1. Materials for Reference Kit #1

1.1. Chemicals and Reagents

1.1.1. Pre-digested human plasma spiked with SIS peptides (7 vials, 98 µL each; UVic – Genome BC Proteomics Centre; Victoria, BC, Canada)

1.1.2. Formic acid (FA; Sigma Aldrich; St. Louis, MO, USA; part no. 56302)

1.1.3. LC/MS water (Sigma-Aldrich; part no. 39253)

1.1.4. LC/MS acetonitrile (ACN; Sigma-Aldrich; part no. 34967)

2. Materials for Reference Kit #2

2.1. Chemicals and Reagents

2.1.1. Human plasma (1 vial, 40 µL; UVic – Genome BC Proteomics Centre; Victoria, BC, Canada; see **Note 1**)

2.1.2. Ammonium bicarbonate (Sigma Aldrich; part no. A6141)

2.1.3. Sodium deoxycholate (Sigma Aldrich; part no. D6750)

2.1.4. Tris(2-carboxyethyl)phosphine (TCEP; Thermo Scientific; Rockford, Il, USA; catalogue no. 77720)

2.1.5. Iodoacetamide (Sigma Aldrich; part no. 57670)

2.1.6. Dithiothreitol (DTT; Sigma Aldrich; part no. 43815)

2.1.7. Trypsin (2 vials, 100 µL each; UVic – Genome BC Proteomics Centre; see **Note 2**)

2.1.8. SIS peptide mixture (1 vial, 110 µL at 250 fmol/µL; UVic – Genome BC Proteomics Centre; see **Note 2**)

2.1.9. Formic acid (Sigma Aldrich)

2.1.10. LC/MS water (Sigma-Aldrich)

2.1.11. LC/MS acetonitrile (Sigma-Aldrich)

2.1.12. Methanol (Sigma-Aldrich; part no. 34860)

2.2. Sample Preparation Products

2.2.1. Ultra-Low Temperature Upright Freezer (Thermo Scientific; Forma 900 series; model no. 995 or the equivalent, capable of storing samples at -80ºC)

2.2.2. Vortex mixer (Thermo Scientific; catalogue no. 02-215-365 or the equivalent)

2.2.3. Minicentrifuge (Thermo Scientific; catalogue no. 05-090-100 or the equivalent, capable of achieving a relative centrifugal force of 2,200 x g)

2.2.4. Incubators (Barnstead Lab-Line; Melrose Park, IL, USA; model 120 for 60°C applications and Binder; Tuttlingen, Germany; model BD23 for 30°C applications)

2.2.5. Non-refrigerated bench-top centrifuge (Eppendorf; Hamburg, Germany; model 5415D or the equivalent, capable of achieving a relative centrifugal force of 12,000 x g)

2.2.6. 10 mg Oasis HLB cartridges for solid phase extractions (Waters; Milford, MA, USA; part no. 186000383)

2.2.7. PrepSep 12-port vacuum manifold for the extractions (Thermo Scientific; catalogue no. 60104-232)

2.2.8. Lyophilizer and accessories (Thermo Scientific; SuperModulyo freeze dryer; catalogue no. SuperModulyo220 and 16-port drum manifold with wide-mouth borosilicate glass flasks; catalogue no. F056 28 000 and F056 57 000)

3. Instrument Platform for Standardization

3.1. Standard-flow UHPLC-MRM/MS on Agilent's 6490

3.1.1. 1290 Infinity UHPLC system (Agilent Technologies; Santa Clara, CA, USA)

3.1.2. Reversed-phase UHPLC column (Agilent Technologies; part no. 959759-902; 150 x 2.1 mm i.d., Zorbax RRHD Eclipse Plus C_{18}, 1.8 μm particles)

3.1.3. LC gradient mobile phase A: 0.1% v/v FA

3.1.4. LC gradient mobile phase B: 90% v/v ACN with 0.1% v/v FA

3.1.5. Standard-flow ESI source (Agilent Technologies)

3.1.6. 6490 triple quadrupole mass spectrometer (Agilent Technologies)

3.1.7. MassHunter Quantitative Analysis software (Agilent Technologies)

4. Methods

The methods described in this section outline the specific steps required to prepare the human plasma samples for accreditating the mass spectrometer (Kit #1), as well as the entire bottom-up proteomic workflow (Kit #2).

4.1. Solution Preparation for Standard Kit #1

4.1.1. Thaw the standard plasma samples (7 supplied - standards A to G; see **Note 3**).

4.1.2. Place the samples in the autosampler rack for LC/MRM-MS analyses (concentration: 1 μg/μL; see **Tables 2** and **3** for method details).

4.2. Solution Preparation for Standard Kit #2

Begin by preparing the solutions indicated in steps **4.2.1** to **4.2.6** then proceed to ***Section 4.3***. The solutions outlined in steps **4.2.7** to **4.2.10** need not be prepared until after the second trypsin addition in step **4.3.13**, but prior to digestion completion.

4.2.1. Dissolve 44.35 mg of ammonium bicarbonate in 22.45 mL of LC-MS water for a 25 mM ammonium bicarbonate solution. Vortex briefly to ensure a fully mixed solution.

4.2.2. After thawing the supplied human plasma, vortex briefly and spin down quickly in the minicentrifuge before diluting 24 µL with 216 µL of 25 mM ammonium bicarbonate. Store the diluted plasma on ice.

4.2.3. Dissolve 200 mg of sodium deoxycholate in 2 mL of 25 mM ammonium bicarbonate for a 10% w/v sodium deoxycholate solution. Vortex until fully solubilized.

4.2.4. Add 100 µL of 0.5 M TCEP solution to 900 µL of 25 mM ammonium bicarbonate for a 50 mM TCEP solution. Vortex briefly.

4.2.5. Dissolve 36.99 mg of iodoacetamide in 2 mL of 25 mM ammonium bicarbonate for a 100 mM iodoacetamide solution. Vortex briefly.

4.2.6. Dissolve 30.85 mg of DTT in 2 mL of 25 mM ammonium bicarbonate for a 100 mM DTT solution. Vortex briefly and proceed to step **4.3.1**.

4.2.7. Prepare 10 mL of 0.1% FA by combining 10 µL of FA with 9.99 mL of water. Vortex briefly and store on ice.

4.2.8. Make serial dilutions of the supplied 250 fmol/µL SIS peptide mixture (see **Table 1** for details). Vortex briefly and store on ice.

4.2.9. Prepare 1% FA by combining 20 µL of FA with 1.98 mL of water. Vortex briefly and store on ice.

4.2.10. Prepare 50% ACN 0.1% FA by combining 5 µL FA with 2.497 mL of water and 2.497 mL of ACN.

4.3. Sample Preparation for Standard Kit #2

4.3.1. In three separate 1.5 mL Eppendorf tubes, add 60 µL of diluted plasma to 354.8 µL of 25 mM ammonium bicarbonate and 54 µL of 10% w/v sodium deoxycholate for protein denaturation. Vortex briefly.

4.3.2. Reduce the disulfide bonds with a 52.4 µL addition of 50 mM TCEP for a final concentration of 5 mM (concentration of sodium deoxycholate: 1%). Vortex briefly.

4.3.3. Incubate the plasma samples at 60ºC for 30 min.

4.3.4. Alkylate the free sulfhydryl groups with 58 µL of 100 mM iodoacetamide to give a final concentration of 10 mM. Vortex briefly.

4.3.5. Incubate at 37ºC for 30 min in the dark.

4.3.6. Quench the remaining iodoacetamide by adding 58 µL of 100 mM DTT (final concentration: 10 mM). Vortex briefly.

4.3.7. Incubate at 37ºC for 30 min.

4.3.8. Thaw one trypsin vial just minutes prior to its addition to reduce autolysis.

4.3.9. Add 23.3 µL of the freshly thawed trypsin to each sample for a 20:1 substrate:enzyme ratio. Vortex briefly.

4.3.10. Allow digestion to proceed for 4 h at 37°C.

4.3.11. Thaw the second supplied trypsin vial moments before its addition.

4.3.12. Add 23.3 µL of trypsin to each sample for a 20:1 substrate:enzyme ratio.

4.3.13. Allow digestion to proceed for an additional 5 h at 37°C. During this time, return to ***Section 4.2*** and prepare the solutions indicated in steps **4.2.7** to **4.2.10**.

4.3.14. Upon completion, combine the three digests into a single 10 mL falcon tube. Vortex briefly and place in ice.

4.3.15. Prepare 7 standard samples (labeled A to G) by first combining sequential aliquots of the plasma tryptic digest (200 µL) and the SIS peptide mixture (49 µL; see **Note 4**). Following thorough mixing, add 1% FA (251 µL) to each standard.

4.3.16. Pellet the acid insoluble sodium deoxycholate by centrifugation at 12,000 x g for 10 min.

4.3.17. Desalt and concentrate 400 µL of the peptide supernatants by solid phase extraction using traditional vacuum manifold processing on 7 separate 10 mg Oasis HLB cartridges. The extractions are performed as follows: (i) wash with 1 mL methanol, (ii) condition with 1 mL water, (iii) load with 400 µL of digest supernatant and 600 µL of 0.1% FA (see **Note 5**), (iv) wash with 1 mL water, and (v) elute with 200 µL of 50% ACN 0.1% FA into 7 separate Eppendorf tubes (see **Note 6**).

4.3.18. Place parafilm over the vials then freeze the samples and lyophilizer container at -80°C.

4.3.19. Once frozen, puncture 2 small holes in the parafilm prior to overnight lyophilization.

4.3.20. Rehydrate with 98 µL of 0.1% FA (final concentration: 1 µg/µL based on an initial plasma protein concentration of 70 mg/mL) and transfer to 7 separate 300 µL autosampler vials.

4.3.21. Prepare for LC/MRM-MS analyses using the parameters indicated in **Tables 2** and **3** and the methods specified in ***Sections 4.5*** and **4.*6***.

4.4. LC/MRM-MS Platform Parameters

The LC and MRM-associated platform-specific parameters for the Agilent 6490 LC-MS platform are listed in **Tables 2** and **3**. The Agilent 6490 mass spectrometer is controlled by Agilent's MassHunter Workstation software (version B.04.01).

4.5. Retention Time Scheduling

4.5.1. Create an acquisition method using the LC/MRM-MS parameters indicated in **Tables 2** and **3**.

4.5.2. Use the precursor/product ion *m/z* values indicated in **Supplemental Material – Table 1** for the SIS and NAT peptides.

4.5.3. Run unscheduled MRM for SIS in buffer then for standard E to confirm retention times.

4.5.4. Determine the SIS and NAT retention times with the aid of data analysis software (see **Note 7**).

4.6. Protein Quantitation

Performance metrics (*e.g.*, lower limit of quantitation, dynamic range, reproducibility, and plasma protein concentration) will be determined from peptide calibration curves. These curves are constructed using the 7 standard samples that contain a variable concentration of SIS peptides (spanning a 4 order-of-magnitude range) and a constant amount of plasma digest.

4.6.1. Create a dynamic MRM/MS acquisition method for the SIS and NAT peptides.

4.6.2. Analyze the standard samples by LC/MRM-MS (n = 5; see **Note 8**).

4.6.3. Upon data collection, manually inspect the integrated peaks in the software program to ensure correct peak detection and accurate integration.

4.6.4. In performing the linear regression of the calibration curves, use a $1/x^2$ (x = concentration) weighting option to assist in covering a wide dynamic range.

4.6.5. The standard points within each sample concentration must be both precise (20% CV) and accurate (80-120%) to remain in the calibration curve. If the points for a given concentration fall outside these ranges, that concentration must be removed from the curve. In the end, a minimum of 3 concentrations must remain in order to construct a calibration curve.

4.6.6. The lower limit of quantitation (LLOQ) is defined as the lowest point on the curve that has a CV below 20% and an average accuracy in determining the expected concentration within 80-120%. The LLOQ (in fmol/µL) can readily be converted to ng/mL by taking into account the protein's molecular weight (see **Supplemental Material – Table 2** for the molecular weights and the target LLOQs).

4.6.7. The dynamic range is defined as the quotient of the upper and lower LOQ.

4.6.8. The concentration of each peptide target (in fmol/µL of plasma) is calculated based on the mean product of the average relative response (NAT/SIS) and the SIS concentration for each qualified concentration level (see **Supplemental Material – Table 2** for the SIS concentrations in plasma for Standard E and the target plasma protein concentrations). The determined concentrations can then be converted to ng/mL by taking into account the molecular weight of the protein.

4.6.9. Assess performance by determining the accuracy of your calculated values to the known values (see **Supplemental Material – Table 2** for the known values).

5. Notes

1. The human plasma was originally purchased from Bioreclamation (Westbury, NY, USA; catalog no. HMPLEDTA2) and represents a pooled sample of whole blood donations collected from 15 male and 15 female race-matched healthy donors, between the ages of 18 and 50. The plasma should be handled with care according to standard biosafety level 2 protocols and be stored at -80°C until use.

2. Store at -80°C until use.

3. Calibration curves are constructed using standard samples that contain a variable concentration of SIS peptides (spanning a 4 order-of-magnitude range) and a constant amount of plasma digest to keep the matrix effect constant. Standard A corresponds to the lowest concentration level (0.01 fmol/µL on column) and is 10,000-fold lower than the highest concentration level. The dilution pattern from standard G is 1:2:5:2:5:10:10.

4. As is the case for the standards in Kit #1, standard sample A is spiked with the lowest concentration of SIS peptides (0.025 fmol/µL; 1000-fold lower SIS peptide than standard E), while standard G is spiked with the highest (250 fmol/µL; 10-fold higher SIS peptide than standard E). Standard E will provide a near equivalent ratio of SIS to endogenous peptides when 25 fmol/µL of the concentration-balanced SIS peptide mixture is spiked into the plasma tryptic digest.

5. Carefully withdraw the supernatant digest along one side of the Eppendorf tube to prevent disrupting the sodium deoxycholate precipitate.

6. The elution step is performed off-line by adding the 50% ACN 0.1% FA solution to the cartridge and applying positive pressure via a syringe.

7. Since the SIS peptides separate, ionize, and fragment identically to their NAT, matching MRM acquisition parameters and retention times can be used for both peptide forms.

8. To minimize sample carryover, we analyse the standard samples in order of increasing concentration, with two blank solvent injections run between each concentration.

Tables

Table 1. Recipe for preparing the SIS peptide dilutions spanning a 4 order of magnitude range of concentrations originating from the 250 fmol/µL provided stock (for Std G).

Stocks	Serial Dilutions of SIS Peptide Mixes (µL)					
	125 fmol/µL (for Std F)[1]	**25 fmol/µL (for Std E)**	**12.5 fmol/µL (for Std D)**	**2.5 fmol/µL (for Std C)**	**0.25 fmol/µL (for Std B)**	**0.025 fmol/µL (for Std A)**
250 fmol/µL	40					
125 fmol/µL		20				
25 fmol/µL			40			
12.5 fmol/µL				20		
2.5 fmol/µL					10	
0.25 fmol/µL						10
0.1% FA	40	80	40	80	90	90

[1] To make a stock of the 125 fmol/µL SIS mix, combine 40 µL of the supplied 250 fmol/µL SIS mix with 40 µL of 0.1% FA. The remaining mixes are prepared in a similar fashion.

Table 2. Standard-flow UHPLC/MRM-MS parameters for Agilent's 6490.

Parameters	Values
RP-UHPLC column temperature	50°C
Autosampler tray temperature	4°C
Injection volume	10 µL
LC gradient	30 min, see **Table 3**
Post-run equilibration time	4 min
Ionization mode	Positive
Capillary voltage	3500 V
Nozzle voltage	300 V
Sheath gas flow	11 L/min ultra high purity nitrogen
Sheath gas temperature	250°C
Drying gas flow	15 L/min ultra high purity nitrogen
Drying gas temperature	150°C
Nebulizer gas flow	30 psi ultra high purity nitrogen
Q1/Q3 mass resolution	Unit
Default fragmentor voltage	380 V
Cell accelerator potential	5 V
Collision energy	see **Supplemental Material – Table 1**
MRM detection window	2 min

Table 3. LC gradient used in the standard-flow UHPLC/MRM-MS platform.

Agilent Standard-flow Platform	
Time (min)	%B
0	3
2	11
15	19
22	29
25	45
27	90
29	90
30	3