



NIJ Forensic Laboratory Needs Technology Working Group (FLN-TWG)

IMPLEMENTATION STRATEGIES: Proteomic Mass Spectrometry for Biology Fluid Identification

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Description and Overview

The proteomic mass spectrometry (MS) body fluid assay described herein is undergoing validation at the New York City Office of Chief Medical Examiner (NYC OCME).¹ It is designed to detect multiple blood, saliva, and semen marker peptides using high performance liquid chromatography (HPLC) followed by multiple reaction monitoring (MRM) MS (employing an AB SCIEX Eksigent HPLC and 6500 QTRAP mass spectrometer respectively). The assay is unbiased, meaning no a priori knowledge of a sample is necessary before testing, because each sample is tested for all marker proteins. The assay is designed for high-throughput sample processing (~72 samples/day). On a volume basis, detection of each body fluid is in the nanoliter range (for both neat and mock forensic samples). According to Yang et al., sensitivity is at least 10 times greater than other presumptive or confirmatory methods, including chemical, immunochemical, enzymatic, and messenger RNA (mRNA) profiling.²

This section contains seven parts:

- Synopsis of the Problem
- Proteomic MS—How it Works
- Criteria for Establishing A Forensic Proteomic MS Body Fluid Assay
- Structure of the NYC OCME Assay
- Assay Validation at the NYC OCME
- Assay Workflow
- Assay Parameters

Synopsis of the Problem: Body fluid identification plays a vital role in forensic investigations, contributing to case evidence and directing the course of investigations—particularly with sexual assault cases. Body fluid identification is becoming progressively more important in this era of increasing DNA assay sensitivity, when an accused individual may acknowledge that their DNA profile is present but that its presence may be the result of a touch, sneeze, or secondary transfer from a doorknob or another object. Clearly, the ability to confirm that the source of an individual’s DNA at a crime scene is from blood, saliva, or semen is becoming increasingly critical, yet the methods used for body fluid detection have—for the most part—failed to keep pace with scientific and technological advances. Consequently, testing

¹ Research for this work was supported by the following National Institute of Justice grants: Establishment of a Fast and Accurate Proteomic Method for Body Fluid/Cell Type, 2008-DN-BX-K011; Development of a Proteomic Assay for Menstrual Blood, Vaginal Fluid and Species Identification, NIJ Award 2010-DN-BX-K192; Use of Novel Chemistry & Microwave Instrumentation to Improve Body Fluid Assay Sensitivity & Speed while Reducing Costs, NIJ Award # 2012-DN-BX-K044; Bioinformatic Analysis of Big Proteomic Data: A New Forensic Tool to Identify Menstrual Blood & Body Fluid Mixtures, NIJ Award # 2017-NE-BX-0003.

² Yang, H., Zhou, B., Deng, H., Prinz, M., & Siegel, D. (2013). Body fluid identification by mass spectrometry. *International Journal of Legal Medicine*, 127(6), 1065–1077. <https://doi.org/10.1007/s00414-013-0848-1>.

currently involves a diverse mixture of techniques that vary dramatically in their sensitivity, reproducibility, speed, cost, and—above all—specificity (i.e., their ability to confirm that test results are reliable). Even relatively quantitative immunoassays, such as enzyme-linked immunosorbent assay (**ELISA**), suffer from limitations imposed by antibody specificity, affinity, narrow working analytical ranges, and manufacturing variability, in addition to relatively high costs and long assay times. Furthermore, no tests are routinely available for many body fluids (e.g., menstrual blood, vaginal fluid).

Forensic DNA analysis relies on a single, dominant method for testing samples that uses polymerase chain reaction (**PCR**) to amplify specific loci on autosomal, X, and Y chromosomes, regardless of their source. Those loci are composed of short tandem repeats (**STRs**)—relatively small segments of DNA bases that repeat multiple times within a locus. The number of repeats within a specific locus can vary between individuals and can therefore be used to distinguish one individual from another. Detection of the variable repeat lengths of a locus between individuals is performed by capillary electrophoresis (**CE**), which separates loci by their size (i.e., DNA length). The combination of PCR and CE for STR analysis is both sensitive and accurate and is supported by robust statistical analyses. However, no single uniform methodology currently exists in public forensic laboratories for routine analysis of an unknown forensic sample to test for blood, saliva, and semen. Consequently, each body fluid test is performed separately. This consumes time, and more importantly evidence, and may require multiple instruments or laboratory personnel trained in the nuances of each method. In short, a single reproducible, confirmatory test that can identify all body fluids simultaneously is needed. Proteomic MS offers a confirmatory, statistically sound, single method for body fluid analysis comparable to DNA STR testing for identifying individuals.

What would constitute an ideal body fluid assay? A single confirmatory method that could simultaneously detect all body fluids accurately, reproducibly, and rapidly with high sensitivity and low cost. Much of the current research—particularly in the areas of mRNA, microRNA (**miRNA**), and DNA methylation—is focused on developing such a method. A proteomic MS assay offers the following advantages over other, RNA- or DNA-based methods for the following reasons:

- Proteins are less susceptible to environmental degradation than RNA and DNA.
- Contaminating proteins from a crime scene, for example from plants, insect, vermin, or other animals (including non-human primates) would not interfere with a proteomic MS assay because, like DNA, proteins carry genetic information unique to a marker's species and are automatically determined during a mass spectrometry run.
- Protein detection does not require amplification; consequently, common contaminants that interfere with DNA detection, for example inhibitors of Taq polymerase that would hinder body fluid identification assay based on nucleic acid methods (i.e., mRNA, miRNA, or DNA methylation), would not impact a protein detection assay.

- Partial protein degradation does not preclude marker identification. In contrast, nucleic acid assays require unbroken sequences from primer to primer.
- Specific protein markers are present in each body fluid and can be simultaneously detected, thereby increasing confidence that test results are correct.
- MS is unbiased with regard to sample type (i.e., no a priori knowledge of a sample is necessary before analysis). Additionally, no body fluid– or species-specific primers are required; MS simply identifies proteins that are present.
- Species determination is inherent in the sample analysis and reported with results.
- A proteomic MS assay would be amenable to high throughput and have low per-sample costs.
- Marker proteins that can be used to identify menstrual blood and vaginal fluid are known and can be included in future testing.

Proteomic MS—How it Works: Mass spectrometers are essentially highly sensitive and accurate molecular scales. Measuring the mass of a molecule can help identify it. Proteomic MS measures protein mass and can effectively sequence them. Knowing the amino acid sequences of proteins from an unknown sample not only identifies the proteins (e.g., hemoglobin, semenogelin, amylase), but it becomes possible to identify the body fluid from which they came if a sufficient number of specific marker proteins are sequenced.

Typically, in proteomic MS, solubilized protein mixtures are extracted from a forensic sample (e.g., cloth or evidence swab). The proteins are then digested into peptides with trypsin. The peptides are separated by HPLC and directly injected into a mass spectrometer for sequencing.³

Sample processing (i.e., protein extraction and digestion) usually takes 4–6 hours. Multiple samples can be processed simultaneously (e.g., in a 96-well plate format). HPLC-MS typically takes ~20 minutes per sample, and data analysis adds a few minutes. Consequently, approximately three samples can be sequenced per hour for 72 samples per day and 360 samples per week with one mass spectrometer and a dual pump HPLC instrument.

The amount of sample analyzed is typically in nanogram range. This translates to (1) sub-nanoliter volumes of blood and semen and (2) nanoliter volumes of saliva. Because saliva contains significantly less protein than blood or semen, greater sample volumes are required.

Criteria for Establishing a Forensic Proteomic MS Body Fluid Assay: Each body fluid is used in specific biological functions; for example, blood delivers oxygen, saliva aids digestion and fights microorganisms (i.e., as part of the innate immune system), and semen is involved in reproduction. Consequently, each body fluid contains specific proteins needed to fulfil that

³ Sequencing is performed by MS/MS in which the parent peptide's mass is measured and then the same peptide is fragmented, and the masses of fragments are measured. This method confirms the peptide's sequence.

fluid's purpose. However, no protein-based biomarkers (mRNA or miRNA) are unique to a specific body fluid or tissue. That said, some proteins are both predominately and abundantly expressed in multiple specific body fluids; examples include hemoglobin in blood, amylase in saliva, and semenogelin in semen. These proteins make good but insufficient markers. For instance, both amylase and prostate-specific antigen (PSA) are well known to be present in blood, albeit in amounts that are orders of magnitude smaller than in saliva or semen. Consequently, additional marker proteins—like proteins that are also present predominantly and abundantly in a specific body fluid—must be used.

The NYC OCME has chosen to use the three most specific and abundant proteins found in blood and semen as markers for these body fluids and the four most specific and abundant proteins found in saliva as markers for saliva (Table 1). Additionally, to ensure accurate detection of these marker proteins, multiple peptides from each protein are targeted, except for saliva proteins histatin-1 (HTN1) and liver-enriched gene 1 homolog (LEG1H), which possess only one peptide.

Structure of the NYC OCME Assay: Proteins can be sequenced using various types of mass spectrometers and MS methods. The NYC OCME has chosen to use a QTRAP instrument from AB SCIEX (QTRAP 6500) and MRM because of its sensitivity and speed.

In MRM, specific peptides from marker proteins (Table 1) are targeted for analysis based on their mass. To confirm that a targeted mass is indeed the peptide of interest (i.e., because another peptide may have a similar total mass but a different amino acid sequence), the peptide is fragmented in a collision chamber that contains a neutral gas, and the resulting mass fragments are then analyzed. If the masses (and, consequently, the amino acid sequences) of the fragments are consistent with the mass of the targeted parent peptide, the peptide's identity is confirmed. The NYC OCME assay analyzes four fragment masses per peptide. Most of the considerable research and development of MRM protein assays are for accurate quantitation of a biomarker in a specific, known proteome and was performed using samples obtained in controlled circumstances (usually human plasma). Under these conditions, confident identification of the target peptide can potentially be achieved with two, or even one, fragment(s) of ion per peptide, if carefully selected and validated.

A forensic application is different because it needs to identify peptide targets confidently from three different body fluids (at present) and differentiate them from an unknowable number of possible contaminants—given the nature of forensic samples. Under these circumstances, the selection of four fragment ions per peptide is a conservative decision to be sure that we can rule out the possibility of false positive signals resulting from contamination. It should be noted that the four fragment ions need not only to be present but present in specific ratios characteristic of the target peptide to identify the target peptide positively. Under these conditions, the odds that an unknown contaminant could achieve the same signal are extremely low. At present, we have no way to put a number to those low odds but have developed a comprehensive plan to do so

both computationally and empirically with our bioinformatics colleagues at New York University.

Table 1. Marker Proteins and Peptides for Blood, Saliva, and Semen

Body Fluid	Marker Proteins	Marker Peptides
Blood	Hemoglobin A (HBA)	VGAHAGEYGAEALER TYFPFDLSHGSAQVK FLASVSTVLTSK
	Hemoglobin B (HBB)	SAVTALWGK VNVDEVGGEALGR LLVVYPWTQR
	Erythrocyte Membrane Protein Band 3 (EMPB3)	IPPDSEATLVLVGR ADFLEQPVLGFVR ASTPGAAAQIQEVK
Saliva	Alpha-amylase 1 (AMY1)	LSGLLDLALGK ALVFVDNHDNQR IYVSDDGK
	Cystatin SA (CST2)	ALHFVISEYNK ATEDEYYR SQPNLDTC[CAM]AFHEQPQLQK
	Liver-Enriched Gene 1 Homolog (LEG1H)	ESPGQLSDYR
	Histatin-1 (HTN1)	EFPFYGDYGSNYLYDN
Semen	Semenogelin 1 (SEMG1)	HLGGSQQLLHNK SQQAPNPK EQDLLSHEQK
	Semenogelin 2 (SEMG2)	GQLPSGSSQFPHGQK LWVHGLSK GSISIQTEEK (2X)
	Prostate-specific antigen (PSA)	IVGGWEC[CAM]EK SVILLGR LSEPAELTDAVK

The NYC OCME assay makes no assumptions about the body fluids present in a forensic sample; thus, each sample is analyzed for all 26 body fluid target peptides. Because each peptide is analyzed for four fragments (transitions), a total of 104 transitions are expected. Confirmed body fluid identification requires detection of all transitions known to correspond to that body fluid. For example, identification of blood requires detection of all 36 transitions from the nine peptides in Table 1. Similarly, saliva detection requires observation of all 32 transitions in the eight peptides in Table 1.

Assay Validation at the NYC OCME: The NYC OCME is currently in the process of validating its proteomic MS body fluid assay for blood, saliva, and semen. Implementation is anticipated in 2021. Once validated, standard operating procedures will be made publicly available through the NYC OCME website and publication in a peer-reviewed journal. Validation of menstrual blood and vaginal fluid assays will follow.

Laboratory Workflow: Samples for body fluid identification (e.g., swabs, clothing, bed coverings) will be taken during evidence examination. When evidence is limited, DNA analysis will be given priority. It should be noted that it is possible to extract proteins and DNA simultaneously from the same sample, which can then be analyzed separately (see Use of Novel Chemistry & Microwave Instrumentation to Improve Body Fluid Assay Sensitivity & Speed while Reducing Costs, National Institute of Justice (NIJ) Award # 2012-DN-BX-K044). However, this would require a separate validation which is not part of this project.

Assay Workflow: Figure 1 shows that samples are extracted, quantified, digested with trypsin, and processed by HPLC-MS. Typical sample cuttings of sample swabs are about 2 mm² and are extracted in 200 µL buffer. Subsequently, quantification is achieved by performing the bicinchoninic acid (BCA) protein assay and reading the optical density using a spectrophotometer. The proteins are then reduced, alkylated, and digested with trypsin for 3 hours. Finally, the resulting peptides are separated by HPLC, with direct injection into a mass spectrometer for analysis. Spectral data are compared to retention times for targeted parent peptide masses and their four transition ions and transition ion intensity ratios, then automatically reported out to the analyst.

Assay Parameters: Each sample is spiked with both a digestion and a quantification standard. The digestion standard confirms that trypsin digestion occurred (i.e., that the amount and quality of trypsin were correct and that no substance in the sample inhibited digestion). The quantification standard allows for semi-quantitative analysis of marker peptides. After each sample is run, the HPLC column is washed, and the washed column is analyzed to confirm the absence of sample carry-over. Each batch is preceded and followed by quantification standards. Validation parameter details are found in Section IV.

Cost–Benefit Analysis

Estimated Cost of Instrumentation and Facility Requirements

This section estimates costs for instruments, equipment, software, consumables, reagents, and standards and also describes facilities requirements. Initial costs for a mass spectrometer and HPLC (including computer workstation and nitrogen generator) are ~\$575,000, with annual service contracts after the first 2 years of ~\$35,000. Annual reagent/consumable costs would run about \$12,000 to process over 18,000 samples, or about \$0.66 per sample. The maximum 5-day weekly throughput for one mass spectrometer and one high-performance dual pump/dual column HPLC instrument is about 350 samples.

It should be noted that a single sample or multiple samples may be queued for a LC-MS run. The \$12,000 annual reagent/consumable costs would be half if only 9,000 samples were run per year and 25% if only a quarter of the number of samples are run. Digestion controls and quantitative standards are spiked into all samples and are therefore included in the reagent/consumable costs.

Instrumentation: All major instruments and software needed are included in the following list. Service contracts are included only for the mass spectrometer and HPLC instrument. In addition to the mass spectrometer and HPLC, a forensic laboratory may already be equipped with many other instruments (e.g., centrifuges, spectrophotometers). The mass spectrometer and HPLC specified below are those used by the NYC OCME; equivalent instruments are available from other manufacturers. The NYC OCME proteomic body fluid assay is being validated using a liquid-handling robot. This piece of equipment is not necessary, especially for lower-throughput laboratories.

- Mass spectrometer—AB SCIEX 6500 QTRAP (~\$410,000)
- HPLC—Dionex 3000 with dual pumps for high throughput (~\$120,000)
- Annual MS and LC service contracts with 24-hour response time (~\$35,000)
- Computer workstation for MS data analysis (~\$6,000)
- Nitrogen generator—necessary for mass spectrometer (~\$25,000)
- Liquid-handling robot—optional (~\$50,000 to \$75,000)
- Spectrophotometer or fluorimeter (~\$20,000 to ~\$35,000)
- SpeedVac vacuum concentrator (~\$15,000)
- Refrigerated centrifuge (~\$15,000)
- Benchtop centrifuge (~\$3,000)
- Ice machine (~\$4,000)
- Incubator shaker (~\$2,000)
- Benchtop vortex (~\$500)

MS Software

- Analyst software license (AB SCIEX), at least two copies (~\$15,000/each—free updates until version changes)
- Skyline (free, open source) <https://skyline.ms/project/home/begin.view?>

Consumables, Reagents, and Standards

- Consumables⁴—HPLC columns, ion sources tips, tubes, pipette tips (~\$6,000/year)
- Reagents⁴ – trypsin, buffers, protein quantification kits, HPLC-grade solvents (~\$5,000)
- Synthetic peptide standards²—isotopically labeled (*not* radioactive) synthetic peptides must be prepared for each of the 26 marker peptides and used to ensure correct spectral matches (a single purchase will last several years (~\$15,000))

Facilities Requirements

- ~500 sq ft of lab space—does not require DNA-like clean room as there no amplification
- Mass spectrometer – is a benchtop instrument (footprint ~25 × 31 in.)
- HPLC is a benchtop instrument (footprint ~21 × 23 in.)
- Mass spec vacuums (2) on floor, typically below mass spec (footprint ~18 × 14 in. each)
- Nitrogen generator, stand alone (footprint ~24 × 34 in.)
- Two dedicated 230-volt/30-ampere power outlets
- Ventilation for the mass spectrometer
- Fume hood for preparing HPLC reagents

Personnel Consideration

Academic Requirements: A minimum of a Master of Science degree is recommended for the scientist responsible for developing and validating the assay and maintaining routine quality assurance/quality control. A Bachelor of Science degree, with sufficient training, is suitable for individuals engaged in routine casework operations.

Training Requirements: The minimum training necessary for proteomics work includes training in protein chemistry, LC separation, and MS operation and data interpretation by in-house personnel. A new hire to a proteomics laboratory with no prior experience would likely require 2–3 months training.

Number of personnel considerations/recommendations:

- One scientist with training in proteomics and MS to oversee validation and training;
- One scientist to oversee HPLC and mass spectrometer functioning, ensure routine maintenance is carried out, evaluate daily HPLC-MS quality control data, and assist team members in data analysis and troubleshooting. For example, a toxicology scientist could serve in this role, given the familiarity and use of HPLC-MS within toxicology units. This could be facilitated by cross-unit collaborations; and

⁴ Additional costs may be incurred when first developing an MS method in your laboratory.

- Two forensic scientists (to accommodate vacations schedules, illnesses, or increased caseloads) to perform routine sample preparation; conduct protein extraction, quantification, digestion, HPLC-MS sample loading, and analysis; and write reports.

Potential Funding Sources and Potential for Offering Service Across Jurisdictions

- Local/state funding
- Federal/Department of Justice funding
- Central Laboratory Model (pooled funding among laboratories or fee-for-service)

Pros and Cons

Pros

- Confirmatory serological assay for body fluids: blood, semen, and saliva
 - A single assay for all three body fluids
 - A single extraction procedure is required
 - A single MS run evaluates a sample for all three body fluids simultaneously
 - A single MS training covers all body fluids
 - Use of a single universal assay eliminates the need for multiple instruments for different body fluid assays
 - Low reagent costs/sample
 - Fast turnaround time

Cons

- New instrumentation costs (if instruments are not readily available in a toxicology laboratory)
- Initial training time
- Novel assay: no guidelines or standards currently in place
- Daubert/Frye hearings

Pros and Cons of Offering the Service In-house Versus Outsourcing the Service

- In-house pros
 - More responsive to customers
 - In-house forensic scientists available to testify
 - Lower cost per sample
 - May be able to offer test to outside jurisdictions on a fee-for-service basis
- Outsourcing cons
 - No outside service currently available
 - No guidelines or standards yet in place

- Wait time for sample analysis
- Must rely on the availability of the outside service's expert witness testimony and pay an additional cost for their testimony

Implications on Current Case Work and Return on Investment to Stakeholders

- Serology and body fluid determination assays are currently in high demand from stakeholders (e.g., law enforcement, District Attorneys' Offices). Results can offer activity-level information that may be important to a case.
- Use of a confirmatory serology and body fluid determination method leads to improved criminal justice for both the defendant and law enforcement.
- Use of proteomic MS can expand body fluid testing to include menstrual blood and vaginal fluid.

Considerations of Implementation Plan

With ever-increasing DNA testing sensitivity and with a growing number of forensic scientists documenting the frequency of secondary DNA transfer, juries, prosecutors, and defense attorneys will demand to know the source of DNA found at a crime scene or on a victim. The use of presumptive tests, including commercially available immunochromatographic assays (e.g., Seratec cards) will fail to be persuasive simply because they cannot be confirmed. A forensic analyst testifying on the statistical soundness and conclusions that can be drawn from confirmatory DNA STR testing will have a limited amount to say about the current presumptive serology testing commonly used in forensic laboratories. As presumptive methods that had been used to identify individuals (e.g., ABO blood typing) gave way to DNA STR testing, so too will presumptive serology tests give way to confirmatory tests. Confirmatory serology tests under development include the use of mRNA, miRNA, specific DNA methylation patterns, and proteomic MS. All of these tests will be expensive, and all will require new instrumentation and staff training. Forensic laboratories with toxicology groups (having LC instruments and mass spectrometers) may be able to serve dual purposes. To meet demands for confirmatory serology testing in New York City, the NYC OCME has chosen to validate a proteomic MS assay for the reasons listed previously. Other jurisdictions may choose other confirmatory serology tests. Laboratories in large jurisdictions are likely to lead the transition to confirmatory serology testing simply because of available resources. However, as these tests become as obligatory as DNA STR testing, smaller jurisdictions may find that the frequency of testing means that they too will need to perform their own confirmatory serology testing in-house.

Funding for validation can be applied for through annual NIJ applied forensic science grants. The NYC OCME is currently in the process of validating its proteomic MS assay for blood, saliva, and semen; the anticipated online date is 2021. The validation documents will be available after completion of the method validation. Menstrual blood and vaginal fluid MS assays will follow.

Timeline

- Secure funding (9–12 months)
- Acquire and install instrumentation (6–9 months)
- Hire new personnel, if necessary (6–12 months; can be concurrent with acquiring and installing the instrumentation)
- Train personnel and implement assay (3–6 months)

Resources Needed

- Instrumentation
 - Mass spectrometer
 - Dual pump/dual column HPLC
- Facilities: ventilation, dedicated electrical power
- Personnel trained to operate equipment
- Reagents, consumables, and standards

Challenges

- Funding
- Staff training
- Data storage—server/information technology (**IT**) challenges
- Court challenges (anticipated Frye/Daubert hearings)
- Lack of standardization and guidelines because the assay is new

Solutions

- Partner with prosecutors' offices to educate policy makers about the need for and importance of a confirmatory body fluid test for a fair criminal justice system
- Admissibility hearings for assay should be simple, because the application of MS is a proven science used in clinical and forensic toxicology
- Scientific Working Group on DNA Analysis Methods (**SWGDM**) is currently working to form a standardization group for proteomic assays
- Funding and personnel can assist in resolving most IT data storage challenges

Validation Plan Considerations

Validation Parameters Based on the Scope of the Method

1. **System Suitability**—Evaluated by running a standard peptide mix through LC-MS.
 - a. Ideally, isotopically labeled marker peptides should be used as standards. Cytochrome C is another option.

- b. Standard peptides are run before and after each run.
 - c. Metrics should be tracked using a system of longitudinal monitoring. The following should be routinely checked:
 - i. MS peak areas: no large jumps or decreases (10 times or more)
 - ii. Ratios of MS transitions to expected ratios
 - iii. MS total ion current (TIC)
 - iv. LC retention time: consistent pattern, no large jumps
 - v. LC peak width: wider peaks indicate poor column performance
 - vi. LC column pressure and flow rate
 - d. See also: Abbatiello, S. E., Mani, D. R., Schilling, B., MacLean, B., Zimmerman, L. J., Feng, X., Cusack, M. P., Sedransk, N., Hall, S. C., Addona, T., Allen, S., Dodder, N. G., Ghosh, M., Held, J. M., Hedrick, V., Inerowicz, H. D., Jackson, A., Keshishian, H., Kim, J. W., ... Carr, S. A. (2013). Design, implementation and multisite evaluation of a system suitability protocol for the quantitative assessment of instrument performance in liquid chromatography-multiple reaction monitoring-MS (LC-MRM-MS). *Molecular & Cellular Proteomics*, 12(9), 2623–2639.
<http://dx.doi.org/10.1074/mcp.M112.027078>
- 2. Specificity of Peptide Identification**—The assay has the ability to positively identify the presence of marker peptides and differentiate them from interferences in the biological matrix and instrument noise. Peptide identification will be determined by the following metrics:
- a. Fragment ion ratios: The peak areas of fragment ions for a given peptide marker have a very consistent and stable pattern in relation to one another. How well the fragment ion ratios match the expected pattern is assessed by calculating the dot product score of the fragment ion peak areas scaled to a standard. This dot product score reflects the number of fragment ions detected. For example, if a peak area is zero, the score will decrease, and a positive identification is unlikely. The standard used to compute the score should be either
 - i. The fragment ion ratios of the corresponding labeled peptide included in the sample, or
 - ii. The means established from a set of technical and biological replicates of pure sample at the optimal loading amount.
 - b. Retention time concordance: Measured by calculating the coefficient of variation (CV) of recorded retention times of fragment ions for a peptide. The retention time CV is expected to be zero or very close to zero. The acceptable range of retention time CV values for each target peptide should be established from a set of technical and biological replicates of pure sample at the optimal loading amount.
- 3. Specificity of Body Fluid Identification**—In some cases, real marker peptides may be detected in the absence of the body fluid because of carry-over on the LC column or low levels of expression in other body fluids. The following metrics should be used to positively establish the presence of the body fluid:
- a. Identification of all target peptides from all protein markers for the body fluid. Identification of all markers is unlikely in the absence of the target body fluid.
 - b. Relative abundance of peptide markers. The peptide markers will all be present in high abundance in the target body fluid, but detection in other body fluids and

resulting from carry-over will be much lower. Relative abundance should be measured by the normalized peak areas of transitions. The thresholds of normalized peak areas used to identify a body fluid should be established based on a set of repeat measurements of non-target body fluids, negative controls, and blanks. Peak areas should be normalized by one of the following:

- i. Ratio to the peak areas of fragment ions of the corresponding labeled peptide included in the sample, or
- ii. Ratio to the median of the peak areas of all transitions in a set of peptide standards, currently cytochrome C.

- 4. Limit of Detection**—The limit of detection for a body fluid should be established by measuring a dilution series repeatedly and determining the amount at which the normalized peak areas of all target transitions are reliably above the threshold for differentiation from carry-over and minor expression in other body fluids, as established in **3b**.

Resources

Laboratories that Have Implemented the Technology

The NYC OCME will be the first accredited public forensic laboratory to validate and implement proteomic MS for biology fluid identification into the laboratory.

- Végvári, A., Sjödin, K., Rezeli, M., Malm, J., Lilja, H., Laurell, T., & Marko-Varga, G. (2013). Identification of a novel proteoform of prostate specific antigen (SNP-L132I) in clinical samples by multiple reaction monitoring. *Molecular & Cellular Proteomics*, 12(10), 2761–2773. <http://dx.doi.org/10.1074/mcp.M112.027078>

Other Available Resources for Laboratories Interested in Implementing Proteomic MS

- See the two following publications for more information on these methods:
 - Jenkins, R., Duggan, J. S., Aubry, A.-F., Zeng, J., Lee, J. W., Cojocar, L., Duffield, D., Garofolo, F., Kaur, S., Schultz, G. a., Xu, K., Yang, Z., Yu, H., Zhang, Y. J., & Vazvaei, F. (2015). Recommendations for validation of LC-MS/MS bioanalytical methods for protein biotherapeutics. *The AAPS Journal*, 17(1), 1-16. <http://dx.doi.org/10.1208/s12248-014-9685-5>
 - Addona, T., Abbatiello, S. E., Schilling, B., Skates, S. J., Mani, D. R., Bunk, D. M., Spiegelman, C. H., Zimmerman, L. J., Ham, A. J. L., Keshishian, H., Hall, S. C., Allen, S., Blackman, R. K., Borchers, C. H., Buck, C., Cardasis, H. L., Cusack, M. P., Dodder, N. G., Gibson, B. W., ... Carr, S. A. (2009). Multi-site assessment of the precision and reproducibility of multiple reaction monitoring–based measurements of proteins in plasma. *Nature Biotechnology*, 27(7), 633–641. <http://dx.doi.org/10.1038/nbt.1546>
- The NYC OCME is in the process of validating its proteomic MS assay and will make validation documents available to all interested laboratories through our website: www.nyc.gov/ocme.
- The NYC OCME will publish its validation results in peer-reviewed journals.

- The following are publications about body fluid identification from the NYC OCME:
 - Yang, H., Zhou, B., Deng, H., Prinz, M., & Siegel, D. (2013). Body fluid identification by mass spectrometry. *International Journal of Legal Medicine*, 127, 1065–1077. <http://dx.doi.org/10.1007/s00414-013-0848-1>
 - Yang, H., Zhou, B., Prinz, M., & Siegel, D. (2012). Proteomic analysis of menstrual blood. *Molecular & Cellular Proteomics*, 11(10), 1024–1035. <http://dx.doi.org/10.1074/mcp.M112.018390>

Per the [Description and Overview section](#), Table 2 lists NIJ-funded body fluid ID projects. All the abstracts can be found by searching the title on the NIJ website.

Table 2. NIJ-Funded Fluid ID Projects

Title	Date Range (Start Date–End Date)
Nanobiosensor Arrays for On-Site Multiplexed Detection of Protein Markers to Identify Forensically Relevant Body Fluids	1/1/2020–9/30/2022
Verification and Evaluation of a miRNA Panel for Body Fluid Identification Using DNA Extracts	1/1/2020–6/30/2021
Completion of the SONIC-DE 2.0 System for Implementation in Forensic Laboratories	1/1/2020–12/31/2020
Body Fluid Analysis Detection and Identification by Surface Enhanced Raman Spectroscopy for Forensic Scientists	1/1/2019–12/31/2021
A Low-Cost Isothermal Amplification Microdevice for Rapid Colorimetric Detection Applied to Body Fluid Identification and Y-screening	1/1/2018–12/31/2019
A Confirmatory Test for Sperm in Sexual Assault Samples using a Microfluidic-Integrated Cell Phone Imaging System	1/1/2018–12/31/2020
Bioinformatic Analysis of Big Proteomic Data: A New Forensic Tool to Identify Menstrual Blood & Body Fluid Mixtures	1/1/2018–12/31/2019
Implementation of Epigenetics Into Forensic Science: Development of a PCR Based Multiplex for the Simultaneous Analysis of age and Body Fluid Identification	1/1/2018–12/31/2019
Raman Spectroscopy for Analyzing Body Fluid Traces: Universal Method Development	1/1/2018–12/31/2020
Human Organ Tissue Identification by Targeted RNA Deep Sequencing to Aid in the Investigation of Shooting and Other Traumatic Bodily Injury Incidents	1/1/2017–12/31/2019
Developmental Validation of a miRNA Panel for Forensic Body Fluid Identification	1/1/2017–12/31/2019
Forensic Body Fluid Identification using Microbiome Signature Attribution	1/1/2017–12/31/2019

(continued)

Table 2. NIJ-Funded Fluid ID Projects (continued)

Title	Date Range (Start Date–End Date)
Body Fluid Analysis by Surface Enhanced Raman Spectroscopy for Forensic Applications	1/1/2015–3/31/2017
How It Got There: Associating Individual DNA Profiles with Specific Body Fluids in Mixtures using Targeted Digital Gene Expression and RNA-SNP Identification	1/1/2015–12/31/2018
Raman Spectroscopy for Analyzing Body Fluid Traces: Moving Towards a Practical Forensic Application	1/1/2015–12/31/2017
Use of Novel Chemistry & Microwave Instrumentation to Improve Body Fluid Assay Sensitivity & Speed while Reducing Costs	10/1/2012–3/31/2017
Developmental Validation of a High-Specificity Multiplex Assay for Human Body Fluid Identification	10/1/2012–5/31/2016
Proximity Ligation Real Time PCR for the Detection of Spermatozoa	1/1/2013–10/31/2015
Body Fluid Identification using Epigenetic Methylation Markers and Pyrosequencing	1/1/2013–6/30/2017
Deep Sequencing for Identification and Characterization of MicroRNAs in Forensically Important Biological Fluids	1/1/2013–5/31/2016
Automated Multianalyte Screening Tool for Classification of Forensic Samples	1/1/2012–3/31/2014
Raman Spectroscopy for Analyzing Body Fluid Traces, Stain Aging, Differentiation Between Races, Genders and Species	1/1/2012–12/31/2015
Development of a Proteomic Assay for Menstrual Blood, Virginal Fluid and Species Identification	10/1/2010–6/30/2016
Identification of Forensically Relevant Fluids and Tissues by Small RNA Profiling	11/1/2009–12/31/2013
Application of Raman Spectroscopy for an Easy-to-Use, on-Field, Rapid, Nondestructive, Confirmatory Identification of Body Fluids	11/1/2009–2/29/2012
Validation of Highly-Specific Protein Markers for the Identification of Biological Stains	10/1/2009–2/28/2013
Detection and Quantitation of Spermatozoa	11/1/2008–10/31/2011
Development of a DNA-Based Real-Time PCR Assay for Identification of Semen, Blood and Saliva	11/1/2008–2/28/2013
Establishment of a Fast and Accurate Proteomic Method for Body Fluid/Cell Type	9/1/2008–4/30/2011
De-convolution of Body Fluid Mixtures: Cell Type Identification and Single Source Genetic Profiling of Micro-Dissected Cells	10/1/2008–2/28/2014
Forensic Stain Identification by RT-PCR Analysis	1/1/2008–4/30/2011
Isolation of Highly-Specific Protein Markers for the Identification of Biological Stains: Adapting Comparative Proteomics to Forensics	10/1/2006–7/31/2010



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