Study 9-2 SOP (Phase III) for NCI CPTAC Consortium-Wide Multiple Reaction Monitoring (MRM) Experiment

9-Point Calibration Curve of 27 ^{15}N labeled target proteins monitoring 125 target peptides in 3 different isotopically labeled forms

Experimental Design and Statistics Verification Studies Working Group

Overview

In Study 9.2, there will be 27 undigested ANL target proteins and 7 undigested "Study 7" proteins that will be centrally spiked into depleted human K_2EDTA plasma. Preparation of a standard curve will be performed at Vanderbilt prior to distribution of the sample kits. A detailed SOP for digestion, subsequent sample handling, and instrumental analysis will be included for each lab (also see Appendix A for chemical reagents needed). For Study 9-2, the 9-point calibration curve will span from 10 amol/µL to 100 fmol/µL in a background of MARS-14 depleted human K_2EDTA plasma (500 ng/µL). The assay will monitor for three isotopic forms of 115 peptides from 27 cancer-relevant proteins (light, $^{13}C/^{15}N$, and uniformly labeled ^{15}N, the latter from here on referred to as U-^{15}N), as well as two isotopic forms (light and $^{13}C/^{15}N$) of 10 control peptides by time-scheduled LC-MRM-MS, for a total of 1095 transitions. Each participating CPTAC site must strictly follow the specifications of the SOP as outlined. Results from Study 9.2 will mimic a "real world" verification study in which each site is responsible for sample preparation. Study 9.2 also rigorously tests the transferability and reproducibility of MRM-MS-based assays for target proteins in plasma across multiple institutions and across 4 major MS instrument vendors and multiple platforms.

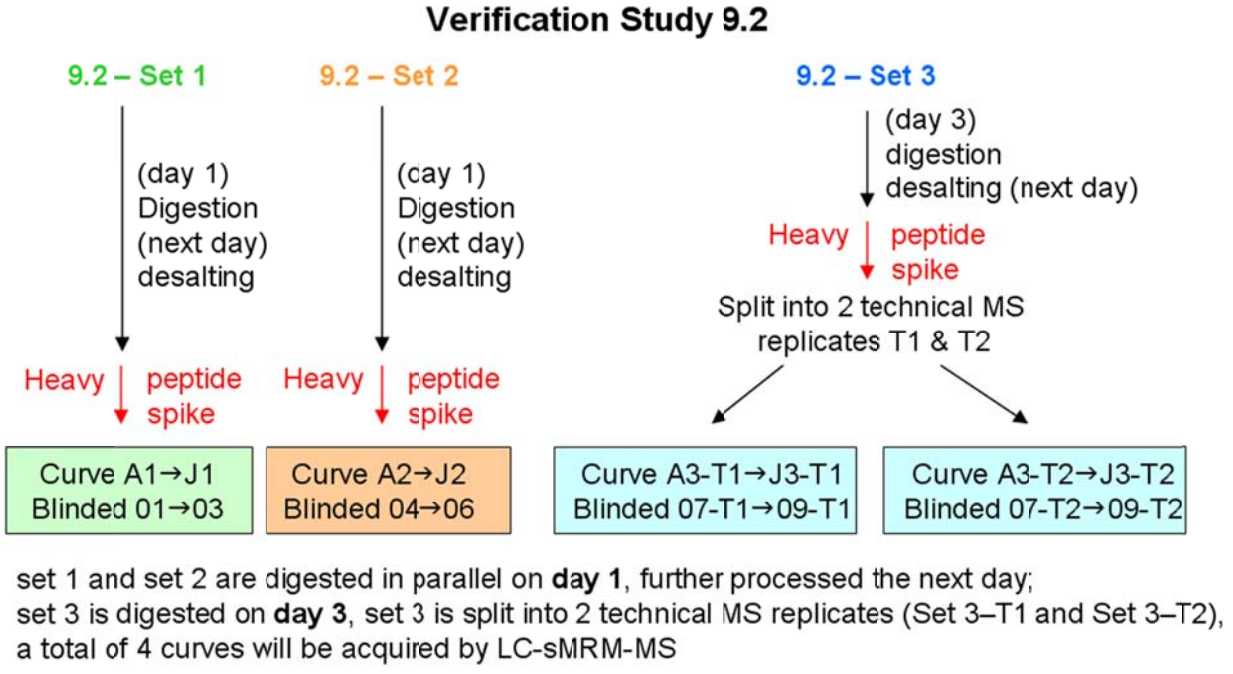
Overall, 27 U-^{15}N -labeled proteins will be spiked into depleted plasma at a constant spike level, which after digestion and dilution (that will be performed at each CPTAC site) will give rise to 115 target peptides at a concentration of 25 fmol equivalent on column. Twenty-seven light proteins will be spiked in at varying levels with final concentrations after digestion/dilution ranging from 10 amol to 100 fmol on column. Seven light "Study 7" proteins will be spiked in at constant levels providing a digestion reproducibility control, and after digestion and dilution yield final concentrations of 2.5 fmol equivalent on column. One hundred twenty-five synthetic $^{13}C/^{15}N$ signature peptides will be spiked in by each operator after digestion and desalting at concentrations that will yield final concentrations of 10 fmol on column.
As an additional phase of the study, three blinded samples will be included with each curve replicate, in which the spiked concentration of the light target proteins is unknown to the operators. As with the curve samples, each site will be responsible for the sample preparation of the blinded samples.

Unlike previous studies, we will ship 3 sets of samples (Set1, Set2 and Set3) with undigested proteins spiked into undigested depleted plasma (each set consists of a curve and blinded samples).
Even though only 3 sets of samples will be shipped, there will be 4 singlicate curves to be acquired. Set 3 will be split in half after processing as described in detail below and it will yield technical replicates Set3T1 and Set3T2 (also see Scheme 1). This will reduce digestion efforts for operators. The digestions (similar to study 7.3) will be performed at each participating

CPTAC site. Digestion replicates (sets) 1 and 2 will be digested in parallel at the same time on "day 1", and then analyzed on the triple quadrupole instruments, as singlicate curves and blinded samples 1 and 2 (digestion replicates one and two). The sample set 3 should be digested shortly after sample sets 1 and 2, preferably on "day 3" (for logistical reasons it can also be later than "day 3", it just should be done on a separate day). For details on how to split Set 3 after processing: Set 3 will be split in half <u>after</u> completion of the following steps i) digestion, ii) desalting, and iii) heavy $^{13}C^{15}N$ peptide spike, and will be acquired as technical replicates Set3T1 and Set3T2. Set3T1 and Set3T2 will be frozen separately and acquired later independently as curves 3 and 4, respectively.

The schematic illustrating the workflow for each 9.2 sample set is shown below in Scheme 1. Please note: Sets 1 and 2 should be digested on the same day, while Set 3 should be digested on a later day.

<u>Scheme 1:</u>



For all sample sets, after digestion, desalting, and heavy peptide spike operators will be asked to aliquot the solutions (for details see below).

It would be ideal if, at each site the same person could do all digestions for an individual instrument platform in order to keep variability to a minimum (otherwise, please keep track of personnel doing the different digestion steps).

Aims: The study described in this SOP is designed to accomplish the following:

1. To generate a 9-point standard curve for 27 cancer-relevant protein targets spanning 10 amol/µL to 100 fmol/µL in a depleted plasma background (500 ng/µL) that will be analyzed by LC-MRM-MS on four different triple quadrupole platforms at CPTAC sites.

2. To enable calculations of LOD, LOQ, accuracy and precision from the calibration curve for all 115 peptides that will be monitored after protein digestion performed at each site.

3. To monitor digestion reproducibility and efficiency across all samples by addition of seven "Study 7" proteins at constant levels in all samples.

4. To introduce a set of 3 blinded samples that mimic real biomarker samples into the sample handling workflow and assess the quantitative reproducibility across all sites.

5. To evaluate the level of endogenous and interfering signals for all peptides by replicate measurements of blank plasma samples. As in this study 115 different peptides are monitored the aspect of endogenous and interfering signals can be assessed more comprehensively than comparison to Study 7.

6. To observed the improvement in protein-level quantification through use of U-15N proteins as internal standards.

The number of transitions for 3 different label types that will be targeted in this method (1095) is a critical challenge and will require the use of retention time scheduled LC-MRM-MS. To achieve success, the following protocol is designed to assess the system suitability and retention time stability of the LC-MRM-MS instrument, as well as design a single MRM-MS method empirically. The protocol outlined herein should be followed as strictly as possible and all deviations from this protocol must be outlined in detail on the last sheet. The experimental design is outlined below.

Experimental Outline

1. Digest samples according to the digestion protocol.
2. Run System Suitability Sample 5 times to condition column and assess LC-MRM-MS performance. Only proceed to the following steps if the system suitability samples pass defined criteria.
3. Using a Skyline template for heavy target peptides (download from NIST FTP server), create 6 MRM-MS transition lists for the heavy target peptides only (maximum of 63 transitions in each unscheduled method)
4. Analyze the heavy-only peptide sample using the 6 different MRM-MS methods
   i. Import the 6 raw files into Skyline
   ii. Obtain retention times for each heavy target peptide
   iii. Export a scheduled MRM-MS transition list (or MRM-MS method) with a 4 min RT window

iv. Run the scheduled LC-MRM-MS method twice using one processed sample vial 9.2-A that will be digested at each CPTAC site.
v. Import the 2 raw files into Skyline
vi. Download a second Skyline template from the NIST server containing **heavy ($^{13}C/^{15}N$), light (unlabeled) and U-^{15}N -labeled peptides** (download the new template rather than adding in new label types into the existing heavy only template!).
vii. Export a new scheduled LC-MRM-MS method with all 1095 transitions and a 2 min RT window. For instruments that allow for flexible RT window scheduling (i.e. Waters Xevo QQQ, Agilent 6400 series and Thermo Vantage instruments), adjustments can be made deviating from the 2 min window for selected problematic peptides. Please make sure the number of concurrent transitions does not exceed 100.
viii. Run sample 9.2-A with the 2 min RT window method prior to running the cal curve.

5. Execute calibration curve and blinded samples according to specified run order.
6. Import data into Skyline and integrate.
   i. Import System Suitability Sample files into System Suitability Skyline file and integrate. Ensure peak area CVs and retention times are within specified error limits.
   ii. Import Calibration Curve files into Study 9-2 Curve Skyline file and integrate.
   iii. Import Blinded Sample files into Study 9-2 Blinded Sample Skyline file and integrate.
7. Export reports from each Skyline file <u>(note: a new, different Study 9.2 Skyline report template will be provided)</u> and process data using QuaSAR to monitor for data quality. Make any adjustments necessary to data integration and re-run samples as necessary.
8. Upload the 3 generated Skyline reports to the NIST FTP server along with the 3 different Skyline files and all raw MRM-MS data files (see separate document).

Materials and Reagents Provided in Kit

A. Study 9-2-SSS (System Suitability Sample)
- a. Digest of 6 equimolar proteins (Michrom Bioresources, #PTD/00001/63)
  - i. Four 10 µL aliquots, 1 pmol/µL, supplied in 30% acetonitrile/0.1 % formic acid in water

B. Heavy-Only IS Peptide Mixture for Scheduling only (in 25 fmol/uL 6-protein mix)
- a. 125 heavy IS peptides at **800 fmol/µL each**
  - i. Two 10 µL aliquots, supplied in 30% acetonitrile/0.1% formic acid

C. QC Sample (Sample 9-2-QC)
- a. Equimolar mixture of the 125 unlabeled and 125 labeled synthetic peptides in 25 fmol/µL 6-protein mix matrix
  - i. Four 25 µL aliquots supplied at 10 fmol/µL of each peptide in 0.1% formic acid in water. Background of 25 fmol/µL of 6-protein mix added to minimize adsorption/loss of hydrophobic peptides.

D. Sample 9.2-A that will be digested at each CPTAC site (on-site digestion), for scheduling and as "blank in between blinded samples" use
- a. Each 9.2-A vial contains depleted plasma at ~4.5 µg/µL spiked with 27 U-^{15}N-labeled proteins and 7 light digestion control proteins. Depleted digested plasma will be diluted to a final concentration after digestion of approximately 0.5 µg/µL
  - i. **Four 9.2-A vials, 35 µL each; vials A should be digested along with the other study samples. Referring to Scheme 1, two A vials can be digested on day 1 (along with sample sets 1 and 2), while the other two A vials can be digested on day 3 (along with sample set 3).**
  - ii. After desalting the sample will be spiked with 125 heavy ^{13}C/^{15}N IS peptides (stock at 500 fmol/µl, see Reagent F).
  - **iii.** After dilution, the sample will ultimately contain 125 ^{13}C/^{15}N-labeled peptides at 10 fmol/µL, 115 U-^{15}N-labeled peptides at 25 fmol/ul equivalent in 500 ng/µL digested depleted plasma (no light ANL proteins were spiked).

E. Samples 9-2-B to 9-2-J: Non-digested human depleted plasma spiked with 27 light and 27 U-^{15}N-labeled ANL proteins, and 10 light digestion control proteins
- a. Depleted plasma, 500 ng/µL
  - i. Three sets of 35 µL aliquots of each spike level are supplied (of which 25 µL from each vial will be digested on site). Set 3 will be split into 2 technical replicates T1 and T2 after digestion/desalting to yield a final number of 4 singlicate curves (see Scheme 1 above).
  - ii. 27 U-^{15}N-labeled proteins (yielding 115 target peptides after digestion) spiked at a concentration of 25 fmol/µL on column.
  - iii. 27 unlabeled proteins (yielding 115 target peptides after digestion) were spiked in at the following concentrations (note, these concentrations are different from the 9.1 concentration points). *Concentrations in the table below refer to shipped light target protein concentrations, and then after digestion resulting final on-column peptide equivalent concentrations, assuming theoretical 100% recovery).*

Sample (Study 9-2)	Spiked light target Protein Concentration (fmol/µL)	On-column Peptide Equivalent Concentration (fmol/µL)
9-2-J	900	100
9-2-I	213	24
9-2-H	51	5.6
9-2-G	12	1.3
9-2-F	7.4	0.82
9-2-E	2.8	0.32
9-2-D	0.68	0.075
9-2-C	0.16	0.018
9-2-B	0.09	0.010

Note: the above protein/depleted plasma concentration points also contain 7 undigested light "Study 7" proteins spiked at constant concentration yielding a final of 2.5 fmol equivalent on column, assuming 100% recovery.
After digestion/desalting and spiking of the heavy synthetic $^{13}C^{15}N$-labeled IS peptides (see reagent F), each of the resulting 4 different singlicate calibration curves can be acquired: Specific naming including the first blank (A sample) is required, for details also see run order below:
9-2-A1 to 9-2-J1; 9-2-A2 to 9-2-J2;
9-2-A3-T1 to 9-2-J3-T1; 9-2-A3-T2 to 9-2-J3-T2.

F. Heavy IS Peptide for Spike after Digestion, 500 fmol/µL

- **a.** One aliquot of 200 µl aliquot of 125 heavy synthetic $^{13}C^{15}N$-labeled IS peptides
- **b.** Supplied at **500 fmol/µL** in 30% acetonitrile/0.1% formic acid
- **c.** 4.5 µl need to be added (at this undiluted concentration 500 fmol/ul) by the operator to all samples after digestion and desalting to yield final concentrations of 10 fmol on column.

G. Blinded Samples

- **a.** **Nine blinded samples, 35 µL in depleted plasma,** (Total: Samples 9-2-01Blinded to 9-2-09Blinded)
- b. To be digested and to be run after each singlicate calibration curve
- **c.** Three sets of three Blinded samples (each provided in 35 µl aliquot, of which 25 µl are digested), with Set 3 being split into two technical replicates T1 and T2 after digestion and desalting (refer to Scheme 1):
  - **i.** first singlicate: 9-2-01Blinded to 9-2-03Blinded
  - **ii.** second singlicate: 9-2-04Blinded to 9-2-06Blinded
  - **iii.** third singlicate: 9-2-07-T1Blinded to 9-2-09-T1Blinded
  - **iv.** fourth singlicate: 9-2-07-T2Blinded to 9-2-09-T2Blinded
- **d.** For each singlicate: the three blinded samples will be run twice as runs a) and b), see runorder.

H. Wash Samples

- **a.** To be prepared from the 6-protein mix / Michrom sample at stock concentration of 1 pmol/µL (Reagent A, Study 9-2 SSS vial, see above).

b. **Wash Samples** will be 10 fmol/µL of the 6-protein mix (Michrom sample). Samples will be diluted from provided stock concentration of 1 pmol/µL).
c. Will be used to avoid carryover between blinded samples, the run order defines running a "wash" gradient in between blinded samples. "

I. Chemical Reagents for Sample Preparation, Digestion and Buffer Solutions for digestion are detailed in Appendix A. Several but not all of these reagents are included in the sample kit, please review which additional reagents your laboratory may need to obtain (Appendix A)

J. HPLC solvents
a. Acetonitrile, HPLC grade
b. Water, HPLC grade
c. Formic Acid

K. HPLC columns
a. General specifications: C18 packing material in column dimensions of 75 µm ID x 12 cm with 10 µm ID tip
b. AB SCIEX and Thermo operators: Prepacked New Objective Reprosil Columns may be requested from New Objective. Limited to a total of 5 per site, including those used in Study 9.1. If more than 5 columns are required, the cost for additional columns will be the responsibility of the site.
c. Waters UPLC operators: a Waters specific column will be used (1.7µm BEH130 C18, 75 µm X 150 mm column)
d. Agilent 6400 series operators with ChipCube sources, please use Chips packed with Zorbax C18 (5 um and 300 angstrom beads) with 160 nL enrichment column and 75 µm ID x 15 cm analytical column.

L. Autosampler loop for HPLC systems: 1 µL PEEKsil loop provided in sample kit, 100 µm ID PEEKsil; newer design 1 µL loops are provided for Eksigent Ultra systems.

M. Fused silica
a. 25 µm ID between gradient pump and autosampler (for direct inject configuration)
b. 25 µm ID between autosampler and PicoFrit column (keep tubing as short as possible)

N. High voltage contact
a. For liquid-liquid junction, Upchurch part P-888.

O. Autosampler vials
a. Polypropylene autosampler vials with conical insert, 250 µL maximum volume

P. Column heater
a. If the system is equipped with a column heater, please set to 35 degrees Celsius.

Q. **Oasis HLB desalt cartridge** (Waters)
a. Off-line desalting will use Waters Oasis HLB 1 cc, 30 mg cartridges (Product # WAT094225, box of 100).
b. A vacuum manifold (Product # WAT200677) and vacuum source will be required for the cartridges. Each participating lab is responsible for ordering the necessary equipment. *For operators who are experienced with Oasis elution plates those can be used as well. The product number for the Waters Oasis HLB uElution Plate (30 um) is 186001828BA (Qty/Box=1) with corresponding extraction manifold with product number 186001831. (however as these plates are not as trivial to use we rather recommend to use the individual cartridges, see above).*

Required Software

A. **Skyline Daily Software**, most recent version (please note version used)
B. **Platform-specific Skyline document** (*.sky), provided by CPTAC VWG on NIST ftp site (**ftp://chemdata.nist.gov/verification_main_study9.2/Skyline_templates/**)
C. **QuaSAR Quantatitive Statistical Analysis for Reaction Monitoring Experiments** (available at GenePattern test site: http://genepatterntest.broadinstitute.org/gp/pages/login.jsf)

Overview

In Study 9-2 (9-point calibration curve), 27 U-^{15}N-labeled proteins will be spiked into depleted human K_2EDTA plasma at a constant spike level, which after digestion, desalting, and dilution (performed at each CPTAC site) will result in 115 U-^{15}N-labeled target peptide equivalents at a concentration of 25 fmol on column (assuming 100% digestion efficiency and recovery). The U-^{15}N-labeled proteins will serve as internal standards to provide more accurate protein quantification. Twenty-seven light proteins will be spiked in at varying levels with final concentrations after digestion/dilution ranging from 10 amol to 100 fmol on-column. Seven light "Study 7" proteins will be spiked in at constant levels providing a digestion reproducibility control, and after digestion and dilution will yield final concentrations of 2.5 fmol on column (assuming 100% recovery). One hundred twenty-five synthetic ^{13}C/^{15}N-labeled signature peptides will be spiked in by each operator after digestion and desalting to yield final concentrations of 10 fmol on column. Their use is to assess digestion recovery.
Digestion of each prepared concentration point and blinded samples will be performed at individual CPTAC sites. Results from Study 9-2 from all CPTAC sites will provide the following metrics:

- LOD/LOQ values for the 115 peptide targets in depleted plasma based on ^{13}C/^{15}N synthetic internal standard peptides
- Overall recovery of each peptide from the sample handling process, based on ^{13}C/^{15}N synthetic internal standard peptides
- Improvements in quantitation precision by the use of U-^{15}N-labeled proteins as internal standards
- Digestion reproducibility across assays (intralaboratory) and across sites (interlaboratory), based on the 7 proteins used as digestion standards
- Overall reproducibility both intralaboratory and interlaboratory for the quantification of blinded samples with unknown concentrations

The following paragraphs provide a description of the experimental procedure, with each major step separated into its own paragraph and heading. Paragraphs are followed by outlined "checklists" that have matching headings for reference.

1. Sample Preparation: Denaturation, Reduction, Alkylation, Digestion, Desalt

All protein samples (calibration curve points A-J, and blinded samples) are provided in a

matrix of undigested depleted plasma. All samples will have to be denatured with urea, reduced with DTT, alkylated with iodoacetamide, and diluted prior to addition of proteolytic enzymes. Stock solutions of Lys-C and Trypsin will be provided to allow a double digestion protocol. It is recommended to begin the denaturation process early in the day (at least by noon) so that the reduction, alkylation, and addition of Lys-C with 2 hour incubation can be completed before the end of the day. Final addition of Trypsin should occur approximately 16 hours before you are able to quench the reaction. Each sample should be quenched with formic acid so that the final concentration in solution is 1%, then subjected to desalting on an Oasis HLB cartridge (Waters). The $^{13}C/^{15}N$ internal standard peptide mix will be added after desalting and lyophylization, immediately prior to analysis by LC-MRM-MS. After addition of the IS peptides, sample aliquotting should be performed as described below. Curve replicates 1 and 2 (along with associated blinded samples) should be digested at the same time, while curve replicate 3 should be reserved for preparation on a separate day (to assess day-to-day variability in sample handling). Replicate 3 should also be split in half after reconstitution with the $^{13}C/^{15}N$ internal peptide standards and will be analyzed twice to assess technical variability in the assay (generating curves 3 and 4).

2. System Suitability Sample, Column Conditioning and Instrument Performance

System Suitability Sample (SSS) runs using the Michrom 6-protein mix (based on Study 9S) will be periodically interspersed into Study 9-2 to guarantee system suitability and performance and, in particular, to monitor peak area stability and RT drift. The SSS will first be analyzed in an unscheduled LC-MRM-MS method (Study 9S) to condition the column and assess the performance of the LC-MS instrument platform prior to initiation of the quantitative assay. Upon obtaining the specified results for retention time and peak area (**RT drift should be <1min; peak area CV should be <20%, certainly <30%**), sites may proceed to the generation of the scheduled LC-MRM-MS runs for Study 9-2.

The SSS will also be run every 6-8 runs in the sample queue, in order to track instrument performance. Data will be analyzed in Skyline and will be submitted in a separate "Michrom Study 9S" file.

3. Generation of Scheduled LC-MRM-MS Method for Study 9 Peptides

In preparation for the scheduled calibration curve LC-MRM-MS runs, in which 1095 transitions will be monitored in one run, participating sites will monitor all heavy synthetic labeled peptides with 375 transitions (125 peptides with 3 transitions each) in six unscheduled LC-MRM-MS runs (~63 transitions per run), with each run represented by a separate LC-MRM-MS method. Retention times for scheduling will be determined empirically using these six runs and will be verified by analysis with a single scheduled LC-MRM-MS method (acquired twice) prior to analyzing the samples from which the calibration curve will be generated. All method building and data analysis will be performed using Skyline (please make sure to have downloaded the most recent Skyline daily version as of your data acquisition date).

4. Data Analysis

Operators will import all acquired data files into Skyline to check and, if necessary, adjust peak integration parameters. Check "Integrate All" (Menu, Settings, Integrate all), so that light, heavy

and U-15N transitions will be integrated together. While checking peak integration open RT replicate views (Menu, View, Retention Time, Replicate Comparison), and Peak Area replicate views (Menu, View, Peak Areas, Replicate Comparison) to visually help confirm proper peak integration. In Study 9-2, there will be three forms of each peptide monitored, and all should co-elute. The $^{13}C/^{15}N$ and U-^{15}N-labeled forms of the peptides should be present at consistent levels across all samples. Check for RT drift problems during scheduling, and ensure that the correct peak has been integrated (helpful hint: look for co-elution of the three forms of each peptide, and specifically, the $^{13}C/^{15}N$ and U-^{15}N-labeled forms should always be coeluting). It is recommended to not "fine tune" the automatic Skyline peak integration, unless it is obvious that the wrong peak has been integrated (more likely in the lower concentration samples). Use Skyline Custom Annotation features to annotate any observations or notes for Study Statisticians to consider (Menu, View, Results Grid; and annotate observations on the precursor or transition level). Finally use "QuaSAR" to analyze data before submitting data to statisticians (see separate SOP). The experimentally determined molar concentration of the spiked peptide or protein will be calculated and compared to its theoretical value for accuracy. QuaSAR will generate linear plots of response versus known concentration from each of the 9-point standard curves and will be used to evaluate the linearity of the MRM measurements across the range of spiked peptide concentrations, thus providing evidence of a quantitative measurement process. Samples with high %CV (>20%) and/or interferences will be flagged on the plots. Replicate analyses of the spiked plasma samples will provide estimates of assay precision (standard deviation and % CV), and LOQ and LOD will be determined. Blank runs of digested plasma with labeled peptides and ^{15}N ANL proteins (referred to as sample 9.2-A) will provide estimates of chemical background levels in the absence of unlabeled signature peptide peaks as well as the presence of endogenous peptides in the sample (*i.e.*, CRP). Furthermore, an estimate of carryover will be determined by running a series of gradient HPLC washout runs. Finally, variation across CPTAC sites will be assessed for each of these characteristic analytical metrics.

5. Troubleshooting

This study contains a large number of samples that need to be run in a defined order and requires very reproducible peptide RTs. Problems may arise that will affect RT stability, including, but not limited to, increased column pressure, tip blockage, significant change in ambient temperature or injection of air into the system. The troubleshooting section provides some suggested routes of diagnosis and an outline of how to resolve problems and continue with data acquisition. Importantly, operators are instructed to use the SSS and Skyline to observe any problems with retention time shifting, asymmetric peaks, and low signal. These problems can be caught and rectified without much down time and with minimal re-running of sample.

Procedures

1. Step by Step Sample Preparation/Digestion Procedures:

Note: at each of the steps perform appropriate vortex and spin-down steps (particularly to assemble the sample at the bottom of the tube after incubations). Under appendix A point II, page 37, you can find further details on procedures for Digestion Reagent Preparation (please review before starting, specifically how to prepare digestion reagent B, 9M urea in 100 mM Tris).

A. Digestion Protocol for Plasma Samples.

A. Each undigested protein/depleted plasma sample vial will contain a volume of 35 µL. Let the sample thaw at room temperature. Centrifuge briefly in a benchtop centrifuge and remove 25 µL out of each vial (that originally contained 35 µL) and transfer to a new tube.
B. To those 25 µL of the undigested protein/depleted plasma mixture, add 50 µL of 9M Urea in 100 mM Tris pH 8 (digestion reagent B, Appendix A). The final concentration of Urea will be 6M. [Approximate protein concentration prior to dilution is ~4.5 µg/µL].
C. Add 8.3 µL of 200 mM DTT [final concentration of 20 mM]; incubate for 30 min at 37°C. (refer to digestion reagent D, Appendix A)
D. Add 9.3 µL of 400 mM M IAM [final concentration of 40 mM]; alkylate at room temperature for 30 min in the dark. (refer to digestion reagent E, Appendix A)
E. Add 135 µL of 100 mM Tris, pH 8 (refer to digestion reagent C, Appendix A) to to reduce the urea concentration to 2M.
F. Add 6.4 µL of 0.4 µg/µL LysC solution. (about 135 ug total protein to digest)
G. Check the pH of the digestion solution with pH strips and if needed adjust it to 8.0-8.5 with 1M Tris solution, pH 8 (refer to digestion reagent A, appendix A). If you do need to adjust, please carefully add 1-2 µL 1M Tris, pH 8 at a time (digestion reagent A) so the solution does not get too basic).
H. Incubate at 30°C for 2 hours with shaking at 850 rpm.
I. After 2 hours add 600 µL of 100 mM Tris pH 8 to each tube to reduce the urea concentration to < 1 M.
J. Dissolve one trypsin vial (100 ug per vial) of Promega Trypsin Gold in 1000 uL of 100 mM Tris, pH 8.0 (digestion reagent C, Appendix A). Keep trypsin solution on ice and use quickly after preparation to avoid autolysis. Add 25.5 µL of this freshly prepared 0.1 µg/µL trypsin solution to each digest sample with gentle mixing. Use one fresh tryspin vial for digestion of sample sets 1 and 2 on day 1 and a second fresh trypsin vial for digestion set 3 (on a separate day, i.e. day 3).
K. Check the pH of digest solution with pH strips and if needed adjust it to 8.0 with 1M Tris solution, pH 8 (refer to digestion reagent A, appendix A). If you do need to adjust, please carefully add 1-2 µL 1M Tris at a time (digestion reagent A) so the solution does not get too basic).
L. Incubate overnight (16 h) at 37 °C with shaking at 850 rpm.
M. Add 9 µL concentrated formic acid to each digest to quench enzyme activity for a final acid concentration of 1%.

NOTE the difference between Reagent B (Heavy-Only IS Peptide Mixture for Scheduling only with 125 heavy IS peptides at 800 fmol/µL) each, and Reagent F (Heavy IS Peptide for Spike after Digestion: 125 heavy synthetic $^{13}C^{15}N$-labeled IS peptides, supplied at 500 fmol/µL). While Reagent B is initially used for RT scheduling (after dilution to 80 fmol/µl), Reagent F will be used at the given concentration (500 fmol/µL) to spike in 4.5 µl for each sample after digestion, desalting, reconstitution.

B. Offline Desalting of Digest Solutions via Oasis HLB SPE Cartridges

i. Each digest requires off-line desalting using Waters Oasis HLB 1 cc, 30 mg cartridges (Product # WAT094225, box of 100). A vacuum manifold (Product # WAT200677) and vacuum source will be required for the cartridges. Each participating lab is responsible for ordering the necessary equipment. Part numbers were provided previously to help each lab procure these items.

ii. Condition cartridge with 3 x 400 µL of 0.1 % formic acid in 80 % ACN.

iii. Equilibrate cartridge with 4 x 400 µL of 0.1 % formic acid in 100 % water.

iv. Reduce flow rate by lowering vacuum. A slower flow rate during sample loading, washing and eluting will minimize sample loss and maximize salt removal.

v. Add sample to cartridge.

vi. Wash cartridge with 4 x 400 µL of 0.1 % formic acid in 100 % water.

vii. Elute plasma digest peptides with 3 x 400 µL 0.1 % formic acid in 80 % acetonitrile into 1.7 mL Eppendorf tubes.

viii. Freeze eluates on dry ice or at -80 °C for approximately 1 hour. Lyophilize to dryness. Samples can be stored lyophilized at -80 °C until ready for MRM-MS analysis.

C. Sample Reconstitution and $^{13}C/^{15}N$ IS Peptide Spikes, Reagent F (500 fmol/µL), to be performed just prior to executing LC-MRM/MS

i. Reconstitute dried and desalted plasma digests with 25 µL of 5% formic acid, 3% acetonitrile, 92% water and vortex. To make this solution prepare 1 ml aliquots as needed (v/v/v: start with 920 ul water, then carefully add 50 ul formic acid, and finally add 30 ul acetonitrile).

ii. Add 4.5 µL of 500 fmol/µL $^{13}C/^{15}N$ IS peptide mixture (see Reagent F above) to each of the digestion solutions.

iii. Add 195.5 µL of water to achieve final $^{13}C/^{15}N$ peptide concentration of 10 fmol/µL and the plasma digest concentration is 0.5 µg/µL.

iv. This will yield a total volume of 225 µL per each sample after digestion, desalting, reconstitution, $^{13}C/^{15}N$ IS peptide spike, and final dilution.

v. **Aliquot** the final dilution volume into **50 µL aliquots** and freeze aliquots that are not immediately subjected to LC-MRM/MS in the -80 °C freezer.

vi. **Aliquoting of Reagent F:** After Reagent F (with a total volume of 200 µl) is thawed for the first time in order to add reagent F to each of the samples of **Sets 1 and 2**, please generate aliquots of 50 µl volume each to be used for sample **Set 3** to avoid further freeze thaw cycles.

2. <u>System Suitability Sample – Column Conditioning</u>

A. Sample preparation

i. Dilute SSS from 1 pmol/µL to 50 fmol/µL:

ii. Add 95 µL of 0.1% formic acid/3% acetonitrile to an Eppendorf tube

iii. Add 5 µL of MichromMix (SSS) to the vial and vortex

iv. Centrifuge sample for 1 min in a benchtop centrifuge

v. Transfer to an autosampler vial and place in autosampler

B. Method Preparation
   i. Prepare SSS transition list from the appropriate Skyline file
      1. 115 transitions, 22 peptides
      2. Q1, Q3 resolution = unit
      3. Dwell time = 10 msec
      4. Interscan delay (where applicable) = 5 msec (3 msec for QTRAP 5500)
   ii. LC Gradient: use the **Study 9S** LC gradient (also see Table 2b)
      1. 0-5 min, 3% B; 5-8 min, 3-15% B; 8-42 min 15-35% B; 42-45 min, 35-90% B, 45-49 min, 90% B hold; 49-50 min, 90-3% B; 50-80 min, 3% B. *Note: operators with Waters Aquity UPLC will use a slightly adjusted gradient (due to the use of Mobile phase B: 100% acetonitrile (v/v)/0.1% formic acid in water (v/v) only for Waters UPLC, see Table 3b).*
      2. Flow rate: 300 nL/min
      3. Mobile phase A: 0.1% formic acid in water (v/v); Mobile phase B: 90% acetonitrile (v/v)/0.1% formic acid in water (v/v). *Note: operators with Waters Aquity UPLC will use Mobile phase B: 100% acetonitrile (v/v)/0.1% formic acid in water (v/v) only for Waters UPLC).*
   iii. Autosampler Settings
      1. Direct Injection, full-loop injection, 1 µL sample loop
      2. Pick up 2 µL of sample sandwiched between mobile phase A (0.1% formic acid)
      3. See Table 1 for example
   ii. Column heater Settings
      1. If using a column heater, please set to 35 degrees Celsius.
   iii. Recommended MS Source Conditions
      1. AB SCIEX (4000 QTRAP and QTRAP 5500)
         a. ESI voltage: 2200 ± 200 V ; Curtain gas: 20 ± 5; GS1: 5 ± 5; IHT: 150 ˚C
      2. ThermoFisher Scientific (Vantage)
         a. ESI voltage: 1300-1400 V; Declustering voltage: -1 V; Capillary Temperature: 210 ˚C
      3. Waters Xevo
         a. Capillary voltage: 2800 V; Cone voltage: 35V; Ion source temperature: 150 ˚C; Cone gas flow: 15 L/Hr; Nanoflow gas flow: 0.20 bar
      4. Agilent 6410/6460/6490 Chip Cube
         a. ESI voltage: 1700-1800 V; gas flow: 2.5 L/min (11 L/min for 6490); temperature: 325˚C (150˚C for 6490); Fragmentor voltage: 125 V (380 for 6490); Cell Accelerator Voltage: 4 V for 6490
   iv. Inject sample 5 times
C. Data Analysis
   i. Import data files into Skyline System Suitability Sample file
   ii. Check automatic integration of all peaks
   iii. Manually adjust integration of peaks, if necessary
   iv. Make sure integration start and stop is identical for all transitions of a precursor (go to “Settings”, and check “Integrate All” to enable this feature automatically)
   v. Document any peak that looks problematic by utilizing the “note” feature in Skyline
      1. Excessive tailing or fronting

2. Drop-out of electrospray
3. Poor peak shape
4. Missing transitions

ii. Check that peaks pass criteria
1. RT shift is isolated to first 3 injections
2. Peak area CV is less than 30% for all peaks
3. All peaks are detected in the last 2 sample runs

iii. If data do not pass criteria, troubleshoot the LC system and re-run SSS column conditioning procedure

iv. If necessary, contact CPTAC VWG members for advice or assistance
1. Susan Abbatiello, susana@broadinstitute.org; 617-714-7653
2. Birgit Schilling, bschilling@buckinstitute.org; 415-209-2079

3. Retention Time Scheduling for Heavy Peptides

A. Sample preparation

i. Dilute Heavy-Only IS peptide mixture from 800 fmol/µL to 80 fmol/µL
1. Add 45 µL 3% acetonitrile/0.1% formic acid to an Eppendorf tube
2. Add 5 µL of the 800 fmol/mL Heavy-Only IS peptide stock to the vial, vortex
3. Transfer to an autosampler vial and place in autosampler

B. Method Preparation

i. Prepare the Heavy-Only IS peptide transition list from the appropriate Skyline file
1. 375 transitions, 125 peptides, 125 precursors
2. Q1, Q3 resolution = unit
3. Dwell time = 10 msec (can be lower for QTRAP 5500 instrument, minimum is 5 msec)
4. Interscan delay (where applicable) = 5 msec (3 msec for QTRAP 5500)
5. Maximum number of transitions per individual unscheduled method: 63
6. Export transition list as multiple methods, ignore proteins

ii. Use the **Study 9-2** LC gradient (also see Table 2a)
1. 0-5 min, 3% B; 5-8 min, 3-7% B; 8-35 min 7-25% B; 35-42 min, 25-40% B, 42-45 min, 40-90% B; 45-49 min, 90% B hold; 49-50 min, 90-3% B; 50-80 min, 3% B. *Note: operators with Waters Aquity UPLC will use a sligthly adjusted gradient (due to the use of Mobile phase B: 100% acetonitrile (v/v)/0.1% formic acid in water (v/v) only for Waters UPLC, see Table 3a).*
2. Flow rate: 300 nL/min
3. Mobile phase A: 0.1% formic acid in water (v/v); Mobile phase B: 90% acetonitrile (v/v)/0.1% formic acid in water (v/v). *Note: operators with Waters Aquity UPLC will use Mobile phase B: 100% acetonitrile (v/v)/0.1% formic acid in water (v/v) only for Waters UPLC).*

iii. Autosampler Settings
1. Direct Injection, full-loop injection, 1 µL sample load
2. Pick up 2 µL of sample sandwiched between mobile phase A (0.1% formic acid)
3. See Table 1 for example

iv. Recommended MS Source Conditions (use as guidelines, see above, Section 2.B.iii.)

C. Inject the sample 6 times, one time for each method created in Skyline

i. Name each injection with its own file name/sample name so that each injection is its own, individual file

1. Study9-2_SiteXX_HeavyOnly_method01
2. Study9-2_SiteXX_HeavyOnly_method02
3. Study9-2_SiteXX_HeavyOnly_method03
4. Study9-2_SiteXX_HeavyOnly_method04
5. Study9-2_SiteXX_HeavyOnly_method05
6. Study9-2_SiteXX_HeavyOnly_method06

ii. Follow with 1-5 injections of SSS

1. Do not allow the system to sit idle after the 6 HeavyOnly sample runs

4. Data Analysis

i. Import data files into the appropriate Skyline Platform-specific Study 9-2 file

1. Import multiple files as one replicate
2. Name replicate (HeavyOnly_rep1)

ii. Check automatic integration of all peaks

1. Manually adjust integration of peaks, if necessary
2. Make sure integration start and stop is identical for all transitions of a precursor (check integrate all in Skyline)
3. Document any peak that looks problematic using the "note" feature in Skyline - Use Skyline **Custom Annotation** to note any of the above features as described below (see separate document)
   a. Excessive peak tailing or fronting
   b. Drop-out of electrospray
   c. Poor peak shape
   d. Missing transitions
      i. In the case of missing peptides or missing transitions, first, make a note in Skyline at the peptide level (right click peptide sequence, then "Edit Note" and supply description such as "no signal", "only 2 transitions detected", "not sure which peak is peptide", etc. The Skyline files on the NIST server may already contain "notes" indicating which peptides are weak or poorly detected. If you encounter additional peptides, consider creating an unscheduled method to target the few peptides that were undetectable. If they remain to be undetectable, please contact Birgit Schilling and Sue Abbatiello for further advice.

iii. Under Peptide Settings, set window to 4 minutes

1. Export the transition list in scheduled mode – heavy only, ~375 transitions with 4 min RT window. Double check that all 375 transitions are exported and added into the MS method appropriately.
2. Please set target cycle time to be approximately 1.5 seconds
   a. 4000 QTRAP users: please set to 2.0 sec
   b. 5500 QTRAP users: please set to 1.2 sec (1.5 if using Nanoflex)

c. Agilent ChipCube users: please adjust cycle time so that the minimum dwell time calculated in MassHunter is >10 msec.
d. Use the Autodwell feature of Waters software to determine dwell times based on two inputs: point-per-peak and average peak width.
e. Other platforms: please adjust cycle time such that you will get ~15 points across your chromatographic peak (baseline to baseline) and >10 msec dwell time minimum for all transitions.

3. Utilize the "scheduling" feature in Skyline which will show how many concurrent transitions there are in the most complex part of the gradient (highest number of concurrent transitions). Assess the transition distribution spanning the gradient evenly, aiming for a Gaussian-like distribution of the concurrent transitions in the Skyline scheduling view. This will help to see problems of early eluting hydrophilic peptides that might "bleed through", gradient problems might visually become evident, i.e., if everything elutes very early or very late or within a very small RT range.
4. Run two replicates of on-site digested/spiked sample Study9-2-A (which contains on site digested U-^{15}N-labeled protein plus operator-spiked heavy ^{13}C/^{15}N synthetic IS peptides in depleted plasma), in which the method monitors for only the 13C/15N heavy peptide forms with a 4 min RT window, and import into Skyline.
5. Ensure all 13C/15N peptide peaks are detected.

iv. Download a second, different Skyline template from the NIST server containing heavy (^{13}C/^{15}N), light (unlabeled) and U-^{15}N-labeled peptides (download the new template rather than adding in new label types into the existing heavy only template! Don't use Edit->Refine to add new label types) Note: appropriate corresponding Skyline templates for all three label types can be found on the FTP server in the Study 9.2 folder. Recommendation: Use one dedicated Skyline file with all three label types always for scheduling.
ftp://chemdata.nist.gov/verification_main_study9.2/Skyline_templates/

1. Import your last two acquired scheduled Study9-2-A samples into this newly downloaded Skyline template that includes all three label types
2. There should now be 1095 transitions, 125 peptides, 365 precursors
3. View the RT plot for Scheduling
4. Ensure the maximum number of concurrent transitions throughout the gradient is < 100
5. If there are incidents of concurrent transitions >100 anywhere in the gradient, contact CPTAC VWG members for assistance
6. Set the RT window for 2 minutes (Settings, Peptide Settings, Prediction Tab).

5. Export all transitions (~1095) in Scheduled mode, with 2 min RT window. Check that your two (4 min)-scheduled Study9-2-A samples that you have imported above into your all inclusive Skyline template (3 label forms) do not show much RT drift, and use the second, most recent Study9-2-A run for scheduling (with a 2 min scheduling window). Double check that all 1095 transitions are exported and added into the MS method

appropriately (i.e., the total number of transitions is correct and that each light, $^{13}C/^{15}N$, U-^{15}N set have the same retention times for all 9 transitions). Acquire 1- 4 replicates of sample Study9-2 A with the scheduled LC-MRM-MS method (now using the 2 min scheduling window)

i. Import data back into Skyline and observe any shift in retention times
ii. Use RT graph, peptide replicate view
iii. If peaks shift by > 30 seconds (0.5 minutes), do not proceed and contact CPTAC VWG members for assistance
iv. Look at data to make sure peak apexes are in or near the center of the RT window and not getting cut off during detection.
v. The Study 9-2-A sample contains heavy peptide signal ($^{13}C^{15}N$ peptides), but also U-^{15}N peptides that were generated from your digestion on-site. Look for the U-^{15}N peptide signal and if you don't see the U-^{15}N peptide signal possibly something was unusual with the performed digestion. In such case, please pause and assess and troubleshoot. The last 7 proteins in the Skyline file are for the digestion controls. You should see consistent peak area ratios (light : $^{13}C/^{15}N$) for the peptides across your standards. If these vary greatly, also pause and troubleshoot.

6. Continue acquiring Study 9-2 samples according to the sample chart shown below.

3. Important additional Notes for Sample Preparation and Acquisitions:

a) The four replicate concentration points in Study 9.1 and here in Study 9.2 will be acquired in **4x singlicate curves** (with blanks and SSS in between to avoid carryover). Also note each non-digested Study 9.2 singlicate concentration point was prepared individually at the central preparation site (Vanderbilt), so for each concentration point there will be 3 tubes supplied (the 3rd replicate supplied will be split into to technical replicates after reconstitution, and will be acquired twice on the LC-MRM-MS platform as curve 3 and curve 4, also see Scheme 1 above).

b) **After digestion, desalting, and reconstitution (including adding in the heavy synthetic peptides, as described below)**, take a 25 µl aliquot of each reconstituted sample for immediate LC-MRM-MS acquisition (the total volume per sample after digestion, desalting, lyophilizing and reconstitution is **225 µL**). There will be a remainder of each sample of 200 µl, aliquot this remainder into 50 µl aliquots and freeze those at -80 ºC (the latter is very important, please freeze the non-used digested reconstituted sample in aliquots so one can retrieve them later for possible reruns).

c) **Adding Calibration Curve Samples and Blinded Samples into Autosampler Vials:** Add one Calibration Curve and corresponding Blinded Samples at a time into the autosampler. Transfer a 25 µL aliquot from each Calibration Curve concentration point tube per singlicate curve (as described above) into an autosampler vial, and store the remaining 50 µL aliquots generated in the -80 ºC freezer (there should be 4x 50 µL aliquots leftover per concentration point and singlicate curve / and half of that for the split 3rd set). Make sure to finish each singlicate curve (including initial data analysis and review of data points for quality, or for retention time drift of peaks outside the RT scheduling window) before starting the next singlicate curve. If data points need to be rerun from the current singlicate curve use remaining sample that is still in autosampler

vials or if multiple reruns are needed use one of the aliquots that was transferred into the freezer as was described above. At the beginning of a singlicate curve, and as blank in between blinded samples transfer 25 µL of the 9.2 A aliquot into an autosampler vial.

d) **In Case of Acquisition Problems and Re-runs of Samples:** In case there are problems with the data acquisition or RT drift, and possible reruns that may become necessary, please maintain the run number at the end of the filename to keep track of the run order. The run number should agree with the chronological order in which the samples were analyzed on the LC-MRM-MS. In addition, please re-run the blank 9.2-A sample prior to any re-run, and please re-run from lowest to highest concentration for the calibration curve samples.

e) **Binded Samples**

i. There are three sets of three Blinded samples (each provided in 35 µl aliquot, of which 25 µl are digested), with Set 3 being split into two technical replicates T1 and T2 after digestion and desalting:

1. First singlicate: 9-2-01Blinded to 9-2-03Blinded
2. Second singlicate: 9-2-04Blinded to 9-2-06Blinded
3. Third singlicate: 9-2-07-T1Blinded to 9-2-09-T1Blinded
4. Fourth singlicate: 9-2-07-T2Blinded to 9-2-09-T2Blinded

ii. For each singlicate: the three blinded samples will be run **twice**, i.e., as runs 9-2-01aBlinded and 9-2-01bBlinded, see runorder.

iii. Blinded samples will be run with 1 wash run (10 fmol 6-protein mix) and 1 blank run in between. The blank run consists of Sample 9-2-A, that was digested on-site and which contains no unlabeled peptides, only the $^{13}C/^{15}N$ IS peptides and U-^{15}N-labeled peptide equivalents in depleted plasma.

iv. For Blinded Samples use Study 9-2 gradient (Table 2a) and "Study 9 scheduled MRM-MS, light and heavy" MS-method. For Wash Samples use Wash gradient (Table 2c) and "Study 9S, SSS"-adjusted MS-method. For Blank Samples use Study 9-2 gradient (Table 2a) and "Study 9 scheduled MRM-MS, light and heavy" MS-method. Blanks (in between blinded samples) can be injected from the same autosampler vial

7. **Fresh Preparation of System Suitability Samples (SSS) for Each Singlicate Curve:** For each Singlicate Calibration Curve a fresh autosampler vial of the SSS will should be prepared to prevent degradation of the sample (note in a notebook when fresh SSS are added).
8. **Please adhere closely to sample naming as defined in the run order** (with chronological runorder number at the end).
9. **Note that System Suitability samples (SSS) indicate which singlicate curve they belong to:** S1 for Singlicate Curve 1, S2 for Singlicate Curve 2, S3 for Singlicate Curve 3, and S4 for Singlicate Curve 4.

Please remember: there are 3 different gradients to be used in the run orders below:

- **Study 9S** gradient for System Suitability Samples
- **Study 9-2** gradient for the main study (all concentration curve points as well as blinded samples)
- **Study 9 wash** gradient (in between blinded samples)

Gradients are described in detail in Tables 2a-c, and 3a-c for Waters UPLC (see below)

First singlicate curve plus blinded samples (01-03blinded)

Run Number	Number of injections	Sample Description	Filename	Method	Notes
1	1	SSS, Michrom Mix	9-2_SiteX_SSS_run_001	Study 9S (SSS)	Column Conditioning
2	1	SSS, Michrom Mix	9-2_SiteX_SSS_run_002	Study 9S (SSS)	
3	1	SSS, Michrom Mix	9-2_SiteX_SSS_run_003	Study 9S (SSS)	
4	1	SSS, Michrom Mix	9-2_SiteX_SSS_run_004	Study 9S (SSS)	
5	1	SSS, Michrom Mix	9-2_SiteX_SSS_run_005	Study 9S (SSS)	
6	1	Study9-2 Heavy Only	9-2_SiteX_heavy_method01_run_006	Study 9 method 01	For scheduling
7	1	Study9-2 Heavy Only	9-2_SiteX_heavy_method02_run_007	Study 9 method 02	
8	1	Study9-2 Heavy Only	9-2_SiteX_heavy_method03_run_008	Study 9 method 03	
9	1	Study9-2 Heavy Only	9-2_SiteX_heavy_method04_run_009	Study 9 method 04	
10	1	Study9-2 Heavy Only	9-2_SiteX_heavy_method05_run 010	Study 9 method 05	
11	1	Study9-2 Heavy Only	9-2_SiteX_heavy_method06_run_011	Study 9 method 06	
12-16	5	SSS, Michrom Mix	9-2_SiteX_SSS_S1_run_012 9-2_SiteX_SSS_S1_run_013 9-2_SiteX_SSS_S1_run_014 9-2_SiteX_SSS_S1_run_015 9-2_SiteX_SSS_S1_run_016	Study 9S (SSS)	At least 3 SSS samples and if necessary place holder
17	1	Study9-2 A, IS peptides in plasma	9-2_SiteX_A_sMRM_run_017	Study 9 scheduled MRM-MS, heavy only	Scheduled run, 4 min RT window
18	1	Study9-2 A, IS peptides in plasma	9-2_SiteX_A_sMRM_run_018	Study 9 scheduled MRM-MS, heavy only	
19	1	Study9-2 A, IS peptides in plasma	9-2_SiteX_A_sMRM_run_019	Study 9 scheduled MRM-MS, light heavy, and U-^{15}N	Scheduled run, 2 min RT window
20	1	Study9-2 A, IS peptides in plasma	9-2_SiteX_A_sMRM_run_020	Study 9 scheduled MRM-MS, light heavy, and U-^{15}N	
21	1	Study9-2 A, IS peptides in plasma	9-2_SiteX_A_sMRM_run_021	Study 9 scheduled MRM-MS, light heavy, and U-^{15}N	
22	1	Study9-2 A, IS peptides in plasma	9-2_SiteX_A_sMRM_run_022	Study 9 scheduled MRM-MS, light heavy, and U-^{15}N	

23	**1**	**Study9-2 A1, curve proteins in plasma**	**9-2_SiteX_A1_CalCurve_run_023**	**Study 9 scheduled MRM-MS, light heavy, and U-^{15}N**	**Calibration Curve**
24	**1**	**Study9-2 B1, curve proteins in plasma**	**9-2_SiteX_B1_CalCurve_run_024**	**Study 9 scheduled MRM-MS, light heavy, and U-^{15}N**	
25	**1**	**Study9-2 C1**	**9-2_SiteX_C1_CalCurve_run_025**	**Study 9 scheduled MRM-MS, light, heavy, U-^{15}N "Study9 sMRM"**	**CalCurve**
26	**1**	**Study9-2 D1**	**9-2_SiteX_D1_CalCurve_run_026**	**Study9 sMRM**	**CalCurve**
27	**1**	**SSS**	**9-2_SiteX_SSS_S1_run_027**	**Study 9S (SSS)**	**System Suitability**
28	**1**	**Study9-2 E1**	**9-2_SiteX_E1_CalCurve_run_028**	**Study9 sMRM**	**CalCurve**
29	**1**	**Study9-2 F1**	**9-2_SiteX_F1_CalCurve_run_029**	**Study9 sMRM**	**CalCurve**
30	**1**	**Study9-2 G1**	**9-2_SiteX_G1_CalCurve_run_030**	**Study9 sMRM**	**CalCurve**
31	**1**	**Study9-2 H1**	**9-2_SiteX_H1_CalCurve_run_031**	**Study9 sMRM**	**CalCurve**
32	**1**	**Study9-2 QC1**	**9-2_SiteX_QC1_CalCurve_run_032**	**Study9 sMRM**	**QC**
33	**1**	**Study9-2 I1**	**9-2_SiteX_I1_CalCurve_run_033**	**Study9 sMRM**	**CalCurve**
34	**1**	**Study9-2 J1**	**9-2_SiteX_J1_CalCurve_run_034**	**Study9 sMRM**	**CalCurve**
35	**1**	**Study9-2 wash**	**9-2_SiteX_wash_run_35**	**Study 9 wash**	
36	**1**	**SSS**	**9-2_SiteX_SSS_S1_run_036**	**Study 9S (SSS)**	**System suitability**
37	**1**	**SSS**	**9-2_SiteX_SSS_S1_run_037**	**Study 9S (SSS)**	**System suitability**
38	**1**	**SSS**	**9-2_SiteX_SSS_S1_run_038**	**Study 9S (SSS)**	**System suitability**
39	**1**	**Study9-2 A, IS peptides in plasma**	**9-2_SiteX_A_blank_run_039**	**Study 9 scheduled MRM-MS, light and heavy**	
40	**1**	**Study9-2 01blinded-Sample**	**9-2_SiteX_01a_blinded_run_040**	**Study 9 scheduled MRM-MS, light and heavy**	**Blinded**
41	**1**	**Study9-2 wash**	**9-2_SiteX_wash_run_041**	**Study 9 wash**	
42	**1**	**Study9-2 A, IS peptides in plasma**	**9-2_SiteX_A_blank _run_042**	**Study 9 scheduled MRM-MS, light and heavy**	
43	**1**	**Study9-2 02blinded**	**9-2_SiteX_02a_blinded_run_043**	**Study 9 scheduled**	**Blinded**

				MRM-MS, light and heavy	
44	1	Study9-2 wash	9-2_SiteX_wash_run_044	Study 9 wash	
45	1	Study9-2 A, IS peptides in plasma	9-2_SiteX_A_blank _run_045	Study 9 scheduled MRM-MS, light and heavy	
46	1	SSS	9-2_SiteX_SSS_S1_run_046	Study 9S (SSS)	System suitability
47	1	Study9-2 03blinded	9-2_SiteX_03a_blinded_run_047	Study 9 scheduled MRM-MS, light and heavy	Blinded
48	1	Study9-2 wash	9-2_SiteX_wash_run_048	Study 9 wash	
49	1	Study9-2 A, IS peptides in plasma	9-2_SiteX_A_blank _run_049	Study 9 scheduled MRM-MS, light and heavy	
50	1	Study9-2 01blinded-Sample	9-2_SiteX_01b_blinded_run_050	Study 9 scheduled MRM-MS, light and heavy	Blinded
51	1	Study9-2 wash	9-2_SiteX_wash_run_051	Study 9 wash	
52	1	Study9-2 A, IS peptides in plasma	9-2_SiteX_A_blank _run_052	Study 9 scheduled MRM-MS, light and heavy	
53	1	Study9-2 02blinded	9-2_SiteX_02b_blinded_run_053	Study 9 scheduled MRM-MS, light and heavy	Blinded
54	1	Study9-2 wash	9-2_SiteX_wash_run_054	Study 9 wash	
55	1	Study9-2 A, IS peptides in plasma	9-2_SiteX_A_blank _run_055	Study 9 scheduled MRM-MS, light and heavy	
56	1	SSS	9-2_SiteX_SSS_S1_run_056	Study 9S (SSS)	System suitability
57	1	Study9-2 03blinded	9-2_SiteX_03b_blinded_run_057	Study 9 scheduled MRM-MS, light and heavy	Blinded
58	1	Study9-2 wash	9-2_SiteX_wash_run_058	Study 9 wash	

59	1	Study9-2 A, IS peptides in plasma	9-2_SiteX_A_blank _run_059	Study 9 scheduled MRM-MS, light and heavy	

Second singlicate curve plus blinded samples (04-06blinded)

60-62	3	SSS, Michrom Mix	9-2_SiteX_SSS_S2_run_060 9-2_SiteX_SSS_S2_run_061 9-2_SiteX_SSS_S2_run_062	Study 9S (SSS)	3 SSS samples
63	1	Study9-2 A2, proteins in plasma	9-2_SiteX_A2_CalCurve_run_063	Study 9 scheduled MRM-MS, light and heavy	Scheduled run, 2 min RT window, Calibration Curve
64	1	Study9-2 B2	9-2_SiteX_B2_CalCurve_run_064	Study 9 scheduled MRM-MS, light and heavy	
65	1	Study9-2 C2	9-2_SiteX_C2_CalCurve_run_065	Study 9 scheduled MRM-MS, light and heavy, "Study9 sMRM"	CalCurve
66	1	Study9-2 D2	9-2_SiteX_D2_CalCurve_run_066	Study9 sMRM	CalCurve
67	1	SSS	9-2_SiteX_SSS_S2_run_067	Study 9S (SSS)	System Suitability
68	1	Study9-2 E2	9-2_SiteX_E2_CalCurve_run_068	Study9 sMRM	CalCurve
69	1	Study9-2 F2	9-2_SiteX_F2_CalCurve_run_069	Study9 sMRM	CalCurve
70	1	Study9-2 G2	9-2_SiteX_G2_CalCurve_run_070	Study9 sMRM	CalCurve
71	1	Study9-2 H2	9-2_SiteX_H2_CalCurve_run_071	Study9 sMRM	CalCurve
72	1	Study9-2 QC2	9-2_SiteX_QC2_CalCurve_run_072	Study9 sMRM	QC
73	1	Study9-2 I2	9-2_SiteX_I2_CalCurve_run_073	Study9 sMRM	CalCurve
74	1	Study9-2 J2	9-2_SiteX_J2_CalCurve_run_074	Study9 sMRM	CalCurve
75	1	Study9-2 wash	9-2_SiteX_wash_run_075	Study 9 wash	
76	1	SSS	9-2_SiteX_SSS_S2_run_076	Study 9S (SSS)	System suitability
77	1	SSS	9-2_SiteX_SSS_S2_run_077	Study 9S (SSS)	System suitability
78	1	SSS	9-2_SiteX_SSS_S2_run_078	Study 9S (SSS)	System suitability
79	1	Study9-2 A, IS peptides in plasma	9-2_SiteX_A_blank_run_079	Study 9 scheduled MRM-MS, light	

				and heavy	
80	1	Study9-2 04blinded-Sample	9-2_SiteX_04a_blinded_run_080	Study 9 scheduled MRM-MS, light and heavy	Blinded
81	1	Study9-2 wash	9-2_SiteX_wash_run_081	Study 9 wash	
82	1	Study9-2 A, IS peptides in plasma	9-2_SiteX_A_blank _run_082	Study 9 scheduled MRM-MS, light and heavy	
83	1	Study9-2 05blinded	9-2_SiteX_05a_blinded_run_083	Study 9 scheduled MRM-MS, light and heavy	Blinded
84	1	Study9-2 wash	9-2_SiteX_wash_run_084	Study 9 wash	
85	1	Study9-2 A, IS peptides in plasma	9-2_SiteX_A_blank _run_085	Study 9 scheduled MRM-MS, light and heavy	
86	1	SSS	9-2_SiteX_SSS_S2_run_086	Study 9S (SSS)	System suitability
87	1	Study9-2 06blinded	9-2_SiteX_06a_blinded_run_087	Study 9 scheduled MRM-MS, light and heavy	Blinded
88	1	Study9-2 wash	9-2_SiteX_wash_run_088	Study 9 wash	
89	1	Study9-2 A, IS peptides in plasma	9-2_SiteX_A_blank _run_089	Study 9 scheduled MRM-MS, light and heavy	
90	1	Study9-2 04blinded-Sample	9-2_SiteX_04b_blinded_run_090	Study 9 scheduled MRM-MS, light and heavy	Blinded
91	1	Study9-2 wash	9-2_SiteX_wash_run_091	Study 9 wash	
92	1	Study9-2 A, IS peptides in plasma	9-2_SiteX_A_blank _run_092	Study 9 scheduled MRM-MS, light and heavy	
93	1	Study9-2 05blinded	9-2_SiteX_05b_blinded_run_093	Study 9 scheduled MRM-MS, light and heavy	Blinded
94	1	Study9-2 wash	9-2_SiteX_wash_run_094	Study 9 wash	

95	1	Study9-2 A, IS peptides in plasma	9-2_SiteX_A_blank _run_095	Study 9 scheduled MRM-MS, light and heavy	
96	1	SSS	9-2_SiteX_SSS_S2_run_096	Study 9S (SSS)	System suitability
97	1	Study9-2 06blinded	9-2_SiteX_06b_blinded_run_097	Study 9 scheduled MRM-MS, light and heavy	Blinded
98	1	Study9-2 wash	9-2_SiteX_wash_run_098	Study 9 wash	
99	1	Study9-2 A, IS peptides in plasma	9-2_SiteX_A_blank _run_099	Study 9 scheduled MRM-MS, light and heavy	

Third singlicate curve plus blinded samples (07-09blinded), first technical replicate 3-T1

100-102	3	SSS, Michrom Mix	9-2_SiteX_SSS_S3_run_100 9-2_SiteX_SSS_S3_run_101 9-2_SiteX_SSS_S3_run_102	Study 9S (SSS)	3 SSS samples
103	1	Study9-2 A3-T1, proteins in plasma	9-2_SiteX_A3-T1_CalCurve_run_103	Study 9 scheduled MRM-MS, light and heavy	Scheduled run, 2 min RT window, Calibration Curve
104	1	Study9-2 B3-T1	9-2_SiteX_B3-T1_CalCurve_run_104	Study 9 scheduled MRM-MS, light and heavy	
105	1	Study9-2 C3-T1	9-2_SiteX_C3-T1_CalCurve_run_105	Study 9 scheduled MRM-MS, light and heavy, "Study9 sMRM"	CalCurve
106	1	Study9-2 D3-T1	9-2_SiteX_D3-T1_CalCurve_run_106	Study9 sMRM	CalCurve
107	1	SSS	9-2_SiteX_SSS_S3_run_107	Study 9S (SSS)	System Suitability
108	1	Study9-2 E3-T1	9-2_SiteX_E3-T1_CalCurve_run_108	Study9 sMRM	CalCurve
109	1	Study9-2 F3-T1	9-2_SiteX_F3-T1_CalCurve_run_109	Study9 sMRM	CalCurve
110	1	Study9-2 G3-T1	9-2_SiteX_G3-T1_CalCurve_run_110	Study9 sMRM	CalCurve

111	1	Study9-2 H3-T1	9-2_SiteX_H3-T1_CalCurve_run_111	Study9 sMRM	CalCurve
112	1	Study9-2 QC3	9-2_SiteX_QC3_CalCurve_run_112	Study9 sMRM	QC
113	1	Study9-2 I3-T1	9-2_SiteX_I3-T1_CalCurve_run_113	Study9 sMRM	CalCurve
114	1	Study9-2 J3-T1	9-2_SiteX_J3-T1_CalCurve_run_114	Study9 sMRM	CalCurve
115	1	Study9-2 wash	9-2_SiteX_wash_run_115	Study 9 wash	
116	1	SSS	9-2_SiteX_SSS_S3_run_116	Study 9S (SSS)	System suitability
117	1	SSS	9-2_SiteX_SSS_S3_run_117	Study 9S (SSS)	System suitability
118	1	SSS	9-2_SiteX_SSS_S3_run_118	Study 9S (SSS)	System suitability
119	1	Study9-2 A-T1, IS peptides in plasma	9-2_SiteX_A-T1_blank_run_119	Study 9 scheduled MRM-MS, light and heavy	
120	1	Study9-2 07-T1blinded-Sample	9-2_SiteX_07a-T1blinded_run_120	Study 9 scheduled MRM-MS, light and heavy	Blinded
121	1	Study9-2 wash	9-2_SiteX_wash_run_121	Study 9 wash	
122	1	Study9-2 A, IS peptides in plasma	9-2_SiteX_A _blank _run_122	Study 9 scheduled MRM-MS, light and heavy	
123	1	Study9-2 08-T1blinded	9-2_SiteX_08a-T1blinded_run_123	Study 9 scheduled MRM-MS, light and heavy	Blinded
124	1	Study9-2 wash	9-2_SiteX_wash_run_124	Study 9 wash	
125	1	Study9-2 A, IS peptides in plasma	9-2_SiteX_A_blank _run_125	Study 9 scheduled MRM-MS, light and heavy	
126	1	SSS	9-2_SiteX_SSS_S3_run_126	Study 9S (SSS)	System suitability
127	1	Study9-2 09-T1-blinded	9-2_SiteX_09a-T1blinded_run_127	Study 9 scheduled MRM-MS, light and heavy	Blinded
128	1	Study9-2 wash	9-2_SiteX_wash_run_128	Study 9 wash	
129	1	Study9-2 A, IS peptides in	9-2_SiteX_A_blank _run_129	Study 9 scheduled	

		plasma		MRM-MS, light and heavy	
130	1	Study9-2 07-T1blinded-Sample	9-2_SiteX_07b-T1blinded_run_130	Study 9 scheduled MRM-MS, light and heavy	Blinded
131	1	Study9-2 wash	9-2_SiteX_wash_run_131	Study 9 wash	
132	1	Study9-2 A, IS peptides in plasma	9-2_SiteX_A _blank _run_132	Study 9 scheduled MRM-MS, light and heavy	
133	1	Study9-2 08-T1blinded	9-2_SiteX_08b-T1blinded_run_133	Study 9 scheduled MRM-MS, light and heavy	Blinded
134	1	Study9-2 wash	9-2_SiteX_wash_run_134	Study 9 wash	
135	1	Study9-2 A, IS peptides in plasma	9-2_SiteX_A_blank _run_135	Study 9 scheduled MRM-MS, light and heavy	
136	1	SSS	9-2_SiteX_SSS_S3_run_136	Study 9S (SSS)	System suitability
137	1	Study9-2 09-T1-blinded	9-2_SiteX_09b-T1blinded_run_137	Study 9 scheduled MRM-MS, light and heavy	Blinded
138	1	Study9-2 wash	9-2_SiteX_wash_run_138	Study 9 wash	
139	1	Study9-2 A, IS peptides in plasma	9-2_SiteX_A_blank _run_139	Study 9 scheduled MRM-MS, light and heavy	

Technical replicate T2 of third singlicate curve plus blinded samples (07-09blinded, technical replicate2, T2)

140-142	3	SSS, Michrom Mix	9-2_SiteX_SSS_S4_run_140 9-2_SiteX_SSS_S4_run_141 9-2_SiteX_SSS_S4_run_142	Study 9S (SSS)	3 SSS samples
143	1	Study9-2 A3_T2, proteins in plasma	9-2_SiteX_A3_T2_CalCurve_run_143	Study 9 scheduled MRM-MS, light and heavy	Scheduled run, 2 min RT window,

144	1	Study9-2 B3_T2	9-2_SiteX_B3_T2_CalCurve_run_144	Study 9 scheduled MRM-MS, light and heavy	Calibration Curve
145	1	Study9-2 C3_T2	9-2_SiteX_C3_T2_CalCurve_run_145	Study 9 scheduled MRM-MS, light and heavy, "Study9 sMRM"	CalCurve
146	1	Study9-2 D3_T2	9-2_SiteX_D3_T2_CalCurve_run_146	Study9 sMRM	CalCurve
147	1	SSS	9-2_SiteX_SSS_S4_run_147	Study 9S (SSS)	System Suitability
148	1	Study9-2 E3_T2	9-2_SiteX_E3_T2_CalCurve_run_148	Study9 sMRM	CalCurve
149	1	Study9-2 F3_T2	9-2_SiteX_F3_T2_CalCurve_run_149	Study9 sMRM	CalCurve
150	1	Study9-2 G3_T2	9-2_SiteX_G3_T2_CalCurve_run_150	Study9 sMRM	CalCurve
151	1	Study9-2 H3_T2	9-2_SiteX_H3_T2_CalCurve_run_151	Study9 sMRM	CalCurve
152	1	Study9-2 QC4	9-2_SiteX_QC4_CalCurve_run_152	Study9 sMRM	QC
153	1	Study9-2 I3_T2	9-2_SiteX_I3_T2_CalCurve_run_153	Study9 sMRM	CalCurve
154	1	Study9-2 J3_T2	9-2_SiteX_J3_T2_CalCurve_run_154	Study9 sMRM	CalCurve
155	1	Study9-2 wash	9-2_SiteX_wash_run_155	Study 9 wash	
156	1	SSS	9-2_SiteX_SSS_S4_run_156	Study 9S (SSS)	System suitability
157	1	SSS	9-2_SiteX_SSS_S4_run_157	Study 9S (SSS)	System suitability
158	1	SSS	9-2_SiteX_SSS_S4_run_158	Study 9S (SSS)	System suitability
159	1	Study9-2 A, IS peptides in plasma	9-2_SiteX_A_blank_run_159	Study 9 scheduled MRM-MS, light and heavy	
160	1	Study9-2 07_T2blinded-Sample	9-2_SiteX_07a_T2blinded_run_160	Study 9 scheduled MRM-MS, light and heavy	Blinded
161	1	Study9-2 wash	9-2_SiteX_wash_run_161	Study 9 wash	
162	1	Study9-2 A, IS peptides in plasma	9-2_SiteX_A_blank _run_162	Study 9 scheduled MRM-MS, light and heavy	
163	1	Study9-2 08_T2blinded	9-2_SiteX_08a_T2blinded_run_163	Study 9 scheduled MRM-MS, light and heavy	Blinded

164	1	Study9-2 wash	9-2_SiteX_wash_run_164	Study 9 wash	
165	1	Study9-2 A, IS peptides in plasma	9-2_SiteX_A_blank _run_165	Study 9 scheduled MRM-MS, light and heavy	
166	1	SSS	9-2_SiteX_SSS_S4_run_166	Study 9S (SSS)	System suitability
167	1	Study9-2 09_T2blinded	9-2_SiteX_09a_T2blinded_run_167	Study 9 scheduled MRM-MS, light and heavy	Blinded
168	1	Study9-2 wash	9-2_SiteX_wash_run_168	Study 9 wash	
169	1	Study9-2 A, IS peptides in plasma	9-2_SiteX_A_blank _run_169	Study 9 scheduled MRM-MS, light and heavy	
170	1	Study9-2 07_T2blinded-Sample	9-2_SiteX_07b_T2blinded_run_170	Study 9 scheduled MRM-MS, light and heavy	Blinded
171	1	Study9-2 wash	9-2_SiteX_wash_run_171	Study 9 wash	
172	1	Study9-2 A, IS peptides in plasma	9-2_SiteX_A_blank _run_172	Study 9 scheduled MRM-MS, light and heavy	
173	1	Study9-2 08_T2blinded	9-2_SiteX_08b_T2blinded_run_173	Study 9 scheduled MRM-MS, light and heavy	Blinded
174	1	Study9-2 wash	9-2_SiteX_wash_run_174	Study 9 wash	
175	1	Study9-2 A, IS peptides in plasma	9-2_SiteX_A_blank _run_175	Study 9 scheduled MRM-MS, light and heavy	
176	1	SSS	9-2_SiteX_SSS_S4_run_176	Study 9S (SSS)	System suitability
177	1	Study9-2 09_T2blinded	9-2_SiteX_09b_T2blinded_run_177	Study 9 scheduled MRM-MS, light and heavy	Blinded
178	1	Study9-2 wash	9-2_SiteX_wash_run_178	Study 9 wash	
179	1	Study9-2 A, IS peptides in	9-2_SiteX_A_blank _run_179	Study 9 scheduled	

		plasma		**MRM-MS, light and heavy**	
180	**1**	**SSS**	**9-2_SiteX_SSS_S4_run_180**	**Study 9S (SSS)**	**System suitability**
181	**1**	**SSS**	**9-2_SiteX_SSS_S4_run_181**	**Study 9S (SSS)**	**System suitability**
182	**1**	**SSS**	**9-2_SiteX_SSS_S4_run_182**	**Study 9S (SSS)**	**System suitability**

1. **Data Analysis**
    a. All data should be analyzed in "real time," or as close to its acquisition time as possible to catch the chance of peaks drifting outside the 2 min RT windows.
    b. Data analysis must be conducted through Skyline using templates provided for each instrument platform
    c. Once data are imported, check integration of all peaks
        i. Integrate transitions with the same start and stop time: Under "Settings" in Skyline, enable "Integrate All"
        ii. Integrate light, heavy, and U-^{15}N peptides with the same start and stop time
        iii. If an interference is present, document the transition-level note for that transition
    d. Export data for submission to VWG statisticians using the report template provided (note the new Study 9.2 Skyline report template is slightly different from the 9.1 template, and can be found on the NIST server, ftp://chemdata.nist.gov/verification_main_study9.2/Skyline_templates/)
    e. Under Settings, click on "custom annotation" and make sure that all settings are selected.
        i. If a transition, precursor, or peptide is deemed unusable because of a bad injection, loss of electrospray or another explainable reason and the sample is re-run, please click the "do not use" box for the sample that is to be excluded from subsequent analysis for LOD/LOQ.

Even if a specific replicate needs to be rerun, the original and new (rerun) replicate are to both be imported into Skyline, and "custom annotation" capabilities of Skyline should be used to annotate what data points to use for statistical analysis.

4. Trouble Shooting Section

b. Symptom: No data (missing peaks) or poor quality peaks for Study 9 peptides

i. Look at pressure trace to make sure sample (not air) was injected onto column.

ii. Open up the last SSS acquired before file with missing peaks

1. In Skyline or in vendor specific software

iii. Are 22 peaks present?

1. Yes: go on to point iv.
2. No: identify which peaks are missing
    a. Check pressure trace to make sure air was not injected onto column and that column pressure isn't too high
    b. Is there enough sample in all vials (sample vials and reagent vials)?

iv. Are peak shapes symmetric and well defined as opposed to mis-shapen and jagged?

1. Yes: go on to point v.
2. No: check ESI tip of column, clean if necessary.
    a. Also check pressure trace to make sure pressure is not too high.

v. Is column pressure too high?

1. Compare column pressure of bad runs to previous runs in the sample list
    a. If pressure is too high, remove column and clean tip
        i. Wipe tip with gloved hand, wet with water and wipe again. Check tip under microscope if possible.

ii. If this does not work, check for blockages in transfer lines closest to column and work backwards to LC.
iii. If this does not work, consider replacing column.

vi. Is column pressure too low?

1. If pressure is too low, check all connections between column and LC.

vii. Are flow rates calibrated correctly? (potentially check or recalibrate flow rates)
viii. Are samples being picked up properly from the autosampler vials?

c. Symptom: Retention Time Drift

i. Observe RT shift of the SSS samples in a single Skyline document

1. Does RT shift in one direction or randomly?

ii. If in one direction, are the peaks migrating out of the 2 min RT window for the Study 9 method? Solution: Re-scheduling because of shifting retention times

1. Re-run Study 9-2_A with the "heavy only" scheduled method that has a 4 minute RT window.
2. Import data into an empty Skyline template for Study 9 peptides and export a new scheduled method based on the RT data for light and heavy peptides (750 transitions).
3. Keep close eye on RT shifts.
4. Consider running SSS runs at points when you would not be able to check the RT shift and if peaks are in danger of shifting out of RT window (like overnight).

d. **If any concentration points must be re-run, it is important to follow this procedure**:

i. Run SSS sample to ensure system is working properly. Look for smooth, symmetrical peaks with stable retention times.
ii. Inject Sample Study9-2_A before re-running a calibration point to make sure there is little carryover.
iii. Follow with another SSS run so that you have time to analyze the data before running the next concentration point.

HPLC Chromatography Conditions for Studies 9-2:

Individual CPTAC sites are expected to implement these HPLC conditions for the duration of the study. Prepacked New Objective HPLC Columns are provided.

- HPLC-plumbing: <u>DIRECT INJECTION</u>
- Sample Loop: 1 µL sample loop
- Columns for AB SCIEX and Thermo HPLC systems: (New Objective custom-packed as previously shipped to all sites): PicoFrit 75 µm ID / 10 µm tip (ReproSil-Pur C18-AQ, 3µm, 120Å, length of 12 cm)
- Columns for Waters UPLC systems: a Waters specific column will be used (1.7µm BEH130 C18, 75 µm X 150 mm column)
- Columns for Agilent ChipCube systems: NanoChip
- Mobile phases: (A) 0.1% Formic acid (v/v); (B) 90% Acetonitrile / 0.1% Formic acid (v/v)

- Flow rate: 300 nL/min
- Injection volume: 1 µL on column (full loop injection with overloading the loop with 2 µL)
- Loop for Eksigent LC: 1 µL PEEKsil loop provided in sample kit, 100 µm ID PEEKsil.
- Injection Amount: ~0.5 µg total protein on-column
- Gradient: for details see below, **NOTE: Study 9S system suitability has a slightly different gradient than the new Study 9-2 gradient. Please use the appropriate gradient for these different applications.**

Table 1: Eksigent/Tempo Notes: Autosampler set-up, direct injection mode (representative example)

Autosampler Program with Standard Injection*

#	Function	Command		
1	Output	1-Off		
2	Output	2-Off		
3	Valve	Injector Load		
4**	Aspirate	10 µL Reagent-1	Speed:1	Height:5
5	Aspirate	2 µL Sample	Speed:1	Height:2
6**	Aspirate	2.3 µL Reagent-1	Speed:1	Height:5
7	Output	2-On		
8	Valve	Injector Inject		
9	Dispense	14.3 µL Waste	Speed:5	Height:0
10 **	Needle Wash	200 µL		
11	End			

NOTE: Different gradients are used for Study 9-2 calibration curve (Study 9-2 gradient) vs. system suitability samples (Study 9S gradient) vs. Wash gradient used in between blinded samples (Study 9 Wash gradient).

Table 2a. 9-2 HPLC gradient for all analyses of 125 target synthetic peptides.

Time (min)	%A	%B	Flow Rate (nL/min)
0	97	3	300
5	97	3	300
8	93	7	300
35	75	25	300
42	60	40	300
45	10	90	300
49	10	90	300
50	97	3	300
80	97	3	300

Mobile phase A: 0.1% formic acid (v/v) in water
Mobile phase B: 90% acetonitrile/0.1% formic acid (v/v) in water

Table 2b. 9S HPLC gradient to be used for System Suitability Sample (Michrom 6 protein mix).

Time (min)	%A	%B	Flow Rate (nL/min)
0	97	3	300
5	97	3	300
8	85	15	300
42	65	35	300
45	10	90	300
49	10	90	300
50	97	3	300
80	97	3	300

Mobile phase A: 0.1% formic acid (v/v) in water
Mobile phase B: 90% acetonitrile/0.1% formic acid (v/v) in water

Table 2c. 9-Wash HPLC gradient to be used for washing the column in between Blinded Samples.

Time (min)	%A	%B	Flow Rate (nL/min)
0	97	3	300
15	10	90	300
16	97	3	300
31	10	90	300
32	97	3	300
62	97	3	300

Mobile phase A: 0.1% formic acid (v/v) in water
Mobile phase B: 90% acetonitrile/0.1% formic acid (v/v) in water

NOTE: Total peptide amount greater than 1 µg injected onto nanoLC columns can result in poor chromatographic peak shape and poor reproducibility from run to run. The MARS-14 depleted plasma samples (Samples 9-2-A through 9-2-J, and 9-2-Blank) have been diluted such that a 1 µL injection results in approximately 0.5 µg of total protein on-column. Therefore, they should be analyzed without any additional dilution.

For Waters operators only:

Operators with Waters Aquity UPLC will use a slighly adjusted gradient, due to the use of Mobile phase B: 100% acetonitrile (v/v)/0.1% formic acid in water (v/v) only for Waters UPLC.

Adjusted Table 3a. for Waters UPLC only. Waters 9-2 HPLC gradient for all analyses of 125 target synthetic peptides.

Time (min)	%A	%B	Flow Rate (nL/min)
0	97.3	2.7	300
5	97.3	2.7	300
8	93.7	6.3	300
35	77.5	22.5	300
42	64	36	300
45	19	81	300
49	19	81	300
50	97.3	2.7	300
80	97.3	2.7	300

Mobile phase A: 0.1% formic acid (v/v) in water
Mobile phase B: 100% acetonitrile/0.1% formic acid (v/v) in water

Adjusted Table 3b. for Waters UPLC only. Waters 9S HPLC gradient to be used for System Suitability Sample (Michrom 6-protein mix).

Time (min)	%A	%B	Flow Rate (nL/min)
0	97.3	2.7	300
5	97.3	2.7	300
8	86.5	13.5	300
42	68.5	31.5	300
45	19	81	300
49	19	81	300
50	97.3	2.7	300
80	97.3	2.7	300

Mobile phase A: 0.1% formic acid (v/v) in water
Mobile phase B: 100% acetonitrile/0.1% formic acid (v/v) in water

Adjusted Table 3c. for Waters UPLC only. Waters 9-Wash HPLC gradient to be used for washing the column in between Blinded Samples.

Time (min)	%A	%B	Flow Rate (nL/min)
0	97.3	2.7	300
15	19	81	300
16	97.3	2.7	300
31	19	81	300
32	97.3	2.7	300
62	97.3	2.7	300

Mobile phase A: 0.1% formic acid (v/v) in water
Mobile phase B: 100% acetonitrile/0.1% formic acid (v/v) in water

Appendix A

Preparation of buffer solutions for digestion:
I. Chemical Reagents for Sample Preparation and Recommended Source

The following chemicals are NOT provided in the sample kits:

1. Tris Base – Sigma
2. Water, HPLC grade
3. Acetonitrile, HPCL grade
4. Formic acid
5. Hydrochloric acid (12M, for adjusting pH of Tris solution)

The following chemicals are <u>included</u> in the sample kits:

1. Urea – SigmaUltra
2. 1,4-Dithiothreitol [DTT]- Pierce No-Weight Format, (pre-weighed in 7.7 mg aliquots to be used fresh for each experiment.
3. Iodoacetamide – Sigma
4. Promega Trypsin Gold (2 vials)
5. LysC
6. $^{13}C/^{15}N$ IS peptide mixture supplied at 500 fmol/uL in 30% acetonitrile/0.1% formic acid.

II. Digestion Reagent Preparation for Plasma/target Protein Digestions

A. 1 M Tris, pH 8.0 – 250 mL

1. To a 500 mL beaker, add 30.3 g solid Tris-base and add 150 mL deionized water and stir until dissolved.
2. Adjust pH of solution to 8.0 with concentrated HCl (12 M).
3. Transfer solution to 250 mL or 500 mL graduated cylinder and bring volume to 250mL with deionized water.

B. 9M urea, 100 mM Tris, pH 8.0 Stock - 50 mL
(This solution must be prepared fresh for each process replicate.)

1. To a 100 mL beaker, add 27 g solid urea
2. Add 15 mL of water and 5 mL of 1 M Tris, pH 8.0 (Reagent A).
3. Add stir bar and place beaker in larger beaker of warm water. Stir until dissolved. Keep temperature at or below 37 °C. Do not overheat!
4. Measure pH and adjust to 8.0 if necessary.
5. Transfer to 50 or 100 mL graduated cylinder and bring volume to 50 mL with deionized water.

C. 100 mM Tris, pH 8.0 – 100 mL

1. Add 10 mL of 1 M Tris pH 8.0 stock to a 100 mL graduated cylinder and add water to a final volume 100 mL.

D. 200 mM 1,4-Dithiothreitol (DTT)
(This solution must be prepared fresh for each process replicate.)

1. Transfer the pre-weighed 7.7 mg aliquot of DTT to a larger 1.5 mL Eppendorf tube by adding 100 ul of water, wait until the DTT dissolves, pipette this liquid to the new tube, and then add another 150 μL of water resulting in a final concentration of 200 mM (don't transfer the dry solid).

E. 400 mM Iodoacetamide (IAM)
(Prepare immediately before use and keep out of the light)

1. To one 56 mg vial of iodoacetamide, add 757 μL of water for a final concentration of 400 mM.