Detailed Method for Performing the ExSTA Approach in Quantitative Bottom-Up Plasma Proteomics

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Abstract

The use of stable isotope-labeled standards (SIS) is an analytically valid means of quantifying proteins in biological samples. The nature of the labeled standards and their point of insertion in a bottom-up proteomic workflow can vary, with quantification methods utilizing curves in analytically sound practices. A promising quantification strategy for low sample amounts is external standard addition (ExSTA). In ExSTA, multipoint calibration curves are generated in buffer using serially diluted natural (NAT) peptides and a fixed concentration of SIS peptides. Equal concentrations of SIS peptides are spiked into experimental sample digests, with all digests (control and experimental) subjected to solid-phase extraction prior to liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. Endogenous peptide concentrations are then determined using the regression equation of the standard curves. Given the benefits of ExSTA in large-scale analysis, a detailed protocol is provided herein for quantifying a multiplexed panel of 125 high-to-moderate abundance proteins in undepleted and non-enriched human plasma samples. The procedural details and recommendations for successfully executing all phases of this quantification approach are described. As the proteins have been putatively correlated with various noncommunicable diseases, quantifying these by ExSTA in large-scale studies should help rapidly and precisely assess their true biomarker efficacy.

Key words Forward curve, Human plasma, Protein, Proteomics, Stable isotope-labeled standard, Standard curve, Quantification

1 Introduction

The quantitative proteomics field has advanced tremendously during the past decade. This includes the method workflows, automation schemes, analysis regimens, and scope of applications. Improvements have been made in sample throughput and analytical metrics (e.g., precision, sensitivity) in MS-based quantitation [1–3]. The reproducibility of the methodologies and techniques has also been enhanced, as has its utility in interlaboratory studies [2, 4, 5]. These merits are collectively needed for the widespread assessment of protein disease biomarkers toward clinical diagnostic implementation [6].

While MS-based proteomic measurements are increasingly performed in a label-free manner [7, 8] (*see* Chaps. 8, 20–24), higher identification confidence and improved analytics can be obtained through the use of stable isotope-labeled protein or peptide standards (*see* Chaps. 8, 11). These standards can be inserted at a number of points of the analytical workflow and can be constructed in various formats with different isotope-labeling patterns (commonly $^{13}\text{C}/^{15}\text{N}$, with a minimum mass shift of 6 Da from its unlabeled counterpart) [9]. The classical workflow is “bottom-up,” where the enzymatically cleaved peptides serve as molecular surrogates of the proteins of interest. The types of standard(s) used have included recombinant proteins [10, 11], tryptic peptides [12–14], winged (referred to also as extended, flanked, or cleavable) peptides [15, 16], concatenated peptides (abbreviated QconCATs for quantification concatemers) [17, 18], and protein epitope signature tags (PrESTs) [19–21]. In all cases, the labeled standards are designed to resemble the chemical structure of its endogenous (natural or NAT) analogue. Their quantitative effectiveness has been evaluated and compared/contrasted in different sample matrices (e.g., plasma, cerebrospinal fluid, urine), using both protein [22] and peptide [23] standards. These studies have demonstrated the utility of labeled standards, with the selection ultimately guided by experimental design and quantitative application.

Protein quantification with standards can be accomplished in a number of ways. While single-point measurements (using area ratios of unlabeled peptides, from a sample, to labeled peptides, from a spiked-in standard) are the easiest to perform, this method is not analytically accurate for large-scale analyses (due, for example, to plate-to-plate variation) and over wide dynamic ranges. Better quantitative methods utilize standard curves prepared in buffer or in a pooled control sample. These can be generated in a forward or reverse manner [24–27]. Forward curves involve a dilution series of NAT peptide concentrations (derived synthetically or from an endogenous sample) and constant SIS peptide concentrations, while reverse curves are the converse involving a dilution series of SIS peptide concentrations with constant NAT across the calibrant levels. There are advantages and disadvantages of curve-based quantitative strategies, as described and demonstrated previously [28]. One promising and applicable approach uses external standard addition (ExSTA), where forward standard curves are prepared in buffer and constant SIS peptide concentrations are spiked into the experimental samples. The peptide concentrations in the endogenous sample can then be determined by applying the experimental response ratios (i.e., NAT vs. spiked-in SIS) to the regression equation of their peptide-specific standard curves (plot of NAT/SIS peptide response vs. NAT peptide concentration). This strategy has been demonstrated to be a robust means for precisely quantifying endogenous proteins in human plasma

samples. Moreover, it is well suited for routine LC-MS/MS processing, large-scale proteomic applications, and sample-limited analyses.

Detailed here is a procedure for preparing samples (both controls and experimental), via the ExSTA approach, for robust, MS-based quantification of human plasma proteins without the use of upfront depletion, enrichment, or LC fractionation. The controls are generated in a BSA/PBS (bovine serum albumin/phosphate-buffered saline) surrogate matrix. These surrogate controls encompass standard-curve samples comprising eight concentration-level calibrants (spanning a 1000-fold range) and three quality-control (QC) samples (at low, medium, and high levels). The mixtures utilized in these samples are NAT and SIS tryptic peptides, with the SIS mixes also implemented in the plasma sample analysis ($n = 20$). The peptides correspond to a panel of medium-to-high abundance human plasma proteins (125 in total, with one peptide per protein) and were initially carefully selected according to a series of peptide/protein selection rules [29, 30]. The peptide mixtures and the described methods have been validated according to the Clinical Proteomic Tumor Analysis Consortium (CPTAC) guidelines [31, 32] and are verified to be fit-for-purpose for Tier 2, research-based analysis [33]. The assays utilize reversed-phase liquid chromatography (RPLC) in conjunction with tandem MS (MS/MS), performed in the selected/multiple reaction monitoring (SRM/MRM) or parallel reaction monitoring (PRM) acquisition mode. Performing the procedures described herein should produce precise quantitative results for putative biomarker analysis (at the discovery or verification stage) while opening the door for expanded target panels and sample sizes.

2 Materials

2.1 Sample and Solution Preparation Supplies

1. Analytical balance.
2. Adjustable pipettes and pipette tips.
3. Polypropylene Falcon tubes (15 and 50 mL) and screw caps (all from Fisher Scientific; Suwanee, GA, USA).
4. LoBind (i.e., low-binding) microcentrifuge tubes (1.5 mL; Eppendorf; Hauppauge, NY, USA).
5. Glass beaker (250 mL).
6. Glass graduated cylinder (100 mL).
7. Ultralow temperature freezer (capable of -80°C).
8. Vortex mixer.
9. Minicentrifuge (capable of $\geq 1000 \times g$).
10. Benchtop centrifuge (compatible with 96-well plates).

11. Incubator.
12. Aluminum foil.
13. 96-well deep well plates (1.1 mL) and accessories (e.g., silicone sealing mat, Axygen; Union City, CA, USA; AluminaSeal II™ sealing film; EXCEL Scientific; Victorville, CA, USA).
14. 96-well Oasis HLB (i.e., hydrophilic-lipophilic balance) μ Elution plates (2 mg sorbent, 30 μ m particles; part number 186001828BA; Waters; Milford, MA, USA).
15. Skirted 96-well (150 μ L) PCR collection plates (Eppendorf).
16. Positive pressure vacuum manifold (compatible with 96-well plates, e.g., part number 186006961; Waters).
17. Lyophilizer (or SpeedVac) and accessories (e.g., wide-mouth borosilicate glass flasks).
18. Autosampler vials and screw caps.

2.2 Control and Experimental Sample Preparations

1. 10 mg/mL BSA in PBS: In a 1.5 mL LoBind microcentrifuge tube, dissolve 10 mg BSA in 1 mL of PBS solution (*see Note 1*). The pH of this solution should be approximately 7.5.
2. Reagent A: 1 M Tris (pH 8.0). In a 250 mL glass beaker, dissolve 12.12 g of Tris in 90 mL of LC-MS grade water and adjust the pH to 8.0 with dropwise addition of 12 M HCl. Transfer the solution to a 100 mL glass graduated cylinder and bring the volume to 100 mL with LC-MS grade water (*see Note 1*). Vortex briefly.
3. Reagent B: 9 M urea in 300 mM Tris (pH 8.0). In a 15 mL screw cap polypropylene tube, dissolve 5.4 g of urea in 3 mL of Reagent A and 3 mL of LC-MS grade water (*see Note 1*). Vortex until fully solubilized (may take up to 5 min; *see Note 2*).
4. Reagent C: 9 M urea and 20 mM DL-dithiothreitol (DTT) in 300 mM Tris (pH 8.0). In a 1.5 mL LoBind microcentrifuge tube, dissolve 15.4 mg of DTT in 200 μ L of Reagent B, to prepare a 500 mM DTT solution. Using the 500 mM DTT solution, dilute this 25 $\times$ by transferring 40 μ L to 960 μ L of Reagent B (*see Note 1*). Vortex briefly.
5. Reagent D: 100 mM iodoacetamide (IAA). In a 1.5 mL LoBind microcentrifuge tube, dissolve 18.5 mg of IAA in 1 mL of LC-MS grade water. Vortex until completely solubilized, and then wrap the tube in aluminum foil to prevent deactivation by light (*see Note 1*).
6. Reagent E: 100 mM Tris (pH 8.0). In a 15 mL screw cap polypropylene tube, add 1 mL of Reagent A to 9 mL of LC-MS grade water (*see Note 1*). Vortex briefly.

7. 1 mg/mL trypsin. In a 1.5 mL LoBind microcentrifuge tube, dissolve 1 mg of trypsin in 1 mL of Reagent E (*see Note 1*).
8. 2% aqueous formic acid (FA) (v/v). In a 15-mL screw cap polypropylene tube, add 200 μ L FA to 9.8 mL of LC-MS grade water.
9. 0.1% aqueous FA (v/v). In a 50-mL screw cap polypropylene tube, add 50 μ L of FA to 49.95 mL of LC-MS grade water. Vortex briefly.
10. 0.1% FA in 30% LC-MS grade acetonitrile (ACN)/water (v/v/v). In a 1.5 mL LoBind microcentrifuge tube, add 3 μ L of FA to 899 μ L of LC-MS grade ACN and 2.098 mL of LC-MS grade water. Vortex briefly.
11. 0.1% FA in 50% LC-MS grade ACN/water (v/v/v). In a 15 mL screw cap polypropylene tube, add 5 μ L of FA to 2.497 mL of LC-MS grade ACN and 2.497 mL of LC-MS grade water. Vortex briefly.
12. NAT peptide mixture. Rehydrate a lyophilized NAT mix aliquot (*see Note 3*) in 60 μ L of 0.1% FA/30% ACN (v/v). Vortex and centrifuge briefly, and then store on ice. This stock solution represents level H of the 8-point standard curve and is to be later diluted serially to produce the calibrant levels and three curve QC samples.
13. SIS peptide mixture. Rehydrate a lyophilized SIS mix aliquot (*see Note 3*) in 450 μ L of 0.1% FA/30% ACN (v/v). Vortex and centrifuge briefly, and then store on ice. This creates a concentration-balanced solution (i.e., MS response signals have been tested to be within an order of magnitude of their unlabeled peptide analogues) for direct addition to experimental and control samples. For alternative SIS peptide configurations, refer to **Note 4**.

2.3 LC-MS Equipment

1. LC system: 1290 Infinity UHPLC (Agilent Technologies; Santa Clara, CA, USA).
2. Analytical column: Zorbax Eclipse Plus RRHD C₁₈ RP-UHPLC (150 $\times$ 2.1 mm i.d., 1.8 μ m particles; Agilent Technologies).
3. Eluent A: 0.1% aqueous FA in LC-MS grade water.
4. Eluent B: 0.1% FA in LC-MS grade ACN.
5. 6495 QqQ (Agilent Technologies) with standard-flow ESI source.
6. Q ExactiveTM Plus (Thermo Fisher Scientific) with standard-flow ESI source.
7. Mass spectrometer calibrant: ESI-tuning mix.

2.4 Data Collection and Analysis Software

1. MassHunter Quantitative Analysis (Agilent Technologies).
2. Xcalibur™ (Thermo Fisher Scientific).
3. Skyline-daily.
4. Microsoft Excel.

3 Methods

3.1 Digest Preparations (for Control and Experimental Samples)

1. Using a 96-well deep well plate, add 20 μ L of Reagent C to the highlighted wells in Fig. 1a (red wells, A1-H1, A2-H2, and A3-D3 for the experimental samples; green wells, A11 and A12 for the control samples).
2. Add 10 μ L of undiluted human plasma to the red wells (i.e., A1-H1, A2-H2, and A3-D3).
3. Add 10 μ L of the BSA/PBS solution (at 10 mg/mL) to the green wells (i.e., A11 and A12).
4. Cover the plate with a silicone sealing mat, and then mix, centrifuge briefly, and incubate for 30 min at 37 °C. The concentrations of urea and DTT during the denaturation/reduction are 4.5 mM and 10 mM, respectively.
5. Following this incubation, alkylate the free sulfhydryl groups by adding 20 μ L of Reagent D to each of the aforementioned wells (22 in total). The reaction concentration of IAA is 33 mM.
6. Cover the plate with a silicone sealing mat, and then mix, centrifuge briefly, and incubate for 30 min at ambient

Fig. 1 Map of 96-well plate illustrating the suggested placement of the control and experimental samples. Part (a) is for the digest preparations and (b) for the standard spiking to the digests. In both cases, the green/blue wells are for the control samples and red wells for the experimental samples ($n = 20$ human plasma). Regarding the controls in (b), the green wells are for the calibration curve sample digests (A, or low NAT peptide concentration, to H, or high), and the blue wells are for the curve QC digests (low, QC-A; medium, QC-B; and high, QC-C)

temperature (i.e., 20–25 °C) in the dark. During this incubation, prepare the trypsin solution as described in **item 7** of Subheading 2.2.

7. After alkylation, prepare the protein solutions for digestion by adding 272 μL of Reagent E and 35 μL of trypsin (at 1 mg/mL for a 20:1 w/w substrate/enzyme ratio; *see Note 5*) to the 22 aforementioned wells. The reagent concentrations at digestion are 0.5 M urea (>1 M inhibits trypsin activity), 1 mM DTT, and 6 mM IAA in 100 mM Tris (pH 8.0).
8. Cover the plate with a silicone sealing mat, and then mix, centrifuge briefly, spin-down, and incubate for 18 h at 37 °C. Prior to the completion of this incubation period (e.g., 2 h beforehand), we recommend to prepare the NAT/SIS peptide standard solutions outlined in Subheading 2.2.
9. After the 18-h incubation, quench the proteolytic digestion by adding 343 μL of 2% FA. This brings the total volume of each well to 700 μL .
10. Cover the plate with a silicone sealing mat and then mix and centrifuge briefly.
11. Combine the two BSA/PBS digests into a 1.5 mL LoBind microcentrifuge tube. This solution will be used in the control sample preparations (for the calibration curve and the QC samples; Subheading 3.3).
12. Store the plate (containing the plasma samples) and microcentrifuge tube (containing the surrogate matrix) on ice until standard spiking (Subheading 3.3).

3.2 Standard Solution Preparations (for the Control Samples)

1. Label seven 1.5 mL LoBind microcentrifuge tubes from Standard G to A. These form a 7-concentration dilution series for the 8-point standard curve, with the highest concentration level being Standard H. This standard reflects the undiluted, concentrated NAT stock that was prepared earlier (in **item 12** of Subheading 2.2).
2. To the Standard G microcentrifuge tube, add 14 μL of Standard H to 21 μL of 0.1% FA/30% ACN. Vortex and centrifuge briefly, and then store on ice. Subsequent concentration levels (i.e., Standards F to A) are to be prepared as outlined in Table 1.
3. For the curve-based QC samples, label five LoBind microcentrifuge tubes as follows: QC-C, ID-1, QC-B, ID-2, and QC-A. The IDs reflect intermediate dilutions and are used only in these QC-sample preparations.
4. Prepare QC-C by adding 18 μL of Standard H to 42 μL of 0.1% FA/30% ACN. Vortex and centrifuge briefly, and then store on ice.

Table 1
Serial dilution of the NAT peptide mix toward an 8-point standard curve (1000-fold range)

Standard	H	G	F	E	D	C	B	A
Level-to-level dilution	1	2.5	2.5	4	2	4	2	2.5
Necessary additions	N/A	14 µL of H	12 µL of G	10 µL of F	15 µL of E	10 µL of D	15 µL of C	10 µL of B
	N/A	21 µL solvent	18 µL solvent	30 µL solvent	15 µL solvent	30 µL solvent	15 µL solvent	15 µL solvent
Total volume (µL)	46	36	40	40	30	40	30	25

Dilutions involve combining an aliquot of a more concentrated standard with a volume of solvent (0.1% FA/30% ACN). Each mixture is to be vortexed and centrifuged before proceeding, with final storage on ice until use.

Table 2
Preparation of three curve QC samples (high, QC-C; medium, QC-B; low, QC-A)

QC sample	QC-C	ID-1	QC-B	ID-2	QC-A
Dilution from standard H	3.33×	6.66×	33.3×	83.3×	250×
Necessary additions	18 µL of H 42 µL of solvent	15 µL of QC-C 15 µL of solvent	15 µL of ID-1 60 µL of solvent	16 µL of QC-B 24 µL of solvent	20 µL of ID-2 40 µL of solvent
Total volume (µL)	60	30	75	40	60

The dilutions involve combining an aliquot of a more concentrated standard with a volume of solvent (0.1% FA/30% ACN). Each mixture is to be vortexed and centrifuged before proceeding, with final storage on ice. The intermediate dilutions (ID-1 and ID-2) can be discarded following the QC-B and QC-A preparations.

5. The subsequent ID (ID-1 and ID-2) and QC (QC-B and QC-A) samples are to be prepared as outlined in Table 2. The ID samples can be discarded following the QC-B and QC-A preparations.

3.3 Standard Spiking to Control/Experimental Digests

The steps outlined in this section are to be conducted in a fresh 96-well deep well plate.

1. To prepare the curve samples (comprises eight concentration levels, 1:2.5:2.5:4:2:4:2:2.5 dilutions from standard H to A), add the following solutions in succession to wells A12–H12 (see green wells in Fig. 1b):
 - (a) 40 µL of the BSA/PBS digest (from step 12 of Subheading 3.1)
 - (b) 10 µL of the SIS peptide mixture (rehydrated earlier in item 13 of Subheading 2.2)

- (c) 10 μL of a NAT standard mix (i.e., add standard A to well A12, down to standard H to well H12; prepared in **step 2** of Subheading 3.2)
 - (d) 540 μL of 0.1% aqueous FA.
2. To prepare the curve QC samples, add the following solutions in succession to wells H10 and A11 to H11 (*see* blue wells in Fig. 1b):
 - (a) 40 μL of the BSA/PBS digest (from **step 12** of Subheading 3.1)
 - (b) 10 μL of the rehydrated SIS peptide mixture
 - (c) 10 μL of a NAT QC mix (QC-A to wells H10, A11, B11; QC-B to C11, D11, E11; QC-C to F11, G11, H11; prepared in **step 4** of Subheading 3.2)
 - (d) 540 μL of 0.1% aqueous FA.
 3. To prepare the experimental plasma samples ($n = 20$), add the following solutions in succession to wells A1–H1, A2–H2, and A3–D3 (*see* red wells in Fig. 1b and **Note 6**):
 - (a) 40 μL of a human plasma digest (from **step 12** of Subheading 3.1)
 - (b) 10 μL of the rehydrated SIS peptide mixture
 - (c) 10 μL of 0.1% FA/30% aqueous ACN
 - (d) 540 μL of 0.1% aqueous FA.
 4. Cover the plate with a sealing film (AlumaSeal IITM) and then mix and centrifuge briefly.
 5. Store on ice until performing sample cleanup by solid-phase extraction.

3.4 Extraction and Reconstitution for LC-MS/MS

1. Desalt and concentrate the peptide solutions by solid-phase extraction using a positive pressure vacuum manifold on a 96-well Oasis HLB $\mu\text{Elution}$ plate. The extractions are to take place in 37 wells (*see* Fig. 1b for example locations) and involve the following sequential steps (*see* **Note 7**):
 - (a) Wash with 600 μL of LC-MS grade methanol.
 - (b) Condition with 600 μL of 0.1% aqueous FA.
 - (c) Load with 510 μL of each sample (i.e., standard curve samples A–H, QC samples A–C, plasma samples 1–20).
 - (d) Wash with 600 μL of LC-MS grade water or 0.1% FA.
 - (e) Elute with 75 μL of 0.1% FA/50% ACN into a skirted 96-well microplate.
2. Cover the plate of eluate with a sealing film (AluminaSeal IITM) and centrifuge briefly.

3. Puncture the sealing film with a hole (using an 18-gauge needle) at the designated well positions (37 in total), and then cover with parafilm.
4. Freeze the plate and lyophilizer container at -80°C .
5. Once frozen, remove the parafilm, and then dry down (with lyophilizer) to completion overnight.
6. Rehydrate the 37 samples in $34\ \mu\text{L}$ of 0.1% FA (this will give a final concentration of $1\ \mu\text{g}/\mu\text{L}$ based on an initial plasma protein concentration of $70\ \text{mg}/\text{mL}$).
7. Cover the plate with a sealing film, mix, and centrifuge briefly.
8. Remove the sealing film and replace with a silicone sealing mat.
9. Place the sample plate and an autosampler vial containing $\sim 200\ \mu\text{L}$ eluent A (for blank injections) into the LC autosampler (*see* **Note 8**).
10. Prepare for the LC-MS/MS analysis (Subheading 3.5).

3.5 LC System Setup

1. Thermostat the column and autosampler compartments to 50 and 4°C , respectively.
2. Set up the autosampler for acquisition at a $20\ \mu\text{L}/\text{min}$ draw, $40\ \mu\text{L}/\text{min}$ eject, and vial/well bottom sensing activated.
3. To facilitate the RPLC peptide separations, use mobile phase compositions of 0.1% aqueous FA in water for A and 0.1% aqueous FA in ACN for B.
4. Use the following ACN gradient (time in min, eluent B composition in %): 0, 2; 2, 7; 50, 30; 53, 45; 53.5, 80; 55.5, 80; 56, 2.
5. To help preserve instrument cleanliness, divert the solvent stream (2% eluent A) to waste at the conclusion of the ACN gradient (at 56 min), and then hold for 4 min to allow column equilibration.
6. Use a $0.4\ \text{mL}/\text{min}$ flow rate.
7. Use $10\ \mu\text{L}$ injection volumes for all sample types.

3.6 MRM/MS System Setup

1. Operate the ESI source in the positive ion mode with capillary voltage at 3.5 kV.
2. Use the following general parameters: 300 V nozzle voltage, 11 L/min sheath gas at 250°C , 15 L/min drying gas at 150°C , 30 psi nebulizer, and iFunnel RF pressures of 200 V (high) and 110 V (low). Ultrahigh-purity nitrogen serves as the carrier gas in all settings.
3. Using the MRM mode (for the 6495 QqQ, *see* **Note 9**), enter the specific acquisition parameters. This encompasses the precursor/product ion m/z values, retention times (RTs), and collision energies (*see* Table 3).

Table 3
Specific acquisition parameters for the bottom-up LC-MRM/MS analysis

Protein	Peptide	Product Ion/ Charge	NAT		SIS		RT (min)	CE (V)
			Precursor m/z	Product m/z	Precursor m/z	Product m/z		
78 kDa glucose-regulated protein	ITPSYVAFTEGER	y ₁₂ ²⁺	783.8936	676.8277	788.8977	681.8318	24.1	17
Adipocyte plasma membrane-associated protein	LLEYDVTVR	y ₇ ⁺	555.2955	883.4156	560.2996	893.4239	24.21	13
Adiponectin	IFYNQNHVDGSGTK	y ₁₃ ²⁺	591.2727	756.3291	593.9441	760.3362	8.91	13
Afamin	DADPDTEFAK	y ₇ ⁺	563.7562	825.4141	567.7633	833.4283	20.36	9
α-1-acid glycoprotein 1	NWGLSVVADKPETTK	y ₁₃ ²⁺	570.2895	704.8696	572.9609	708.8767	21.15	13
α-1-Antichymotrypsin	EIGELYLPK	y ₂ ⁺	531.2975	244.1656	535.3046	252.1798	24.8	9
α-1-antitrypsin	LSITGYDLK	y ₇ ⁺	555.8057	797.4040	559.8128	805.4182	21.98	17
α-1B-glycoprotein	LQSLFDSPDFSK	b ₂ ⁺	619.3268	243.1339	623.3339	243.1339	29.68	21
α-2-Antiplasmin	LGNQEPGGQTALK	b ₅ ⁺	656.8570	542.2575	660.8464	542.2575	16.65	17
α-2-HS-glycoprotein	FSVVYAK	y ₅ ⁺	407.2289	579.3501	411.2360	587.3643	14.29	9
α-2-macroglobulin	AIGYLVNTGYQR	y ₇ ⁺	628.3251	851.4370	633.3293	861.4453	16.75	21
Antithrombin-III	DDLVSDAFHK	y ₈ ²⁺	437.2068	483.7376	439.8782	487.7447	18.34	9
Apolipoprotein A-I	ATEHLSTLSEK	y ₁₀ ²⁺	405.8787	572.7959	408.5501	576.8030	5.68	9
Apolipoprotein A-II	EQLTPLIK	y ₄ ⁺	471.2869	470.3337	475.2940	478.3479	19.36	9
Apolipoprotein A-IV	LGEVNTYAGDLQK	b ₃ ⁺	704.3594	300.1554	708.3665	300.1554	18.95	25
Apolipoprotein B-100	FPEVDVLTK	y ₈ ²⁺	524.2897	450.7555	528.2968	454.7626	25.8	13
Apolipoprotein C-I	EWSETFQK	y ₇ ⁺	601.2798	886.4305	605.2869	894.4447	25.16	13
Apolipoprotein C-II	TYLPAVDEK	b ₂ ⁺	518.2715	265.1183	522.2786	265.1183	14.64	13

(continued)

Table 3
(continued)

Protein	Peptide	Product Ion/ Charge	NAT		SIS		RT (min)	CE (V)
			Precursor m/z	Product m/z	Precursor m/z	Product m/z		
Apolipoprotein C-III	GWVTDGFSSLK	b ₂ ⁺	598.8009	244.1081	602.8080	244.1081	28.36	17
Apolipoprotein C-IV	ELLETVVNR	y ₄ ⁺	536.8082	487.3075	541.8082	497.3075	19.52	17
Apolipoprotein D	VLNQELR	y ₅ ⁺	436.2534	659.3471	441.2576	669.3554	7.43	13
Apolipoprotein E	LGPLVEQGR	y ₇ ²⁺	484.7798	399.7271	489.7840	404.7312	7.23	13
Apolipoprotein L1	VAQELEEK	y ₆ ⁺	473.2480	775.3832	477.2551	783.3974	4.26	17
Apolipoprotein M	AFLTPR	y ₅ ⁺	409.2502	599.3875	414.2543	609.3958	21.77	9
Apolipoprotein(a)	TPAYYPNAGLIK	y ₇ ⁺	654.3533	712.4352	658.3604	720.4494	22.46	25
Attractin	SVNNVVVR	y ₆ ⁺	443.7589	700.4100	448.7630	710.4183	6.69	13
β-2-glycoprotein 1	ATVWYQGER	y ₆ ⁺	511.7669	751.3733	516.7710	761.3816	5.84	17
β-Ala-his dipeptidase	ALEQDLPVNIK	y ₅ ⁺	620.3508	570.3610	624.3579	578.3752	24.17	17
Biotinidase	SHLIIAQVAK	b ₄ ⁺	360.5572	451.2663	363.2286	451.2663	13.82	9
Cadherin-13	INENTGSVSVTR	b ₂ ⁺	638.8282	228.1343	643.8324	228.1343	7.18	21
Carbonic anhydrase I	VLDALQAIK	y ₇ ⁺	485.8002	758.4407	489.8073	766.4549	23.62	9
Carboxypeptidase B2	IAWHVIR	y ₄ ²⁺	298.8518	262.6729	302.1851	267.6729	16.37	9
Cathelicidin antimicrobial peptide	AIDGINQR	y ₆ ⁺	443.7407	702.3529	448.7448	712.3612	5.82	17
Cation-independent mannose-6-phosphate receptor	GHQAFDVGQPR	y ₄ ⁺	404.5354	457.2518	407.8715	467.2600	7.79	13
CD5 antigen-like	LVGGLHR	y ₅ ⁺	376.2323	539.3049	381.2364	549.3131	4.87	13
Ceruloplasmin	IYHSHIDAPK	y ₈ ²⁺	394.2085	452.7354	396.8799	456.7425	4.29	9

Cholinesterase	YLTLNTESTR	y ₈ ⁺	599.3091	921.4636	604.3133	931.4719	13.96	25
Clusterin	ELDESLQVAER	y ₃ ⁺	644.8226	375.1987	649.8267	385.2069	15.69	17
Coagulation factor IX	SALVLQYLR	y ₅ ⁺	531.8189	692.4090	536.8231	702.4173	30.11	17
Coagulation factor V	SEAYNTFSER	y ₈ ⁺	602.2675	987.4530	607.2716	997.4613	9.33	17
Coagulation factor VIII	LHPTHYSIR	y ₇ ²⁺	375.2051	437.2325	378.5412	442.2366	12.5	9
Coagulation factor X	TGIVSGFGR	y ₅ ⁺	447.2456	523.2623	452.2497	533.2706	15.37	13
Coagulation factor XII	EQPPSLTR	y ₆ ²⁺	464.2483	335.6978	469.2525	340.7019	6.74	9
Complement C1q subcomponent subunit B	IAFSATR	y ₅ ⁺	383.2163	581.3042	388.2205	591.3125	9.49	9
Complement C1q subcomponent subunit C	FNAVLTNPQGDYD TSTGK	y ₁₁ ²⁺	643.3059	584.7595	645.9773	588.7666	19.86	9
Complement C1r subcomponent	GLTLHLK	y ₅ ²⁺	261.1692	306.1974	263.8406	310.2045	13.81	5
Complement C1r subcomponent-like protein	VVVHPDYR	y ₇ ²⁺	328.8469	443.2325	332.1830	448.2366	5.75	5
Complement C1s subcomponent	TNFDNDIALVR	b ₂ ⁺	639.3279	216.0979	644.3320	216.0979	23.87	17
Complement C2	HAFILQDTK	b ₄ ⁺	358.1977	469.2558	360.8691	469.2558	11.94	5
Complement C3	TGLQEVEVK	y ₆ ⁺	501.7769	731.3934	505.7840	739.4076	11.71	17
Complement C5	VFQFLEK	y ₅ ⁺	455.7553	664.3665	459.7624	672.3807	24.42	9
Complement component C7	LIDQYGTHYLQSG SLGGEYR	b ₂ ⁺	753.0343	227.1754	756.3704	227.1754	4.94	25
Complement component C9	LSPINYNLVPVK	y ₉ ²⁺	621.8765	521.8184	625.8836	525.8255	33.22	17
Complement factor B	EELLPAQDIK	y ₆ ⁺	578.3164	671.3723	582.3235	679.3865	19.39	13
Complement factor H	SSQESYAHGTK	y ₉ ²⁺	398.8510	510.7409	401.5224	514.7480	2.30	5
Complement factor I	VFSLQWGEVK	y ₈ ⁺	596.8217	946.4993	600.8288	954.5135	30.84	13
Corticosteroid-binding globulin	WSAGLTSSQVDLYIPK	y ₂ ⁺	882.9620	244.1656	886.9691	252.1798	34.46	29

(continued)

Table 3
(continued)

Protein	Peptide	Product Ion/ Charge	NAT		SIS		RT (min)	CE (V)
			Precursor m/z	Product m/z	Precursor m/z	Product m/z		
Cystatin-C	ALDFAVGEYNK	y ₆ ⁺	613.8062	709.3515	617.8133	717.3657	22.72	13
Endothelial protein C receptor	TLAFPLTIR	y ₇ ⁺	516.3160	817.4931	521.3202	827.5013	34.09	13
Fetuin-B	LVVLPEPK	y ₆ ⁺	456.7995	700.4392	460.8066	708.4534	32.26	9
Fibrinogen α chain	ESSSHHPGIAEEPSR	y ₄ ⁺	546.5937	506.2722	549.9298	516.2804	9.76	21
Fibrinogen β chain	HQLYIDETVNSNIP TNLR	b ₁₃ ²⁺	709.6992	764.3755	713.0352	764.3755	25.7	9
Fibrinogen γ chain	YEASILTHDSSIR	y ₁₁ ²⁺	497.9195	600.3226	501.2555	605.3267	16.33	13
Fibronectin	HTSVQTTSSGSGPF TDVR	y ₆ ⁺	621.9659	734.3832	625.3020	744.3914	12.13	13
Fibulin-1	TGYFFDGISR	y ₆ ⁺	589.7775	694.3519	594.7816	704.3601	20.64	17
Galectin-3-binding protein	SDLAVPSELALLK	y ₈ ⁺	678.3927	870.5295	682.3998	878.5437	35.83	13
Gelsolin	AGALNSNDAFVLK	b ₃ ⁺	660.3513	200.1030	664.3584	200.1030	22.97	25
Glutathione peroxidase 3	QEPGENSEILPTLK	y ₁₂ ²⁺	777.9041	649.3535	781.9112	653.3606	23.17	25
Haptoglobin	DIAPTLTLVVGK	y ₉ ²⁺	645.8688	496.2948	649.8759	500.3019	31.67	9
Hemoglobin subunit α	VGAHAGEYGAEALER	y ₄ ⁺	510.5829	488.2827	513.9190	498.2910	10.94	9
Hemopexin	NFPSPVDAAFR	y ₉ ²⁺	610.8066	480.2509	615.8107	485.2550	27.04	13
Heparin cofactor 2	TLEAQLTPR	y ₇ ⁺	514.7904	814.4417	519.7945	824.4500	13.89	17
Hepatocyte growth factor-like protein	SPLNDFQVLR	y ₇ ⁺	594.8222	891.4683	599.8263	901.4766	28.23	17
Hyaluronan-binding protein 2	VVLGDQDLK	y ₇ ⁺	493.7795	788.4149	497.7866	796.4291	13.42	17

Ig mu chain C region	GFPSVLR	y ₅ ²⁺	388.2267	286.1817	393.2308	291.1859	20.33	9
Insulin-like growth factor I	GFYFNKPTGYGSSSR	y ₁₃ ²⁺	556.5986	732.3493	559.9347	737.3535	16.87	13
Inter-α-trypsin inhibitor heavy chain H2	SLAPTAALK	y ₆ ⁺	415.2425	558.3246	419.2496	566.3388	5.12	9
Intercellular adhesion molecule 1	LLGIETPLPK	y ₈ ⁺	540.8368	854.4982	544.8439	862.5124	15.88	17
Interleukin-10	AHVNSLGENLK	y ₅ ⁺	394.5473	560.3039	397.2187	568.3181	7.12	9
Kallistatin	VGSALFLSHNLK	y ₁₁ ²⁺	429.2470	593.8326	431.9183	597.8397	20.93	9
Keratin-type I cytoskeletal 10	SLLEGEGSSGGGGR	b ₂ ⁺	631.8022	201.1234	636.8063	201.1234	7.92	25
Keratin-type II cytoskeletal 2 epidermal	YEELQVTVGR	b ₂ ⁺	597.3117	293.1132	602.3158	293.1132	17.33	17
Kininogen-1	TVGSDTFYSFK	y ₉ ⁺	626.2982	1051.4731	630.3053	1059.4873	22.22	13
Leucine-rich α-2-glycoprotein	DLLLPQPDLR	y ₆ ⁺	590.3402	725.3941	595.3444	735.4023	30.62	9
Lipopolysaccharide-binding protein	ITLPDFTGDLR	y ₈ ⁺	624.3352	920.4472	629.3393	930.4555	34.27	17
L-selectin	AEIEYLEK	y ₆ ⁺	497.7582	794.4294	501.7653	802.4436	14.16	9
Lysozyme C	AWVAWR	y ₄ ⁺	394.7137	531.3038	399.7179	541.3121	21.00	9
Mannan-binding lectin serine protease 1	TGVITSPDFNPYPK	b ₃ ⁺	816.9170	258.1448	820.9241	258.1448	26.88	25
Mannan-binding lectin serine protease 2	WPEPVFGR	y ₅ ⁺	494.2560	575.3300	499.2601	585.3383	25.84	17
Metalloproteinase inhibitor 2	EYLIAGK	y ₃ ⁺	397.2264	275.1714	401.2335	283.1856	11.14	9
Myeloblastin	LVNVVLGAHNVR	y ₁₀ ²⁺	430.9262	539.8094	434.2623	544.8136	17.45	5
Peroxiredoxin-2	GLFIIDGK	y ₆ ⁺	431.7553	692.3978	435.7624	700.4120	27.03	9
Phosphatidylinositol-glycan-specific phospholipase D	FGSSLITVR	y ₄ ⁺	490.2822	488.3191	495.2863	498.3274	21.52	13
Phospholipid transfer protein	AVEPQLQEEER	y ₈ ²⁺	664.3281	514.7540	669.3322	519.7581	9.36	17
Pigment epithelium-derived factor	LQSLFDSPDFSK	y ₁₀ ⁺	692.3432	1142.5364	696.3503	1150.5506	28.44	17
Plasma protease C1 inhibitor	FQPTLLTLPR	y ₈ ⁺	593.3531	910.5720	598.3573	920.5803	32.61	17
Plasma serine protease inhibitor	AVVEVDESGTR	y ₉ ²⁺	581.2909	496.2382	586.2951	501.2423	7.64	13

(continued)

Table 3
(continued)

Protein	Peptide	Product Ion/ Charge	NAT		SIS		RT (min)	CE (V)
			Precursor m/z	Product m/z	Precursor m/z	Product m/z		
Plasminogen	LFLEPTR	b ₂ ⁺	438.2529	261.1598	443.2570	261.1598	34.25	9
Plasminogen activator inhibitor 1	VFQQVAQASK	y ₅ ⁺	553.3037	504.2776	557.3108	512.2918	7.53	13
Pregnancy zone protein	ISEITNIVSK	y ₆ ⁺	552.3190	661.3879	556.3261	669.4021	18.45	17
Protein AMBP	HHGPTITAK	y ₂ ⁺	321.1786	218.1499	323.8500	226.1641	2.62	13
Protein S100-A9	DLQNELK	y ₅ ⁺	439.2425	649.3668	443.2496	657.3810	23.70	9
Protein Z-dependent protease inhibitor	ETSNFGFSLR	y ₆ ⁺	635.8250	692.4090	640.8291	702.4173	33.06	17
Prothrombin	ELLESYIDGR	y ₆ ⁺	597.8037	710.3468	602.8078	720.3550	24.47	21
Retinol-binding protein 4	YWGVASFLQK	y ₈ ⁺	599.8164	849.4829	603.8235	857.4971	34.68	13
Serotransferrin	DGAGDVAFVK	y ₇ ⁺	489.7482	735.4036	493.7553	743.4178	14.06	13
Serum albumin	LVNEVTEFAK	y ₂ ⁺	575.3111	218.1499	579.3182	226.1641	19.45	37
Serum paraoxonase/arylesterase 1	IFFYDSENPPASEVLR	y ₈ ⁺	942.4623	868.4887	947.4665	878.4970	34.14	37
Serum paraoxonase/lactonase 3	IQNVLSEKPR	y ₈ ²⁺	395.2313	471.7720	398.5674	476.7761	9.01	9
SPARC	LEAGDHPVELLAR	y ₁₁ ²⁺	473.9245	589.3198	477.2606	594.3240	19.17	9
Tenascin	FTTDLSPR	y ₇ ⁺	526.2564	803.3894	531.2605	813.3976	12.24	17
Tenascin-X	ILISGLEPSTPYR	b ₂ ⁺	723.4036	227.1754	728.4077	227.1754	28.20	21
Thrombospondin-1	GTLALER	y ₄ ⁺	436.7636	488.2827	441.7678	498.2910	19.99	13
Thrombospondin-4	KPQDFLEELK	y ₄ ⁺	416.2274	518.2821	418.8988	526.2963	23.95	9
Thyroxine-binding globulin	AVLHIGEK	y ₆ ²⁺	289.5080	348.7056	292.1794	352.7127	7.95	5

Tissue factor pathway inhibitor	FYNSVIGK	y ⁷⁺	545.7820	780.4250	549.7891	788.4392	20.09	9
Tissue-type plasminogen activator	VVPGEEEQK	y ⁷⁺	507.7587	816.3734	511.7658	824.3876	3.49	9
Transferrin receptor protein 1	GFVEPDHYVVVGAQR	y ₁₃ ²⁺	558.2862	734.8808	561.6223	739.8849	21.50	13
Transthyretin	GSPAINVAHVFR	y ₁₁ ²⁺	456.2578	611.8564	459.5939	616.8605	25.73	5
Vascular cell adhesion protein 1	NTVISVNPSTK	y ₈ ⁺	580.3195	845.4727	584.3266	853.4869	11.48	17
Vasorin	ESHVTLASPEETR	y ₅ ⁺	485.9073	631.3046	489.2434	641.3128	8.14	9
Vitamin K-dependent protein S	SFQTGLFTAAR	y ₈ ⁺	599.8144	836.4625	604.8185	846.4707	24.59	21
Vitamin K-dependent protein Z	GLLSGWAR	y ₅ ⁺	430.2429	576.2889	435.2470	586.2971	24.20	9
Vitronectin	FEDGVLDPPDYPR	y ₅ ⁺	711.8304	647.3148	716.8346	657.3230	23.14	33
Zinc-α-2-glycoprotein	EIPAWVPEDPAAQITK	y ₁₀ ⁺	891.9749	1087.5782	895.9820	1095.5924	41.70	25

For simplicity, shown are the NAT and SIS transition details for the quantifier ions. The PRM acquisitions target the precursor ions, with alternative collision energies stemming from empirical optimizations on the Q Exactive Plus.

4. Use the following general MRM parameters for all transitions in the final method: unit mass resolution (for first and third quadrupole), 1.5 min RT windows, 380 V fragmentor voltage, 5 V cell accelerator potential, and 10 ms dwell times. The total cycle time for this acquisition method run on this LC-MS platform is 700 ms.

3.7 PRM/MS System Setup

1. Operate the ESI source in the positive ion mode.
2. Use the following general parameters: 350 °C capillary temperature, 50 L/min sheath gas, 20 L/min auxiliary gas at 350 °C, 0 sweep gas, 3 kV spray voltage, and S-lens RF level at 50. Ultrahigh-purity nitrogen serves as the carrier gas in all settings.
3. Additional PRM parameters include 17.5 k resolution, 2e5 automatic gain control (AGC) target, 60 ms maximum injection time (IT), and 30 loop count.
4. Using the PRM mode, enter the specific acquisition parameters. This comprises an inclusion list consisting of peptide precursor m/z values and RTs (inserted as “Start” and “End” times over 1.5 min window; *see* Table 3).

3.8 LC-MS/MS Platform Performance Test

1. Purge the pumps with 50% mobile phase B for 5 min at 10 mL/min.
2. Once the UHPLC system is re-equilibrated to the starting conditions (i.e., 2% eluent B), run a solvent blank (at 0.1% FA) to flush the 20 µL loop and connecting tubing.
3. Using the ESI tuning mix, run the automated tuning program to tune or realign a set of m/z values for positive ESI-MS/MS analysis.
4. Following a successful instrument tune, confirm the peptide RTs by injecting 10 µL of a calibrant standard (*see* Note 10, for scheduling, and Note 11, for data analysis).
5. Input the updated RTs into the acquisition method and use 1.5 min in all target acquisition windows.

3.9 Sample Injection

1. After the performance tests (for LC RTs and mass analyzer tuning, *see* Subheading 3.7) have been successful, a sample batch can be queued for analysis. Worklists vary, but in our practice, the first sample to be injected (all at 10 µL) is the first replicate of the curve QCs (order: A in well H10, B in C11, then C in F11).
2. Inject two solvent blanks after the three QC samples (replicate 1), and then inject a single replicate of the standard curve samples from low concentration (Standard A in well A12) to high concentration (Standard H in H12).

3. Inject two solvent blanks after the calibration-curve samples; then inject the second replicate of the curve QC samples (in the order: A in A11, B in D11, then C in G11).
4. Inject two solvent blanks after the three QC samples (replicate 2), and then inject the experimental samples ($n = 20$), individually, from well A1 to D3.
5. Inject two solvent blanks after the experimental samples, and then inject the final replicate of the curve QCs (order: A in B11, B in E11, C in H11).
6. After the completion of the sample batch, set the LC to an isocratic flow of 0.02 mL/min with 50% eluent B. Divert the solvent stream to waste to help preserve the MS ion source until the next worklist is initiated.

3.10 Data Analysis

Although the analysis of the MRM or PRM data can be performed with several vendors' software programs, for the purposes here, a guide for executing this in Skyline-daily is outlined (*see* [34] for login and installation instructions).

1. Starting with an empty Skyline document set in the "Proteomics Interface," build the MRM or PRM target list. This can be inserted or copied directly from Excel, provided that the column names and order are the same as in the "Skyline Transition List" window. After pasting, click on "Check for Errors," and then save the populated Skyline Window.
2. In the main screen, import the raw data by clicking on "File," "Import," and then "Results." In the "Import Results" window, select "Add single-injection replicates in files" and enable "Show chromatograms during import." After selecting "OK," locate and highlight the appropriate data files (i.e., .d for Agilent, .raw for Thermo) to be imported.
3. In the document grid, select "Views" and "Replicates." In that tab, input the "Analyte Concentration" values for the curve calibrants and QC samples, as illustrated in Fig. 2.
4. In the main screen, select "View," "Document Grid," and then "Peptide Quantification." Enter the peptide-specific concentration multipliers, as outlined in Table 4 (*see* Note 12).
5. In the "Quantification" tab, adjust the peptide settings to allow the quantification of the experimental samples by linear regression using the NAT/SIS peptide ratios with a regression weighting of $1/x^2$ (x = concentration).
6. Manually inspect the extracted ion chromatograms (XICs) and calibration curves for the control samples to ensure correct peak selection and accurate integration (*see* Fig. 3 and Note 13 for projected observations).

Replicate	Sample Type	Analyte Concentration
QC-A_R01	Quality Control	4
QC-A_R02	Quality Control	4
QC-A_R03	Quality Control	4
QC-B_R01	Quality Control	30
QC-B_R02	Quality Control	30
QC-B_R03	Quality Control	30
QC-C_R01	Quality Control	300
QC-C_R02	Quality Control	300
QC-C_R03	Quality Control	300
STD-A	Standard	1
STD-B	Standard	2.5
STD-C	Standard	5
STD-D	Standard	20
STD-E	Standard	40
STD-F	Standard	160
STD-G	Standard	400
STD-H	Standard	1000
Experimental Sample	Unknown	

Fig. 2 Peptide concentrations to be entered in Skyline's "Document Grid: Replicates." These fmol/ μ L values reflect the dilution series of this methods protocol, which comprises an 8-point standard curve (1000-fold range) and 3-point QCs (labeled -A to -C). No concentrations are to be entered for the experimental samples

7. Review the accuracy of the control-based samples, as calculated automatically by Skyline, against the batch acceptance criteria (*see* **Note 14**).
8. Using the control XICs as reference, manually inspect the experimental sample XICs (e.g., for peak symmetry, retention time, interference) and its relation to the curve (*see* Fig. 4).
9. Export the collective Skyline results for further review and analysis. Data extraction pertains to the peaks (e.g., SIS and NAT responses, retention times) and quantified peptides (*see* **Note 15**).

Table 4
Target details for the quantification calculations

Protein	UniProt acc. no.	Peptide	MW (Da)	Concentration multiplier
78 kDa glucose-regulated protein	P11021	ITPSYVAFTPEGER	72,333	15
Adipocyte plasma membrane-associated protein	Q9HDC9	LLEYDTVTR	46,480	4.4
Adiponectin	Q15848	IFYNQNHYDG STGK	26,414	3.7
Afamin	P43652	DADPDITFFAK	69,069	32.1
α -1-acid glycoprotein 1	P02763	NWGLSVYADKPE TTK	23,512	168.5
α -1-Antichymotrypsin	P01011	EIGELYLPK	47,651	37.5
α -1-antitrypsin	P01009	LSITGTYDLK	46,737	49.4
α -1B-glycoprotein	P04217	LETPDFQLFK	54,254	85.2
α -2-Antiplasmin	P08697	LGNQEPGGQTALK	54,566	7.6
α -2-HS-glycoprotein	P02765	FSVVYAK	39,325	27.6
α -2-macroglobulin	P01023	AIGYLNTGYQR	16,3291	41.3
Antithrombin-III	P01008	DDLIVSDAFHK	52,602	3.3
Apolipoprotein A-I	P02647	ATEHLSTLSEK	30,778	132.3
Apolipoprotein A-II	P02652	EQLTPLIK	11,175	133.2
Apolipoprotein A-IV	P06727	LGEVNTYAGDLQK	45,399	49.7
Apolipoprotein B-100	P04114	FPEVDVLTK	515,605	17.8
Apolipoprotein C-I	P02654	EWFSETFQK	9332	33.3
Apolipoprotein C-II	P02655	TYLPAVDEK	11,284	9.9
Apolipoprotein C-III	P02656	GWVTDGFSSLK	10,852	70.6
Apolipoprotein C-IV	P55056	ELLETVVNR	14,553	9.5
Apolipoprotein D	P05090	VLNQELR	21,276	5.9
Apolipoprotein E	P02649	LGPLVEQGR	36,154	14.9
Apolipoprotein L1	O14791	VAQELEEK	43,974	7.2
Apolipoprotein M	O95445	AFLLTTPR	21,253	9.8
Apolipoprotein(a)	P08519	TPAYYPNAGLIK	501,319	4.8
Attractin	O75882	SVNNVVVR	158,537	4.2
β -2-glycoprotein 1	P02749	ATVVYQGER	38,298	38.8
β -Ala-his dipeptidase	Q96KN2	ALEQDLPVNIK	56,706	15.4
Biotinidase	P43251	SHLIIAQVAK	61,133	2.9

(continued)

Table 4
(continued)

Protein	UniProt acc. no.	Peptide	MW (Da)	Concentration multiplier
Cadherin-13	P55290	INENTGSVSVTR	78,287	60.5
Carbonic anhydrase 1	P00915	VLDALQAIK	28,870	6.1
Carboxypeptidase B2	Q96IY4	IAWHVIR	48,424	37.9
Cathelicidin antimicrobial peptide	P49913	AIDGINQR	19,301	15.7
Cation-independent mannose-6-phosphate receptor	P11717	GHQAFDVGQPR	274,375	1.9
CD5 antigen-like	O43866	LVGGLHR	38,088	89.8
Ceruloplasmin	P00450	IYHSHIDAPK	122,205	51.4
Cholinesterase	P06276	YLTNTESTR	68,418	5.3
Clusterin	P10909	ELDESLQVAER	52,495	4.1
Coagulation factor IX	P00740	SALVLQYLR	51,778	70.6
Coagulation factor V	P12259	SEAYNTFSER	251,703	52.3
Coagulation factor VIII	P00451	LHPTHYSIR	267,009	97
Coagulation factor X	P00742	TGIVSGFGR	54,732	6.4
Coagulation factor XII	P00748	EQPPSLTR	67,792	7.8
Complement C1q subcomponent subunit B	P02746	IAFSATR	26,722	11.7
Complement C1q subcomponent subunit C	P02747	FNAVLTNPQGDYD TSTGK	25,774	42.8
Complement C1r subcomponent	P00736	GLTLHLK	80,119	3.7
Complement C1r subcomponent-like protein	Q9NZP8	VVVHPDYR	53,498	3.9
Complement C1s subcomponent	P09871	TNFDNDIALVR	76,684	51.2
Complement C2	P06681	HAFILQDTK	83,268	5.3
Complement C3	P01024	TGLQEVEVK	187,148	161.5
Complement C5	P01031	VFQFLEK	188,305	12.1
Complement component C7	P10643	LIDQYGTHYLQSG SLGGEYR	93,518	293
Complement component C9	P02748	LSPIYNLVPVK	63,173	33.6
Complement factor B	P00751	EELLPAQDIK	85,533	13.8
Complement factor H	P08603	SSQESYAHGTK	139,096	19.1
Complement factor I	P05156	VFSLQWGEVK	65,750	75.8

(continued)

Table 4
(continued)

Protein	UniProt acc. no.	Peptide	MW (Da)	Concentration multiplier
Corticosteroid-binding globulin	P08185	WSAGLTSSQVDL YIPK	45,141	2570.3
Cystatin-C	P01034	ALDFAVGEYNK	15,799	13.8
Endothelial protein C receptor	Q9UNN8	TLAFPLTIR	26,671	12.6
Fetuin-B	Q9UGM5	LVVLPFPK	42,055	81.9
Fibrinogen α chain	P02671	ESSSHHPGIAEFPSR	94,973	45.9
Fibrinogen β chain	P02675	HQLYIDETVNSNIP TNLR	55,928	35.4
Fibrinogen γ chain	P02679	YEASILTHDSSIR	51,512	256.4
Fibronectin	P02751	HTSVQTTSSGSGPF TDVR	262,625	35.1
Fibulin-1	P23142	TGYYFDGISR	77,214	10.1
Galectin-3-binding protein	Q08380	SDLAVPSELALLK	65,331	62.3
Gelsolin	P06396	AGALNSNDAFVLK	85,698	52.7
Glutathione peroxidase 3	P22352	QEPGENSEILPTLK	25,552	6.7
Haptoglobin	P00738	DIAPTLTLYVGK	45,205	515.8
Hemoglobin subunit α	P69905	VGAHAGEYGAE ALER	15,258	11.2
Hemopexin	P02790	NFPSPVDAAFR	51,676	73.2
Heparin cofactor 2	P05546	TLEAQLTPR	57,071	14
Hepatocyte growth factor-like protein	P26927	SPLNDFQVLR	80,320	51.1
Hyaluronan-binding protein 2	Q14520	VVLGDQDLK	62,672	5.7
Ig mu chain C region	P01871	GFPSVLR	49,440	39.6
Insulin-like growth factor I	P05019	GFYFNKPTGYGSSSR	21,841	36.3
Inter- α -trypsin inhibitor heavy chain H2	P19823	SLAPTAAAK	106,463	37.3
Intercellular adhesion molecule 1	P05362	LLGIETPLPK	57,825	10.5
Interleukin-10	P22301	AHVNSLGENLK	20,517	4.8
Kallistatin	P29622	VGSALFLSHNLK	48,542	77.9
Keratin-type I cytoskeletal 10	P13645	SLLEGEGSSGGGGR	58,827	887.6
Keratin-type II cytoskeletal 2 epidermal	P35908	YEELQVTVGR	65,433	326.7

(continued)

Table 4
(continued)

Protein	UniProt acc. no.	Peptide	MW (Da)	Concentration multiplier
Kininogen-1	P01042	TVGSDTFYSEK	71,957	69.9
Leucine-rich α -2-glycoprotein	P02750	DLLLPQPDLR	38,178	5.5
Lipopolysaccharide-binding protein	P18428	ITLPDFTGDLR	53,384	42.8
L-selectin	P14151	AEIEYLEK	42,187	9.3
Lysozyme C	P61626	AWVAWR	16,537	5.3
Mannan-binding lectin serine protease 1	P48740	TGVITSPDFNPYPK	79,247	70.5
Mannan-binding lectin serine protease 2	O00187	WPEPVFGR	75,702	64.7
Metalloproteinase inhibitor 2	P16035	EYLIAGK	24,399	3.8
Myeloblastin	P24158	LVNVVLGAHNVR	27,807	97.1
Peroxiredoxin-2	P32119	GLFIIDGK	21,892	17
Phosphatidylinositol-glycan-specific phospholipase D	P80108	FGSSLITVR	92,336	4.9
Phospholipid transfer protein	P55058	AVEPQLQEEER	54,739	5.6
Pigment epithelium-derived factor	P36955	LQSLFDSPDFSK	46,312	42.4
Plasma protease C1 inhibitor	P05155	FQPTLLTLPR	55,154	26.1
Plasma serine protease inhibitor	P05154	AVVEVDESGTR	45,675	2.9
Plasminogen	P00747	LFLEPTR	90,569	9.5
Plasminogen activator inhibitor 1	P05121	VFQQVAQASK	45,060	15.3
Pregnancy zone protein	P20742	ISEITNIVSK	163,863	4.1
Protein AMBP	P02760	HHGPTITAK	38,999	175.1
Protein S100-A9	P06702	DLQNFLK	13,242	10.6
Protein Z-dependent protease inhibitor	Q9UK55	ETSNFGFSLLR	50,707	56.5
Prothrombin	P00734	ELLESYIDGR	70,037	33.3
Retinol-binding protein 4	P02753	YWGVASFLQK	23,010	70.4
Serotransferrin	P02787	DGAGDVAFVK	77,064	352.5
Serum albumin	P02768	LVNEVTEFAK	69,367	1396.9
Serum paraoxonase/arylesterase 1	P27169	IFFYDSENPPASEVL R	39,731	46.7
Serum paraoxonase/lactonase 3	Q15166	IQNVLSEKPR	39,607	4.8

(continued)

Table 4
(continued)

Protein	UniProt acc. no.	Peptide	MW (Da)	Concentration multiplier
SPARC	P09486	LEAGDHPVELLAR	34,632	10
Tenascin	P24821	FTTDLDSR	240,853	5.7
Tenascin-X	P22105	ILISGLEPSTPYR	458,220	10.5
Thrombospondin-1	P07996	GTLLALER	129,383	4.2
Thrombospondin-4	P35443	KPQDFLEELK	105,869	42
Thyroxine-binding globulin	P05543	AVLHIGEK	46,325	6.1
Tissue factor pathway inhibitor	P10646	FYNSVIGK	35,015	7.8
Tissue-type plasminogen activator	P00750	VVPGEEEQK	62,917	90.4
Transferrin receptor protein 1	P02786	GFVEPDHYVVVGA QR	84,871	25.5
Transthyretin	P02766	GSPAINVAVHVFR	15,887	24.6
Vascular cell adhesion protein 1	P19320	NTVISVNPSTK	81,276	4.4
Vasorin	Q6EMK4	ESHVTLASPEETR	71,713	27.6
Vitamin K-dependent protein S	P07225	SFQTGLFTAAR	75,123	26.7
Vitamin K-dependent protein Z	P22891	GLLSGWAR	44,744	38.7
Vitronectin	P04004	FEDGVLDPDYPR	54,306	75.2
Zinc- α -2-glycoprotein	P25311	EIPAWVPFDPAQI TK	34,259	30.8

The peptide-specific concentration multipliers are to be inserted in the Skyline method builder, while the resulting fmol/ μ L concentrations can be converted to ng/mL through aid of the protein molecular weights (MWs)

4 Notes

1. The stability of the prepared reagents (i.e., Reagents A to E, BSA/PBS, and trypsin) is predicated based on their time of preparation and storage conditions. Reagent A (stock Tris solution) can be stored at 4 °C for 1 month. The remaining reagents noted above are to be prepared fresh, on day of use. Reagent C (comprising urea and DTT in Tris), Reagent D (IAA), and trypsin are to be prepared immediately prior to use and stored on ice until dispensed.
2. Do not heat the urea solution in an effort to hasten its solubilization. Elevated temperature (i.e., >37 °C) accelerates urea decomposition, with its product (isocyanic acid) resulting in

Fig. 3 Theoretical plot of control and experimental sample analysis by bottom-up LC-MS/MS. **(a)** XICs of expected NAT/SIS peptide ratios (NAT in red, SIS in blue) in a buffer-generated, standard curve. **(b)** Placement of 8-point calibrant levels (in green diamonds) and 3-point QCs (in blue diamonds) relative to a plasma sample measurement. Quantitation of this sample is then accomplished through linear regression analysis of the peptide relative response (i.e., NAT/SIS peak area ratio)

carbamylation at ϵ -amine lysine residues and protein N-termini [35]. To reduce this artifact, dissolve the urea at room temperature with the aid of vortexing only.

3. The NAT and SIS peptides were synthesized (at the University of Victoria-Genome BC Proteomics Centre; Victoria, BC, Canada) on a robotic peptide synthesizer using Fmoc chemistry. The labeled protected amino acids ($^{13}\text{C}_6/^{15}\text{N}_2$ L-lysine, CNLM-4754-H; $^{13}\text{C}_6/^{15}\text{N}_4$ L-arginine, CNLM-8474-H), used in the C-terminal labeling of tryptic peptide residues, were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). After synthesis, the standard peptides were purified (by HPLC) and characterized (for composition and concentration) and then formulated into mixtures, in a manner similar to that described previously [36]. Lyophilized aliquots of the mixtures are stored at -80°C , with established stabilities of at least 6 months. Overall, the peptides selected are proteotypic and abide by a series of sequence-specific selection rules (e.g., unique within plasma proteome, devoid of cysteine and methionine, 6–20 residues in length), which makes them well suited to serve as external or internal standards.

4. If an alternative SIS peptide labeling scheme is desired, as would be the case with double isotopologue peptide standards (i.e., isotope labeling at a C-terminal amino acid as well as an internal residue), the protocol described herein would need to be amended. The methods article by Eshghi et al. serves as a useful reference guide for the quantitative application of the double SIS peptide approach to different sample types [37]. Regardless of the type of peptide standard, these should be prepared, stored, and handled according to established guidelines for best practices in MS-based proteomics [38].
5. The ideal quantity of trypsin added was previously determined in empirical measurements. The 20:1 (w/w) substrate/enzyme ratio implemented enables efficient tryptic digestion in pooled human plasma samples. Appropriate substrate/enzyme ratios for alternative sample types will need to be determined before application to real experimental samples.
6. The current protocol is amenable to be scaled up to 30 samples. For larger-scale analysis, the procedure will need to be adapted. This affects the quantity of surrogate digest initially prepared (in Subheading 3.1) and the quantity of SIS peptide mixture to be reconstituted (in Subheading 2.3).
7. For all extraction steps, add solvent or sample before applying vacuum. The flow through and washes (extraction steps in Subheading 3.4) are to be collected in an empty basin and can be discarded. In the sample loading step, start at the lowest vacuum setting and then gradually increase the vacuum to load the full sample onto the sorbent bed. Flow rates for sample loading and elution should not exceed 1 mL/min. Also, for improved throughput and precision, all SPE steps should be performed with a multichannel pipette or with a liquid handling robot (e.g., Tecan Freedom EVO 150).
8. Ensure that the sample container of the LC autosampler is configured to read a 96-well plate in one tray (for the digests) and vials in a second tray (with one designated for the solvent blank, e.g., at position A1). This latter tray will need to be loaded first with a vial plate to accommodate the autosampler vial(s).
9. The specific acquisition parameters (i.e., transitions, collision energies, and retention times) will need to be optimized if alternative mass spectrometers are used in place of the 6495 or Q Exactive instrument. It is recommended that these be selected based on empirical measurements using the SIS peptide mixtures as MS infusion solutions.
10. Although we have observed minimal RT shifts in method transfer between laboratories when identical LC-MS conditions/parameters are employed, it is recommended that a

broader detection window of 3 min be initially used for peptide RT verification. This test should require one run only, with standard E being an example standard for injection. After the peptide rescheduling, confirm that the cycle/dwell times enable 10–15 data points across the chromatographic peaks for optimum and accurate peak shapes. It must be noted that although the RT verification is conducted in buffer, in our experience, minimal shifts (maximally 0.5 min) are typically observed when the assay is transferred to human plasma.

11. Analysis of the peptide scheduling data can be accomplished with vendor software, Skyline [39], or other (e.g., Qualis-SIS [40]). If Skyline is used, as advocated here, refer to Skyline's tutorial section for guidance on method refinement for efficient acquisitions. After the retention times have been confirmed for all targets, export the data file to Excel and then update the acquisition method accordingly.
12. The analyte concentration refers to the initial peptide concentration in the standard mixture and is used as a basis for the quantitation calculations. The concentration multipliers are used to automatically convert the calculated values to protein concentrations (expressed in fmol/ μ L of human plasma).
13. The NAT (synthetic and endogenous) and SIS peptides have identical physicochemical properties and therefore exhibit the same behavior during extraction, separation, and mass analysis (e.g., ionization, fragmentation). The resulting XICs for each peptide pair should therefore co-elute in all sample analyses, with the responses of the NAT standards differing from SIS in the curves and QC samples only (*see* Fig. 3a). Regarding the ratios, the calibrant levels should approximate a 1:2.5:2.5:4:2:4:2:2.5 dilution from standard H to A (e.g., standard E is 25-fold diluted from standard H), with the QC samples being x-fold from standard H (i.e., 3.33-fold, in QC-C; 33.3-fold, in QC-B; 250-fold, in QC-A; *see* Fig. 3b). The relative responses and ratios should be confirmed for all sample types.
14. The accuracies of the standard curve and curve QC samples are reported in Skyline as a percent theoretical value. For each peptide, each measured NAT standard in the curve should be within 20% of the theoretical concentration, and at least 6 of the 9 measured NAT concentrations in the QC samples should be within 20% of the theoretical concentrations. In addition, for each peptide, at least 1 of 3 of the QC samples (QC-A, QC-B, or QC-C) should be within 20% of its theoretical concentration. Finally, for each peptide quantified, at least 6 of the 8 measured in the standard curve should be within 20% of the expected concentration.

15. To convert the peptide concentrations (in fmol/ μ L) to protein concentrations (in ng/mL), multiply the calculated concentrations from Skyline by the molecular weight of a given protein (*see* Table 4) and then divide by 1000. For example, the concentration of ATVVYQGER in a de-identified human plasma sample (from Bioreclamation, lot BRH1447352) was determined to be 1897.5 fmol/ μ L, which equates to 72.6 μ g/mL for β -2-glycoprotein 1 (also referred to as apolipoprotein H or Apo-H).

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Competing Interests: The target panel and protocols described in this article are similar to those used in the PeptiQuant™ Plus biomarker assessment kit for protein quantification in human plasma. These kits are commercially available through MRM Proteomics Inc. (Montreal, QC, Canada) where Dr. Borchers is the Chief Scientific Officer, and its partner, Cambridge Isotope Laboratories, Inc., where Dr. Percy is the Senior Applications Chemist for Mass Spectrometry.

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