Study 9-1 SOP (Phase II) for NCI CPTAC Consortium-Wide Multiple Reaction Monitoring (MRM) Experiment

9-Point Calibration Curve of 123 target peptides

Experimental Design and Statistics Verification Studies Working Group

Overview

Study 9-1, the 9 point calibration curve will entail monitoring 123 signature peptides and corresponding stable-isotope labeled internal standard (SIS) peptides (~750 total transitions) that will be quantitatively assayed by time-scheduled LC-MRM-MS against a background of MARS-14 depleted human K_2EDTA plasma (0.5 μg/μL).

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

1. To generate a 9 point standard curve for 123 potentially cancer relevant peptide targets spanning 1 amol/μL to 100 fmol/μL in a depleted digested plasma background (500 ng/μL) that will be analyzed by LC-MRM-MS on four different triple quadrupole platforms at volunteer CPTAC sites.

2. To enable calculations of LOD, LOQ, accuracy and precision for all peptides from the calibration curve.

3. A set of 6 blinded samples is provided that mimic real biomarker samples.

4. To evaluate the level of endogenous and interfering signals for all peptides by replicate measurements of blank plasma samples.

The number of transitions that will be targeted in this method (~750) 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. Run System Suitability Sample 5 times to condition column and assess LC-MRM-MS performance
2. Using Skyline, create 6 MRM-MS transition lists for the heavy target peptides only (maximum of 60 transitions in each unscheduled method)
3. 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 the sample containing heavy peptides spiked into depleted, digested plasma.
    v. Import the 2 raw files into Skyline
    vi. Add in the light (unlabeled) peptides into the Skyline document
    vii. Export a new scheduled LC-MRM-MS method with all ~750 transitions and a 2 min RT window
4. Execute calibration curve and blinded samples according to specified run order.
5. 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 9-1 Skyline file and integrate.
6. Export reports from each Skyline file and upload to the NIST ftp server (see separate document).

Materials and Reagents

A. System Suitability Sample (Study 9-1-SSS)
- 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 (in 25 fmol/uL 6 protein mix)
- a. 123 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 9-1-QC)
- a. Equimolar mixture of the 123 unlabeled and 123 labeled synthetic peptides in 25 fmol/uL 6 Protein mix matrix
  - i. Four 25 µL aliquot supplied at 10 fmol/µL each peptide in 0.1% formic acid in water. Background of 25 fmol/uL of 6 protein mix added to minimize adsorption/loss of hydrophobic peptides.

D. Digested human plasma spiked with labeled IS peptides only, (Sample 9-1-A)
- a. Depleted plasma diluted to a final concentration after digestion of approximately 0.5 µg/µL
  - i. 123 labeled IS peptides spiked at a concentration of 10 fmol/µL (Sample 9-1-A)
  - ii. Ten 25 µL aliquots supplied in 0.1 % formic acid in water (after desalting by SPE, note: desalting was already performed at Vanderbilt University)

E. Digested human depleted plasma spiked with 123 unlabeled synthetic peptides and 123 labeled IS peptides (Samples 9-1-B to 9-1-J)
- a. Depleted plasma diluted to a final concentration after digestion of ~ 0.5 µg/µL
  - i. 123 labeled IS peptides spiked at a concentration of 10 fmol/µL
  - ii. Four 25 µL aliquot of each spike level supplied for 4 singlicate curves
  - iii. 123 unlabeled synthetic peptides are spiked in at the following approximate concentrations:

Sample (Study 9-1)	Spiked [$^{12}C/^{14}N$] peptide Concentration (fmol/µL)
9-1-J	100.00
9-1-I	23.71
9-1-H	5.62
9-1-G	1.33
9-1-F	0.316
9-1-E	0.075
9-1-D	0.018
9-1-C	0.004
9-1-B	0.001

Important note: each of the 4 different singlicate calibration curves will be completed before the next replicate curve will be started: Specific naming including the first blank (A sample) is required, for details also see run order below:

9-1-A1 to 9-1-J1; 9-1-A2 to 9-1-J2; 9-1-A3 to 9-1-J3; 9-1-A4 to 9-1-J4.

F. **Six Blinded Samples to be run in four replicates in between each singlicate calibration curve** (Total: Samples 9-1-01Blinded to 9-1-24Blinded)

Four replicates of 6 Blinded samples (each provided in 25 aliquot):

first replicate:	9-1-01Blinded	to	9-1-06Blinded
second replicate:	9-1-07Blinded	to	9-1-12Blinded
third replicate:	9-1-13Blinded	to	9-1-18Blinded
fourth replicate:	9-1-19Blinded	to	9-1-24Blinded

mixed run order for the Blinded Samples within each replicate set

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

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

I. **PicoFrit columns**
   a. 75 µm ID with 10 µm ID tip
   b. For ABSciex and Thermo operators: Prepacked New Objective Reprosil Columns that were sent to all laboratories from Vanderbilt University (in case the operator runs out of prepacked columns please contact Susan Abbatiello or Birgit Schilling)
   c. For Waters UPLC operators: a Waters specific column will be used (1.7µm BEH130 C18, 75um X 150mm column)

J. **Loop for Eksigent LC:** 1 µL PEEKsil loop provided in sample kit, 100 µm ID PEEKsil; newer design 1 µL loops are provided for Eksigent Ultra systems.

K. **Fused silica**
   a. 20 µm ID between gradient pump and autosampler (for direct inject configuration)
   b. 20 µm ID between autosampler and PicoFrit column (keep tubing as short as possible)

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

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

N. **Autosampler loop**
   a. 1 µL loop for direct inject set-up (fused silica or PEEKsil)

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

Required Software

A. **Skyline Daily Software**, most recent version (please note version used)
B. **Platform-specific Skyline document** (*.sky), provided by CPTAC VWG
C. **Microsoft Excel** (or similar)
D. **AuDIT Software** (available at http://www.broadinstitute.org/cancer/software/genepattern/modules/AuDIT.html)

Overview

In Main Study 9-1 (9-point calibration curve), synthetic [$^{12}C/^{14}N$] and [$^{13}C/^{15}N$] signature peptides will be spiked into MARS-14 depleted, digested human K_2EDTA plasma and analyzed by scheduled LC-MRM-MS at all sites. All sample preparation will be performed at Vanderbilt University prior to distribution of the sample kits. Results from Study 9-1 from all CPTAC sites, will provide LOD/LOQ values for each peptide (this design contains minimal sample handling, and no target protein digestion and eliminates key factors of experimental variability). The Study 9-1 scheduled LC-MRM-MS samples will consist of a 9-point standard curve ranging in concentration from 100 fmol/µL to 1 amol/µL, with all concentrations in the presence of 0.5 µg/µL depleted digested plasma. Synthetic signature internal standard (IS) peptides uniformly labeled with a $^{13}C/^{15}N$ amino acid will be spiked into all plasma samples at a constant concentration of 10 fmol/µL.

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. 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 interspersed into Study 9-1 (to guarantee system suitability and performance and, in particular, monitor peak area stability and potential 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. Upon obtaining the specified results for retention time and peak area, sites may proceed to the generation of the scheduled LC-MRM-MS runs for Study 9.

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.

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

In preparation for the scheduled calibration curve LC-MRM-MS runs, in which 738 transitions will be monitored in one run, participating sites will monitor all heavy synthetic labeled peptides with about 369 transitions (123 peptides with 3 transitions each) in six unscheduled LC-MRM-MS runs (~60 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 run prior to analyzing the samples from which the calibration curve will be generated. All method building and data analysis will be performed using Skyline.

3. Data Analysis

The experimentally determined molar concentration of the spiked peptide or protein will be calculated and compared to its theoretical value for accuracy. Operators will import all acquired data files into Skyline and check and if necessary adjust peak integration. Check "Integrate All" (Menu, Settings, Integrate all), so that heavy and light 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. Specifically, check for interferences and RT drift problems during scheduling. 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 "AuDIT" to analyze data for interferences before submitting data to statisticians. Linear plots of response versus known concentration from each of the 9-point standard curves 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. Replicate analyses of the spiked plasma samples will provide estimates of assay precision (standard deviation and % CV), plus LOQ and LOD will be determined at defined signal-to-noise ratio (S/N) values. Blank runs of digested plasma with labeled peptides 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.

4. 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 the problem 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, low signal, and asymmetric peak shape. These problems can be caught and rectified without much down time and with minimal re-running of sample.

Procedures

1. **System Suitability Sample – Column Conditioning**
    a. Sample preparation
        i. Dilute SSS from 1 pmol/µL to 50 fmol/µL
            1. Add 95 µL of 0.1% formic acid/3% acetonitrile to an Eppendorf tube
            2. Add 5 µL of MichromMix (SSS) to the vial and vortex
            3. Centrifuge sample for 1 min in a benchtop centrifuge
            4. 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 AB Sciex 5500)
        ii. 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 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, 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
        iv. Column heater Settings
            1. If using a column heater, please set to 35 degrees Celsius.
        v. 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)
                b. ESI voltage: 1300-1400 V; Declustering voltage: -1 V; Capillary Temperature: 210 ˚C
            3. Waters Xevo
                c. 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

d. ESI voltage: 1700-1800 V; gas flow: 2.5 L/min; temperature: 325˚C; fragmentor voltage: 125 V

For all instruments:
Q1, Q3 resolution = unit
Dwell time = 10 msec
Interscan delay (where applicable) = 5 msec (3 msec for AB Sciex 5500)

c. Inject sample 5 times
d. Data Analysis
   i. Import data files into Skyline System Suitability Sample file
   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 (go to "Settings", and check "Integrate All" to enable this feature automatically)
      3. Document any peak that looks problematic by utilizing the "note" feature in Skyline
         a. Excessive tailing or fronting
         b. Drop-out of electrospray
         c. Poor peak shape
         d. Missing transitions
   iii. 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
   iv. If data do not pass criteria, troubleshoot the LC system and re-run SSS column conditioning procedure
   v. If necessary, contact CPTAC VWG members for advice or assistance
      1. Susan Abbatiello, susana@broadinstitute.org
      2. Birgit Schilling, bschilling@buckinstitute.org

2. 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. 369 transitions, 123 peptides, 123 precursors
      2. Q1, Q3 resolution = unit
      3. Dwell time = 10 msec (can be lower for AB Sciex 5500 instrument, minimum is 5 msec)
      4. Interscan delay (where applicable) = 5 msec (3 msec for AB Sciex 5500)
      5. Maximum number of concurrent transitions: 60
      6. Export transition list as multiple methods, ignore proteins

ii. Use the **Study 9-1** 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 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, 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 1.b.iv.)

c. Inject the sample 6 times, one time for each method created in Skyline
    i. Name each injection with its own filename/samplename so that each injection is its own, individual file
        1. Study9-1_SiteXX_HeavyOnly_method01
        2. Study9-1_SiteXX_HeavyOnly_method02
        3. Study9-1_SiteXX_HeavyOnly_method03
        4. Study9-1_SiteXX_HeavyOnly_method04
        5. Study9-1_SiteXX_HeavyOnly_method05
        6. Study9-1_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

d. Data Analysis
    i. Import data files into the appropriate Skyline Platform-specific Study 9-1 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 tailing or fronting
            b. Drop-out of electrospray
            c. Poor peak shape
            d. Missing transitions

iii. Under Peptide Settings, set window to 4 minutes
    1. Export the transition list in scheduled mode – heavy only, ~325 transitions with 4 min RT window. Double check that all 325 transitions are exported and added into the MS method appropriately.
    2. Run two replicates of Study 9-1 A and import into Skyline
    3. Ensure all peaks are detected
iv. Under Peptide Settings, set RT window to 2 minutes.
v. Under Edit->Refine, add light labeled peptides to the document
    1. There should now be 738 transitions, 123 peptides, 246 precursors
    2. View the RT plot for Scheduling
    3. Ensure the maximum number of concurrent transitions throughout the gradient is < 80
    4. If there are incidents of concurrent transitions > 80 anywhere in the gradient, contact CPTAC VWG members for assistance

e. Export all transitions (~750) in Scheduled mode, with 2 min RT window. Double check that all 738 transitions are exported and added into the MS method appropriately.
f. Acquire 4 replicates of sample Study9-1 A with the scheduled LC-MRM-MS method
    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.
g. Continue acquiring Study9-1 samples according to the sample chart shown below.

Important Note:
Different than in Study 7, the four replicate concentration points will be acquired in **4x singlicate curves** (with blanks and SSS in between to avoid carryover). Also note each singlicate concentration point was prepared individually at the central preparation site (Vanderbilt), so for each concentration point there will be 4 tubes supplied.

Adding Calibration Curve samples and Blinded Samples into Autosampler Vials:
Add one Calibration Curve and corresponding Blinded Samples at a time into the Autosampler. Each Calibration Curve concentration point tube contains 25 µl sample, for each singlicate curve remove the appropriate singlicate aliquot tube from the freezer, fill 12 µl into an autosampler vial, and store the remainder of that aliquot in the +4 degrees centigrade refrigerator (to avoid additional freeze-thaw cycles). 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 the saved other half of the specific singlicate aliquot that was transferred into the refrigerator as described above. For the 9-1-A sample used as blank (A-sample) at the beginning of a singlicate curve, and as blank in between blinded samples fill the entire A aliquot into an autosampler vial.

In Case of Acquisition Problems:
In case there are problems with the data acquisition or RT drift, and possible reruns that may become necessary, please keep 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 by LC-MRM-MS.

Blinded Samples:
Six blinded samples will be run with 1 wash run (10 fmol 6 protein mix) and 1 blank run (heavy peptides in plasma matrix, 9-1-A) in between. A total of 4 replicates of the 6 blinded samples will be acquired, one set of six in between each of the four singlicate calibration curves yielding a total of 24 blinded samples (Samples 9-1-01Blinded to 9-1-24Blinded). The wash runs use a different wash gradient **9-1-Wash** (see Table 2c below).
For Blinded Samples use Study 9-1 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, and for Blank Samples use Study 9-1 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.

Fresh Preparation of System Suitability samples (SSS) for each Singlicate Curve:
For each Singlicate Calibration Curve a fresh autosampler vial of the SSS will be prepared to prevent degradation of the sample (note in a notebook when fresh SSS are added).

Please adhere closely to sample naming as defined in the run order (with chronological runorder number at the end).

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

- **Study 9S** gradient for System Suitability Samples
- **Study 9-1** 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 (01-06blinded)

Run Number	Number of injections	Sample Description	Filename	Method	Notes
1	1	SSS, Michrom Mix	9-1_SiteX_SSS_run_001	Study 9S (SSS)	Column Conditioning
2	1	SSS, Michrom Mix	9-1_SiteX_SSS_run_002	Study 9S (SSS)	
3	1	SSS, Michrom Mix	9-1_SiteX_SSS_run_003	Study 9S (SSS)	
4	1	SSS, Michrom Mix	9-1_SiteX_SSS_run_004	Study 9S (SSS)	
5	1	SSS, Michrom Mix	9-1_SiteX_SSS_run_005	Study 9S (SSS)	
6	1	Study9-1 Heavy Only	9-1_SiteX_heavy_method01_run_006	Study 9 method 01	For scheduling
7	1	Study9-1 Heavy Only	9-1_SiteX_heavy_method02_run_007	Study 9 method 02	
8	1	Study9-1 Heavy Only	9-1_SiteX_heavy_method03_run_008	Study 9 method 03	
9	1	Study9-1 Heavy Only	9-1_SiteX_heavy_method04_run_009	Study 9 method 04	
10	1	Study9-1 Heavy Only	9-1_SiteX_heavy_method05_run 010	Study 9 method 05	
11	1	Study9-1 Heavy Only	9-1_SiteX_heavy_method06_run_011	Study 9 method 06	
12-16	5	SSS, Michrom Mix	9-1_SiteX_SSS_run_012 9-1_SiteX_SSS_run_013 9-1_SiteX_SSS_run_014 9-1_SiteX_SSS_run_015 9-1_SiteX_SSS_run_016	Study 9S (SSS)	At least 3 SSS samples and if necessary place holder
17	1	Study9-1 A, IS peptides in plasma	9-1_SiteX_A_sMRM_run_017	Study 9 scheduled MRM-MS, heavy only	Scheduled run, 4 min RT window
18	1	Study9-1 A, IS peptides in plasma	9-1_SiteX_A_sMRM_run_018	Study 9 scheduled MRM-MS, heavy only	
19	1	Study9-1 A1, IS peptides in plasma	9-1_SiteX_A1_CalCurve_run_019	Study 9 scheduled MRM-MS, light and heavy	Scheduled run, 2 min RT window, Calibration Curve
20	1	Study9-1 B1	9-1_SiteX_B1_CalCurve_run_020	Study 9 scheduled MRM-MS, light and heavy	
21	1	Study9-1 C1	9-1_SiteX_C1_CalCurve_run_021	Study 9 scheduled MRM-MS, light and heavy, “Study9 sMRM”	CalCurve
22	1	Study9-1 D1	9-1_SiteX_D1_CalCurve_run_022	Study9 sMRM	CalCurve
23	1	SSS	9-1_SiteX_SSS_run_023	Study 9S (SSS)	System Suitability
24	1	Study9-1 E1	9-1_SiteX_E1_CalCurve_run_024	Study9 sMRM	CalCurve

25	1	Study9-1 F1	9-1_SiteX_F1_CalCurve_run_025	Study9 sMRM	CalCurve
26	1	Study9-1 G1	9-1_SiteX_G1_CalCurve_run_026	Study9 sMRM	CalCurve
27	1	Study9-1 H1	9-1_SiteX_H1_CalCurve_run_027	Study9 sMRM	CalCurve
28	1	Study9-1 QC1	9-1_SiteX_QC1_CalCurve_run_028	Study9 sMRM	QC
29	1	Study9-1 I1	9-1_SiteX_I1_CalCurve_run_029	Study9 sMRM	CalCurve
30	1	Study9-1 J1	9-1_SiteX_J1_CalCurve_run_030	Study9 sMRM	CalCurve
31	1	Study9-1 wash	9-1_SiteX_wash_run_31	Study 9 wash	
32	1	SSS	9-1_SiteX_SSS_run_032	Study 9S (SSS)	System suitability
33	1	SSS	9-1_SiteX_SSS_run_033	Study 9S (SSS)	System suitability
34	1	SSS	9-1_SiteX_SSS_run_034	Study 9S (SSS)	System suitability
35	1	Study9-1 A, IS peptides in plasma	9-1_SiteX_A_blank_run_035	Study 9 scheduled MRM-MS, light and heavy	
36	1	Study9-1 01blinded-Sample	9-1_SiteX_01blinded_run_036	Study 9 scheduled MRM-MS, light and heavy	Blinded
37	1	Study9-1 wash	9-1_SiteX_wash_run_037	Study 9 wash	
38	1	IS peptides in plasma, blank	9-1_SiteX_A_blank _run_038	Study 9 scheduled MRM-MS, light and heavy	
39	1	Study9-1 02blinded	9-1_SiteX_02blinded_run_039	Study 9 scheduled MRM-MS, light and heavy	Blinded
40	1	Study9-1 wash	9-1_SiteX_wash_run_040	Study 9 wash	
41	1	IS peptides in plasma, blank	9-1_SiteX_A_blank _run_041	Study 9 scheduled MRM-MS, light and heavy	
42	1	SSS	9-1_SiteX_SSS_run_042	Study 9S (SSS)	System suitability
43	1	Study9-1 03blinded	9-1_SiteX_03blinded_run_043	Study 9 scheduled MRM-MS, light and heavy	Blinded
44	1	Study9-1 wash	9-1_SiteX_wash_run_044	Study 9 wash	

45	**1**	**IS peptides in plasma, blank**	**9-1_SiteX_A_blank _run_045**	**Study 9 scheduled MRM-MS, light and heavy**	
46	**1**	**Study9-1 04blinded**	**9-1_SiteX_04blinded_run_046**	**Study 9 scheduled MRM-MS, light and heavy**	**Blinded**
47	**1**	**Study9-1 wash**	**9-1_SiteX_wash_run_047**	**Study 9 wash**	
48	**1**	**IS peptides in plasma, blank**	**9-1_SiteX_A_blank _run_048**	**Study 9 scheduled MRM-MS, light and heavy**	
49	**1**	**SSS**	**9-1_SiteX_SSS_run_049**	**Study 9S (SSS)**	**System suitability**
50	**1**	**Study9-1 05blinded**	**9-1_SiteX_05blinded_run_050**	**Study 9 scheduled MRM-MS, light and heavy**	**Blinded**
51	**1**	**Study9-1 wash**	**9-1_SiteX_wash_run_051**	**Study 9 wash**	
52	**1**	**IS peptides in plasma, blank**	**9-1_SiteX_A_blank _run_052**	**Study 9 scheduled MRM-MS, light and heavy**	
53	**1**	**Study9-1 06blinded**	**9-1_SiteX_06blinded_run_053**	**Study 9 scheduled MRM-MS, light and heavy**	**Blinded**
54	**1**	**Study9-1 wash**	**9-1_SiteX_wash_run_054**	**Study 9 wash**	
55	**1**	**IS peptides in plasma, blank**	**9-1_SiteX_A_blank _run_055**	**Study 9 scheduled MRM-MS, light and heavy**	
			Run second, third and fourth singlicate curves (see below)		

Second singlicate curve plus blinded samples (07-12blinded)

56-58	3	SSS, Michrom Mix	9-1_SiteX_SSS_run_056 9-1_SiteX_SSS_run_057 9-1_SiteX_SSS_run_058	Study 9S (SSS)	3 SSS samples
59	1	Study9-1 A2, IS peptides in plasma	9-1_SiteX_A2_CalCurve_run_059	Study 9 scheduled MRM-MS, light and heavy	Scheduled run, 2 min RT window, Calibration Curve
60	1	Study9-1 B2	9-1_SiteX_B2_CalCurve_run_060	Study 9 scheduled MRM-MS, light and heavy	
61	1	Study9-1 C2	9-1_SiteX_C2_CalCurve_run_061	Study 9 scheduled MRM-MS, light and heavy, "Study9 sMRM"	CalCurve
62	1	Study9-1 D2	9-1_SiteX_D2_CalCurve_run_062	Study9 sMRM	CalCurve
63	1	SSS	9-1_SiteX_SSS_run_063	Study 9S (SSS)	System Suitability
64	1	Study9-1 E2	9-1_SiteX_E2_CalCurve_run_064	Study9 sMRM	CalCurve
65	1	Study9-1 F2	9-1_SiteX_F2_CalCurve_run_065	Study9 sMRM	CalCurve
66	1	Study9-1 G2	9-1_SiteX_G2_CalCurve_run_066	Study9 sMRM	CalCurve
67	1	Study9-1 H2	9-1_SiteX_H2_CalCurve_run_067	Study9 sMRM	CalCurve
68	1	Study9-1 QC2	9-1_SiteX_QC2_CalCurve_run_068	Study9 sMRM	QC
69	1	Study9-1 I2	9-1_SiteX_I2_CalCurve_run_069	Study9 sMRM	CalCurve
70	1	Study9-1 J2	9-1_SiteX_J2_CalCurve_run_070	Study9 sMRM	CalCurve
71	1	Study9-1 wash	9-1_SiteX_wash_run_071	Study 9 wash	
72	1	SSS	9-1_SiteX_SSS_run_072	Study 9S (SSS)	System suitability
73	1	SSS	9-1_SiteX_SSS_run_073	Study 9S (SSS)	System suitability
74	1	SSS	9-1_SiteX_SSS_run_074	Study 9S (SSS)	System suitability
75	1	Study9-1 A, IS peptides in plasma	9-1_SiteX_A_blank_run_075	Study 9 scheduled MRM-MS, light and heavy	
76	1	Study9-1 07blinded-Sample	9-1_SiteX_07blinded_run_076	Study 9 scheduled MRM-MS, light and heavy	Blinded
77	1	Study9-1 wash	9-1_SiteX_wash_run_077	Study 9 wash	

78	**1**	**IS peptides in plasma, blank**	**9-1_SiteX_A_blank _run_078**	**Study 9 scheduled MRM-MS, light and heavy**	
79	**1**	**Study9-1 08blinded**	**9-1_SiteX_08blinded_run_079**	**Study 9 scheduled MRM-MS, light and heavy**	**Blinded**
80	**1**	**Study9-1 wash**	**9-1_SiteX_wash_run_080**	**Study 9 wash**	
81	**1**	**IS peptides in plasma, blank**	**9-1_SiteX_A_blank _run_081**	**Study 9 scheduled MRM-MS, light and heavy**	
82	**1**	**SSS**	**9-1_SiteX_SSS_run_082**	**Study 9S (SSS)**	**System suitability**
83	**1**	**Study9-1 09blinded**	**9-1_SiteX_09blinded_run_083**	**Study 9 scheduled MRM-MS, light and heavy**	**Blinded**
84	**1**	**Study9-1 wash**	**9-1_SiteX_wash_run_084**	**Study 9 wash**	
85	**1**	**IS peptides in plasma, blank**	**9-1_SiteX_A_blank _run_085**	**Study 9 scheduled MRM-MS, light and heavy**	
86	**1**	**Study9-1 10blinded**	**9-1_SiteX_10blinded_run_086**	**Study 9 scheduled MRM-MS, light and heavy**	**Blinded**
87	**1**	**Study9-1 wash**	**9-1_SiteX_wash_run_087**	**Study 9 wash**	
88	**1**	**IS peptides in plasma, blank**	**9-1_SiteX_A_blank _run_088**	**Study 9 scheduled MRM-MS, light and heavy**	
89	**1**	**SSS**	**9-1_SiteX_SSS_run_089**	**Study 9S (SSS)**	**System suitability**
90	**1**	**Study9-1 11blinded**	**9-1_SiteX_11blinded_run_090**	**Study 9 scheduled MRM-MS, light and heavy**	**Blinded**
91	**1**	**Study9-1 wash**	**9-1_SiteX_wash_run_091**	**Study 9 wash**	
92	**1**	**IS peptides in plasma, blank**	**9-1_SiteX_A_blank _run_092**	**Study 9 scheduled MRM-MS, light and heavy**	

93	1	Study9-1 12blinded	9-1_SiteX_12blinded_run_093	Study 9 scheduled MRM-MS, light and heavy	Blinded
94	1	Study9-1 wash	9-1_SiteX_wash_run_094	Study 9 wash	
95	1	IS peptides in plasma, blank	9-1_SiteX_A_blank _run_095	Study 9 scheduled MRM-MS, light and heavy	
			Run third and fourth singlicate curves (see below)		

Third singlicate curve plus blinded samples (13-18blinded)

96-98	3	SSS, Michrom Mix	9-1_SiteX_SSS_run_096 9-1_SiteX_SSS_run_097 9-1_SiteX_SSS_run_098	Study 9S (SSS)	3 SSS samples
99	1	Study9-1 A3, IS peptides in plasma	9-1_SiteX_A3_CalCurve_run_099	Study 9 scheduled MRM-MS, light and heavy	Scheduled run, 2 min RT window, Calibration Curve
100	1	Study9-1 B3	9-1_SiteX_B3_CalCurve_run_100	Study 9 scheduled MRM-MS, light and heavy	
101	1	Study9-1 C3	9-1_SiteX_C3_CalCurve_run_101	Study 9 scheduled MRM-MS, light and heavy, "Study9 sMRM"	CalCurve
102	1	Study9-1 D3	9-1_SiteX_D3_CalCurve_run_102	Study9 sMRM	CalCurve
103	1	SSS	9-1_SiteX_SSS_run_103	Study 9S (SSS)	System Suitability
104	1	Study9-1 E3	9-1_SiteX_E3_CalCurve_run_104	Study9 sMRM	CalCurve
105	1	Study9-1 F3	9-1_SiteX_F3_CalCurve_run_105	Study9 sMRM	CalCurve
106	1	Study9-1 G3	9-1_SiteX_G3_CalCurve_run_106	Study9 sMRM	CalCurve
107	1	Study9-1 H3	9-1_SiteX_H3_CalCurve_run_107	Study9 sMRM	CalCurve
108	1	Study9-1 QC3	9-1_SiteX_QC3_CalCurve_run_108	Study9 sMRM	QC
109	1	Study9-1 I3	9-1_SiteX_I3_CalCurve_run_109	Study9 sMRM	CalCurve
110	1	Study9-1 J3	9-1_SiteX_J3_CalCurve_run_110	Study9 sMRM	CalCurve

111	**1**	**Study9-1 wash**	**9-1_SiteX_wash_run_111**	**Study 9 wash**	
112	**1**	**SSS**	**9-1_SiteX_SSS_run_112**	**Study 9S (SSS)**	**System suitability**
113	**1**	**SSS**	**9-1_SiteX_SSS_run_113**	**Study 9S (SSS)**	**System suitability**
114	**1**	**SSS**	**9-1_SiteX_SSS_run_114**	**Study 9S (SSS)**	**System suitability**
115	**1**	**Study9-1 A, IS peptides in plasma**	**9-1_SiteX_A_blank_run_115**	**Study 9 scheduled MRM-MS, light and heavy**	
116	**1**	**Study9-1 13blinded-Sample**	**9-1_SiteX_13blinded_run_116**	**Study 9 scheduled MRM-MS, light and heavy**	**Blinded**
117	**1**	**Study9-1 wash**	**9-1_SiteX_wash_run_117**	**Study 9 wash**	
118	**1**	**IS peptides in plasma, blank**	**9-1_SiteX_A_blank _run_118**	**Study 9 scheduled MRM-MS, light and heavy**	
119	**1**	**Study9-1 14blinded**	**9-1_SiteX_14blinded_run_119**	**Study 9 scheduled MRM-MS, light and heavy**	**Blinded**
120	**1**	**Study9-1 wash**	**9-1_SiteX_wash_run_120**	**Study 9 wash**	
121	**1**	**IS peptides in plasma, blank**	**9-1_SiteX_A_blank _run_121**	**Study 9 scheduled MRM-MS, light and heavy**	
122	**1**	**SSS**	**9-1_SiteX_SSS_run_122**	**Study 9S (SSS)**	**System suitability**
123	**1**	**Study9-1 15blinded**	**9-1_SiteX_15blinded_run_123**	**Study 9 scheduled MRM-MS, light and heavy**	**Blinded**
124	**1**	**Study9-1 wash**	**9-1_SiteX_wash_run_124**	**Study 9 wash**	
125	**1**	**IS peptides in plasma, blank**	**9-1_SiteX_A_blank _run_125**	**Study 9 scheduled MRM-MS, light and heavy**	
126	**1**	**Study9-1 16blinded**	**9-1_SiteX_16blinded_run_126**	**Study 9 scheduled MRM-MS, light**	**Blinded**

				and heavy	
127	1	Study9-1 wash	9-1_SiteX_wash_run_127	Study 9 wash	
128	1	IS peptides in plasma, blank	9-1_SiteX_A_blank _run_128	Study 9 scheduled MRM-MS, light and heavy	
129	1	SSS	9-1_SiteX_SSS_run_129	Study 9S (SSS)	System suitability
130	1	Study9-1 17blinded	9-1_SiteX_17blinded_run_130	Study 9 scheduled MRM-MS, light and heavy	Blinded
131	1	Study9-1 wash	9-1_SiteX_wash_run_131	Study 9 wash	
132	1	IS peptides in plasma, blank	9-1_SiteX_A_blank _run_132	Study 9 scheduled MRM-MS, light and heavy	
133	1	Study9-1 18blinded	9-1_SiteX_18blinded_run_133	Study 9 scheduled MRM-MS, light and heavy	Blinded
134	1	Study9-1 wash	9-1_SiteX_wash_run_134	Study 9 wash	
135	1	IS peptides in plasma, blank	9-1_SiteX_A_blank _run_135	Study 9 scheduled MRM-MS, light and heavy	
			Run fourth singlicate curve (see below)		

Fourth singlicate curve plus blinded samples (19-24blinded)

136-138	3	SSS, Michrom Mix	9-1_SiteX_SSS_run_136 9-1_SiteX_SSS_run_137 9-1_SiteX_SSS_run_138	Study 9S (SSS)	3 SSS samples
139	1	Study9-1 A4, IS peptides in plasma	9-1_SiteX_A4_CalCurve_run_139	Study 9 scheduled MRM-MS, light and heavy	Scheduled run, 2 min RT window, Calibration Curve
140	1	Study9-1 B4	9-1_SiteX_B4_CalCurve_run_140	Study 9 scheduled MRM-MS, light and heavy	

141	1	Study9-1 C4	9-1_SiteX_C4_CalCurve_run_141	Study 9 scheduled MRM-MS, light and heavy, "Study9 sMRM"	CalCurve
142	1	Study9-1 D4	9-1_SiteX_D4_CalCurve_run_142	Study9 sMRM	CalCurve
143	1	SSS	9-1_SiteX_SSS_run_143	Study 9S (SSS)	System Suitability
144	1	Study9-1 E4	9-1_SiteX_E4_CalCurve_run_144	Study9 sMRM	CalCurve
145	1	Study9-1 F4	9-1_SiteX_F4_CalCurve_run_145	Study9 sMRM	CalCurve
146	1	Study9-1 G4	9-1_SiteX_G4_CalCurve_run_146	Study9 sMRM	CalCurve
147	1	Study9-1 H4	9-1_SiteX_H4_CalCurve_run_147	Study9 sMRM	CalCurve
148	1	Study9-1 QC4	9-1_SiteX_QC4_CalCurve_run_148	Study9 sMRM	QC
149	1	Study9-1 I4	9-1_SiteX_I4_CalCurve_run_149	Study9 sMRM	CalCurve
150	1	Study9-1 J4	9-1_SiteX_J4_CalCurve_run_150	Study9 sMRM	CalCurve
151	1	Study9-1 wash	9-1_SiteX_wash_run_151	Study 9 wash	
152	1	SSS	9-1_SiteX_SSS_run_152	Study 9S (SSS)	System suitability
153	1	SSS	9-1_SiteX_SSS_run_153	Study 9S (SSS)	System suitability
154	1	SSS	9-1_SiteX_SSS_run_154	Study 9S (SSS)	System suitability
155	1	Study9-1 A, IS peptides in plasma	9-1_SiteX_A_blank_run_155	Study 9 scheduled MRM-MS, light and heavy	
156	1	Study9-1 19blinded-Sample	9-1_SiteX_19blinded_run_156	Study 9 scheduled MRM-MS, light and heavy	Blinded
157	1	Study9-1 wash	9-1_SiteX_wash_run_157	Study 9 wash	
158	1	IS peptides in plasma, blank	9-1_SiteX_A_blank _run_158	Study 9 scheduled MRM-MS, light and heavy	
159	1	Study9-1 20blinded	9-1_SiteX_20blinded_run_159	Study 9 scheduled MRM-MS, light and heavy	Blinded
160	1	Study9-1 wash	9-1_SiteX_wash_run_160	Study 9 wash	
161	1	IS peptides in plasma, blank	9-1_SiteX_A_blank _run_161	Study 9 scheduled	

				MRM-MS, light and heavy	
162	1	SSS	9-1_SiteX_SSS_run_162	Study 9S (SSS)	System suitability
163	1	Study9-1 21blinded	9-1_SiteX_21blinded_run_163	Study 9 scheduled MRM-MS, light and heavy	Blinded
164	1	Study9-1 wash	9-1_SiteX_wash_run_164	Study 9 wash	
165	1	IS peptides in plasma, blank	9-1_SiteX_A_blank _run_165	Study 9 scheduled MRM-MS, light and heavy	
166	1	Study9-1 22blinded	9-1_SiteX_22blinded_run_166	Study 9 scheduled MRM-MS, light and heavy	Blinded
167	1	Study9-1 wash	9-1_SiteX_wash_run_167	Study 9 wash	
168	1	IS peptides in plasma, blank	9-1_SiteX_A_blank _run_168	Study 9 scheduled MRM-MS, light and heavy	
169	1	SSS	9-1_SiteX_SSS_run_169	Study 9S (SSS)	System suitability
170	1	Study9-1 23blinded	9-1_SiteX_23blinded_run_170	Study 9 scheduled MRM-MS, light and heavy	Blinded
171	1	Study9-1 wash	9-1_SiteX_wash_run_171	Study 9 wash	
172	1	IS peptides in plasma, blank	9-1_SiteX_A_blank _run_172	Study 9 scheduled MRM-MS, light and heavy	
173	1	Study9-1 24blinded	9-1_SiteX_24blinded_run_173	Study 9 scheduled MRM-MS, light and heavy	Blinded
174	1	Study9-1 wash	9-1_SiteX_wash_run_174	Study 9 wash	
175	1	IS peptides in plasma, blank	9-1_SiteX_A_blank _run_175	Study 9 scheduled MRM-MS, light and heavy	
			Study 9-1finished !!		

1. **Data Analysis**
    a. All data should be analyzed in "real time," or as close to its acquisition time as possible
    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 and heavy 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
    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 should both be imported into Skyline, and "custom annotation" capabilities of Skyline should be used to annotate what data points should be used for statistical analysis.

4. **Trouble Shooting Section**
    a. **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?

b. 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 9-1_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 (738 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).

c. **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 Study9-1_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 9-1:

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: DIRECT INJECTION
- Sample Loop: 1 µL sample loop
- Columns for AB 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, 75um X 150mm 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 9-1 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 9-1 calibration curve (Study 9-1 gradient) vs. system suitability samples (Study 9S gradient) vs. Wash gradient used in between blinded samples (Study 9 Wash gradient).

Table 2a. 9-1 HPLC gradient for all analyses of 123 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 9-1-A through 9-1-J, and 9-1-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 9-1 HPLC gradient for all analyses of 123 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