely in dryer. Cutting of stain and control region taken for analysis.
	LS01	Semen on Bed Sheet	
	LS02	Semen on Underwear	
	LS03	Peripheral Blood on Towel	
	LS04	Peripheral Blood on Denim	
	Digital Swabs		Dry swab of finger, dried at RT overnight prior to analysis. Full swab resuspended in 1 mL of deionized water. Single Source.
	DS01.1; DS01.2	Saliva	
	DS02.1; DS02.2	Vaginal Fluid	
	DS03.1; DS03.2	Menstrual Blood	

**APPENDIX D: REPEATABILITY AND REPRODUCIBILITY CALCULATED COEFFICIENT OF
VARIATION FOR PEAK AREA RESPONSE**

Fluid	Protein	Peptide	Analyst 1	Analyst 2	Combined
Peripheral Blood	Alpha-1 Antitrypsin	LSITGTYDLK	11.845	10.800	11.530
		SVLGQLGITK	9.366	9.210	9.336
	Hemoglobin	SAVTALWGK	7.054	6.768	6.792
		LLVVYPWTQR	7.457	5.374	6.463
	Hemopexin	NFPSPVDAFR	10.990	9.664	10.756
		LLDNWDSVTSTFSK	13.664	9.755	11.945
	Apolipoprotein	VSFLSALEEYTK	7.843	15.313	12.289
		DGAGDVAFK	12.350	10.574	11.615
Saliva	Serotransferrin	SASDLTWDNLK	6.351	7.044	7.045
		LSGLLDLALGK	6.434	7.201	9.067
	Alpha Amylase	IAEYMNHLIDIGVAGFR	3.954	9.962	9.079
		FGYGYGPYQPVPEQPLYPQPYQPYQQYTF	12.736	11.058	13.988
	Statherin	GPYPGGLAPPQFPGFVPPPPPTGPGR	7.189	9.944	10.139
		IPPPPPAPYGPGFPPPPPPQ	5.941	8.414	9.873
	Submaxillary Protein	IIEGGYDADLNDER	5.811	10.255	12.909
		ALHFVISEYNK	5.991	8.555	10.719
Vaginal Fluid	Cystatin	LLDEDHTGTVEFK	6.647	15.880	13.902
		ISPQIQLSGQTEQTQK	6.635	17.521	14.595
		AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR	4.567	10.743	8.064
	Neutrophil Gelatinase	WYVVGLAGNAILR	7.926	11.252	10.790
		MYATIELK	9.313	12.546	12.265
	Ly6/PLAUR	GLDHGLLAFIQLQQCAQDR	10.027	7.458	8.874
		GCVQDEFCTR	7.244	16.604	14.520
	Suprabasin	ALGDINSGITHAGR	4.790	12.886	11.096
		FGQGVHGLSEGWK	10.922	13.427	13.655
	Periplakin	AQSLQSAK	5.933	11.403	11.056
		NLLDEIASR	9.246	15.735	16.460
		NQGPQESVVR	7.738	13.654	12.771
	Small Proline Rich Protein 3	VPEPGCTK	8.700	7.030	8.070
		VPVPGYTK	9.925	11.226	10.284
	Involucrin	HLVQQEGQLEQQR	5.846	18.955	15.417
		QEAQLELPEQVQGPK	4.357	15.600	12.361
		GEVLLPVEHQQK	9.393	10.984	10.677

Fluid	Protein	Peptide	Analyst 1	Analyst 2	Combined
Seminal Fluid	Acid Phosphatase	FVTLVFR	87.638	41.252	95.688
		FQELESETLK	19.405	14.717	26.483
		ELSELSLLSLYGIHK	5.787	10.988	8.773
	Prostate Specific Antigen	FLRPGDDSSHDLMLLR	54.820	21.388	62.553
		LSEPAELTDAVK	34.849	17.358	45.393
	Semenogelin 2	GSISIQTEEK	38.678	19.467	51.764
		GSISIQTEEQIHGK	10.208	10.259	15.247
		DVSQSSISFQIEK	2.677	11.744	8.304
	Semenogelin 1	DIFSTQDELLVYNK	5.340	10.368	8.063
		QITIPSQEQEHSQK	6.822	7.261	7.747
Menstrual Blood	Epididymal Secretory	DCGSVDGVIK	6.243	11.758	9.125
		SGINCPQK	9.318	9.948	9.425
	Cornulin	LLDEDHTGTVEFK	14.904	12.570	14.500
		ISPQIQLSGQTEQTQK	N/D	N/D	N/D
		AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR	N/D	N/D	N/D
	Neutrophil Gelatinase	WYVVGLAGNAILR	24.821	15.549	20.181
		MYATIYELK	22.655	24.441	23.949
	Ly6/PLAUR	GLDHGLLAFIQLQQCAQDR	N/D	N/D	N/D
		GCVQDEFCTR	N/D	N/D	N/D
	Suprabasin	ALGDINSGITHAGR	N/D	N/D	N/D
		FGQGVHHGLSEGWK	N/D	N/D	N/D
	Periplakin	AQSLQSAK	N/D	N/D	N/D
		NLLDEIASR	N/D	N/D	N/D
		NQGPQESVVR	N/D	N/D	N/D
	Involucrin	HLVQQEGQLEQQR	N/D	N/D	N/D
		QEAQLELPEQVVGQPK	N/D	N/D	N/D
		GEVLLPVEHQQQK	N/D	N/D	N/D
	Small Proline Rich Protein 3	VPEPGCTK	8.312	10.234	10.224
		VPVPGYTK	8.304	4.714	8.635
	Alpha-1 Antitrypsin	LSITGTYDLK	19.303	11.382	16.432
		SVLGQLGITK	13.169	11.973	12.729
	Hemoglobin	SAVTALWGK	9.186	6.718	8.131
		LLVVYPWTQR	8.966	5.508	7.739
	Hemopexin	NFPSPVDAEFR	15.337	12.862	14.875
	Apolipoprotein	LLDNWDSVTSTFSK	21.725	13.442	19.061
		VSFLSALEEYTK	22.803	17.151	21.686
	Serotransferrin	DGAGDVAFVK	15.868	11.908	14.349
		SASDLTWDNLK	12.424	11.779	12.704

**APPENDIX E: REPEATABILITY AND REPRODUCIBILITY CALCULATED COEFFICIENT OF
VARIATION FOR RELATIVE RETENTION TIME**

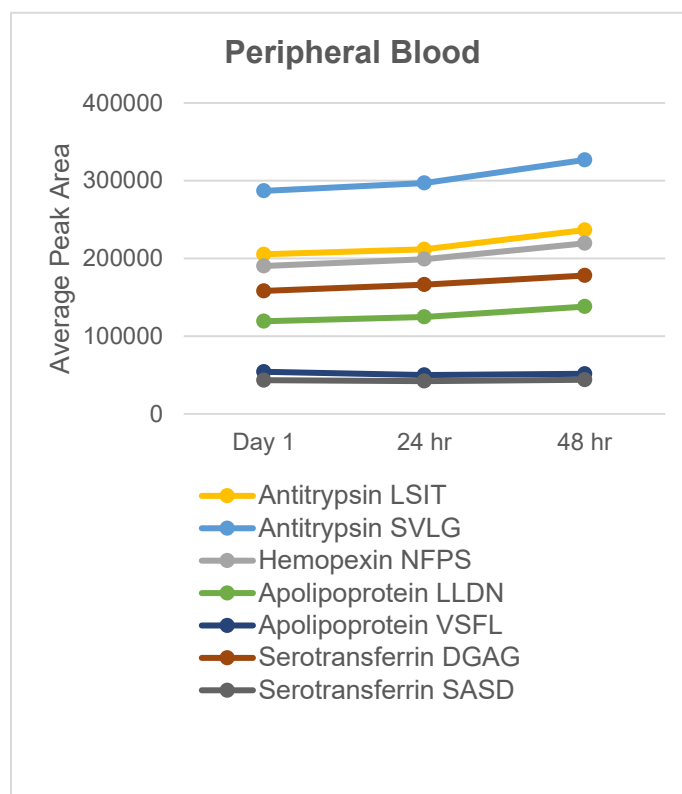
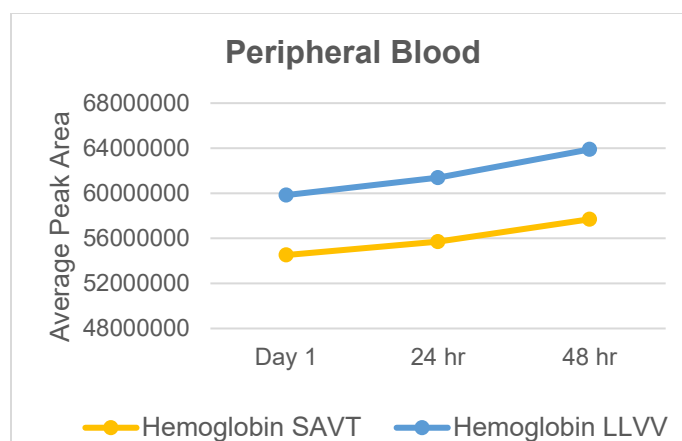
Fluid	Protein	Peptide	Analyst 1	Analyst 2	Combined
Peripheral Blood	Alpha-1 Antitrypsin	LSITGTYDLK	0.093	0.093	0.090
		SVLGQLGITK	0.077	0.043	0.060
	Hemoglobin	SAVTALWGK	0.106	0.132	0.118
		LLVVYPWTQR	0.184	0.124	0.157
	Hemopexin	NFPSPVDAAFR	0.062	0.059	0.059
	Apolipoprotein	LLDNWDSVTSTFSK	0.109	0.070	0.089
		VSFLSALEEYTK	0.106	0.025	0.075
	Serotransferrin	DGAGDVAFVK	0.000	0.000	0.000
		SASDLTWDNLK	0.102	0.076	0.088
Saliva	Alpha Amylase	LSGLLDLALGK	0.044	0.036	0.040
		IAEYMNHLIDIGVAGFR	0.058	0.036	0.047
		FGYGYGPYQVPVEQPLYPQYQPYQQYTF	0.044	0.032	0.039
	Statherin	GPYPPGPLAPPQPFPGFVPPPPPTGPGR	0.076	0.055	0.064
	Submaxillary Protein	IPPPPPAPYGP GIFFPPPPQP	0.072	0.073	0.074
		IIEGGIYDADLNDER	0.053	0.053	0.051
		ALHFVISEYNK	0.000	0.000	0.000
Vaginal Fluid	Cystatin	LLDEDHTGTVEFK	0.118	0.065	0.093
		ISPQIQLSGQTEQTQK	0.128	0.107	0.115
		AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR	0.176	0.264	0.226
	Cornulin	WYVVGLAGNAILR	0.064	0.036	0.052
		MYATIELK	0.066	0.066	0.064
	Neutrophil Gelatinase	GLDHGLLAFIQLQQCAQDR	0.029	0.022	0.025
		GCVQDEFCTR	0.193	0.170	0.185
	Ly6/PLAUR	ALGDINSGITHAGR	0.135	0.171	0.152
		FGQGVHGLSEGWK	0.248	0.159	0.215
	Suprabasin	AQSLQSAK	0.000	0.269	0.190
		NLLDEIASR	0.038	0.061	0.053
		NQGPQESVVR	0.186	0.295	0.258
	Periplakin	VPEPGCTK	0.324	0.342	0.331
		VPVPGYTK	0.172	0.163	0.163
	Small Proline Rich Protein 3	HLVQQEGQLEQQER	0.000	0.225	0.165
		QEAQLELPEQQVGQPK	0.100	0.118	0.107
	Involucrin	GEVLLPVEHQQQK	0.155	0.110	0.136

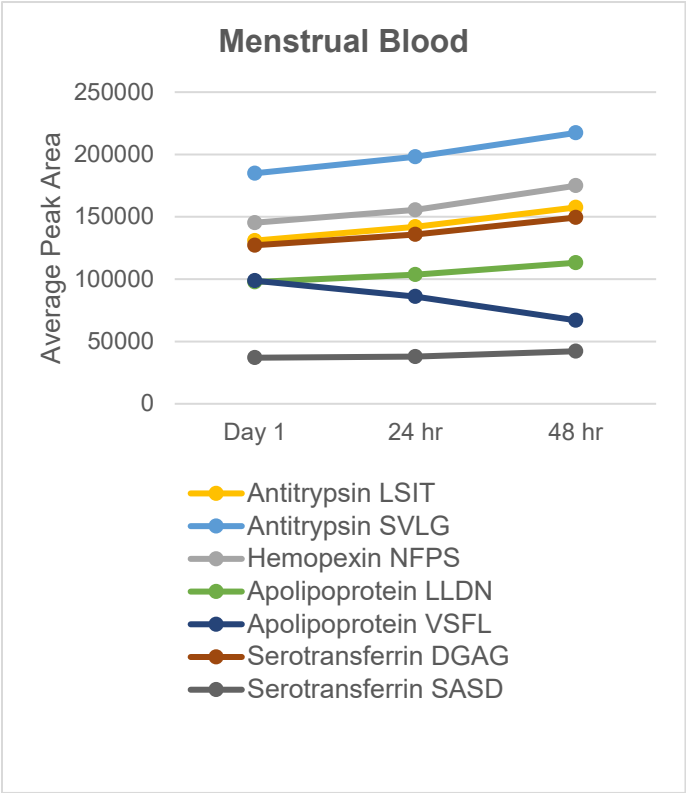
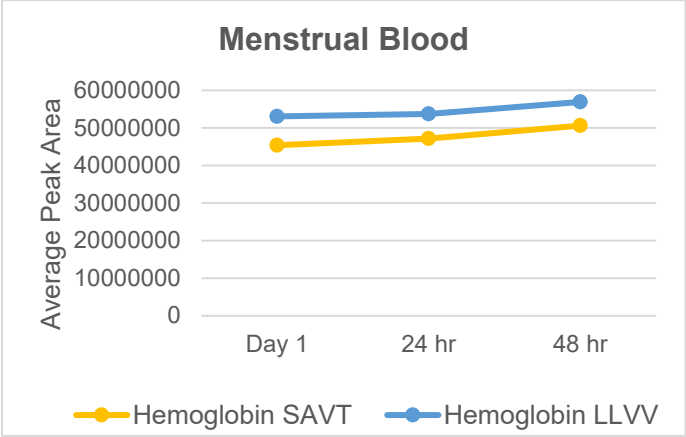
Fluid	Protein	Peptide	Analyst 1	Analyst 2	Combined
Seminal Fluid	Acid Phosphatase	FVTLVFR	0.049	0.059	0.054
		FQELESETLK	0.069	0.069	0.067
		ELSELSLLSLYGIHK	0.058	0.059	0.058
	Prostate Specific Antigen	FLRPGDDSSHDLMLLR	0.081	0.081	0.078
		LSEPAELTDAVK	0.085	0.071	0.078
		GSISIQTEEK	0.124	0.124	0.120
	Semenogelin 2	GSISIQTEEQIHGK	0.134	0.160	0.147
		DVSQSSISFQIEK	0.075	0.079	0.076
		DIFSTQDELLVYNK	0.063	0.042	0.052
	Semenogelin 1	QITIPSQEQEHSQK	0.174	0.132	0.151
		DCGSVDGVK	0.138	0.157	0.148
	Epididymal Secretory	SGINCPIQK	0.147	0.000	0.104
Menstrual Blood	Cornulin	LLDEDHTGTVEFK	0.104	0.118	0.113
		ISPQIQLSGQTQTK	N/D	N/D	N/D
		AHQGTGETVTGSGTQTQAGATQVEQDSSHQTGR	N/D	N/D	N/D
	Neutrophil Gelatinase	WYVVGLAGNAILR	0.089	0.083	0.084
		MYATIYELK	0.066	0.114	0.093
	Ly6/PLAUR	GLDHGLLAFIQLQQCAQDR	N/D	N/D	N/D
		GCVQDEFCTR	N/D	N/D	N/D
	Suprabasin	ALGDINSGITHAGR	N/D	N/D	N/D
		FGQGVHHGLSEGWK	N/D	N/D	N/D
	Periplakin	AQSLQSAK	N/D	N/D	N/D
		NLLDEIASR	N/D	N/D	N/D
		NQGPQESVVR	N/D	N/D	N/D
	Involucrin	HLVQQEGQLEQQR	N/D	N/D	N/D
		QEAQLELPEQQVGQPK	N/D	N/D	N/D
		GEVLLPVEHQQQK	N/D	N/D	N/D
	Small Proline Rich Protein 3	VPEPGCTK	0.000	0.000	0.000
		VPVPGYTK	0.231	0.163	0.202
	Alpha-1 Antitrypsin	LSITGTYDLK	0.078	0.082	0.078
		SVLGQLGITK	0.064	0.085	0.073
	Hemoglobin	SAVTALWGK	0.066	0.128	0.099
		LLVVYPWTQR	0.176	0.094	0.137
	Hemopexin	NFPSPVDAAFR	0.114	0.071	0.094
	Apolipoprotein	LLDNWDSVTSTFSK	0.106	0.043	0.078
		VSFLSALEEYTK	0.054	0.062	0.057
	Serotransferrin	DGAGDVAFAVK	0.100	0.136	0.122
		SASDLTWDLNK	0.102	0.051	0.078

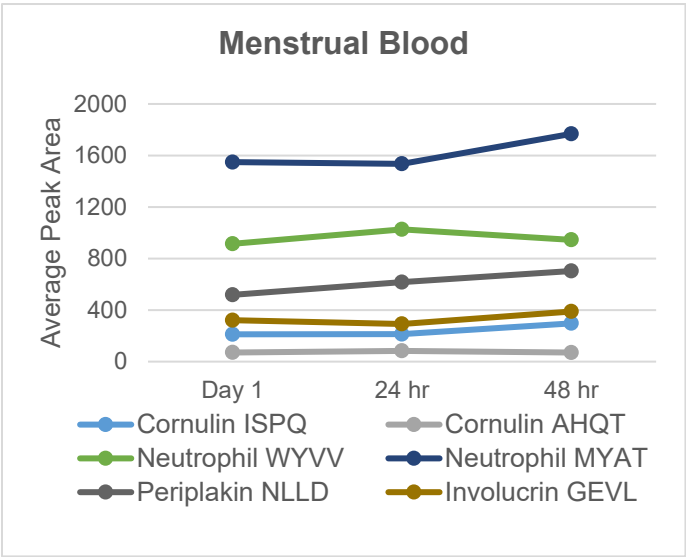
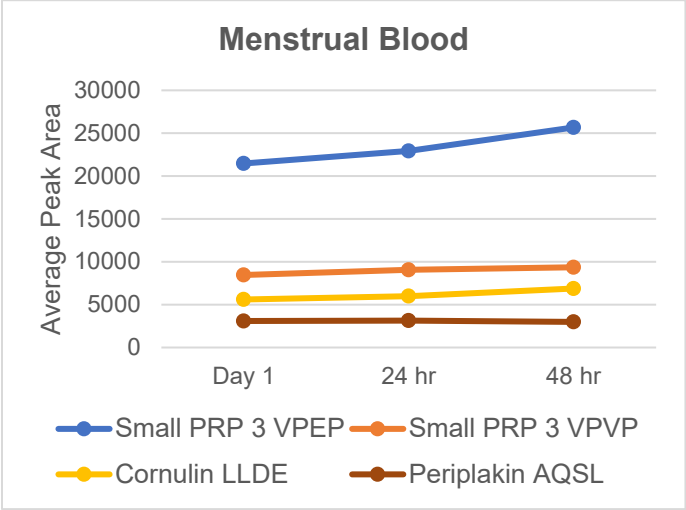
**APPENDIX F: REPEATABILITY AND REPRODUCIBILITY CALCULATED COEFFICIENT OF
VARIATION FOR ION RATIO RESPONSE**

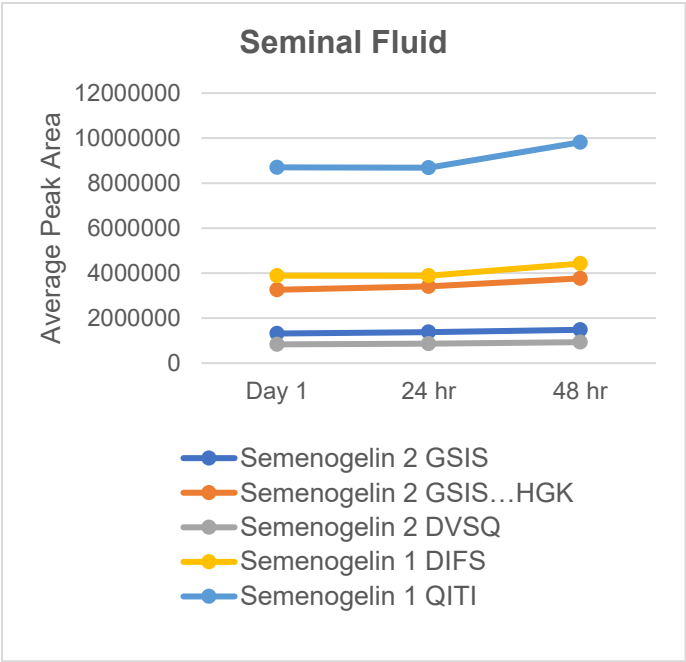
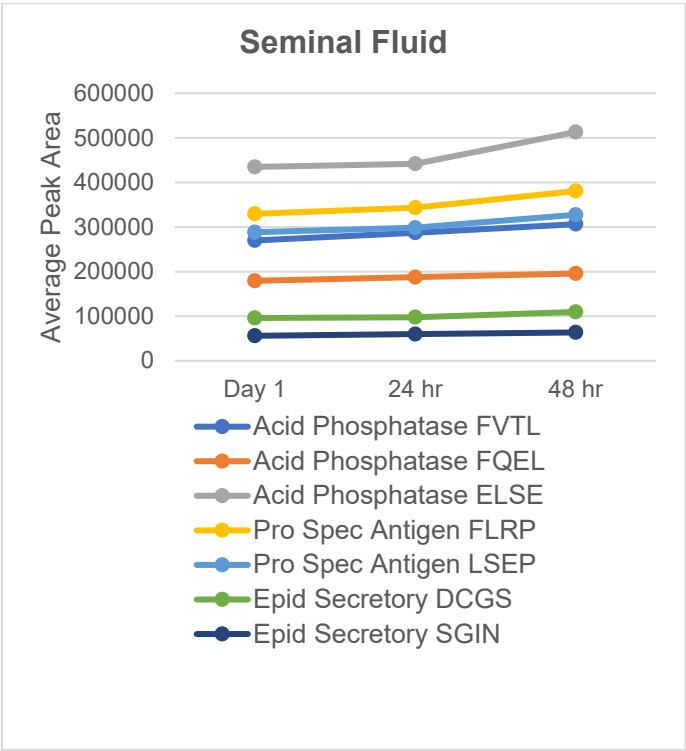
Fluid	Protein	Peptide	Analyst 1	Analyst 2	Combined
Peripheral Blood	Alpha-1 Antitrypsin	LSITGTYDLK	7.520	5.011	6.315
		SVLGQLGITK	3.103	3.745	3.341
	Hemoglobin	SAVTALWGK	4.820	4.842	4.743
		LLVVYPWTQR	1.963	1.824	1.969
	Hemopexin	NFPSPVDAAFR	3.156	3.490	3.496
	Apolipoprotein	LLDNWDSVTSTFSK	1.830	3.817	3.088
		VSFLSALEEYTK	2.973	2.785	2.796
	Serotransferrin	DGAGDVAFVK	3.095	2.746	2.853
SASDLTWDLNK		4.843	5.640	5.469	
Saliva	Alpha Amylase	LSGLLDLALGK	3.647	2.038	3.099
		IAEYMNHLIDIGVAGFR	4.826	4.782	4.867
	Statherin	FGYGYGPYQPVPEQPLYPQYQPYQQYTF	3.617	6.615	5.362
	Submaxillary Protein	GPYPPGPLAPPQFPGFVPPPPPPPTGPGR	0.864	1.209	1.044
		IPPPPPAPYGPFGIFPPPPQP	0.511	0.588	0.586
	Cystatin	IIEGGIYDADLNDER	4.112	4.217	4.541
ALHFVISEYNK		2.928	4.286	3.695	
Vaginal Fluid	Cornulin	LLDEDHTGTVEFK	4.193	3.883	4.534
		ISPQQLSGQTEQTQK	2.287	2.340	2.333
		AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR	7.412	6.244	7.175
		WYVVGLAGNAILR	6.804	5.743	6.108
	Neutrophil Gelatinase	MYATIELK	4.088	3.308	3.753
		GLDHGLLAFIQLQQCAQDR	2.913	1.805	2.364
	Ly6/PLAUR	GCVQDEFCTR	5.220	4.690	5.500
		ALGDINSGITHAGR	3.605	5.569	4.568
	Suprabasin	FGQGVHHLSEGWK	6.678	6.419	6.370
		AQSLQSAK	2.477	2.819	2.896
	Periplakin	NLLDEIASR	4.053	3.911	4.113
		NQGPQESVVR	8.886	3.902	7.232
		VPEPGCTK	4.396	3.693	3.954
	Small Proline Rich Protein 3	VPVPGYTK	5.035	2.408	4.044
		HLVQQEGQLEQQR	5.630	7.285	6.368
	Involucrin	QEAQLELPEQQVGQPK	1.731	2.578	2.309
		GEVLLPVEHQQQK	2.940	2.489	2.651

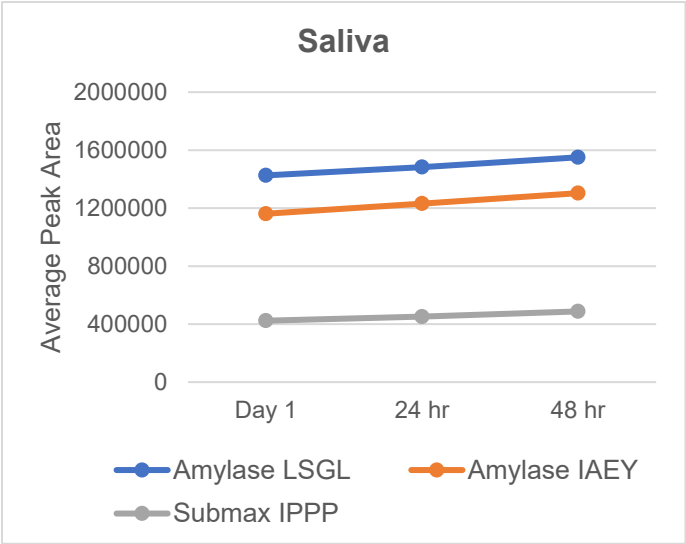
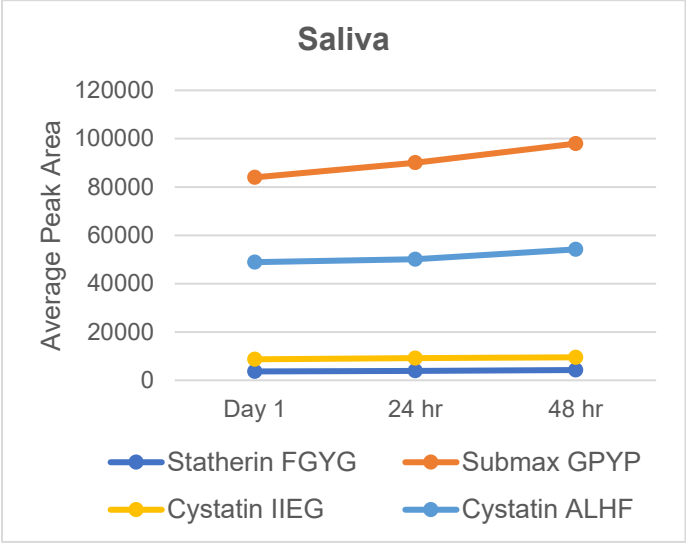
Fluid	Protein	Peptide	Analyst 1	Analyst 2	Combined
Seminal Fluid	Acid Phosphatase	FVTLVFR	1.503	1.240	1.342
		FQELESETLK	4.248	3.127	3.636
		ELSELSSLSLYGIHK	4.844	2.577	4.455
	Prostate Specific Antigen	FLRPGDDSSHDMLLR	3.828	3.360	3.677
		LSEPAELTDAVK	1.918	1.032	1.601
	Semenogelin 2	GSISIQTEEK	4.360	3.249	4.025
		GSISIQTEEQIHGK	3.749	3.127	3.505
		DVSQSSISFQIEK	6.209	3.842	5.066
	Semenogelin 1	DIFSTQDELLVYNK	3.455	4.174	3.801
		QITIPSQEQEHSQK	2.578	3.036	2.810
Menstrual Blood	Epididymal Secretory	DCGSVDGVK	5.835	5.021	5.501
		SGINCPQIK	3.971	3.225	3.863
	Cornulin	LLDEDHTGTVEFK	5.145	7.970	6.519
		ISPQIQLSGQTEQTQK	N/D	N/D	N/D
		AHQTGETVTGSGTQTQAGATQTVEQDSSHQTGR	N/D	N/D	N/D
	Neutrophil Gelatinase	WYVVLGNAILR	11.812	14.081	12.682
		MYATIELK	8.859	29.230	20.998
	Ly6/PLAUR	GLDHGLLAFIQLQCAQDR	N/D	N/D	N/D
		GCVQDEFCTR	N/D	N/D	N/D
	Suprabasin	ALGDINSGITHAGR	N/D	N/D	N/D
		FGQGVHGLSEGWK	N/D	N/D	N/D
	Periplakin	AQSLQSAK	N/D	N/D	N/D
		NLLDEIASR	N/D	N/D	N/D
		NQGPQESVVR	N/D	N/D	N/D
	Involucrin	HLVQQEGQLEQQR	N/D	N/D	N/D
		QEAQLELPEQQVGQPK	N/D	N/D	N/D
		GEVLLPVEHQQQK	N/D	N/D	N/D
	Small Proline Rich Protein 3	VPEPGCTK	4.830	1.963	3.579
		VPVPGYTK	5.886	7.259	6.432
	Alpha-1 Antitrypsin	LSITGTDLK	4.598	4.323	4.418
		SVLGQLGITK	3.147	2.257	2.661
	Hemoglobin	SAVTALWGK	4.661	3.597	4.200
		LLVVYPWTQR	3.446	2.150	2.820
	Hemopexin	NFPSPVDAFR	3.964	2.758	3.320
		LLDNWDSVTSTFSK	3.863	2.246	3.070
	Apolipoprotein	VSFLSALEEYTK	3.316	3.242	3.805
		DGAGDVAFVK	2.730	3.418	3.001
	Serotransferrin	SASDLTWDNLK	3.875	6.952	5.488

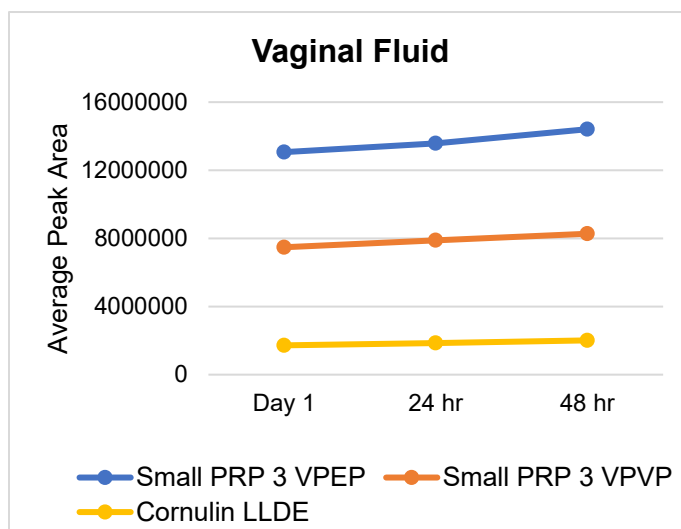
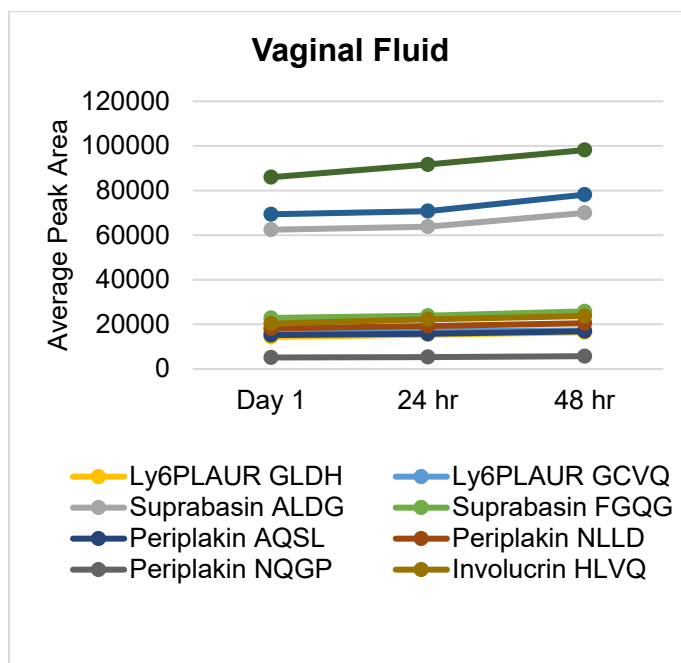
APPENDIX G: AUTOSAMPLER STABILITY COMPREHENSIVE RESULTS

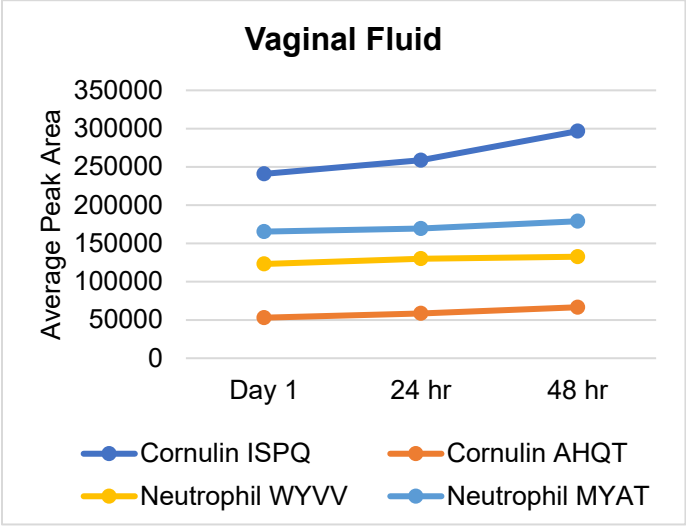












APPENDIX H: BLIND SAMPLE ANALYSIS RESULTS

Sample Number	Sample Description	Quant Value (µg/mL)	Experimental Fluid Identification	True Fluid Identification	Supplemental Info	Points of Discrepancy
1	Cloth swatch No visible staining	ND	Peripheral Blood	Peripheral Blood	100 µL of neat blood, laundered (w/o bleach)	NTR
2	Condom	29.318	ND	ND	Blank	NTR
		25.381	ND	ND	Blank	NTR
3	Cotton swab (1/2) No visible staining	454.335	Seminal Fluid Saliva Vaginal Fluid	Vaginal Fluid Seminal Fluid Saliva	10 µL each semen & saliva in 100 µL V _T on pre-collected and dried vaginal swab	NTR
4	Cloth swatch No visible staining	ND	Seminal Fluid	Seminal Fluid	100 µL neat semen, laundered (w/ bleach)	NTR
5	Cotton swab (1/2) Pale yellow staining	420.607	Seminal Fluid Vaginal Fluid	Vaginal Fluid Seminal Fluid	100 µL of 1:100 diluted semen on a pre-collected and dried vaginal swab	NTR
6	Cotton swab (1/2) No visible staining	382.187	Saliva Vaginal Fluid	Vaginal Fluid Saliva	100 µL 1:10 diluted saliva on a pre-collected and dried vaginal swab	NTR
7	Cloth swatch No visible staining	130.684	Seminal Fluid Saliva	Saliva Seminal Fluid	100 µL 10:1 mixture of saliva:semen on cloth	NTR
8	Cotton swab (1/2) Red-brown staining	607.767	Peripheral Blood Vaginal Fluid	Menstrual Blood	Single-source swab collected from donor, no associated volume	NTR
9	Cotton swab (1/2) No visible staining	ND	ND	ND	Blank	NTR
10	Cloth swatch No visible staining	68.632	Seminal Fluid	Seminal Fluid	100 µL neat semen on cloth, laundered (w/o bleach)	NTR

Sample Number	Sample Description	Quant Value (µg/mL)	Experimental Fluid Identification	True Fluid Identification	Supplemental Info	Points of Discrepancy
11	Cotton swab (1/2) No visible staining	1120.000	Seminal Fluid Saliva	Seminal Fluid Saliva	100 µL 1:1 mixture of saliva and semen on cotton swab	NTR
12	Cotton swab (1/2) Pale yellow staining	4144.280	Seminal Fluid	Seminal Fluid	100 µL neat fluid per swab	NTR
13	Cotton swab (1/2) Red-brown staining	953.931	Peripheral Blood Seminal Fluid Vaginal Fluid	Menstrual Blood Seminal Fluid	100 µL of 1:10 diluted semen on a pre-collected and dried menstrual blood swab	NTR
14	Cloth swatch No visible staining	51.953	Seminal Fluid Saliva	Saliva Seminal Fluid	100 µL of 100:1 mixture of saliva:semen on cloth	NTR
15	Cotton swab (1/2) Pale yellow staining	778.717	Seminal Fluid Vaginal Fluid	Vaginal Fluid Seminal Fluid Saliva	100 µL of mixture including 10 µL semen, 1 µL saliva, pipetted onto a previously collected and dried vaginal swab.	False Negative Missed Saliva
16	Cloth swatch No visible staining	1027.320	Seminal Fluid	Seminal Fluid	100 µL neat fluid per swab	NTR
17	Cotton swab (1/2) Red-brown staining	1215.418	Peripheral Blood Seminal Fluid Vaginal Fluid	Menstrual Blood	Single-source swab collected from donor, no associated volume	False Positive False Semen
18	Cloth swatch No visible staining	38.773	Saliva	Saliva	100 µL neat fluid per swab	NTR
19	Cotton swab (1/2) Red-brown staining	3941.220	Peripheral Blood Seminal Fluid Vaginal Fluid	Menstrual Blood Seminal Fluid	100 µL of 1:100 semen dilution on a previously collected and dried menstrual swab	NTR
20	Cotton swab (1/2) Pale yellow staining	331.883	Peripheral Blood Seminal Fluid Vaginal Fluid	Vaginal Fluid Seminal Fluid	100 µL of a 1:100 semen dilution on a previously collected and dried vaginal swab	NTR

Sample Number	Sample Description	Quant Value (µg/mL)	Experimental Fluid Identification	True Fluid Identification	Supplemental Info	Points of Discrepancy
21	Cotton swab (1/2) No visible staining	440.115	Seminal Fluid Vaginal Fluid	Vaginal Fluid Seminal Fluid	100 µL of a 1:10 semen dilution on a previously collected and dried vaginal swab	NTR
22	Cotton swab (1/2) No visible staining	110.124	Saliva Vaginal Fluid	Saliva	100 µL neat fluid per swab	False Positive: Incorrect Vaginal Fluid Identification
23	Cotton swab (1/2) No visible staining	221.366	ND	Urine	100 µL neat fluid per swab	NTR
24	Cotton swab (1/2) No visible staining	219.389	ND	Urine	100 µL neat fluid per swab	NTR
25	Cotton swab (1/2) No visible staining	386.845	Vaginal Fluid	Vaginal Fluid	Swab collected from donor, no associated volume	NTR
26	Condom	33.993	ND	ND	Sample on outside	NTR
		33.295	Seminal Fluid	Seminal Fluid Saliva	100 µL of a 1:1 mixture of semen and saliva on a lubricated condom	False Negative: Missed Saliva Identification
27	Cotton swab (1/2) Faint Red-brown staining	368.870	Peripheral Blood Vaginal Fluid	Rectal Swab	Swab collected from donor, no associated volume	False Positive: Incorrect Vaginal Fluid Identification
28	Cloth swatch Red-brown staining	4367.860	Peripheral Blood	Peripheral Blood	100 µL neat fluid per swab	NTR
29	Cotton swab (1/2) Pale yellow staining	398.438	Vaginal Fluid	Vaginal Fluid	Swab collected from donor, no associated volume	NTR
30	Cotton swab (1/2) No visible staining	267.377	Peripheral Blood Saliva	Nasal Secretions	100 µL neat fluid per swab	Analyst Note: Consistent with Nasal Secretions

Sample Number	Sample Description	Quant Value (µg/mL)	Experimental Fluid Identification	True Fluid Identification	Supplemental Info	Points of Discrepancy
31	Cotton swab (1/2) Pale yellow staining	578.119	Seminal Fluid Vaginal Fluid	Vaginal Fluid Seminal Fluid	100 µL of a 1:10 semen dilution on a previously collected and dried vaginal swab	NTR
32	Cotton swab (1/2) Yellow staining	1159.981	Peripheral Blood Seminal Fluid Vaginal Fluid	Vaginal Fluid Seminal Fluid	100 µL neat semen on a previously collected and dried vaginal swab.	NTR
33	Cotton swab (1/2) No visible staining	2997.140	Seminal Fluid Saliva	Seminal Fluid Saliva	100 µL of a 1:10 mixture of semen:saliva on a cotton swab	NTR
34	Condom	ND	ND	ND	Sample on outside	NTR
		4861.345	Seminal Fluid Saliva	Seminal Fluid Saliva	100 µL of a 1:10 mixture of saliva:semen on a lubricated condom	NTR
35	Cloth swatch No visible staining	564.706	Seminal Fluid	Seminal Fluid	100 µL neat fluid per swab	NTR
36	Cotton swab (1/2) Pale yellow staining	724.519	Saliva Vaginal Fluid	Vaginal Fluid Saliva	100 µL neat saliva on a previously collected and dried vaginal swab	NTR
37	Cotton swab (1/2) No visible staining	367.790	Vaginal Fluid	Vaginal Fluid Saliva	100 µL of a 1:100 saliva dilution on a previously collected and dried vaginal swab.	False Negative: Missed Saliva Identification
38	Cotton swab (1/2) Pale yellow staining	3511.905	Seminal Fluid Vaginal Fluid	Vaginal Fluid Seminal Fluid	100 µL neat semen on a previously collected and dried vaginal swab.	NTR
39	Cotton swab (1/2) No visible staining	524.051	ND	Urine	100 µL neat fluid per swab	NTR
40	Cotton swab (1/2) Yellow staining	577.667	Seminal Fluid Saliva Vaginal Fluid	Vaginal Fluid Saliva Seminal Fluid	100 µL containing 1 µL semen/ 10 µL saliva pipetted onto a previously collected and dried vaginal swab	NTR

Sample Number	Sample Description	Quant Value (µg/mL)	Experimental Fluid Identification	True Fluid Identification	Supplemental Info	Points of Discrepancy
41	Cotton swab (1/2) No visible staining	1473.296	Seminal Fluid	Seminal Fluid Saliva	100 µL of a 1:100 mixture of semen: saliva on a cotton swab	False Negative: Missed Saliva Identification
42	Cloth swatch No visible staining	ND	ND	ND	Blank	NTR
43	Condom	ND	ND	ND	Sample on outside	NTR
		340.854	Seminal Fluid Saliva	Seminal Fluid Saliva	100 µL of a 1:10 mixture of saliva: semen on a lubricated condom	NTR
44	Cotton swab (1/2) Red-brown staining, saturated	7414.495	Peripheral Blood Seminal Fluid	Menstrual Blood Seminal Fluid	100 µL neat semen on a previously collected and dried menstrual swab	False Negative: Missed Vaginal Fluid Identification
45	Cotton swab (1/2) Red-brown staining, saturated	10172.420	Peripheral Blood	Peripheral Blood	100 µL neat fluid per swab	NTR
46	Cotton swab (1/2) No visible staining	2301.795	Seminal Fluid	Seminal Fluid	100 µL neat fluid per swab	NTR
47	Condom	25.050	ND	ND	Sample on outside	NTR
		1314.222	Seminal Fluid Saliva	Saliva Seminal Fluid	100 µL of a 1:1 mixture of semen and saliva on a non-lubricated condom	NTR
48	Cloth swatch No visible staining	ND	Peripheral Blood	Peripheral Blood	100 µL on cloth, laundered (w/ bleach)	NTR
49	Cotton swab (1/2) No visible staining	843.944	Undetermined	Breast Milk	100 µL neat fluid per swab	NTR
50	Cotton swab (1/2) No visible staining	50.705	ND	Sweat	100 µL neat fluid per swab	NTR

APPENDIX I: INTERPRETATION GUIDELINES

INTERPRETATION GUIDELINES

Introduction:

The interpretation of data from LC-MS/MS analyses is a matter of professional training and expertise. The following objective criteria are to be used by analysts to guide most routine data interpretation scenarios. Not every situation, however, may be fully covered by these interpretation guidelines. The treatment of samples that appear to fall outside of these guidelines should be addressed through discussion with the Technical Reviewer in order to reach agreement on a reportable opinion. In the event that agreement on a reportable opinion cannot be reached, the laboratory's Technical Leader should be consulted to issue a final decision on a reportable opinion or other course of action. These interpretation guidelines are based upon validation studies, the peer-reviewed scientific literature, and professional training and expertise. These interpretation guidelines establish a solid framework of quality criteria to ensure that:

- Conclusions in the casework report are scientifically supported by the analytical data, including that obtained from appropriate standards and controls;
- Interpretations are made objectively; and
- Interpretations are consistent and accurate from analyst to analyst and case to case.

Evaluation of Controls:

Internal Positive Control: This control (e.g., bovine myelin basic protein) serves to demonstrate that the sample digest (including digestion, denaturation, reduction and alkylation) performed successfully for each sample in the batch. The internal positive control should be evaluated for each sample to determine if it meets the laboratory's established quality criteria. Specifically, both the natural targeted peptide peak as well as the heavy labeled peak should be identified based on the appearance of two MRM transitions per peptide. Additionally, peaks should fall within two peak widths of the acceptable retention times outlined in the table below (± 0.2 min). The area ratio of the heavy labeled peptide to the natural peptide should be $15.0 \pm 30\%$. Area ratios falling outside of this range may indicate the presence of digestion inhibition. Notes: As the required heavy labelled peptide is not currently commercially available, it should be custom synthesized. In

addition, the purity of commercially available myelin basic protein can vary. As such, the area ratio of the heavy labeled peptide to the natural peptide may fluctuate between reagents lots and should be monitored by the analyst. However, within a single preparation of these standards, the ratio will remain stable.

1. If there appears to be an injection or other chromatographic problem, the sample should be re-injected.
2. If the internal positive control fails to generate a peak for the natural/light peptide, or if the ratio falls to meet the response criteria indicated above, the sample should be considered for re-extraction.

Protein	Peptide Sequence	MRM transitions	Retention time (min)
Myelin	DTGILDSLGR (Light)	523.7 → 660.3, 830.4	5.5
	DTGILDSLGR (Heavy)	528.7 → 670.3, 840.4	5.5

Reagent (Negative) Control: This control (e.g., a sample processed in parallel with the casework samples of a batch but to which no protein source material was added) serves to demonstrate that the protein extraction and processing reagents do not contain targeted protein. The negative reagent control should be evaluated and meet the laboratory's quality criteria. Specifically, the negative reagent control should be free of detectable target protein upon analysis. The occurrence of more than one targeted peptide peak should be considered an indication of protein contamination. Such findings should be considered necessary but not sufficient for failing the negative reagent control. The official designation of a failure of the negative reagent control should be reviewed and documented by the laboratory's supervisor or Technical Leader.

1. If protein contamination is observed in a negative reagent control, acknowledgment of the contaminant and subsequent actions should be documented in the case file. In addition, the analyst should endeavor to determine the point at which the contamination was introduced, and the scope of the samples affected by the contamination.
2. If the contaminating source affects all samples in the extraction set, the analysis should be redone from the point at which the contamination was introduced.

3. If it is unclear at what point the contamination was introduced, the analysis should be repeated from the protein sample preparation step forward.
4. If additional negative reagent controls were prepared with the batch and these show no sign of contamination AND the associated samples show no sign of contamination, the incident may be considered tube-specific. The data already derived from these samples can be used for fluid identification purposes.

Additional Considerations:

Carryover – Carryover was observed for Semenogelin-1 and Hemoglobin during the validation studies of the analytical method. Accordingly, any elevated signal from these proteins should be evaluated in subsequent injections. A Blank (*i.e.*, neat methanol) can be run after every sample in order to wash the column and prevent sample carryover.

Peak Designation and Peptide Identification

Proteins are composed of a sequence of amino acids arranged in a linear order. This allows for the prediction, to a given degree of confidence, of the fragmentation pattern and MS/MS spectra that will be produced. To enhance the specificity of the method, up to three MRM transitions for each peptide are employed. Detectable peptide peaks are those that meet the following criteria:

- The peak has a signal to noise ratio greater than 3
- The peak height is greater than 1,000 counts
- The peak for the peptide should fall within two peak widths (± 0.2 min) from the target retention time
- The response ratio for the qualifier ions should be within $\pm 20\%$ of the target. Note: extreme low or high signal intensity can result in a deviation from expected ion ratios.

Body Fluid Identification and Reporting Language

Confirmatory Identification: The presence of at least one confirmatory protein (see Body Fluid Specificity Table below) for a body fluid of interest provides a confirmatory indication of the presence of the corresponding targeted biological fluid. This will be reported as “A confirmatory identification of (blood/saliva/semen) was obtained for item...”

Presumptive Detection: The presence of at least one presumptive protein (see Specificity Table below) for a body fluid of interest provides a presumptive indication of the presence of the corresponding targeted biological fluid. This will be reported as “A presumptive indication of (vaginal fluid) was obtained for item...”

Not Detected: In all cases, a failure to detect a minimum of one targeted peptide for any body fluid represents a negative result. This will be reported as “No targeted biological fluids were detected”.

Statements Regarding Human Specificity: Within the context of a confirmatory result, reporting of the result as human specific requires the detection of a peptide target unique to humans (see Species Specificity Table below). This will be reported as “the confirmatory identification of human (blood/vaginal fluid/saliva/semen) was obtained”

Body Fluid Specificity

Fluid	Protein	Confirmatory	Presumptive	Non-Specific	Notes:
Peripheral Blood	P02647 APOA1	Y			
	P68871 HBB	Y			Trace levels in some saliva/seminal fluid (<1,000 cts.)
	P02790 HEMO	Y			
	P01009 A1AT	Y			Trace levels in some saliva/seminal fluid (<2,000 cts.)
	P02787 TRFE		Y		Detectable in seminal fluid
Saliva	P02808 STAT	Y			
	P02814 SMR3B	Y			
	P04745 AMY1		Y		Trace levels in some vaginal fluid (<1,000 cts.)
	P09228 CYTT	Y			
Vaginal Fluid	O95274 LYPD3		Y		Consistent trace levels in saliva (<1,000 cts.)
	P80188 NGAL		Y		Consistent trace levels in saliva (<1,000 cts.)
	O60437 PEPL		Y		
	P07476 INVO		Y		Consistent trace levels in saliva (<2,000 cts.)
	Q9UBG3 CRNN		Y		Consistent trace levels in saliva (<1,000 cts.)
	Q6UWP8 SBSN			Y	Detectable in saliva
	Q9UBC9 SPRR3			Y	Detectable in saliva
Seminal Fluid	P15309 PPAP		Y		Detectable in vaginal fluid
	P07288 KLK3		Y		Consistent Trace levels in vaginal fluid
	Q02383 SEMG2	Y			
	P04279 SEMG1	Y			
	P61916 NPC2		Y		Consistent Trace levels in vaginal fluid (<1,000 cts.)

Human/Species Specificity

Fluid	Protein	Peptide	Human Specific	Species Cross Reactivity
Peripheral Blood	P02647 APOA1	LLDNWDSVTSTFSK	N	Chimpanzee
	P02647 APOA1	VSFLSALEEYTK	N	Primates
	P68871 HBB	LLVVYPWTQR	N	Primates / mammals
	P68871 HBB	SAVTALWGK	N	Primates / mammals
	P02790 HEMO	NFPSPVDAAFR	N	Orangutan
	P01009 A1AT	SVLGQLGITK	Y	
	P01009 A1AT	LSITGTYDLK	N	Orangutan
	P02787 TRFE	SASDLTWDNLK	N	Chimpanzee
	P02787 TRFE	DGAGDVAFVK	N	Primates
Saliva	P02808 STAT	FGYGYGPYQPVEQPLYPQPYQPQYQYTF	Y	
	P02814 SMR3B	GPYPGGLAPPQPFPGFVPPPPPPYGPGR	Y	
	P02814 SMR3B	IPPPPPAPYGPGFPPPPPPQ	Y	
	P04745 AMY1	IAEYMNHLIDIGVAGFR	Y	
	P04745 AMY1	LSGLLDLALGK	Y	
	P09228 CYTT	ALHFVISEYNK	Y	
	P09228 CYTT	IIEGGIYDADLNDER	Y	
Seminal Fluid	P15309 PPAP	ELSELSLLSLYGIHK	N	Mouse
	P15309 PPAP	FVTLVFR	N	Mouse
	P15309 PPAP	FQELESETLK	Y	
	P07288 KLK3	FLRPGDDSSHDLMLLR	Y	
	P07288 KLK3	LSEPAELTDAVK	Y	
	Q02383 SEMG2	GSISIQTEEK	N	Primates
	Q02383 SEMG2	DVSQSSISFQIEK	Y	
	Q02383 SEMG2	GSISIQTEEQIHGK	N	Primates
	P04279 SEMG1	QITIPSQEQEHSQK	Y	
	P04279 SEMG1	DIFSTQDELLVYNK	Y	
	P61916 NPC2	DC[+57.0]GSVDGVK	Y	
	P61916 NPC2	SGINC[+57.0]PIQK	N	Mammals

Fluid	Protein	Peptide	Human Specific	Species Cross Reactivity
Vaginal Fluid	O95274 LYPD3	GC[+57.0]VQDEFC[+57.0]TR	Y	
	O95274 LYPD3	GLDLHGLLAFIQLQQC[+57.0]AQDR	Y	
	P80188 NGAL	WYVVGLAGNAILR	Y	
	P80188 NGAL	MYATIELK	Y	
	O60437 PEPL	NQGPQESVVR	Y	
	O60437 PEPL	NLLDEIASR	Y	
	O60437 PEPL	AQSLQSAK	Y	
	P07476 INVO	GEVLLPVEHQQQK	Y	
	P07476 INVO	QEAQLELPEQQVGQPK	N	Chimpanzee
	P07476 INVO	HLVQQEGQLEQQR	Y	
	Q9UBG3 CRNN	LLDEDHTGTVEFK	Y	
	Q9UBG3 CRNN	ISPQIQLSGQTEQTQK	Y	
	Q9UBG3 CRNN	AHQGTGETVTGSGTQTQAGATQTVEQDSSHQ TGR	Y	
	Q6UWP8 SBSN	ALDGINSGITHAGR	Y	
	Q6UWP8 SBSN	FGQGVHHGLSEGWK	Y	
	Q9UBC9 SPRR 3	VPVPGYTK	Y	
	Q9UBC9 SPRR 3	VPEPGC[+57.0]TK	Y	

*Data Searched using NCBI Algorithm PHI-BLAST (Pattern Hit Initiated BLAST), Animalia (taxid: 33208), Non-redundant UniProt KB/SwissProt sequences database, Molecule Type: Protein
Update date: 2019/03/26, Number of sequences: 471513.

NOTES:

Body fluid identification is confirmed through the mass spectral identification of multiple protein markers. Those protein markers, in turn, are confirmed through the detection of multiple tryptic peptides per protein. Below is a brief description of the biological function each targeted protein.

Peripheral Blood is identified through the detection of α -1-antitrypsin, hemopexin, apolipoprotein A1, Serotransferrin, and hemoglobin subunit beta. α -1-antitrypsin is a non-specific serine protease inhibitor found in human plasma. This protein's primary role is as an inhibitor of neutrophil elastase which protects tissues from proteolytic damage (Kolarich et al.; Parfrey et al.). Hemopexin is produced in the liver and found in plasma. This protein is responsible for trapping free heme in plasma as well as iron recycling in the liver (Ascenzi et al.; Liebert). Apolipoprotein A1 is a component of HDL particles and is involved with the transport of cholesterol from tissues (Breslow

et al.). Serotransferrin is an iron binding protein responsible for the transport of iron from sites of absorption and heme degradation to sites of storage and utilization (Aisen et al.; Yang et al.). Hemoglobin subunit beta - The metalloprotein hemoglobin is responsible for oxygen transport and is the major protein contained within erythrocytes. Hemoglobin exists as a tetramer containing two beta chains and two alpha chains (Berg et al.).

Vaginal Fluid is identified through the detection of neutrophil gelatinase-associated lipocalin, cornulin, ly6/PLAUR domain-containing protein 3, periplakin, involucrin, and suprabasin. Neutrophil gelatinase-associated lipocalin belongs to the lipocalin family of transport proteins which have been associated with innate immunity through iron sequestration (Goetz et al.). As such, this protein can be found in tissues prone to exposure to bacterial and other microorganisms including the respiratory tract, salivary glands, uterus, and prostate (Goetz et al.; Cowland and Borregaard). Cornulin is also expressed in squamous cells where it plays a role in epithelial cell differentiation. It may also play a role in mucosal-epithelial immune response. The protein has been characterized in the cervix and in esophageal tissues (Contzler et al.; Arnouk et al.). Ly6/PLAUR domain-containing protein 3 is involved in the regulation between extracellular structural support scaffolding and epithelial cell layers (Smith et al.). Suprabasin is expressed in keratinocytes and plays a role in epidermal differentiation. It has been reported to be expressed in the uterus as well as the esophagus (Park et al.). Involucrin is a component of cornified cell envelope (CE) of stratified squamous epithelia and is involved with membrane protein cross-linking (Djian et al.). Periplakin is a component of the cornified envelope of keratinocytes. May link the cornified cell envelope to desmosomes and intermediate filaments (Ruhrberg et al.).

Saliva is confirmed through the detection of statherin, submaxillary gland androgen-regulated protein 3B, Cystatin SA, and alpha amylase. Statherin, as well as submaxillary gland androgen-regulated protein 3B, assist in inhibiting potentially harmful calcium phosphate precipitation in saliva (Schlesinger and Hay; Hay et al.). Alpha amylase is the most abundant protein found in saliva where it digests starches into glucose and maltose (Ramasubbu et al.). While highly abundant in saliva, this protein can be found in a number of alternate body fluids including vaginal fluid, breast

milk, fecal matter, urine, blood and semen. Cystatin SA is a cysteine protease inhibitor located in the cystatin locus. It is found at high levels in saliva and tears.

Seminal Fluid identification is based off the detection of Epididymal secretory protein E1, prostatic acid phosphatase, semenogelin-I/II, and prostate-specific antigen. Epididymal secretory protein E1 is involved with intracellular cholesterol transporters. It acts in concert with NPC1 and plays an important role in the egress of cholesterol from the lysosomal compartment. Prostatic Acid Phosphatase (also known as Seminal Acid Phosphatase or SAP) is a glycoprotein secreted by the epithelial cells of the prostate gland. It is capable of hydrolyzing phosphate groups from substrate molecules (Yam). SAP is a seminal fluid protein which has seen utility as a clinical marker for prostate cancer (Taira et al.). While largely replaced by PSA/p30 for screening purposes, the combination of low expression in non-target tissues and assay detection limits makes this protein useful as a potential marker of seminal fluid. Semenogelin-I/II are the most abundant proteins in seminal plasma and are responsible for the gel-like matrix of human semen. Both isoforms act as substrates for prostate specific antigen (p30), where upon lysis, sperm are able to move freely through the seminal matrix (Kise et al.; Malm and Hellman). Prostate-Specific Antigen (also known as PSA or p30) is a serine protease produced by epithelial cells located in the prostate (Ward et al.). The primary function of prostate-specific antigen is to cleave semenogelin-I/II thus creating a soluble, liquid medium, for spermatozoa movement (Ward et al.). Prostate-specific antigen has been well studied as an indicator for prostate cancer when serum levels reach approximately 4-10 ng/mL (Basch et al.). While this protein is not absolutely seminal fluid-specific, the detection limits of most assays make it difficult to detect it in whole blood (Keshishian et al.). As a result, this protein may have utility, in combination with other biomarkers, for the detection of seminal fluid.

References

- Aisen, P., et al. "Stoichiometric and Site Characteristics of the Binding of Iron to Human Transferrin." *Journal of Biological Chemistry*, vol. 253, no. 6, © 1978 ASBMB. Currently published by Elsevier Inc; originally published by American Society for Biochemistry and Molecular Biology., 1978, pp. 1930–37, doi:10.1016/s0021-9258(19)62337-9.
- Arnouk, Hilal, et al. "Characterization of Molecular Markers Indicative of Cervical Cancer Progression." *Proteomics - Clinical Applications*, vol. 3, no. 5, 2009, pp. 516–27, doi:10.1002/prca.200800068.
- Ascenzi, Paolo, et al. "Hemoglobin and Heme Scavenging." *IUBMB Life*, vol. 57, no. 11, 2005, pp. 749–59, doi:10.1080/15216540500380871.
- Basch, Ethan, et al. "Screening for Prostate Cancer with Prostate-Specific Antigen Testing: American Society of Clinical Oncology Provisional Clinical Opinion." *Journal of Clinical Oncology*, vol. 30, no. 24, 2012, pp. 3020–25, doi:10.1200/JCO.2012.43.3441.
- Berg, Jeremy M., et al. *Biochemistry*. 5th ed., W.H. Freeman and Company, 2002.
- Breslow, J. L., et al. "Isolation and Characterization of cDNA Clones for Human Apolipoprotein A-I." *Proceedings of the National Academy of Sciences of the United States of America*, vol. 79, no. 22 I, 1982, pp. 6861–65, doi:10.1073/pnas.79.22.6861.
- Contzler, Romuald, et al. "Cornulin, a New Member of the 'Fused Gene' Family, Is Expressed during Epidermal Differentiation." *Journal of Investigative Dermatology*, vol. 124, no. 5, Elsevier Masson SAS, 2005, pp. 990–97, doi:10.1111/j.0022-202X.2005.23694.x.
- Cowland, Jack B., and Niels Borregaard. "Molecular Characterization and Pattern of Tissue Expression of the Gene for Neutrophil Gelatinase-Associated Lipocalin from Humans." *Genomics*, vol. 45, no. 1, 1997, pp. 17–23, doi:10.1006/geno.1997.4896.
- Djian, P., et al. "The Involucrin Genes of the Mouse and the Rat: Study of Their Shared Repeats." *Molecular Biology and Evolution*, vol. 10, no. 6, 1993, pp. 1136–49, doi:10.1093/oxfordjournals.molbev.a040069.
- Goetz, David H., et al. "The Neutrophil Lipocalin NGAL Is a Bacteriostatic Agent That Interferes with Siderophore-Mediated Iron Acquisition Ation, Olfaction, Pheromone Transport, Prostaglandin Synthesis, Modulation of Cell Growth and Metabolism, Regulation of the Immune Response,." *Molecular Cell*, vol. 10, 2002, pp. 1033–43, http://ac.els-cdn.com/S1097276502007086/1-s2.0-S1097276502007086-main.pdf?_tid=6cc6a4a8-97b3-11e7-ace0-00000aacb35e&acdnat=1505218434_b0a1649a0a31d05c6cdd04860b5ee95e.
- Hay, D. I., et al. "Relationship between Concentration of Human Salivary Statherin and Inhibition of Calcium Phosphate Precipitation in Stimulated Human Parotid Saliva." *Journal of Dental Research*, vol. 63, no. 6, 1984, pp. 857–63, doi:10.1177/00220345840630060901.
- Keshishian, Hasmik, et al. "Quantitative, Multiplexed Assays for Low Abundance Proteins in Plasma by Targeted Mass Spectrometry and Stable Isotope Dilution." *Molecular and Cellular Proteomics*, vol. 6, no. 12, © 2007 ASBMB. Currently published by Elsevier Inc; originally published by American Society for Biochemistry and Molecular Biology., 2007, pp. 2212–29, doi:10.1074/mcp.M700354-MCP200.

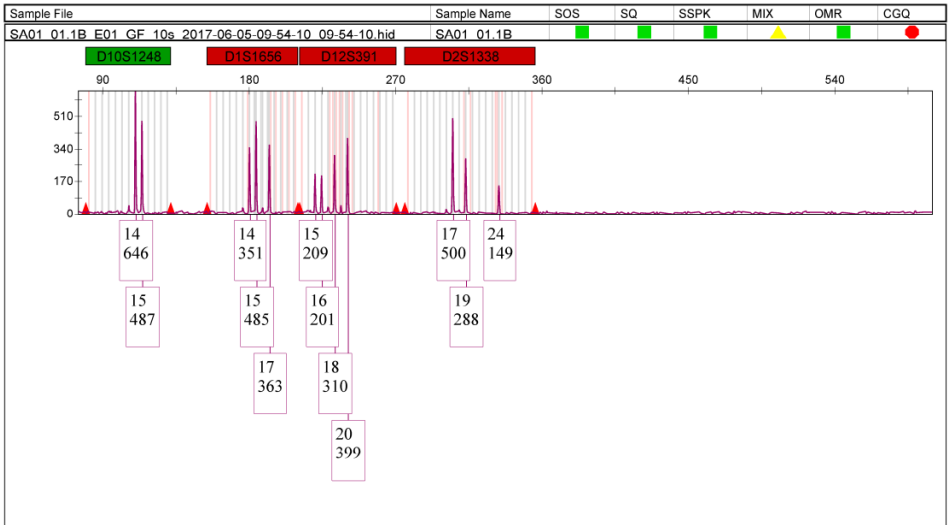
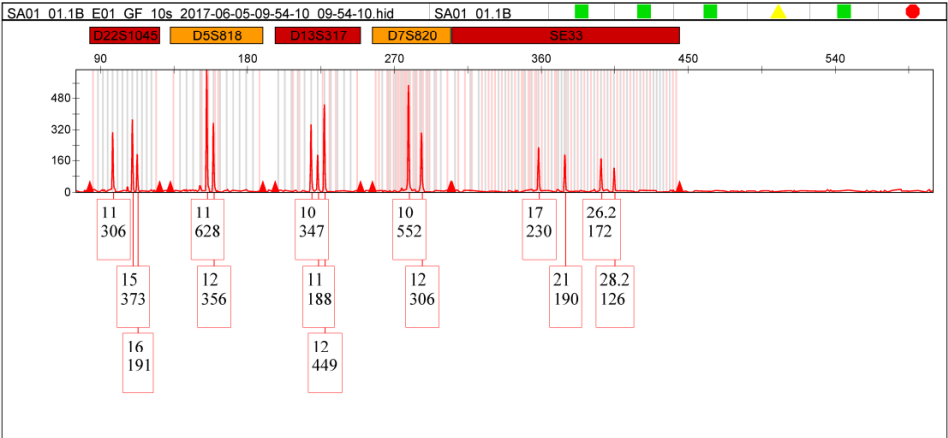
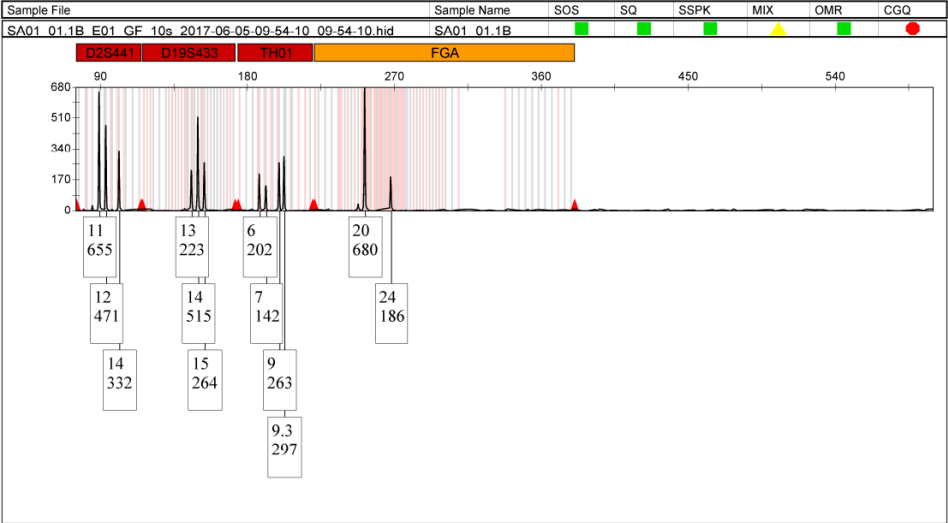
- Kise, Hideaki, et al. "Characterization of Semenogelin II and Its Molecular Interaction with Prostate-Specific Antigen and Protein C Inhibitor." *European Journal of Biochemistry*, vol. 238, no. 1, 1996, pp. 88–96, doi:10.1111/j.1432-1033.1996.0088q.x.
- Kolarich, Daniel, et al. "Comprehensive Glyco-Proteomic Analysis of Human A1- Antitrypsin and Its Charge Isoforms." *Proteomics*, vol. 6, no. 11, 2006, pp. 3369–80, doi:10.1002/pmic.200500751.
- Liebert, Mary Ann. "Hemopexin: Structure, Function, and Regulation." *DNA and Cell Biology*, vol. 21, no. 4, 2002, pp. 297–306.
- Malm, Johan, and Jukka Hellman. "Isolation and Characterization of the Major Gel Proteins in Human Semen, Semenogen I and Semenogen II." *European Journal of ...*, vol. 238, 1996, pp. 48–53, <http://onlinelibrary.wiley.com/doi/10.1111/j.1432-1033.1996.0048q.x/full>.
- Parfrey, Helen, et al. "A1 -Antitrypsin Deficiency, Liver Disease and Emphysema." *International Journal of Biochemistry and Cell Biology*, vol. 35, no. 7, 2003, pp. 1009–14, doi:10.1016/S1357-2725(02)00250-9.
- Park, Geon Tae, et al. "Suprabasin, a Novel Epidermal Differentiation Marker and Potential Cornified Envelope Precursor." *Journal of Biological Chemistry*, vol. 277, no. 47, © 2002 ASBMB. Currently published by Elsevier Inc; originally published by American Society for Biochemistry and Molecular Biology., 2002, pp. 45195–202, doi:10.1074/jbc.M205380200.
- Ramasubbu, Narayanan, et al. "Structure of Human Salivary α -Amylase at 1.6 Å Resolution: Implications for Its Role in the Oral Cavity." *Acta Crystallographica Section D: Biological Crystallography*, vol. 52, no. 3, International Union of Crystallography, 1996, pp. 435–46, doi:10.1107/S0907444995014119.
- Ruhrberg, Christiana, et al. "Periplakin, a Novel Component of Cornified Envelopes and Desmosomes That Belongs to the Plakin Family and Forms Complexes with Envoplakin." *Journal of Cell Biology*, vol. 139, no. 7, 1997, pp. 1835–49, doi:10.1083/jcb.139.7.1835.
- Schlesinger, D. H., and D. I. Hay. "Complete Covalent Structure of Statherin, a Tyrosine Rich Acidic Peptide Which Inhibits Calcium Phosphate Precipitation from Human Parotid Saliva." *Journal of Biological Chemistry*, vol. 252, no. 5, © 1977 ASBMB. Currently published by Elsevier Inc; originally published by American Society for Biochemistry and Molecular Biology., 1977, pp. 1689–95, doi:10.1016/s0021-9258(17)40603-x.
- Smith, Barbara A., et al. "Identification of Genes Involved in Human Urothelial Cell-Matrix Interactions: Implications for the Progression Pathways of Malignant Urothelium." *Cancer Research*, vol. 61, no. 4, 2001, pp. 1678–85.
- Taira, Ai, et al. "Reviving the Acid Phosphatase Test for Prostate Cancer." *Oncology*, vol. 21, no. 8, 2007, pp. 1003–10.
- Ward, A. Milford, et al. "Prostate Specific Antigen: Biology, Biochemistry and Available Commercial Assays." *Annals of Clinical Biochemistry*, vol. 38, no. 6, 2001, pp. 633–51, doi:10.1258/0004563011901055.
- Yam, Lung T. "Clinical Significance of the Human Acid Phosphatases. A Review." *The American Journal of Medicine*, vol. 56, no. 5, 1974, pp. 604–16, doi:10.1016/0002-9343(74)90630-5.

Yang, F., et al. "Human Transferrin: CDNA Characterization and Chromosomal Localization." *Proceedings of the National Academy of Sciences of the United States of America*, vol. 81, no. 9 I, 1984, pp. 2752–56, doi:10.1073/pnas.81.9.2752.

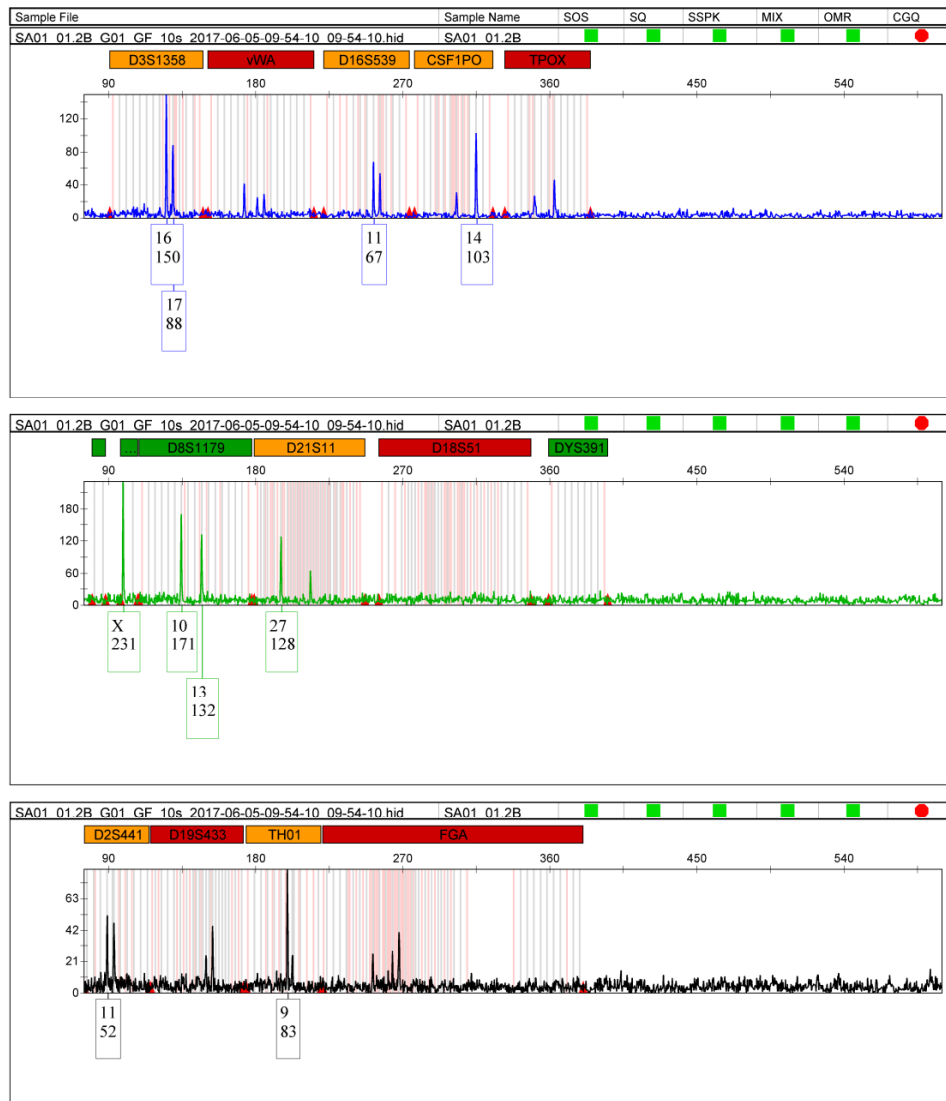
APPENDIX J: GENETIC PROFILES OF CASEWORK SAMPLES

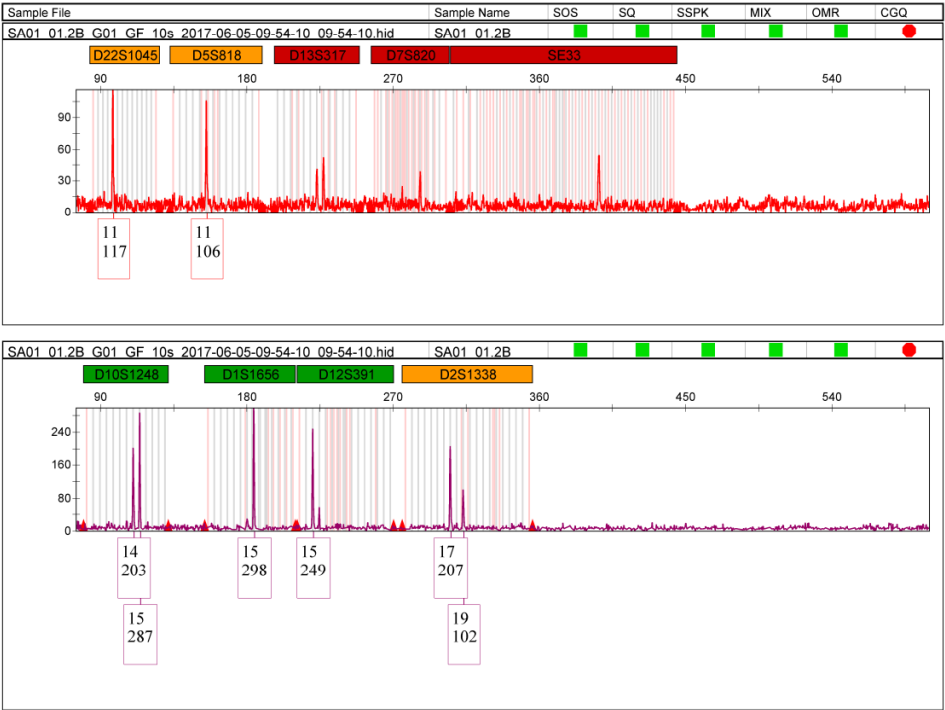
Sample ID: SA01_01.1 Vaginal swab with semen from a non-vasectomized donor (sperm fraction)



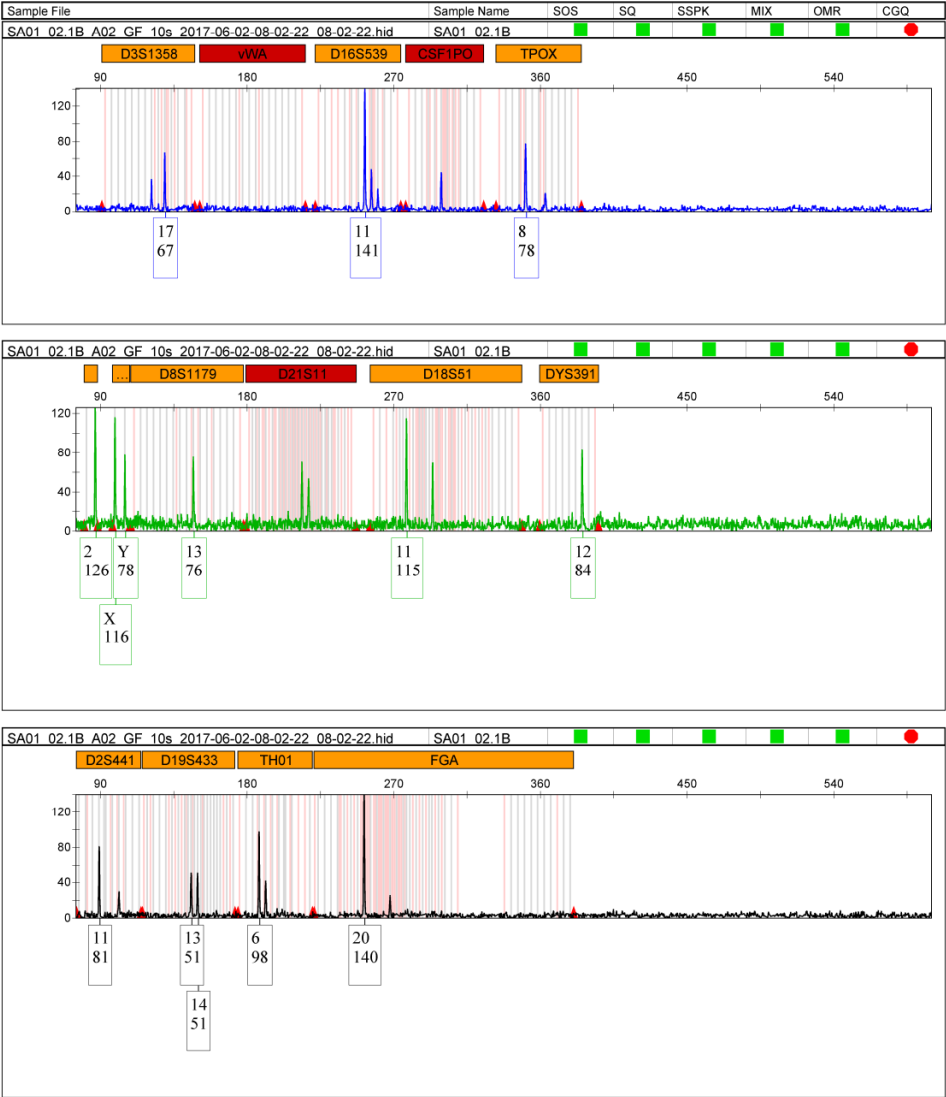


Sample ID: SA01_01.2 Vaginal swab with semen from a non-vasectomized donor (sperm fraction)





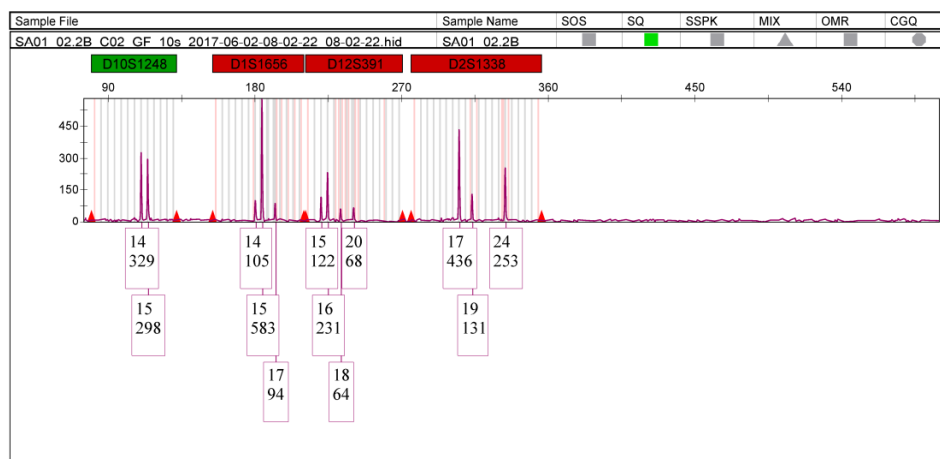
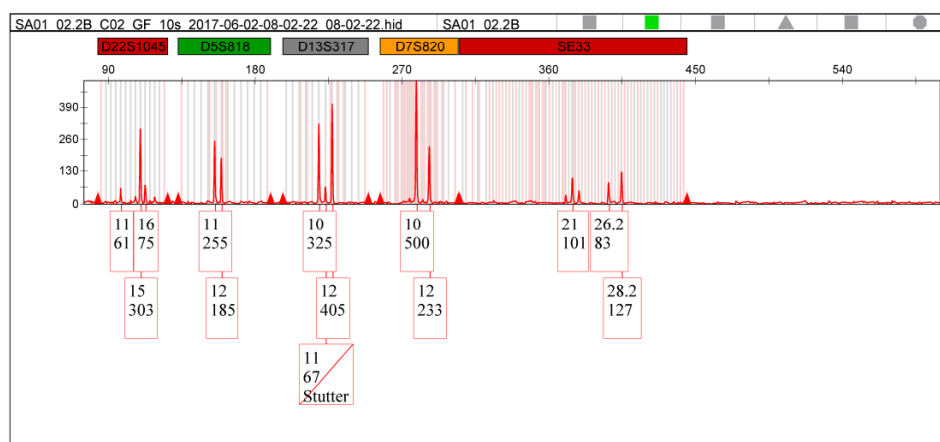
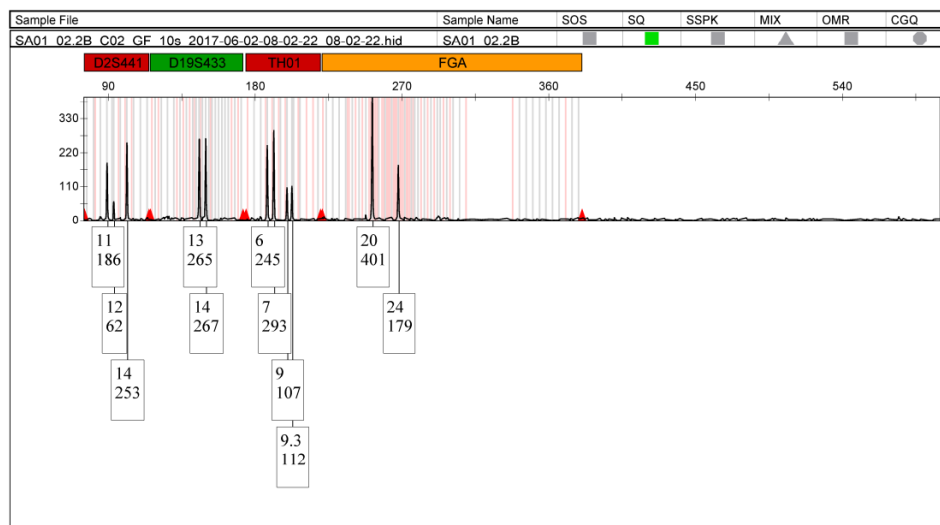
Sample ID: SA01_02.1 Oral swab with semen from a non-vasectomized donor (sperm fraction)



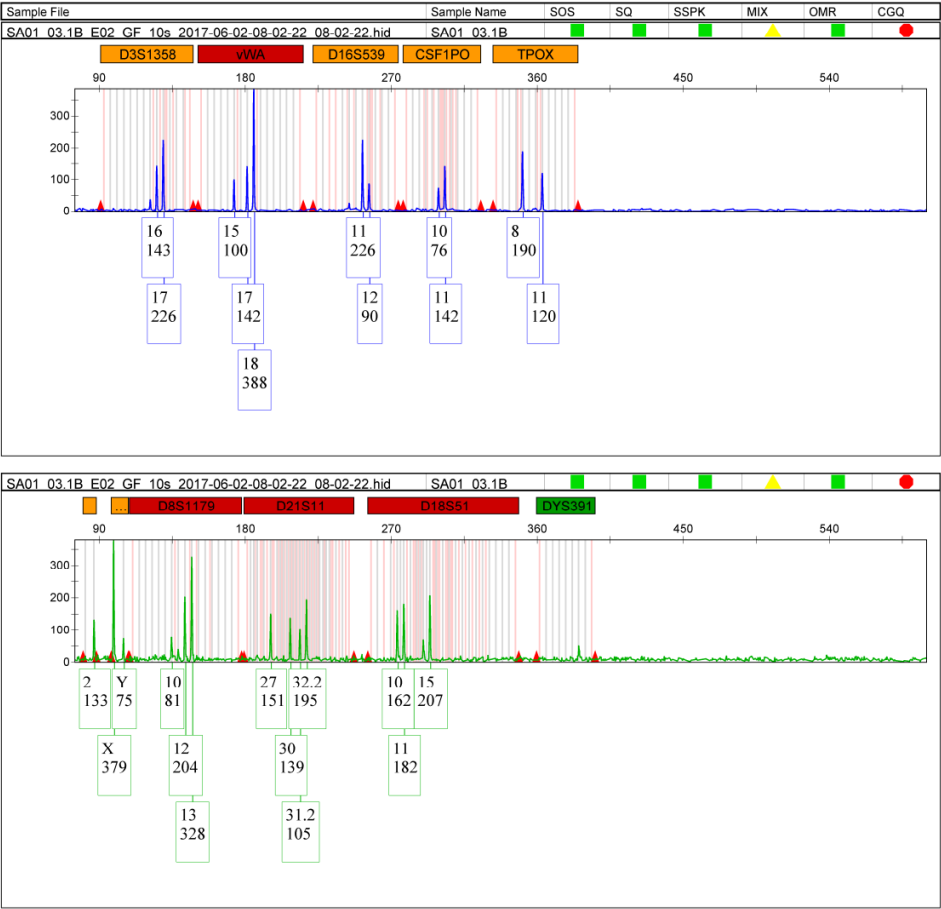


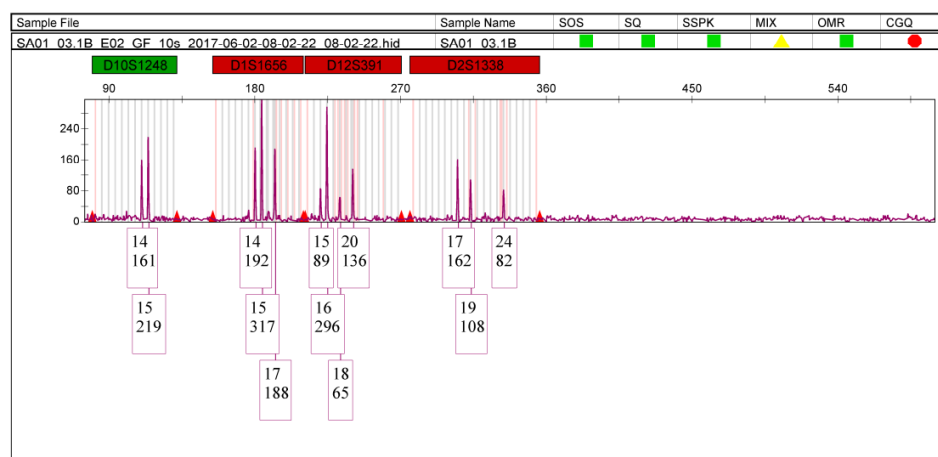
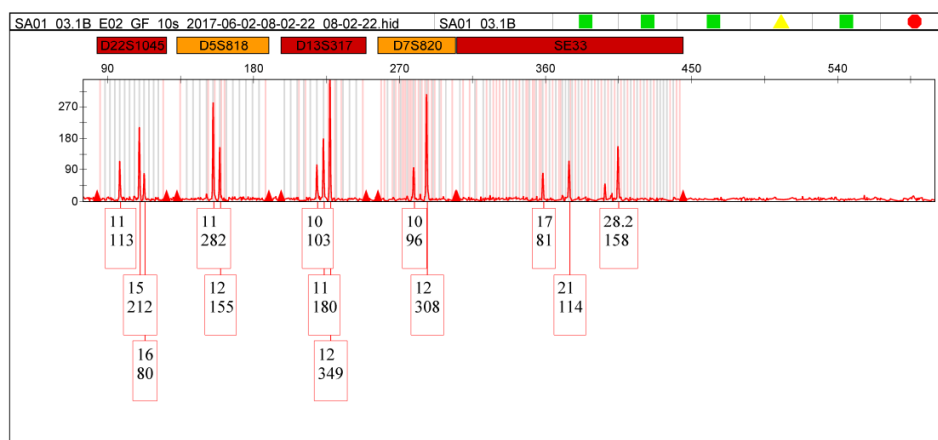
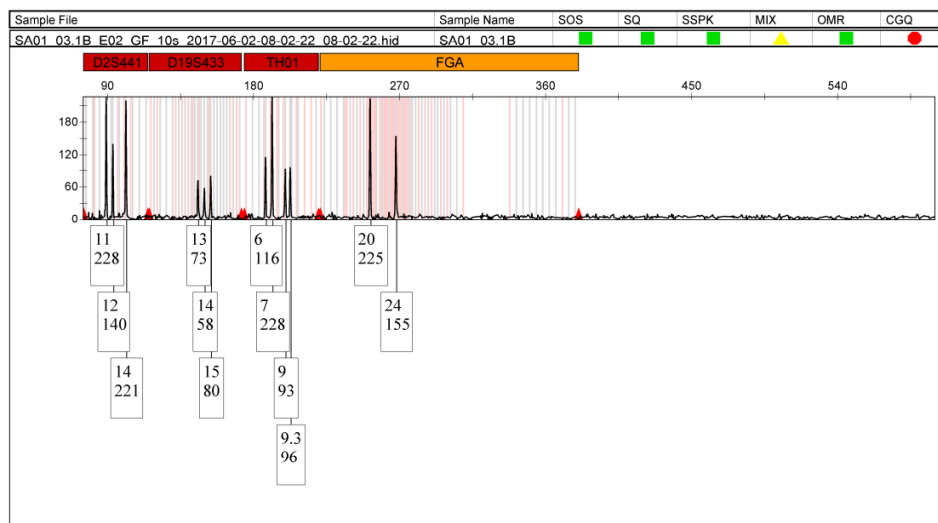
Sample ID: SA01_02.2 Oral swab with semen from a non-vasectomized donor (sperm fraction)





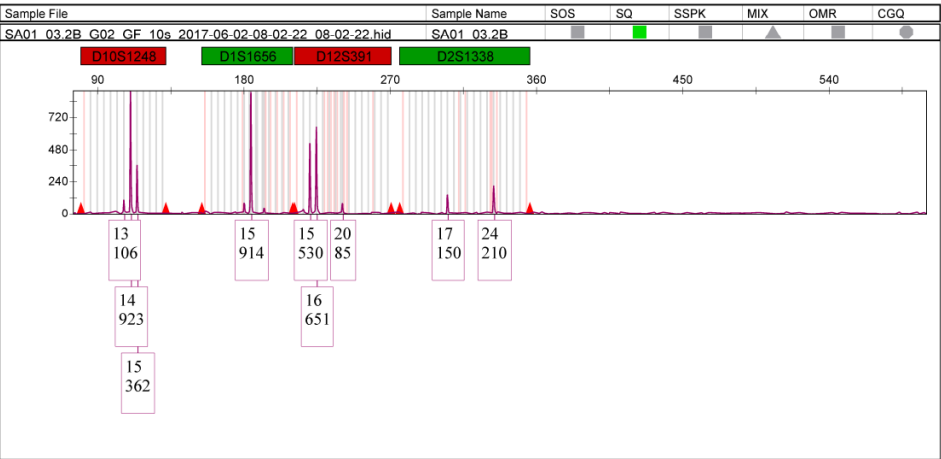
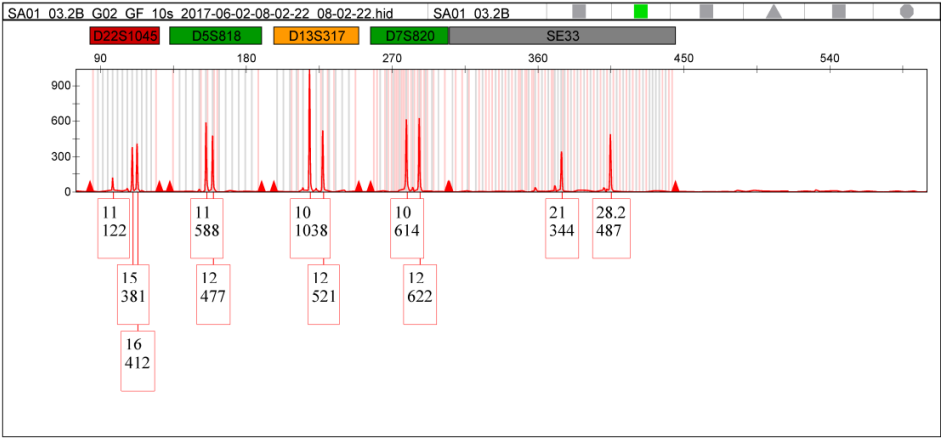
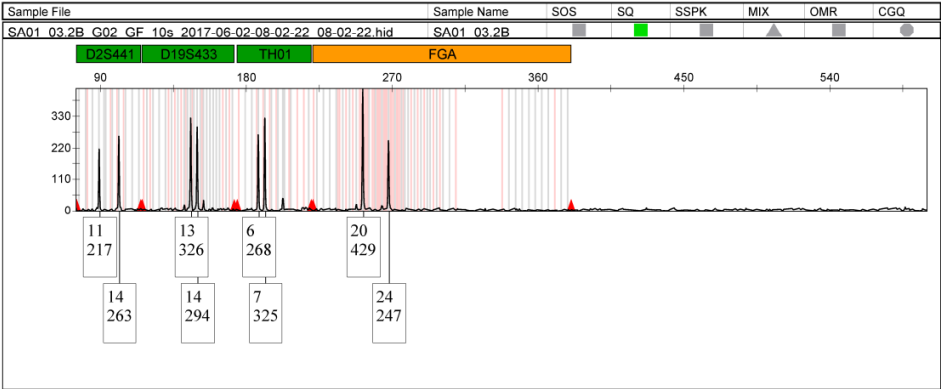
Sample ID: SA01_03.1 Rectal swab with semen from a non-vasectomized donor (sperm fraction)



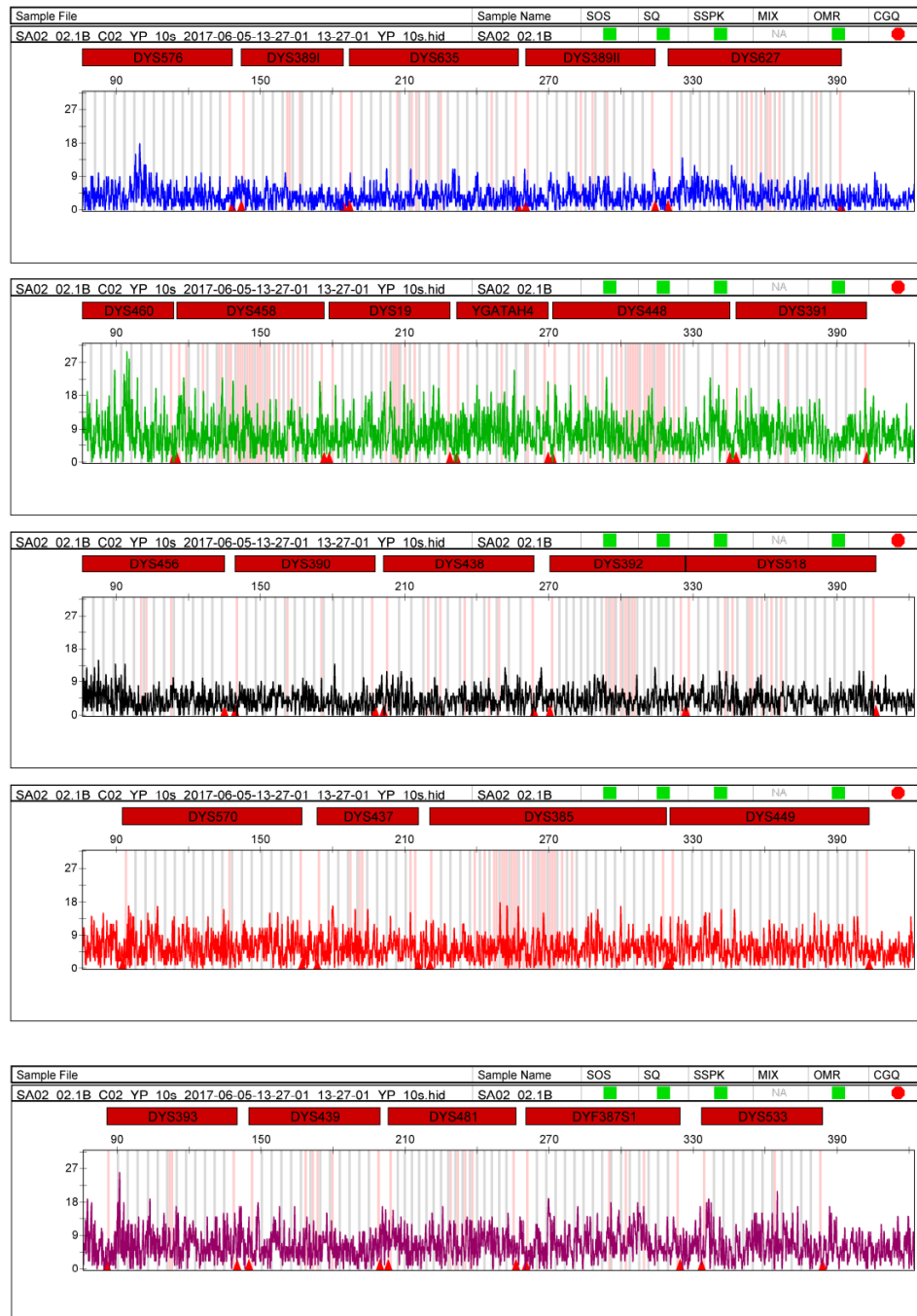


Sample ID: SA01_03.2 Rectal swab with semen from a non-vasectomized donor (sperm fraction)





Sample ID: SA02_02.1 Oral swab with semen from a vasectomized donor (sperm fraction)



APPENDIX K: PROTEIN IDENTIFICATION OF SEMEN POPULATION SAMPLES

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
06C6979	SEMG1	85.71	383	4.30E+09	1.68E+09	1141.90	801.85
	SEMG2	78.69	301	2.39E+09		1350.90	
	KLK3	30.65	8	9.74E+06		296.30	
	PPAP	20.98	9	3.45E+07		418.30	
06C7294	SEMG1	90.04	399	5.05E+09	2.08E+09	1087.90	811.03
	SEMG2	80.58	295	3.24E+09		1404.20	
	KLK3	34.87	7	7.17E+06		330.50	
	PPAP	16.58	6	1.64E+07		421.50	
06C7352	SEMG1	78.57	376	2.86E+09	1.30E+09	1146.30	912.28
	SEMG2	75.94	290	2.04E+09		1321.70	
	KLK3	40.61	22	1.35E+08		482.70	
	PPAP	48.45	64	1.82E+08		698.40	
06C9228	SEMG1	86.80	425	4.00E+09	1.78E+09	1167.50	880.45
	SEMG2	81.27	322	3.00E+09		1366.40	
	KLK3	40.61	19	6.87E+07		542.20	
	PPAP	36.27	18	6.41E+07		445.70	
06C9237	SEMG1	71.43	465	3.66E+09	1.32E+09	1102.90	860.20
	SEMG2	69.42	255	1.57E+09		1301.00	
	KLK3	39.08	15	2.12E+07		496.90	
	PPAP	28.24	24	2.59E+07		540.00	
07C3133	SEMG1	86.36	346	2.92E+09	1.29E+09	1125.00	868.58
	SEMG2	74.57	222	2.14E+09		1368.50	
	KLK3	40.61	16	2.87E+07		484.00	
	PPAP	29.27	17	7.84E+07		496.80	
07C3212	SEMG1	87.01	366	2.60E+09	1.17E+09	1075.90	804.13
	SEMG2	79.21	304	2.06E+09		1364.20	
	KLK3	27.97	6	3.78E+06		445.10	
	PPAP	12.18	3	3.21E+06		331.30	
08C3217	SEMG1	90.26	358	3.48E+09	1.41E+09	1113.70	831.98
	SEMG2	76.46	246	2.12E+09		1373.50	
	KLK3	34.87	9	1.44E+07		456.70	
	PPAP	32.12	10	1.42E+07		384.00	
09C2531	SEMG1	88.31	245	1.65E+09	6.79E+08	1276.10	851.13
	SEMG2	71.65	153	1.05E+09		1236.90	
	KLK3	19.54	4	2.48E+06		478.80	
	PPAP	22.28	8	9.01E+06		412.70	
17_08_505	SEMG1	83.12	245	7.41E+08	3.46E+08	1016.10	729.13
	SEMG2	72.16	188	6.38E+08		1209.20	
	KLK3	21.07	5	2.88E+06		385.80	
	PPAP	15.28	5	3.16E+06		305.40	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
17_08_506	SEMG1	86.15	365	2.62E+09	8.16E+08	1067.30	765.45
	SEMG2	65.81	172	5.57E+08		1112.30	
	KLK3	40.61	18	4.07E+07		469.80	
	PPAP	20.98	16	4.46E+07		412.40	
17_12_513	SEMG1	83.33	400	3.48E+09	1.48E+09	1103.50	818.48
	SEMG2	79.38	265	2.39E+09		1283.00	
	KLK3	30.27	10	1.64E+07		469.00	
	PPAP	20.98	9	3.29E+07		418.40	
18_01_691	SEMG1	77.27	339	2.35E+09	1.17E+09	1145.90	824.23
	SEMG2	77.66	243	2.19E+09		1189.60	
	KLK3	40.61	16	4.91E+07		439.40	
	PPAP	39.38	24	8.46E+07		522.00	
18_03_543	SEMG1	83.55	385	3.64E+09	1.54E+09	1184.40	890.90
	SEMG2	74.23	283	2.48E+09		1345.20	
	KLK3	30.27	12	1.99E+07		460.20	
	PPAP	27.72	26	3.08E+07		573.80	
18_03_579	SEMG1	82.47	354	4.60E+09	1.84E+09	1136.40	913.00
	SEMG2	78.52	253	2.49E+09		1362.80	
	KLK3	40.61	23	1.38E+08		619.50	
	PPAP	36.79	28	1.21E+08		533.30	
18_03_579	SEMG1	92.86	314	4.57E+09	1.84E+09	1345.90	906.78
	SEMG2	77.49	219	2.77E+09		1417.90	
	KLK3	14.94	4	5.54E+06		453.50	
	PPAP	20.98	7	7.11E+06		409.80	
18_03_615	SEMG1	92.42	452	5.98E+09	2.47E+09	1108.50	857.23
	SEMG2	83.33	319	3.82E+09		1400.10	
	KLK3	31.80	12	2.74E+07		479.60	
	PPAP	28.24	17	5.61E+07		440.70	
18_09_594	SEMG1	89.83	376	5.51E+09	2.26E+09	1181.60	861.70
	SEMG2	80.58	287	3.43E+09		1461.10	
	KLK3	40.61	14	3.83E+07		354.10	
	PPAP	25.39	14	5.19E+07		450.00	
19_02_507	SEMG1	83.12	305	2.26E+09	1.14E+09	1128.60	864.40
	SEMG2	82.47	249	2.16E+09		1409.10	
	KLK3	37.93	15	8.07E+07		478.90	
	PPAP	44.04	20	4.97E+07		441.00	
19_03_518	SEMG1	82.90	347	3.02E+09	1.41E+09	1157.50	833.18
	SEMG2	77.49	285	2.61E+09		1298.90	
	KLK3	40.23	8	1.12E+07		480.00	
	PPAP	13.47	4	6.17E+06		396.30	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
19_06_629	SEMG1	86.15	325	2.44E+09	1.21E+09	1149.90	850.25

	SEMG2	74.40	202	2.29E+09		1326.60	
	KLK3	33.33	11	4.13E+07		457.90	
	PPAP	31.09	25	6.81E+07		466.60	
19_07_577	SEMG1	89.39	362	3.43E+09	1.42E+09	1121.00	853.40
	SEMG2	76.98	255	2.22E+09		1390.40	
	KLK3	31.80	11	2.74E+07		490.40	
	PPAP	20.98	11	2.78E+07		411.80	
19_09_609	SEMG1	86.58	395	4.05E+09	1.82E+09	1145.90	822.53
	SEMG2	79.04	243	3.10E+09		1383.80	
	KLK3	40.61	16	5.68E+07		318.90	
	PPAP	38.60	20	7.09E+07		441.50	
19_10_584	SEMG1	88.96	285	1.50E+09	6.58E+08	1075.80	765.08
	SEMG2	74.40	191	1.11E+09		1270.00	
	KLK3	21.84	6	3.25E+06		379.90	
	PPAP	23.83	13	1.23E+07		334.60	
19_11_614	SEMG1	94.16	311	3.87E+09	1.62E+09	1234.90	806.78
	SEMG2	73.20	231	2.59E+09		1371.80	
	KLK3	23.37	6	8.93E+06		237.90	
	PPAP	12.18	4	3.72E+06		382.50	
19_12_573	SEMG1	95.02	398	5.83E+09	2.17E+09	1288.00	884.88
	SEMG2	80.24	264	2.80E+09		1375.90	
	KLK3	31.80	8	1.82E+07		470.60	
	PPAP	20.98	7	2.02E+07		405.00	
20_02_628	SEMG1	88.53	334	4.30E+09	1.72E+09	1242.10	892.28
	SEMG2	75.43	258	2.52E+09		1427.50	
	KLK3	39.08	15	4.04E+07		499.10	
	PPAP	20.98	7	2.15E+07		400.40	
20_02_628	SEMG1	89.18	322	2.83E+09	1.08E+09	1277.20	861.25
	SEMG2	75.43	209	1.44E+09		1303.00	
	KLK3	38.70	11	1.91E+07		462.70	
	PPAP	24.87	14	2.75E+07		402.10	
20_02_694	SEMG1	94.59	278	3.09E+09	1.29E+09	1205.70	691.90
	SEMG2	78.35	209	2.07E+09		1247.70	
	KLK3	11.49	2	2.26E+05		68.80	
	PPAP	13.47	4	1.70E+06		245.40	
20_04_559	SEMG1	91.56	367	5.21E+09	2.09E+09	1250.10	920.75
	SEMG2	79.72	262	3.08E+09		1491.40	
	KLK3	33.33	10	2.66E+07		469.40	
	PPAP	20.98	8	3.14E+07		472.10	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
31978_01	SEMG1	83.55	374	3.74E+09	1.78E+09	1125.10	953.08
	SEMG2	80.24	250	2.70E+09		1417.20	
	KLK3	41.38	33	2.25E+08		600.60	
	PPAP	48.45	54	4.32E+08		669.40	
31978_02	SEMG1	90.04	397	5.76E+09	2.42E+09	1198.10	919.63
	SEMG2	82.30	295	3.56E+09		1391.60	
	KLK3	40.61	21	1.59E+08		503.70	
	PPAP	43.78	30	1.84E+08		585.10	
31978_03	SEMG1	83.98	387	5.71E+09	2.16E+09	1098.60	903.23
	SEMG2	79.72	247	2.67E+09		1360.90	
	KLK3	39.08	20	1.32E+08		577.20	
	PPAP	48.19	40	1.42E+08		576.20	
31978_04	SEMG1	86.58	415	4.22E+09	1.79E+09	1166.70	850.18
	SEMG2	82.30	290	2.84E+09		1353.90	
	KLK3	37.93	12	1.70E+07		343.50	
	PPAP	28.24	12	7.01E+07		536.60	
31978_05	SEMG1	87.88	396	7.31E+09	3.00E+09	1176.00	870.73
	SEMG2	78.35	301	4.64E+09		1395.00	
	KLK3	27.20	8	1.69E+07		478.60	
	PPAP	16.58	5	1.93E+07		433.30	
31978-06	SEMG1	90.9	234	1.58E+09	6.24E+08	1268.5	863.10
	SEMG2	70.6	163	9.09E+08		1359.8	
	KLK3	14.2	4	3.77E+06		443.7	
	PPAP	4.4	2	1.94E+06		380.4	
31978-07	SEMG1	78.8	375	1.80E+09	7.50E+08	1123.1	650.80
	SEMG2	76.6	305	1.20E+09		1273.6	
	KLK3	0	0	0		0	
	PPAP	3.1	2	1.43E+05		206.5	
31978-08	SEMG1	84	390	6.53E+09	2.62E+09	1243.2	813.63
	SEMG2	80.8	272	3.93E+09		1351.5	
	KLK3	8.8	2	3.08E+06		286.4	
	PPAP	25.6	9	8.28E+06		373.4	
31978-09	SEMG1	89.8	418	7.20E+09	2.90E+09	1171.6	843.28
	SEMG2	84.4	327	4.36E+09		1315.8	
	KLK3	27.2	9	1.94E+07		423.4	
	PPAP	25.4	10	2.40E+07		462.3	
31978-10	SEMG1	86.8	330	2.49E+09	1.12E+09	1102.9	831.25
	SEMG2	74.1	289	1.97E+09		1350.6	
	KLK3	31.8	10	1.25E+07		432.5	
	PPAP	21	8	1.11E+07		439	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
31978-11	SEMG1	87.4	373	4.47E+09	1.79E+09	1233.4	816.65

	SEMG2	80.1	272	2.66E+09		1349.2	
	KLK3	31.8	9	1.38E+07		297.3	
	PPAP	21	8	1.47E+07		386.7	
31978-12	SEMG1	83.8	391	3.04E+09	1.37E+09	1095.3	815.05
	SEMG2	78.2	310	2.42E+09		1265.8	
	KLK3	33.3	14	2.30E+07		493.8	
	PPAP	16.6	7	9.46E+06		405.3	
31978-13	SEMG1	90	401	6.14E+09	2.69E+09	1146.9	831.90
	SEMG2	82.1	282	4.61E+09		1332.5	
	KLK3	36	9	1.02E+07		409.4	
	PPAP	21	6	1.27E+07		438.8	
31978-14	SEMG1	90.7	439	9.33E+09	3.60E+09	1172.5	879.85
	SEMG2	79.2	323	4.93E+09		1342.4	
	KLK3	33.3	16	7.47E+07		542.6	
	PPAP	25.4	11	8.03E+07		461.9	
31978-15	SEMG1	89.2	374	4.49E+09	1.99E+09	1217.8	860.88
	SEMG2	80.2	258	3.38E+09		1354.2	
	KLK3	40.2	12	2.47E+07		406.3	
	PPAP	25.4	10	5.91E+07		465.2	
31978-16	SEMG1	83.5	376	2.94E+09	1.25E+09	1136.8	829.40
	SEMG2	77.7	295	2.03E+09		1332.4	
	KLK3	37.9	11	1.04E+07		470.1	
	PPAP	16.6	5	2.97E+06		378.3	
31978-17	SEMG1	90.9	360	3.70E+09	1.57E+09	1097.2	784.13
	SEMG2	79.6	266	2.57E+09		1417.2	
	KLK3	20.7	6	6.00E+06		309	
	PPAP	11.4	2	3.17E+06		313.1	
31978-18	SEMG1	78.6	312	2.70E+09	1.35E+09	1171.3	911.98
	SEMG2	85.6	331	2.59E+09		1354.2	
	KLK3	33.4	30	5.85E+07		605.3	
	PPAP	37.2	11	3.61E+07		517.1	
31978_19	SEMG1	86.58	465	4.56E+09	2.08E+09	1158.20	963.53
	SEMG2	82.13	321	3.37E+09		1443.80	
	KLK3	40.61	23	1.42E+08		564.90	
	PPAP	53.63	61	2.28E+08		687.20	
31978_20	SEMG1	86.80	342	4.73E+09	2.01E+09	1217.90	923.63
	SEMG2	83.16	260	3.08E+09		1359.00	
	KLK3	39.46	21	8.12E+07		543.60	
	PPAP	45.08	31	1.49E+08		574.00	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
20_04_566	SEMG1	81.17	411	2.47E+09	1.18E+09	1112.60	882.85
	SEMG2	79.38	322	2.14E+09		1381.20	
	KLK3	40.23	13	5.41E+07		470.60	
	PPAP	46.63	29	6.43E+07		567.00	

20_05_510	SEMG1	92.21	305	2.96E+09	1.35E+09	1235.10	908.08
	SEMG2	76.80	225	2.26E+09		1300.60	
	KLK3	40.23	17	6.67E+07		502.90	
	PPAP	40.41	27	1.03E+08		593.70	
20_05_515	SEMG1	87.45	236	1.62E+09	6.48E+08	1232.20	817.33
	SEMG2	69.76	166	9.70E+08		1370.80	
	KLK3	9.96	5	3.50E+06		434.40	
	PPAP	3.63	1	7.58E+05		231.90	
20_05_522	SEMG1	89.83	321	3.60E+09	1.62E+09	1173.10	924.98
	SEMG2	79.38	246	2.60E+09		1396.60	
	KLK3	41.76	24	1.57E+08		584.10	
	PPAP	39.12	27	1.09E+08		546.10	
20_05_523	SEMG1	89.39	357	5.89E+09	2.66E+09	1200.10	989.08
	SEMG2	76.12	271	4.42E+09		1424.40	
	KLK3	44.83	27	1.61E+08		705.00	
	PPAP	48.45	43	1.69E+08		626.80	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
18_03_552 (Vasectomized)	SEMG1	87.88	323	2.53E+09	1.08E+09	1171.50	808.35
	SEMG2	74.57	227	1.78E+09		1360.80	
	KLK3	14.18	5	3.64E+06		460.40	
	PPAP	3.63	1	3.76E+05		240.70	
19_09_537 (Vasectomized)	SEMG1	87.88	411	3.99E+09	1.68E+09	1126.10	816.98
	SEMG2	79.38	299	2.72E+09		1391.20	
	KLK3	18.39	7	1.07E+07		443.00	
	PPAP	16.58	6	7.01E+06		307.60	
19_10_565 (Vasectomized)	SEMG1	90.26	334	2.42E+09	1.02E+09	1114.90	852.65
	SEMG2	76.46	210	1.63E+09		1383.20	
	KLK3	23.75	8	1.27E+07		483.60	
	PPAP	20.98	9	1.08E+07		428.90	
17_04_533 (Vasectomized)	SEMG1	90.69	438	4.48E+09	1.78E+09	1146.50	839.95
	SEMG2	76.29	308	2.65E+09		1375.90	
	KLK3	16.86	5	5.24E+06		479.30	
	PPAP	11.66	3	3.17E+06		358.10	
17_02_589 (Vasectomized)	SEMG1	89.83	356	3.21E+09	1.48E+09	1180.30	710.65
	SEMG2	82.65	302	2.71E+09		1350.80	
	KLK3	12.26	2	7.74E+05		137.50	
	PPAP	4.66	2	4.04E+05		174.00	

APPENDIX L: PROTEIN IDENTIFICATION OF SALIVA POPULATION SAMPLES

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
1866	Basic Salivary 1	95.41	181	7.17E+08	2.24E+08	1024.30	667.77
	Basic Salivary 2	45.43	31	1.05E+08		757.10	
	Basic Salivary 3	64.10	78	3.61E+08		976.40	
	Basic Salivary 4	45.16	42	7.03E+07		616.60	
	Salivary Acidic 1/2	90.36	156	7.62E+08		928.70	
	Statherin	54.84	145	2.09E+08		557.80	
	Submaxillary Gland	72.15	76	1.43E+08		496.10	
	Histatin 1	66.67	71	6.83E+07		482.60	
	Histatin 3	62.74	15	1.66E+07		578.90	
	Cystatin-SN	30.50	13	1.26E+07		424.60	
1995	Alpha-Amylase 1	9.59	6	1.86E+06	1.83E+08	502.40	532.11
	Basic Salivary 1	18.11	7	8.74E+06		501.40	
	Basic Salivary 2	85.10	97	2.21E+08		735.90	
	Basic Salivary 3	81.55	96	4.82E+08		824.20	
	Basic Salivary 4	64.52	61	2.32E+08		595.30	
	Salivary Acidic 1/2	90.36	126	7.98E+08		798.10	
	Statherin	45.16	53	4.42E+07		397.60	
	Submaxillary Gland	72.15	80	1.84E+08		500.00	
	Histatin 1	43.86	45	3.62E+07		564.90	
	Histatin 3	31.37	4	1.76E+06		319.50	
2407	Cystatin-SN	11.35	6	2.74E+06	3.22E+08	382.30	601.41
	Alpha-Amylase 1	5.28	4	6.03E+05		234.00	
	Basic Salivary 1	93.62	158	6.61E+08		874.40	
	Basic Salivary 2	45.91	19	4.88E+07		536.50	
	Basic Salivary 3	73.79	76	5.14E+08		927.20	
	Basic Salivary 4	26.45	12	1.63E+07		560.60	
	Salivary Acidic 1/2	90.36	146	1.36E+09		809.90	
	Statherin	54.84	119	2.81E+08		544.90	
	Submaxillary Gland	72.15	98	4.70E+08		609.00	
	Histatin 1	47.37	104	1.87E+08		567.70	
3955	Histatin 3	60.78	7	4.63E+06	4.06E+08	384.00	662.20
	Cystatin-SN	19.86	4	9.64E+05		424.70	
	Alpha-Amylase 1	4.89	3	7.63E+05		376.60	
	Basic Salivary 1	94.90	204	1.40E+09		1032.80	
	Basic Salivary 2	45.43	26	1.23E+08		674.50	
	Basic Salivary 3	70.87	61	3.03E+08		872.50	
	Basic Salivary 4	51.29	51	2.15E+08		1024.80	
	Salivary Acidic 1/2	90.36	149	1.33E+09		923.30	
	Statherin	66.13	96	2.07E+08		482.40	
	Submaxillary Gland	72.15	107	7.15E+08		587.70	
3955	Histatin 1	45.61	90	1.58E+08	4.06E+08	555.00	662.20
	Histatin 3	21.57	3	1.06E+06		338.50	
	Cystatin-SN	10.64	4	1.60E+06		408.90	
	Alpha-Amylase 1	4.89	2	7.41E+05		383.80	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
4646	Basic Salivary 1	93.88	134	6.69E+08	3.72E+08	1050.90	657.98
	Basic Salivary 2	38.70	14	4.16E+07		631.70	
	Basic Salivary 3	76.70	79	6.55E+08		1008.40	
	Basic Salivary 4	34.52	22	8.05E+07		658.80	
	Salivary Acidic 1/2	90.36	131	1.50E+09		961.50	
	Statherin	54.84	109	3.37E+08		623.00	
	Submaxillary Gland	72.15	80	5.42E+08		617.30	
	Histatin 1	66.67	105	2.51E+08		602.10	
	Histatin 3	62.74	14	1.58E+07		405.20	
	Cystatin-SN	5.67	1	2.18E+05		307.50	
4970	Alpha-Amylase 1	2.15	1	8.82E+05	1.37E+08	371.40	514.24
	Basic Salivary 1	53.57	26	2.84E+07		637.90	
	Basic Salivary 2	93.03	140	5.78E+08		1068.00	
	Basic Salivary 3	6.84	1	2.79E+05		125.50	
	Basic Salivary 4	63.23	65	1.49E+08		674.50	
	Salivary Acidic 1/2	81.32	91	3.01E+08		823.90	
	Statherin	54.84	62	7.11E+07		395.70	
	Submaxillary Gland	72.15	91	2.47E+08		504.00	
	Histatin 1	64.91	89	1.09E+08		502.80	
	Histatin 3	62.74	17	1.65E+07		559.10	
5711	Cystatin-SN	13.48	3	7.40E+05	3.98E+08	365.20	631.76
	Alpha-Amylase 1	0.00	0	0.00E+00		0.00	
	Basic Salivary 1	93.37	152	6.73E+08		921.10	
	Basic Salivary 2	42.31	20	5.01E+07		685.00	
	Basic Salivary 3	71.20	77	4.72E+08		927.00	
	Basic Salivary 4	41.61	17	6.32E+07		513.40	
	Salivary Acidic 1/2	90.36	161	1.65E+09		881.30	
	Statherin	54.84	118	4.10E+08		537.60	
	Submaxillary Gland	72.15	100	8.47E+08		633.40	
	Histatin 1	47.37	107	2.05E+08		582.40	
6003	Histatin 3	41.18	5	1.88E+06	2.17E+08	377.30	582.05
	Cystatin-SN	13.48	3	2.25E+06		440.40	
	Alpha-Amylase 1	2.15	1	6.11E+05		450.50	
	Basic Salivary 1	47.70	24	4.15E+07		571.60	
	Basic Salivary 2	90.14	103	3.56E+08		786.40	
	Basic Salivary 3	73.79	54	1.98E+08		783.50	
	Basic Salivary 4	53.87	58	1.25E+08		635.20	
	Salivary Acidic 1/2	90.36	122	8.03E+08		701.00	
	Statherin	54.84	114	1.94E+08		506.30	
	Submaxillary Gland	72.15	94	5.19E+08		543.90	
	Histatin 1	47.37	91	1.36E+08		568.60	
	Histatin 3	60.78	13	9.74E+06		470.30	
	Cystatin-SN	30.50	13	8.03E+06		482.50	
	Alpha-Amylase 1	8.41	4	1.26E+06		353.20	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
6014	Basic Salivary 1	92.35	168	1.22E+09	4.19E+08	1044.50	670.97
	Basic Salivary 2	44.71	22	1.23E+08		597.30	
	Basic Salivary 3	58.90	62	4.93E+08		1051.10	
	Basic Salivary 4	74.84	89	8.56E+08		1027.20	
	Salivary Acidic 1/2	90.36	142	9.72E+08		744.30	
	Statherin	66.13	106	2.63E+08		527.70	
	Submaxillary Gland	67.09	107	5.36E+08		537.30	
	Histatin 1	45.61	69	1.31E+08		532.40	
	Histatin 3	21.57	4	2.12E+06		299.50	
	Cystatin-SN	13.48	9	1.22E+07		520.10	
6066	Alpha-Amylase 1	4.50	2	8.99E+05	2.42E+08	499.30	570.11
	Basic Salivary 1	93.37	152	8.45E+08		1016.50	
	Basic Salivary 2	43.75	21	1.11E+08		592.30	
	Basic Salivary 3	19.94	4	6.27E+06		425.80	
	Basic Salivary 4	67.10	80	3.95E+08		839.80	
	Salivary Acidic 1/2	18.07	5	1.53E+07		529.50	
	Statherin	66.13	102	2.70E+08		535.70	
	Submaxillary Gland	72.15	113	7.10E+08		546.50	
	Histatin 1	54.39	117	3.03E+08		550.70	
	Histatin 3	60.78	12	7.08E+06		353.10	
6163	Cystatin-SN	10.64	4	1.64E+06	3.45E+08	395.90	647.77
	Alpha-Amylase 1	2.15	1	7.39E+05		485.40	
	Basic Salivary 1	95.15	196	1.82E+09		1068.90	
	Basic Salivary 2	54.09	51	3.09E+08		867.80	
	Basic Salivary 3	46.72	34	1.70E+08		806.90	
	Basic Salivary 4	58.39	54	2.72E+08		714.50	
	Salivary Acidic 1/2	90.36	83	6.72E+08		735.90	
	Statherin	53.22	56	8.83E+07		493.10	
	Submaxillary Gland	72.15	94	2.88E+08		635.20	
	Histatin 1	66.67	96	1.47E+08		550.80	
6166	Histatin 3	60.78	9	1.88E+07	2.62E+08	554.70	597.95
	Cystatin-SN	11.35	2	8.45E+05		395.40	
	Alpha-Amylase 1	2.15	1	4.00E+05		302.30	
	Basic Salivary 1	94.90	213	1.16E+09		997.70	
	Basic Salivary 2	50.96	34	1.02E+08		651.30	
	Basic Salivary 3	73.79	67	3.06E+08		788.40	
	Basic Salivary 4	37.74	36	9.86E+07		584.90	
	Salivary Acidic 1/2	90.36	118	6.54E+08		744.70	
	Statherin	54.84	100	1.30E+08		545.90	
	Submaxillary Gland	72.15	93	3.12E+08		521.60	
	Histatin 1	45.61	68	1.07E+08		509.30	
	Histatin 3	58.82	6	1.98E+06		450.60	
	Cystatin-SN	18.44	7	1.32E+06		328.90	
	Alpha-Amylase 1	4.89	2	1.03E+06		454.10	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
6171	Basic Salivary 1	36.67	14	5.80E+07	2.29E+08	637.50	525.11
	Basic Salivary 2	83.41	127	5.17E+08		867.20	
	Basic Salivary 3	54.42	20	9.01E+07		767.90	
	Basic Salivary 4	77.10	94	6.91E+08		895.80	
	Salivary Acidic 1/2	90.36	91	4.43E+08		685.20	
	Statherin	50.00	76	1.90E+08		510.60	
	Submaxillary Gland	72.15	78	4.53E+08		546.00	
	Histatin 1	45.61	49	7.33E+07		480.40	
	Histatin 3	0.00	0.00	0.00E+00		0.00	
	Cystatin-SN	0.00	0.00	0.00E+00		0.00	
	Alpha-Amylase 1	4.89	2	6.38E+05		385.60	
6238	Basic Salivary 1	93.88	162	9.99E+08	2.78E+08	1016.80	606.36
	Basic Salivary 2	44.23	18	9.76E+07		699.00	
	Basic Salivary 3	73.46	65	5.06E+08		823.90	
	Basic Salivary 4	57.10	61	3.59E+08		1068.20	
	Salivary Acidic 1/2	18.07	5	9.13E+06		558.50	
	Statherin	54.84	87	2.49E+08		528.30	
	Submaxillary Gland	72.15	87	7.00E+08		578.80	
	Histatin 1	47.37	82	1.34E+08		565.90	
	Histatin 3	0	0	0.00E+00		0	
	Cystatin-SN	18.44	3	1.44E+06		411.30	
	Alpha-Amylase 1	4.89	2	7.70E+05		419.30	
6239	Basic Salivary 1	37.24	34	6.61E+07	1.28E+08	541.50	459.75
	Basic Salivary 2	2.40	1	5.99E+06		443.70	
	Basic Salivary 3	62.39	39	1.48E+08		765.50	
	Basic Salivary 4	55.47	36	1.00E+08		525.50	
	Salivary Acidic 1/2	81.93	99	5.29E+08		662.80	
	Statherin	61.29	70	7.26E+07		425.70	
	Submaxillary Gland	67.09	56	4.63E+08		500.00	
	Histatin 1	50.88	24	1.55E+07		418.90	
	Histatin 3	0	0	0.00E+00		0	
	Cystatin-SN	24.11	6	1.49E+06		403.30	
	Alpha-Amylase 1	2.74	2	6.76E+05		370.30	
6260	Basic Salivary 1	23.47	7	5.98E+06	1.24E+08	333.10	520.52
	Basic Salivary 2	88.46	77	1.57E+08		715.20	
	Basic Salivary 3	70.87	67	2.43E+08		841.50	
	Basic Salivary 4	49.03	42	6.76E+07		628.90	
	Salivary Acidic 1/2	21.08	7	5.33E+06		283.40	
	Statherin	69.35	127	2.86E+08		491.00	
	Submaxillary Gland	72.15	106	4.79E+08		583.40	
	Histatin 1	47.37	96	1.10E+08		550.70	
	Histatin 3	60.78	9	1.03E+07		473.80	
	Cystatin-SN	12.06	2	2.75E+06		443.30	
	Alpha-Amylase 1	2.15	1	4.18E+05		381.40	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
6310	Basic Salivary 1	0	0	0.00E+00	1.58E+08	0	359.75
	Basic Salivary 2	18.75	10	4.00E+06		299.10	
	Basic Salivary 3	68.95	49	3.36E+08		752.90	
	Basic Salivary 4	30.00	21	2.39E+07		438.60	
	Salivary Acidic 1/2	87.35	94	5.36E+08		707.80	
	Statherin	43.55	55	7.17E+07		460.50	
	Submaxillary Gland	67.09	79	7.37E+08		500.40	
	Histatin 1	42.10	29	2.89E+07		441.10	
	Histatin 3	0	0	0.00E+00		0	
	Cystatin-SN	0	0	0.00E+00		0	
6343	Alpha-Amylase 1	2.74	2	9.66E+05	4.18E+08	356.80	744.31
	Basic Salivary 1	95.92	194	2.01E+09		1115.80	
	Basic Salivary 2	59.62	47	1.77E+08		921.80	
	Basic Salivary 3	55.66	38	2.03E+08		792.00	
	Basic Salivary 4	63.55	48	2.70E+08		961.20	
	Salivary Acidic 1/2	90.36	94	9.83E+08		1105.80	
	Statherin	67.74	101	1.52E+08		527.40	
	Submaxillary Gland	72.15	93	4.47E+08		559.50	
	Histatin 1	66.67	99	2.87E+08		569.90	
	Histatin 3	60.78	15	7.26E+07		670.20	
6462	Cystatin-SN	18.44	6	3.09E+06	3.45E+08	466.10	599.55
	Alpha-Amylase 1	7.04	3	1.18E+06		497.70	
	Basic Salivary 1	89.39	101	5.34E+08		1033.60	
	Basic Salivary 2	25.48	10	2.19E+07		449.20	
	Basic Salivary 3	67.96	70	7.75E+08		924.40	
	Basic Salivary 4	53.87	45	3.74E+08		867.70	
	Salivary Acidic 1/2	90.36	120	1.42E+09		734.20	
	Statherin	51.61	75	1.28E+08		502.10	
	Submaxillary Gland	72.15	92	4.31E+08		600.30	
	Histatin 1	66.67	80	1.08E+08		535.10	
6472	Histatin 3	58.82	7	2.46E+06	1.39E+08	446.70	453.25
	Cystatin-SN	0.00	0.00	0.00E+00		0	
	Alpha-Amylase 1	4.31	2	1.14E+06		501.80	
	Basic Salivary 1	4.08	1	1.09E+06		221.90	
	Basic Salivary 2	51.44	34	3.71E+07		583.20	
	Basic Salivary 3	60.52	47	1.86E+08		741.00	
	Basic Salivary 4	38.71	34	8.53E+07		546.50	
	Salivary Acidic 1/2	86.75	147	6.83E+08		836.70	
	Statherin	50.00	114	2.56E+08		565.20	
	Submaxillary Gland	67.09	95	2.45E+08		494.80	
6472	Histatin 1	42.10	16	2.98E+07	1.39E+08	421.10	453.25
	Histatin 3	0	0	0.00E+00		0	
	Cystatin-SN	30.50	9	4.05E+06		309.40	
	Alpha-Amylase 1	2.15	1	3.97E+05		266.00	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
6503	Basic Salivary 1	92.35	136	3.13E+08	2.07E+08	746.90	511.56
	Basic Salivary 2	21.63	11	1.98E+07		471.90	
	Basic Salivary 3	69.58	54	2.91E+08		768.30	
	Basic Salivary 4	65.16	57	1.62E+08		598.30	
	Salivary Acidic 1/2	21.08	12	7.36E+07		623.40	
	Statherin	66.13	105	2.53E+08		523.70	
	Submaxillary Gland	67.09	134	1.08E+09		540.80	
	Histatin 1	50.88	50	7.65E+07		557.30	
	Histatin 3	0	0	0.00E+00		0	
	Cystatin-SN	15.60	5	6.60E+06		518.30	
6510	Alpha-Amylase 1	2.15	1	3.51E+05	1.59E+08	278.30	573.24
	Basic Salivary 1	95.66	163	5.53E+08		865.50	
	Basic Salivary 2	47.84	30	4.51E+07		624.60	
	Basic Salivary 3	67.64	36	8.87E+07		760.50	
	Basic Salivary 4	47.74	21	3.13E+07		472.80	
	Salivary Acidic 1/2	90.36	85	4.21E+08		903.00	
	Statherin	58.06	100	1.81E+08		412.90	
	Submaxillary Gland	72.15	88	3.36E+08		542.50	
	Histatin 1	43.86	23	9.27E+07		605.20	
	Histatin 3	31.37	3	1.19E+06		394.50	
6554	Cystatin-SN	24.82	5	2.52E+06	3.11E+08	414.30	638.67
	Alpha-Amylase 1	2.15	1	3.44E+05		309.80	
	Basic Salivary 1	91.82	150	7.49E+08		1023.30	
	Basic Salivary 2	51.20	22	5.44E+07		589.70	
	Basic Salivary 3	73.14	68	3.75E+08		859.20	
	Basic Salivary 4	46.45	39	1.47E+08		708.10	
	Salivary Acidic 1/2	90.36	117	1.04E+09		966.20	
	Statherin	69.35	120	1.76E+08		447.40	
	Submaxillary Gland	72.15	89	6.97E+08		557.90	
	Histatin 1	45.61	100	1.77E+08		515.70	
6557	Histatin 3	58.82	12	6.98E+06	2.42E+08	588.50	480.94
	Cystatin-SN	13.48	3	9.48E+05		393.90	
	Alpha-Amylase 1	2.15	1	4.57E+05		375.50	
	Basic Salivary 1	10.97	3	2.72E+06		366.90	
	Basic Salivary 2	71.39	41	3.16E+07		506.10	
	Basic Salivary 3	73.79	73	4.35E+08		787.40	
	Basic Salivary 4	41.61	29	8.25E+07		516.30	
	Salivary Acidic 1/2	90.36	154	1.06E+09		801.10	
	Statherin	54.84	115	2.36E+08		529.10	
	Submaxillary Gland	72.15	108	6.68E+08		523.40	
SA_01	Histatin 1	66.67	92	1.47E+08	1.75E+08	505.30	578.40
	Histatin 3	45.10	3	5.44E+05		276.70	
	Cystatin-SN	0.00	0.00	0.00E+00		0.00	
	Alpha-Amylase 1	2.15	1	9.23E+05		478.00	
	Basic Salivary 1	95.92	127	5.92E+08		921.70	
	Basic Salivary 2	50.48	29	5.34E+07		651.60	
	Basic Salivary 3	75.73	41	1.25E+08		776.10	
	Basic Salivary 4	51.94	26	7.05E+07		521.30	
	Salivary Acidic 1/2	82.53	80	4.50E+08		964.30	
	Statherin	66.13	102	1.47E+08		477.40	
SA_01	Submaxillary Gland	72.15	63	2.45E+08	1.75E+08	507.50	578.40
	Histatin 1	64.91	62	1.14E+08		527.70	
	Histatin 3	62.74	31	1.30E+08		683.30	
	Cystatin-SN	13.48	5	1.93E+06		331.50	
	Alpha-Amylase 1	0.00	0	0.00E+00		0.00	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
IR1	Basic Salivary 1	87.50	48	1.42E+08	4.89E+07	1042.00	412.48
	Basic Salivary 2	7.21	2	1.19E+06		132.00	
	Basic Salivary 3	27.07	13	3.16E+07		555.00	
	Basic Salivary 4	0.00	0	0.00E+00		0.00	
	Salivary Acidic 1/2	62.65	29	1.62E+08		646.60	
	Statherin	53.22	51	6.16E+07		542.10	
	Submaxillary Gland	72.15	53	9.14E+07		493.70	
	Histatin 1	63.16	13	5.14E+06		277.60	
	Histatin 3	62.74	17	4.17E+07		597.90	
	Cystatin-SN	0.00	0	0.00E+00		0.00	
IR2	Alpha-Amylase 1	2.15	1	2.51E+05	3.26E+08	250.40	590.71
	Basic Salivary 1	91.82	101	7.59E+08		1063.60	
	Basic Salivary 2	55.29	26	9.50E+07		791.20	
	Basic Salivary 3	70.55	51	3.01E+08		959.80	
	Basic Salivary 4	51.61	38	7.10E+07		597.80	
	Salivary Acidic 1/2	90.36	100	8.33E+08		999.10	
	Statherin	67.74	176	5.48E+08		535.50	
	Submaxillary Gland	72.15	116	9.29E+08		580.00	
	Histatin 1	63.16	31	4.62E+07		529.20	
	Histatin 3	0.00	0	0.00E+00		0.0	
IR3	Cystatin-SN	12.06	3	1.25E+06	1.34E+08	441.60	445.27
	Alpha-Amylase 1	0.00	0	0.00E+00		0.0	
	Basic Salivary 1	52.55	28	3.50E+07		537.60	
	Basic Salivary 2	6.01	3	2.97E+06		445.70	
	Basic Salivary 3	75.40	52	1.67E+08		815.50	
	Basic Salivary 4	11.29	3	1.06E+06		239.30	
	Salivary Acidic 1/2	90.36	95	6.05E+08		743.80	
	Statherin	69.35	93	1.07E+08		424.70	
	Submaxillary Gland	72.15	75	5.31E+08		496.40	
	Histatin 1	45.61	40	2.62E+07		468.40	
IR4	Histatin 3	54.90	3	5.81E+05	9.20E+07	374.70	423.75
	Cystatin-SN	0.00	0	0.00E+00		0.00	
	Alpha-Amylase 1	2.15	1	4.53E+05		351.90	
	Basic Salivary 1	3.83	1	1.47E+05		203.80	
	Basic Salivary 2	57.93	24	2.28E+07		463.30	
	Basic Salivary 3	66.67	39	8.67E+07		563.10	
	Basic Salivary 4	50.00	21	1.69E+07		547.90	
	Salivary Acidic 1/2	90.36	74	3.77E+08		737.00	
	Statherin	69.35	94	7.75E+07		370.20	
	Submaxillary Gland	72.15	78	3.82E+08		545.80	
IR4	Histatin 1	64.91	40	3.93E+07	9.20E+07	492.70	423.75
	Histatin 3	60.78	9	8.94E+06		520.60	
	Cystatin-SN	0.00	0	0.00E+00		0.00	
	Alpha-Amylase 1	2.15	1	1.65E+05		216.80	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
IR5	Basic Salivary 1	21.68	8	4.46E+06	2.75E+07	403.00	334.59
	Basic Salivary 2	51.68	29	2.81E+07		485.20	
	Basic Salivary 3	68.95	31	7.42E+07		768.30	
	Basic Salivary 4	38.39	18	1.75E+07		419.00	
	Salivary Acidic 1/2	8.43	4	2.02E+07		274.80	
	Statherin	66.13	55	2.54E+07		412.60	
	Submaxillary Gland	72.15	54	1.31E+08		543.50	
	Histatin 1	33.33	5	1.10E+06		291.30	
	Histatin 3	17.65	1	6.24E+04		82.80	
	Cystatin-SN	0.00	0	0.00E+00		0.00	
IR6	Alpha-Amylase 1	0.00	0	0.00E+00	3.94E+08	0.00	489.52
	Basic Salivary 1	95.66	172	1.53E+09		978.30	
	Basic Salivary 2	47.84	18	4.32E+07		430.50	
	Basic Salivary 3	55.66	34	2.97E+08		815.40	
	Basic Salivary 4	85.42	77	6.00E+08		888.90	
	Salivary Acidic 1/2	89.76	108	6.06E+08		692.30	
	Statherin	62.90	85	1.34E+08		536.30	
	Submaxillary Gland	69.62	130	1.11E+09		620.20	
	Histatin 1	24.56	7	1.10E+07		422.80	
	Histatin 3	0.00	0	0.00E+00		0.00	
IR7	Cystatin-SN	0.00	0	0.00E+00	1.87E+08	0.00	621.54
	Alpha-Amylase 1	0.00	0	0.00E+00		0.00	
	Basic Salivary 1	90.61	91	6.77E+08		1052.50	
	Basic Salivary 2	46.15	24	3.50E+07		727.40	
	Basic Salivary 3	44.44	27	1.04E+08		834.50	
	Basic Salivary 4	29.68	14	3.14E+07		549.10	
	Salivary Acidic 1/2	90.36	91	4.58E+08		802.30	
	Statherin	61.29	139	3.17E+08		545.00	
	Submaxillary Gland	72.15	79	3.39E+08		517.90	
	Histatin 1	66.67	53	7.43E+07		535.40	
IR8	Histatin 3	62.74	8	1.63E+07	9.76E+07	568.00	508.85
	Cystatin-SN	5.67	2	1.61E+06		414.50	
	Alpha-Amylase 1	2.15	1	2.02E+05		290.30	
	Basic Salivary 1	93.03	57	1.01E+08		941.00	
	Basic Salivary 2	0	0	0.00E+00		0	
	Basic Salivary 3	41.60	22	4.68E+07		815.90	
	Basic Salivary 4	25.81	10	8.11E+06		529.40	
	Salivary Acidic 1/2	90.36	78	4.75E+08		904.30	
	Statherin	58.06	96	1.58E+08		480.80	
	Submaxillary Gland	72.15	65	2.26E+08		568.40	
IR8	Histatin 1	63.16	29	2.26E+07		453.10	
	Histatin 3	62.74	15	3.45E+07		567.20	
	Cystatin-SN	5.67	2	9.82E+05		337.20	
	Alpha-Amylase 1	0.00	0	0.00E+00		0.00	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
IR9	Basic Salivary 1	66.07	20	1.46E+07	4.28E+07	410.00	350.85
	Basic Salivary 2	0	0	0.00E+00		0	
	Basic Salivary 3	40.74	15	2.33E+07		576.80	
	Basic Salivary 4	12.90	3	1.05E+06		145.00	
	Salivary Acidic 1/2	90.36	76	2.53E+08		679.30	
	Statherin	58.06	55	1.77E+07		309.70	
	Submaxillary Gland	72.15	49	1.54E+08		539.50	
	Histatin 1	66.67	16	3.89E+06		395.10	
	Histatin 3	49.02	5	3.21E+06		496.50	
	Cystatin-SN	0.00	0	0.00E+00		0.00	
IR10	Alpha-Amylase 1	2.15	1	2.38E+05	1.75E+08	307.50	430.98
	Basic Salivary 1	13.52	5	1.58E+06		319.60	
	Basic Salivary 2	30.05	19	2.59E+07		458.80	
	Basic Salivary 3	54.99	32	7.78E+07		549.40	
	Basic Salivary 4	41.70	27	5.73E+07		436.40	
	Salivary Acidic 1/2	83.73	127	5.30E+08		720.90	
	Statherin	61.29	65	4.53E+07		418.30	
	Submaxillary Gland	72.15	140	1.18E+09		581.10	
	Histatin 1	36.84	10	7.23E+06		415.00	
	Histatin 3	0.00	0	0.00E+00		0.00	
IR11	Cystatin-SN	29.79	8	1.33E+06	2.32E+07	286.30	442.00
	Alpha-Amylase 1	3.13	2	1.95E+06		555.00	
	Basic Salivary 1	28.57	10	3.96E+06		360.90	
	Basic Salivary 2	78.37	33	5.22E+07		695.40	
	Basic Salivary 3	21.37	14	8.63E+06		839.40	
	Basic Salivary 4	0.00	0	0.00E+00		0.00	
	Salivary Acidic 1/2	69.28	34	7.56E+07		665.90	
	Statherin	67.74	58	6.92E+07		439.50	
	Submaxillary Gland	72.15	29	2.08E+07		429.80	
	Histatin 1	61.40	19	7.75E+06		443.40	
IR12	Histatin 3	62.74	17	1.67E+07	1.19E+08	626.70	447.31
	Cystatin-SN	5.67	1	7.77E+04		222.80	
	Alpha-Amylase 1	2.15	1	1.74E+05		138.20	
	Basic Salivary 1	12.76	5	1.94E+06		347.80	
	Basic Salivary 2	87.98	49	3.90E+07		572.70	
	Basic Salivary 3	66.95	45	1.43E+08		738.70	
	Basic Salivary 4	43.87	26	1.90E+07		425.90	
	Salivary Acidic 1/2	90.36	87	4.24E+08		712.80	
	Statherin	69.35	158	3.37E+08		480.40	
	Submaxillary Gland	72.15	79	3.10E+08		570.80	
IR12	Histatin 1	61.40	56	3.63E+07	1.19E+08	439.90	447.31
	Histatin 3	0.00	0	0.00E+00		0.00	
	Cystatin-SN	5.67	1	1.71E+05		333.20	
	Alpha-Amylase 1	2.15	1	3.03E+05		298.20	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
IR13	Basic Salivary 1	95.41	116	3.28E+08	8.28E+07	914.70	459.74
	Basic Salivary 2	54.81	19	2.48E+07		460.70	
	Basic Salivary 3	74.76	44	1.45E+08		806.70	
	Basic Salivary 4	51.94	22	7.46E+07		573.40	
	Salivary Acidic 1/2	83.13	70	1.91E+08		713.50	
	Statherin	51.61	52	3.40E+07		401.40	
	Submaxillary Gland	65.82	59	1.09E+08		501.20	
	Histatin 1	35.09	10	4.54E+06		414.90	
	Histatin 3	0.00	0	0.00E+00		0.00	
	Cystatin-SN	0.00	0	0.00E+00		0.00	
IR14	Alpha-Amylase 1	2.15	1	2.37E+05	1.58E+08	270.60	365.89
	Basic Salivary 1	23.21	10	6.26E+06		217.10	
	Basic Salivary 2	2.40	1	2.70E+05		283.40	
	Basic Salivary 3	57.60	24	4.88E+07		533.80	
	Basic Salivary 4	18.71	8	7.22E+06		328.70	
	Salivary Acidic 1/2	83.73	139	6.06E+08		737.40	
	Statherin	64.52	80	7.60E+07		384.40	
	Submaxillary Gland	72.15	103	9.82E+08		499.30	
	Histatin 1	43.86	10	7.60E+06		439.10	
	Histatin 3	0.00	0	0.00E+00		0.00	
IR15	Cystatin-SN	24.82	6	1.41E+06	2.13E+08	232.80	579.25
	Alpha-Amylase 1	21.33	16	3.64E+06		368.80	
	Basic Salivary 1	95.92	110	7.24E+08		872.60	
	Basic Salivary 2	64.90	28	6.04E+07		575.60	
	Basic Salivary 3	72.49	56	2.44E+08		794.00	
	Basic Salivary 4	59.68	52	1.58E+08		989.60	
	Salivary Acidic 1/2	89.76	78	5.84E+08		709.70	
	Statherin	66.13	104	2.07E+08		507.60	
	Submaxillary Gland	72.15	58	2.98E+08		527.40	
	Histatin 1	45.61	46	6.41E+07		477.20	
IR16	Histatin 3	0.00	0	0.00E+00	2.03E+08	0.00	640.42
	Cystatin-SN	13.48	4	2.94E+06		439.50	
	Alpha-Amylase 1	2.15	1	7.86E+05		478.60	
	Basic Salivary 1	92.42	114	7.02E+08		995.50	
	Basic Salivary 2	47.84	23	4.31E+07		602.90	
	Basic Salivary 3	77.67	48	1.61E+08		810.60	
	Basic Salivary 4	54.52	38	9.46E+07		741.30	
	Salivary Acidic 1/2	90.36	77	6.44E+08		882.50	
	Statherin	54.84	78	9.33E+07		459.10	
	Submaxillary Gland	72.15	64	3.70E+08		555.00	
IR16	Histatin 1	45.61	58	9.51E+07	2.03E+08	596.20	640.42
	Histatin 3	45.10	8	2.77E+07		655.90	
	Cystatin-SN	13.48	2	4.79E+05		367.00	
	Alpha-Amylase 1	2.15	1	3.36E+05		378.60	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
IR17	Basic Salivary 1	58.93	44	1.02E+08	1.24E+08	544.40	468.24
	Basic Salivary 2	11.06	4	4.15E+06		416.30	
	Basic Salivary 3	60.68	33	1.08E+08		552.60	
	Basic Salivary 4	45.48	25	4.13E+07		521.10	
	Salivary Acidic 1/2	87.35	66	4.96E+08		734.50	
	Statherin	58.06	158	1.58E+08		393.60	
	Submaxillary Gland	72.15	78	4.43E+08		532.00	
	Histatin 1	63.16	13	5.77E+06		497.50	
	Histatin 3	39.21	3	1.68E+06		429.20	
	Cystatin-SN	12.06	3	1.09E+06		201.10	
IR18	Alpha-Amylase 1	2.15	1	3.17E+05	1.87E+08	328.30	472.75
	Basic Salivary 1	80.61	77	4.80E+08		728.00	
	Basic Salivary 2	10.82	6	1.43E+07		449.10	
	Basic Salivary 3	69.26	39	2.12E+08		564.70	
	Basic Salivary 4	44.84	23	2.80E+07		432.20	
	Salivary Acidic 1/2	90.36	74	5.57E+08		719.70	
	Statherin	59.68	105	9.68E+07		461.70	
	Submaxillary Gland	72.15	69	6.42E+08		543.20	
	Histatin 1	63.16	29	2.84E+07		505.60	
	Histatin 3	47.06	4	3.48E+06		527.00	
IR19	Cystatin-SN	0.00	0	0.00E+00	1.17E+08	0.00	526.48
	Alpha-Amylase 1	2.15	1	2.21E+05		269.00	
	Basic Salivary 1	56.38	34	8.93E+07		704.90	
	Basic Salivary 2	95.43	98	5.78E+08		1014.00	
	Basic Salivary 3	56.13	32	7.63E+07		772.90	
	Basic Salivary 4	39.03	20	4.06E+07		575.30	
	Salivary Acidic 1/2	90.36	66	2.76E+08		684.30	
	Statherin	67.74	130	1.17E+08		372.40	
	Submaxillary Gland	72.15	49	9.52E+07		525.80	
	Histatin 1	36.84	20	5.87E+06		404.60	
IR20	Histatin 3	62.74	9	6.87E+06	1.25E+08	525.10	415.86
	Cystatin-SN	0.00	0	0.00E+00		0.00	
	Alpha-Amylase 1	5.28	2	2.15E+05		212.00	
	Basic Salivary 1	62.24	54	1.61E+08		547.80	
	Basic Salivary 2	11.78	8	2.76E+07		468.90	
	Basic Salivary 3	65.24	43	1.73E+08		574.50	
	Basic Salivary 4	56.45	38	1.03E+08		550.40	
	Salivary Acidic 1/2	86.75	105	5.53E+08		664.90	
	Statherin	61.29	92	8.76E+07		394.10	
	Submaxillary Gland	69.62	64	2.71E+08		494.40	
IR20	Histatin 1	31.58	4	9.17E+05		273.20	
	Histatin 3	0.00	0	0.00E+00		0.00	
	Cystatin-SN	19.15	3	4.64E+05		254.90	
	Alpha-Amylase 1	2.15	1	3.09E+05		351.40	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
IR21	Basic Salivary 1	44.90	29	4.49E+07	8.41E+07	682.50	480.65
	Basic Salivary 2	95.67	95	4.95E+08		1022.60	
	Basic Salivary 3	38.46	29	1.02E+08		524.20	
	Basic Salivary 4	37.42	25	4.89E+07		575.70	
	Salivary Acidic 1/2	74.10	27	7.83E+07		609.40	
	Statherin	67.74	27	1.44E+07		246.80	
	Submaxillary Gland	72.15	37	1.19E+08		491.10	
	Histatin 1	64.91	44	1.81E+07		347.40	
	Histatin 3	62.74	4	4.17E+06		516.50	
	Cystatin-SN	0.00	0	0.00E+00		0.00	
	Alpha-Amylase 1	2.15	1	1.93E+05		271.00	
IR22	Basic Salivary 1	68.62	56	1.75E+08	2.54E+08	703.90	484.06
	Basic Salivary 2	12.74	7	1.04E+07		493.90	
	Basic Salivary 3	75.73	46	2.38E+08		632.80	
	Basic Salivary 4	38.39	20	1.36E+08		508.10	
	Salivary Acidic 1/2	89.76	140	9.88E+08		717.10	
	Statherin	51.61	90	1.62E+08		470.80	
	Submaxillary Gland	72.15	89	1.07E+09		507.00	
	Histatin 1	31.58	13	7.40E+06		423.50	
	Histatin 3	0.00	0	0.00E+00		0.00	
	Cystatin-SN	16.31	7	3.74E+06		507.40	
	Alpha-Amylase 1	2.15	1	4.40E+05		360.20	
IR23	Basic Salivary 1	92.73	152	1.42E+09	2.29E+08	1066.30	566.65
	Basic Salivary 2	52.16	36	8.21E+07		859.80	
	Basic Salivary 3	64.10	42	1.68E+08		783.10	
	Basic Salivary 4	7.10	2	1.69E+07		559.20	
	Salivary Acidic 1/2	83.73	73	4.67E+08		740.00	
	Statherin	69.35	111	1.41E+08		447.00	
	Submaxillary Gland	72.15	70	1.96E+08		537.00	
	Histatin 1	56.14	31	2.67E+07		474.50	
	Histatin 3	0.00	0	0.00E+00		0.00	
	Cystatin-SN	10.64	1	3.35E+05		295.10	
	Alpha-Amylase 1	2.15	1	6.03E+05		471.20	
IR24	Basic Salivary 1	10.97	6	7.90E+06	1.10E+08	554.40	431.72
	Basic Salivary 2	85.34	60	1.59E+08		700.50	
	Basic Salivary 3	70.87	49	3.16E+08		653.80	
	Basic Salivary 4	53.55	42	1.63E+08		579.00	
	Salivary Acidic 1/2	89.76	73	3.57E+08		717.00	
	Statherin	54.84	83	6.36E+07		424.10	
	Submaxillary Gland	72.15	51	1.45E+08		503.70	
	Histatin 1	45.61	10	2.47E+06		339.00	
	Histatin 3	0.00	0.00	0.00E+00		0.00	
	Cystatin-SN	0.00	0.00	0.00E+00		0.00	
	Alpha-Amylase 1	2.15	1	2.71E+05		277.40	
IR25	Basic Salivary 1	92.09	99	3.91E+08	1.50E+08	755.00	446.95
	Basic Salivary 2	22.84	10	1.11E+07		438.00	
	Basic Salivary 3	73.79	47	3.06E+08		778.50	
	Basic Salivary 4	42.58	32	1.40E+08		566.30	
	Salivary Acidic 1/2	86.75	82	4.72E+08		714.80	
	Statherin	61.29	63	5.06E+07		426.50	
	Submaxillary Gland	67.09	56	2.64E+08		496.20	
	Histatin 1	45.61	23	1.21E+07		431.40	
	Histatin 3	0.00	0	0.00E+00		0.00	
	Cystatin-SN	0.00	0	0.00E+00		0.00	
	Alpha-Amylase 1	2.15	1	4.49E+05		309.70	

APPENDIX M: PROTEIN IDENTIFICATION OF VAGINAL FLUID POPULATION SAMPLES

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
3714	CRNN	75.2	90	3.85E+08	1.02E+08	1099.50	605.93
	SBSN	32.5	17	1.52E+07		647.60	
	IVL	23.1	8	8.24E+06		476.70	
	PPL	0.9	1	2.42E+05		199.90	
4669	CRNN	29.1	17	3.04E+06	1.01E+06	201.40	121.10
	SBSN	2.7	2	1.89E+05		67.30	
	IVL	1.9	2	8.06E+05		215.70	
	PPL	0.0	0	0.00E+00		0.00	
5488	CRNN	67.5	93	1.91E+08	5.01E+07	1105.70	434.68
	SBSN	25.8	14	8.49E+06		345.80	
	IVL	2.4	1	7.58E+05		287.20	
	PPL	0.0	0	0.00E+00		0.00	
5560	CRNN	40.0	28	9.83E+06	2.56E+06	629.50	189.83
	SBSN	0.0	0	0.00E+00		0.00	
	IVL	1.9	2	4.26E+05		129.80	
	PPL	0.0	0	0.00E+00		0.00	
5701	CRNN	69.1	68	8.39E+07	2.11E+07	1007.20	313.00
	SBSN	9.8	3	5.23E+05		244.80	
	IVL	0.0	0	0.00E+00		0.00	
	PPL	0.0	0	0.00E+00		0.00	
5854	CRNN	54.3	49	9.91E+07	2.80E+07	956.00	444.35
	SBSN	12.5	5	2.91E+06		146.50	
	IVL	22.4	7	9.34E+06		516.80	
	PPL	0.9	1	5.62E+05		158.10	
6239	CRNN	62.0	69	9.54E+07	2.83E+07	952.80	424.98
	SBSN	15.4	7	4.17E+06		178.60	
	IVL	45.5	33	1.38E+07		568.50	
	PPL	0.0	0	0.00E+00		0.00	
6241	CRNN	63.2	68	1.01E+08	2.65E+07	1022.00	407.98
	SBSN	5.4	3	9.27E+05		75.40	
	IVL	20.7	8	3.34E+06		260.60	
	PPL	0.9	1	5.36E+05		273.90	
6260	CRNN	62.6	42	2.22E+07	5.86E+06	557.30	171.03
	SBSN	15.4	5	1.26E+06		126.80	
	IVL	0.0	0	0.00E+00		0.00	
	PPL	0.0	0	0.00E+00		0.00	
6291	CRNN	51.5	39	4.49E+07	1.29E+07	652.10	284.73
	SBSN	11.7	5	1.72E+06		177.70	
	IVL	6.7	5	5.11E+06		309.10	
	PPL	0.0	0	0.00E+00		0.00	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
6303	CRNN	45.1	46	5.61E+07	1.51E+07	751.50	382.08
	SBSN	10.5	5	4.13E+06		666.10	
	IVL	0.0	0	0.00E+00		0.00	
	PPL	1.1	1	3.04E+05		110.70	
6310	CRNN	45.3	62	1.33E+08	3.50E+07	971.80	438.08
	SBSN	14.1	8	3.01E+06		298.20	
	IVL	10.1	5	3.29E+06		354.80	
	PPL	0.9	1	6.69E+05		127.50	
6311	CRNN	66.3	85	1.11E+08	3.18E+07	766.70	463.78
	SBSN	31.2	16	1.03E+07		415.00	
	IVL	13.9	6	3.33E+06		305.10	
	PPL	2.5	2	2.63E+06		368.30	
6320	CRNN	75.8	94	2.87E+08	8.75E+07	1054.30	596.90
	SBSN	57.3	35	3.85E+07		678.10	
	IVL	24.1	11	1.79E+07		356.10	
	PPL	5.0	6	6.38E+06		299.10	
6343	CRNN	3.2	1	8.44E+04	2.11E+04	36.60	9.15
	SBSN	0.0	0	0.00E+00		0.00	
	IVL	0.0	0	0.00E+00		0.00	
	PPL	0.0	0	0.00E+00		0.00	
6460	CRNN	42.8	41	8.54E+07	2.22E+07	1033.80	307.95
	SBSN	0.0	0	0.00E+00		0.00	
	IVL	10.6	2	3.01E+06		160.00	
	PPL	2.1	2	2.40E+05		38.00	
6551	CRNN	43.6	38	5.54E+07	1.40E+07	936.90	286.93
	SBSN	0.0	0	0.00E+00		0.00	
	IVL	7.4	2	7.40E+05		210.80	
	PPL	0.0	0	0.00E+00		0.00	
6557	CRNN	57.6	59	1.62E+08	5.02E+07	1044.20	514.98
	SBSN	32.7	27	3.73E+07		683.60	
	IVL	2.1	2	6.67E+05		271.80	
	PPL	1.6	2	9.20E+05		60.30	
6587	CRNN	59.6	70	1.53E+08	4.69E+07	690.20	441.00
	SBSN	35.1	26	3.28E+07		604.90	
	IVL	13.9	4	1.60E+06		277.40	
	PPL	0.9	1	4.26E+05		191.50	
6635	CRNN	40.6	38	1.60E+07	9.41E+06	424.40	288.25
	SBSN	15.6	12	3.03E+06		279.60	
	IVL	39.7	42	1.86E+07		449.00	
	PPL	0.0	0	0.00E+00		0.00	
6636	CRNN	52.7	48	3.13E+07	1.23E+07	465.50	226.10
	SBSN	3.4	2	2.47E+05		106.10	
	IVL	44.8	45	1.79E+07		332.80	
	PPL	0.0	0	0.00E+00		0.00	

Sample ID	Protein ID	% Coverage	# Unique Peptides	Intensity	Average Intensity	Best Score	Average Best Score
6654	CRNN	56.6	53	1.95E+08	5.11E+07	1023.70	401.23
	SBSN	12.0	7	3.29E+06		215.90	
	IVL	18.0	5	6.05E+06		365.30	
	PPL	0.0	0	0.00E+00		0.00	
6661	CRNN	68.7	95	2.69E+08	7.34E+07	1059.10	566.78
	SBSN	22.7	12	9.44E+06		382.70	
	IVL	19.5	11	1.49E+07		508.60	
	PPL	1.0	1	6.64E+05		316.70	
6674	CRNN	67.1	84	1.02E+08	2.61E+07	976.80	367.68
	SBSN	13.1	5	1.64E+06		223.80	
	IVL	4.4	2	2.42E+05		270.10	
	PPL	0.0	0	0.00E+00		0.00	
6681	CRNN	58.6	87	1.71E+08	4.48E+07	753.70	382.98
	SBSN	12.0	5	2.05E+06		225.90	
	IVL	13.9	8	5.03E+06		272.50	
	PPL	1.7	2	1.35E+06		279.80	
A1	CRNN	69.9	79	2.44E+08	7.99E+07	990.50	664.75
	SBSN	11.2	7	7.76E+06		519.80	
	IVL	41.2	28	6.61E+07		675.40	
	PPL	2.1	2	1.16E+06		473.30	
B1	CRNN	49.1	32	4.85E+07	1.29E+07	883.30	289.08
	SBSN	21.2	5	3.14E+06		271.90	
	IVL	3.1	1	1.22E+05		1.10	
	PPL	0.0	0	0.00E+00		0.00	
C1	CRNN	0.0	0	0.00E+00	0.00E+00	0.00	0.00
	SBSN	0.0	0	0.00E+00		0.00	
	IVL	0.0	0	0.00E+00		0.00	
	PPL	0.0	0	0.00E+00		0.00	
D1	CRNN	39.4	48	4.34E+07	1.10E+07	760.80	232.90
	SBSN	0.0	0	0.00E+00		0.00	
	IVL	4.6	2	5.87E+05		170.80	
	PPL	0.0	0	0.00E+00		0.00	
E1	CRNN	72.1	79	3.14E+08	8.62E+07	1104.70	646.35
	SBSN	13.9	9	8.66E+06		621.60	
	IVL	23.3	11	2.11E+07		376.90	
	PPL	1.1	1	7.97E+05		482.20	
F1	CRNN	14.6	7	1.33E+06	3.33E+05	357.90	89.48
	SBSN	0.0	0	0.00E+00		0.00	
	IVL	0.0	0	0.00E+00		0.00	
	PPL	0.0	0	0.00E+00		0.00	
G1	CRNN	62.4	62	1.19E+08	3.13E+07	1081.40	529.28
	SBSN	10.2	3	3.57E+06		349.50	
	IVL	6.5	3	2.10E+06		421.10	
	PPL	2.0	2	7.29E+05		265.10	

APPENDIX N: PEPTIDE IDENTIFICATION OF PERFORMANCE ASSESSMENT SAMPLES**TABLE LEGEND:**

Green Box = Positive target peptide identification

Grey Box = Negative target peptide identification, positive protein identification

Red Box = Negative protein identification

NT = Not Tested

[illegible]

Saliva and Vaginal Fluid Contaminants					10% Bleach			Dish Soap			Menstrual Swab			Menstrual Swab + Saliva		
Protein	Sequence	Mass (m/z)	Charge (z)	RT	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3
HIS1	H.SHREFPF.Y	460.2247	2	8.25							NT	NT	NT			
	H.REFFPYGDY.G	597.2682	2	10.58							NT	NT	NT			
	K.HSHREFPF.Y	597.2836	2	6.35							NT	NT	NT			
	S.HREFPYGDY.G	665.7962	2	9.85							NT	NT	NT			
	F.YGDYGSNYLYDN.-	722.2886	2	7.33							NT	NT	NT			
PRP_1	Q.GGNKPGQPPPPGKPKQ.G	518.2792	3	3.09							NT	NT	NT			
	Q.GPPQGGGNRPQ.G	568.2828	2	0.99							NT	NT	NT			
	A.GNPQGSPSQGGNPKQ.G	731.8553	2	1.42							NT	NT	NT			
PRP_2	Q.GPPSPGKPKQ.G	481.2587	2	2.85							NT	NT	NT			
	Q.GGNKPGQPPSPGKPKQ.G	514.9390	3	3.24							NT	NT	NT			
	A.GNPQGAPPQGGNPKQ.G	723.8578	2	1.52							NT	NT	NT			
PRP_4	Q.RPPPPGKPKQ.G	535.8089	2	1.39							NT	NT	NT			
	Q.GPPPPGGRFP.R	577.3093	2	3.77							NT	NT	NT			
	Q.SHRPPPGKPE.R	648.3469	2	1.26							NT	NT	NT			
PRPC	Q.GPPQGSQD.-	448.2170	2	1.23							NT	NT	NT			
	Q.GPPPPPGKPKQ.G	534.7955	2	2.76							NT	NT	NT			
	Q.QGPPPGKPKQ.G	565.8013	2	1.50							NT	NT	NT			
	Q.GPPQGGHPPPPGGRPKQ.G	577.9606	3	2.07							NT	NT	NT			
	Q.GPPQGGHPPPP.R	612.8153	2	2.13							NT	NT	NT			
SMR3B	P.RGPYPGGLA	477.2648	2	7.98							NT	NT	NT			
	P.GIFPPPPQF.-	523.7877	2	10.65							NT	NT	NT			
	G.RIFPPPPAPY.G	562.8137	2	7.69							NT	NT	NT			
	G.FVPPPPPPY.G	564.2973	2	9.58							NT	NT	NT			
	P.GRIPPPPAPY.G	581.3244	2	7.55							NT	NT	NT			
	P.GFVPPPPPPY.G	582.8080	2	10.66							NT	NT	NT			
	Y.GPFIPPPPQF.-	600.8242	2	10.74							NT	NT	NT			
	P.YPPGLAPPQF.G	640.8373	2	12.45							NT	NT	NT			
	L.APPQFPGFVPPPPPPY.G	652.6733	3	13.54							NT	NT	NT			
	Y.GPGRIPPPAPY.G	658.3615	2	8.13							NT	NT	NT			
	F.GPFGVPPPPPPY.G	659.8458	2	11.06							NT	NT	NT			
	R.GPYPPGLAPPQF.G	717.8744	2	12.78							NT	NT	NT			
	F.GPFGVPPPPPPY.GP.G.R	765.3930	2	10.82							NT	NT	NT			
	P.RGPYPGGLAPPQF.G	795.9250	2	11.89							NT	NT	NT			
STAT	P.YQPVPEQLY	535.7795	2	8.39							NT	NT	NT			
	P.EQLYPQPY.Q	567.7769	2	8.34							NT	NT	NT			
	Y.GPYQPVPEQLY	612.8166	2	7.30							NT	NT	NT			
	R.IGRFGYGYQPY.Q	625.3037	2	9.15							NT	NT	NT			
	P.VPEQLYPQPY.Q	665.8375	2	9.19							NT	NT	NT			
	E.QPLYPQPYQPY.Q	679.8406	2	7.81							NT	NT	NT			
	P.YQPVPEQLYPQPYQPY.Q	691.3423	3	9.89							NT	NT	NT			
	Y.QPVPEQLYPQPY.Q	778.3932	2	9.69							NT	NT	NT			
	R.FGYGYGYQPVPEQLY	906.4356	2	11.07							NT	NT	NT			
	Y.QPVPEQLYPQPYQPY.Q	954.9782	2	9.22							NT	NT	NT			
CRNN	F.GYGYGYQPVPEQLYPQPY.Q	1157.0468	2	11.80							NT	NT	NT			
	L.YSYLRSTKP.-	372.2012	3	4.76	NT	NT	NT	NT	NT	NT						
	E.WDDHSRET.V	382.1712	3	2.41	NT	NT	NT	NT	NT	NT						
	A.DVIVKPHDPA.T	545.7982	2	5.19	NT	NT	NT	NT	NT	NT						
	R.SQTSQAVTGGHTQIQAGSH.T	632.3053	3	3.62	NT	NT	NT	NT	NT	NT						
	L.DEDHTGTVEFK.E	639.2859	2	4.6	NT	NT	NT	NT	NT	NT						
	M.PQLLQNINGIE.A	676.3826	2	13.12	NT	NT	NT	NT	NT	NT						
	D.VIVKPHDPA.TVDE.V	710.3775	2	5.25	NT	NT	NT	NT	NT	NT						
	F.ADVIVKPHDPA.TVDE.V	803.4096	2	6.29	NT	NT	NT	NT	NT	NT						
	V.ELPVEVPSKQEEKH.M	412.9690	4	5.98	NT	NT	NT	NT	NT	NT						
IVL	L.KTENPGDASDLQGRQL.L	864.9292	2	5.71	NT	NT	NT	NT	NT	NT						
PPL	R.VVQGLHHGVSAQR.E	722.8920	2	2.31	NT	NT	NT	NT	NT	NT						
SESN	N.NAAGQVGKQADKLIHGVHGHAN.Q	787.4037	3	6.29	NT	NT	NT	NT	NT	NT						

Mixtures					M1			M2			M3			M4		
					10 μL SA + 2.5 μL SE			50 μL SA + 2.5 μL SE			10 μL SA + 25 μL SE			10 μL SA + 10 μL SE		
Protein	Sequence	Mass (m/z)	Charge (z)	RT	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3
HIS1	H.SHREFFP.Y	460.2247	2	8.25												
	H.REFFPYGDY.G	597.2682	2	10.58												
	K.HHSHREFFP.Y	597.2836	2	6.35												
	S.HREFFPYGDY.G	665.7962	2	9.85												
	F.YGDYGSNYLYDN.-	722.2886	2	7.33												
PRP_1	Q.GGNKPQGPPPPGKPQ.G	518.2792	3	3.09												
	Q.GPPQQGGNRPQ.G	568.2828	2	0.99												
	A.GNPQGSPQGGNKPQ.G	731.8553	2	1.42												
PRP_2	Q.GPPSPGKPQ.G	481.2587	2	2.85												
	Q.GGNKPQGPPSPGKPQ.G	514.9390	3	3.24												
	A.GNPQGAPPQGGNKPQ.G	723.8578	2	1.52												
PRP_4	Q.RPPPPGKPQ.G	535.8089	2	1.39												
	Q.GPPPPQGGRPP.R	577.3093	2	3.77												
	Q.SHRPPPPGKPE.R	648.3469	2	1.26												
PRPC	Q.GPPQGQSPQ.-	448.2170	2	1.23												
	Q.GPPPPPGKPQ.G	534.7955	2	2.76												
	Q.QGPPPPGKPQ.G	565.8013	2	1.50												
	Q.GPPQQGGHPPPPQGRPQ.G	577.9606	3	2.07												
	Q.GPPQQGGHPRPP.R	612.8153	2	2.13												
SMR3B	P.RGPYPGFLA	477.2648	2	7.98												
	P.GIFPPPPQP.-	523.7877	2	10.65												
	G.RIPPPPPAPY.G	552.8137	2	7.69												
	G.FVPPPPPPY.G	554.2973	2	9.58												
	P.GRIPPPPPAPY.G	581.3244	2	7.55												
	P.GFVPPPPPPY.G	582.8080	2	10.66												
	Y.GPGIFPPPPQP.-	600.8242	2	10.74												
	P.YPPGGLAPPQPF.G	640.8373	2	12.45												
	L.APPQFPGFVPPPPPPY.G	652.6733	3	13.54												
	Y.GPGRIPPPPPAPY.G	658.3615	2	8.13												
	F.GPGFVPPPPPPY.G	659.8458	2	11.06												
	R.GPYPPGGLAPPQPF.G	717.8744	2	12.78												
	F.GPGFVPPPPPPY.GPG.R	765.3930	2	10.82												
	P.RGPYPGGLAPPQPF.G	795.9250	2	11.89												
STAT	P.YQPVPEQL.Y	535.7795	2	8.39												
	P.EQLYPQPY.Q	567.7769	2	8.34												
	Y.GPYQPVPEQL.Y	612.8166	2	7.30												
	R.IGRFGYGYGPY.Q	625.3037	2	9.15												
	P.VPEQLYPQPY.Q	665.8375	2	9.19												
	E.QPLYPQPYQPQ.Y	679.8406	2	7.81												
	P.YQPVPEQLYPQPYQPQ.Y	691.3423	3	9.89												
	Y.QPVPEQLYPQPY.Q	778.3932	2	9.69												
	R.FGYGYGPYQPVPEQL.Y	906.4356	2	11.07												
	Y.QPVPEQLYPQPYQPQ.Y	954.9782	2	9.22												
F.GYGYGPYQPVPEQLYPQPY.Q	1157.0468	2	11.80													

