

# STANDARD OPERATING PROCEDURE

**Title: Human Plasma Assay Validation on Agilent 6490.**

**Version #: 1**

**Author: UVic GBC Proteomics Centre**

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## Purpose

The purpose of this document is to describe the characterization of a set of assays by response curve (reverse curve version) and repeatability.

## Scope

This procedure covers overall preparation and running of samples for generating the response curve and repeatability validation samples.

## Responsibilities

It is the responsibility of person(s) performing this procedure to be familiar with laboratory safety procedures. The interpretation of results must be done by a person trained in the procedure and familiar with such interpretation.

## Equipment

- Pipettes
- 37°C dry air incubator
- Vortex mixer
- Microcentrifuge
- Balance
- Vacuum manifold
- 1L graduated cylinder
- Agilent 6490
- Skyline software

## Materials

- Worthington Trypsin-TPCK treated (Worthington, LS003744)
- Urea (Sigma, U0631)
- DL-Dithiothreitol (DTT) (Sigma, 43815)
- Iodoacetamide (IAM), (Sigma, I1149)
- Trizma base (Sigma, T6791)
- Sodium Deoxycholate (Sigma, D6750)
- Water, LC-MS grade (Sigma, 39253)
- Acetonitrile, LC-MS grade (Sigma, 34967)
- Methanol, LC-MS grade (Sigma, 34966)
- Formic Acid, LC-MS grade (Sigma, 56302)
- 1.5mL MAXYMum Recovery tubes (Corning Axygen, MCT-150-L-C)
- Falcon 15mL conical centrifuge tubes (Corning, 352096)
- Falcon 50mL conical centrifuge tubes (Corning, 352070)
- 1.5mL screw cap microtubes (Agilent, 5188-5251)
- Oasis HLB 1cc extraction cartridge (10mg) (Waters, 186000383)
- AlumaSeal II foil seals (Fisher, 361006023)
- 300μL PP screw cap vials (Canadian Life Sciences, V0309P-1232)
- Human plasma
- Heavy stable isotope-labeled standards (SIS) and matched light versions were synthesized and purified by HPLC. Heavy peptides incorporated a fully atom labeled <sup>13</sup>C and <sup>15</sup>N isotope at the C-terminal lysine (K) or arginine (R) position of each (tryptic) peptide, resulting in a mass shift of +8 or +10 Da, respectively. Peptides were quantified by amino acid analysis and aliquots were stored in 30% acetonitrile, 0.1% formic acid at -80°C until use.

## Solutions

- 1M Tris, pH 8.0. May be made in advance and stored at 4°C:
  - Add 12.12g solid Trizma base to a 250mL beaker.
  - Add 50mL LC-MS water and mix until dissolved.
  - Adjust pH to 8.0 with concentrated HCl (12M).
  - Transfer solution to 100mL graduated cylinder and bring volume to 100mL with LC-MS water.
- 9M Urea, 300mM Tris, pH 8.0. Must be made fresh daily:
  - Add 2.7g Urea to a 50mL Falcon tube.
  - Add 1.25mL LC-MS water and 1.25mL 1M Tris, pH 8.0 and mix until dissolved.
  - Measure pH and bring to 8.0 if necessary.
  - Bring volume to 5mL with LC-MS water.

- 9M Urea, 300mM Tris, pH 8.0, 20mM DTT. Must be made fresh daily:
  - Dissolve 38.5mg DTT in 500 $\mu$ L 9M Urea, 300mM Tris, pH 8.0 solution (above).
  - Transfer 125 $\mu$ L to 15mL Falcon tube and add 3.0mL 9M Urea, 300mM Tris, pH 8.0m 20mM DTT (above).
  - Mix well.
- 500mM Iodoacetamide (IAM). Prepare immediately before use and keep out of light:
  - Dissolve 56mg IAM in 605 $\mu$ L LC-MS water.
  - Mix well.
- 100mM Tris, pH 8.0:
  - 1 part 1M Tris, pH 8.0 (above).
  - 9 parts LC-MS water.
- 1mg/mL Worthington TPCK- Trypsin. Prepare immediately before use:
  - Dissolve 4mg trypsin in 4mL LC-MS water.
- 6.1 % Formic Acid:
  - Add 305 $\mu$ L Formic acid to 4.695mL LC-MS water.
- 1%(w/v) Sodium Deoxycholate (NaDOC):
  - Add 1g NaDOC to 100mL LC-MS water.
- 50% Acetonitrile, 0.1% Formic Acid:
  - Add 500 $\mu$ L Acetonitrile to 500 $\mu$ L LC-MS water.
  - Add 1 $\mu$ L Formic Acid.
- 30% Acetonitrile, 0.1% Formic Acid:
  - Add 3mL Acetonitrile to 7mL LC-MS water.
  - Add 10 $\mu$ L Formic Acid.
- 0.1% Formic Acid:
  - Add 10 $\mu$ L Formic Acid to 9990 $\mu$ L LC-MS water.

## Procedure

### Preparation of 1% NaDOC-coated tubes and tips

1. Tubes:
  - a. Add 500 $\mu$ L 1% NaDOC to 1.5mL MAXYMum Recovery tubes and vortex to coat.
  - b. To wash, add 500 $\mu$ L LC-MS water, and vortex. Repeat 3X discarding water between washes.
  - c. Lyophilize to dryness.
2. Tips:
  - a. Aspirate/dispense maximum tip volume of 1% NaDOC 3X using multichannel pipette.
  - b. Wash with maximum tip volume of LC-MS water. Repeat 3X discarding water between washes.
  - c. Lyophilize to dryness.

## Matrix Digestion (Experiment 1 & 2)

### Preparation of Samples

3. Prepare 12 replicates (5 for Assay Validation Experiment 1 and 7 for Assay Validation Experiment 2).
4. Dilute human plasma to 14 $\mu$ g/ $\mu$ L with 100mM Tris, pH 8.0 (estimate 70  $\mu$ g/ $\mu$ L human plasma protein concentration).
5. Add 50 $\mu$ L 9M Urea, 300mM Tris, pH 8.0, 20mM DTT to a 1.5mL MAXYMum Recovery tube.
6. Add 25 $\mu$ L diluted plasma.
7. Denature and reduce at 37°C for 30min.
8. Add 6.5 $\mu$ L 500mM IAM.
9. Alkylate at room temperature for 30min in the dark.
10. Add 737 $\mu$ L 100mM Tris, pH 8.0 to decrease urea concentration to 0.55M.
11. Add 17.5 $\mu$ L of 1 $\mu$ g/ $\mu$ L Worthington Trypsin (20:1 substrate:enzyme ratio).
12. Digest at 37°C for 18hrs.
13. Add 164 $\mu$ L of 6.1% Formic Acid to quench digestion.
14. Total volume post digest = 1000 $\mu$ L, plasma protein concentration = 0.35 $\mu$ g/ $\mu$ L
15. The digest replicates prepared for Experiment 2 can be stored at -80°C until required.

## Experiment 1- Response Curves

### Heavy SIS spike preparation

16. Prepare a 10pmol/ $\mu$ L equimolar mix of SIS peptides with 0.1% Formic Acid.
17. Prepare serially diluted SIS peptide mix using 1% NaDOC-coated tubes and tips (table below).

	Std L 10pmol/uL	Std K 1pmol/uL	Std J 100fmol/uL	Std I 50fmol/uL	Std H 10fmol/uL	Std G 5fmol/uL	Std F 1fmol/uL	Std E 0.5fmol/uL	Std D 100amol/uL	Std C 50amol/uL	Std B 25amol/uL	Std A 12.5amol/uL
Std L	stock	10uL										
Std K			10uL									
Std J				25uL								
Std I					14uL							
Std H						25uL						
Std G							14uL					
Std F								25uL				
Std E									14uL			
Std D										25uL		
Std C											25uL	
Std B												25uL
0.1%FA		90	90	25	56	25	56	25	56	25	25	25
Dilution	1	10	10	2	5	2	5	2	5	2	2	2

### Light spike preparation

18. Prepare a 10pmol/ $\mu$ L equimolar mix of Light peptides in 0.1% Formic Acid.
19. Dilute stock Light peptide mix to 100fmol/ $\mu$ L in 0.1% Formic Acid using 1% NaDOC-coated tubes and tips.

### Standard Curve preparation

20. To 12 x 1.5mL uncoated MAXYMum Recovery tubes add:
  - a. 220 $\mu$ L plasma digest
  - b. 15.4 $\mu$ L of 100fmol/ $\mu$ L Light peptide mix
  - c. 15.4 $\mu$ L of level specific SIS peptide mix

### Double blank preparation

21. To an uncoated MAXYMum Recovery tube add:
  - a. 660 $\mu$ L plasma digest
  - b. 92.4  $\mu$ L 0.1% Formic Acid

### SIS+Matrix blank preparation (to estimate endogenous concentration)

22. To an uncoated 1.5mL MAXYMum Recovery tube add:
  - a. 220 $\mu$ L plasma digest
  - b. 15.4 $\mu$ L of 0.1% Formic Acid
  - c. 15.4 $\mu$ L of 100fmol/ $\mu$ L SIS peptide mix

### Desalting and Concentrating Samples Offline by Solid Phase Extraction

23. Set the negative pressure system manifold to -25 kPa.
24. Condition cartridge with 1mL of Methanol.
25. Equilibrate cartridge with 1mL of 0.1% Formic Acid.
26. Add sample to cartridge:
  - a. Response curve & SIS+Matrix blank: 772 $\mu$ L 0.1% Formic Acid then 228 $\mu$ L sample.
  - b. Double blank: 316 $\mu$ L 0.1% Formic Acid then 684 $\mu$ L sample.
27. Wash cartridge with 3 x 1mL of 100% LC-MS water.
28. Elute peptides with 300 $\mu$ L 50% Acetonitrile, 0.1% Formic Acid into a 1.5mL MAXYMum Recovery tube.
29. Freeze eluates using liquid nitrogen, and poke a hole in the lid.
30. Lyophilize samples to dryness overnight.
31. Seal using AlumaSeal II foil. Samples can be stored lyophilized at -80°C until ready for SRM analysis.

### Reconstituting Samples (To be performed just prior to LC/SRM-MS analysis)

32. Reconstitute dried and desalted digests with:
  - a. Std Curve & SIS+Matrix blank: 70 $\mu$ L of 0.1% Formic Acid to each sample (1 $\mu$ g/ $\mu$ L plasma digest).
  - b. Double blank: 210 $\mu$ L of 0.1% Formic Acid (1 $\mu$ g/ $\mu$ L plasma digest).

33. Vortex sample, spin down, and transfer to an autosampler vial for LC/SRM-MS analysis.

## Experiment 2- Mini-Validation of Repeatability

### Heavy SIS spike preparation

1. Prepare a SIS mix 1000X the Lowest point on curve (LPOC) of each peptide in 0.1% Formic Acid, calculated from Experiment 1 data.
2. Prepare serially diluted SIS peptide mix in 0.1% Formic Acid using 1% NaDOC-coated tubes and tips :
  - a. Low SIS spike: 3X LPOC calculated from Experiment 1 data (referred to as 3XLow).
  - b. Medium SIS spike: 50X LPOC calculated from Experiment 1 data (referred to as 50XMedium).
  - c. High SIS spike: 500X LPOC calculated from Experiment 1 data (referred to as 500XHigh).

	L1000X 100pmol/μL	500XHigh 50pmol/μL	50XMedium 5pmol/μL	3XLow 0.3pmol/μL
1000X	stock	20		
500XHigh			7	
50XMedium				12
3XLow				
0.1%FA		20	63	188
Dilution	1	2	10	16.7

### Standard Curve preparation

3. On each of 5 different calendar days:
  - a. To 3 x 1.5mL uncoated MAXYMum Recovery tubes add:
    - i. 100μL plasma digest
    - ii. 7μL of 100fmol/μL Light peptide mix (prepared in Experiment 1)
    - iii. 7μL of 3XLow SIS peptide mix
  - b. To 3 x 1.5mL uncoated MAXYMum Recovery tubes add:
    - i. 100μL plasma digest
    - ii. 7μL of 100fmol/μL Light peptide mix (prepared in Experiment 1)
    - iii. 7μL of 50XMedium SIS peptide mix
  - c. To 3 x 1.5mL uncoated MAXYMum Recovery tubes add:
    - i. 100μL plasma digest
    - ii. 7μL of 100fmol/μL Light peptide mix (prepared in Experiment 1)
    - iii. 7μL of 500XHigh SIS peptide mix

### Double blank preparation

36. On each of 5 different calendar days:
  - a. To an uncoated 1.5mL MAXYMum Recovery tube add:
    - i. 300 $\mu$ L plasma digest
    - ii. 42 $\mu$ L of 0.1% Formic Acid

### Desalting and Concentrating Samples Offline by Solid Phase Extraction

37. Set the negative pressure system manifold to -25 kPa.
38. Condition cartridge with 1mL of Methanol.
39. Equilibrate cartridge with 1mL of 0.1% Formic Acid.
40. Add sample to cartridge:
  - a. Response curve: 912 $\mu$ L 0.1% Formic Acid then 88 $\mu$ L sample.
  - b. Double blank: 674 $\mu$ L 0.1% Formic Acid then 326 $\mu$ L sample.
41. Wash cartridge with 3 x 1mL of 100% LC-MS water.
42. Elute peptides with 300 $\mu$ L 50% Acetonitrile, 0.1% Formic Acid into a 1.5mL MAXYMum Recovery tube.
43. Freeze eluates using liquid nitrogen, and poke a hole in the lid.
44. Lyophilize samples to dryness overnight.
45. Seal using AlumaSeal II foil. Samples can be stored lyophilized at -80°C until ready for SRM analysis.

### Reconstituting Samples (To be performed just prior to LC/SRM-MS analysis)

46. Reconstitute dried and desalted spiked digests with:
  - a. Response curve: 27 $\mu$ L of 0.1% Formic Acid to each sample (1 $\mu$ g/ $\mu$ L plasma digest).
  - b. Double blanks: 100 $\mu$ L of 0.1% Formic Acid (1 $\mu$ g/ $\mu$ L plasma digest).
47. Vortex sample, spin down, and transfer to an autosampler vial for LC/SRM-MS analysis.

### LC and MS Setup (Experiment 1 & 2)

#### LC Setup:

1. Top up both A and B buffers being careful to keep the solvent delivery filters submerged.
2. Purge and flush pumps at 50% A for 5min and 10min respectively if LC has not been recently used.
3. Bring column to start conditions and confirm that pressure is between 400-600bar.

### LC Parameters:

4. Flowrate: 0.4mL/min
5. Column temp: 50°C
6. Gradient:

Time (min)	% Buffer A	% Buffer B
0	98	2
2	93	7
50	70	30
53	55	45
53.5	20	80
55.5	20	80
56	98	2

+ 4min post time

### Agilent 6490 Setup:

7. Perform checktune

### Agilent 6490 SRM Parameters:

8. Transitions:
  - a. Monitor 5 transitions for each light and SIS peptide.
  - b. MS1 and MS2 Resolution: unit
  - c. Fragmentor: 380V
  - d. Cell Accelerator Voltage: 5
9. Dynamic MRM Parameters:
  - a. Cycle time: 850msec
  - b. Min Dwell: ≥10msec
10. Source parameters:
  - a. Gas Temp: 150°C
  - b. Gas flow: 15L/min
  - c. Nebulizer: 30psi
  - d. Sheath Gas Temp: 250°C
  - e. Sheath Gas Flow: 11L/min
  - f. Positive Capillary: 3500V
  - g. Nozzle Voltage: 300V

### Naming of Data Files:

11. Experiment 1:
  - a. Curve levels should be named StdA to StdL, with StdA being the least concentrated sample and StdL being the most concentrated sample. StdA can also be the double blank sample if one is being used.
  - b. Blanks should be named MatrixBlank\_01



- c. Example: Date\_Project\_AssayVal\_Exp1\_StdA\_01
- 12. Experiment 2:
  - a. Curve levels should be named 3XLow, 50XMedium and 500XHigh
  - b. Sample prep replicates should be named R01 to R03
  - c. Injections should be dated
  - d. Example: Date\_Project\_AssayVal\_Exp2\_3XLow\_R01

**Sample injection:**

- 13. Injection volume will be 10 $\mu$ L for all samples.
- 14. Experiment 1:
  - a. A double blank will be injected 5 x prior to the curves to estimate noise.
  - b. Inject samples in singlicate from low concentration to high concentration.
  - c. Three blanks will be injected between curves for carryover.
- 15. Experiment 2:
  - a. Inject samples from low to high concentration.
  - b. Each of the 3 sample prep replicates for the 3XLow, 50XMedium and 500XHigh samples will be injected 1x (for a total of 3 injections per level) on each of 5 different calendar days.
  - c. A double blank will be injected between the 500XHigh and 3XLow samples for carryover.

**Data Analysis (Experiment 1 & 2)**

**Quantitation of Data using Skyline:**

1. Create a new Skyline method
2. Set Internal Standard to light (Settings>Peptide Setting>Modifications)
3. Import proteins, peptide sequences, and transitions from Excel
4. Add protein accession to protein note (View>Document Grid> Proteins)
5. Save as..... validation curve.sky
6. Change Internal Standard to Heavy (Settings>Peptide Setting>Modifications)
7. Save as.... single point.sky
8. Import single point files
9. Inspect and correct integration
10. Export CSV
11. Take PAR X200 to calculate amount in single point measurement
12. Save an close single point skyline file
13. Open .....validation curve.sky file
14. Import data files (File>Import>Result)
15. Set Skyline to integrate all measured transitions (Settings>Integrate All)
16. Add Sample Type and IS (Single point method) concentration (View>Document Grid>Replicates)
17. Inspect and correct integration if necessary
18. Save and upload to Panorama

## Referenced Documents

