**Method and Platform Standardization in MRM-based Quantitative Plasma Proteomics**

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**Abstract**

There exists a growing demand in the proteomics community to standardize experimental methods and liquid chromatography-mass spectrometry (LC/MS) platforms in order to enable the acquisition of more precise and accurate quantitative data. This necessity is heightened by the evolving trend of verifying and validating candidate disease biomarkers in complex biofluids, such as blood plasma, through targeted multiple reaction monitoring (MRM)-based approaches with stable isotope-labeled standards (SIS). Considering the lack of performance standards for quantitative plasma proteomics, we previously developed two reference kits to evaluate the MRM with SIS peptide approach using undepleted and non-enriched human plasma. The first kit tests the effectiveness of the LC/MRM-MS platform (kit #1), while the second evaluates the performance of an entire analytical workflow (kit #2). Here, these kits have been refined for practical use and then evaluated through intra- and inter-laboratory testing on 6 common LC/MS platforms. For an identical panel of 22 plasma proteins, similar concentrations were determined, regardless of the kit, instrument platform, and laboratory of analysis. These results demonstrate the value of the kit and reinforce the utility of standardized methods and protocols.

**INTRODUCTION**

 The proteomics field is rapidly transitioning from a qualitative to a quantitative science to verify/validate protein disease biomarkers and to address questions in systems biology. Although relative quantitative approaches (*e.g.*, isobaric tag for relative and absolute quantitation, iTRAQ; tandem mass tag, TMT) have their merits [1], it is the "absolute" quantitative approaches that can produce results in terms of concentration that are particularly important for clinical studies. A targeted MRM-based method with isotopically labeled standards (be it peptides [2, 3] or proteins [4, 5]) has proven invaluable for quantitating proteins in human biofluids [6-9], such as blood plasma – the most complex, yet most utilized, human proteome sample. As this approach becomes more widely implemented, the importance of performance evaluation or “accreditation” becomes more and more urgent. Standardization is necessary to facilitate more accurate and reproducible quantitative results, which is imperative if the methods are to be transferred between laboratories and eventually employed in a clinical setting.

 Over the past decade, considerable effort has been directed toward standardizing method and system performance in targeted plasma proteomics. In one area, various guidelines have been proposed to reduce the pre-analytical variability that arises from employing different blood collection and processing techniques [10-14]. The idea is that if these guidelines are assembled into a unified set of standard operating procedures (SOPs) and adhered to on a global scale, this source of bias could be eliminated or at least reduced. In addition to biospecimen quality control, the reproducibility and transferability of a targeted quantitative proteomics approach with SIS peptides has also recently been explored [15-18]. The central finding in these multi-site studies is that low intra- and inter-laboratory variability can be attained through quantitative MRM analysis of plasma proteins. While the results are certainly promising, reagents are not available for researchers in the proteomics community to routinely use for quality control (QC) assessment, although it has been pointed out that methods for monitoring the efficiency of digestion are "imperative" [19].

To the best of our knowledge, no standardization “kits” currently exist for quantitative plasma proteomics. If these SOPs were available, MRM might be more widely used in clinical and biomedical laboratories. To address this standardization issue, we have recently developed two reference kits for performance assessment in quantitative plasma proteomics that are centered on the MRM with SIS peptide approach [20]. The first is designed to test the performance of the LC/MS platform, while the second tests the efficacy of one type of sample preparation in addition to the LC/MS platform. Injection-ready quantitative proteomic standards are provided in kit #1 (termed the “LC/MS performance kit for quantitative plasma proteomics”), while the materials required to prepare peptide standards for protein quantitation are supplied in kit #2 (termed the “workflow performance kit for quantitative plasma proteomics”, contains: Bioreclamation plasma, trypsin, and a SIS peptide mixture). Also provided with both kits are SOPs, a performance quality guide, platform-specific LC/MS parameters, and reference values. The effectiveness of kit #2 in revealing procedural or instrumental errors has been demonstrated by Percy et al. *via* intra-laboratory testing [20]. For ultimate utility, however, the kits must be refined for practicality, applied to a variety of LC/MS platforms, and evaluated in external laboratories.

 To that end, we have changed the digestion protocol and extended the stability of two key kit components – SIS peptides and trypsin. Since a new lot of human plasma was employed, the reference values obtained previously first had to be re-established before being applied to alternate LC/MS platforms. Kit evaluations were performed on a total of 6 instrument platforms (3 internal and 3 external). Since the 6 platforms were all different, analysis involved parameter optimization and interference screening prior to MRM- or pseudo MRM-based protein quantitation. By "pseudo MRM", we mean data collected on the hybrid quadrupole-Orbitrap mass spectrometer (the Q Exactive), where full-scan product ion mass spectra instead of selected product-ion masses, are collected from specific precursor ions. The initial panel consisted of 43 peptides (representing 43 high-to-moderate abundance plasma proteins), from which a set of peptides were selected that were interference-free on all platforms tested. The kit advancements, stability testing, and quantitative proteomic evaluations are discussed herein. Note that Kit #2 evaluates the performance of one particular sample preparation workflow in combination with these LC-MS platforms; however, similar kits for other types of sample preparation workflows could also be developed. With continued evaluation and verification, these kits should prove useful to the quantitative proteomics community for standardizing MRM methods and analytical platforms prior to performing small- or large-scale MS-based proteomics studies.

**EXPERIMENTAL**

*Note:* Please refer to the **Supplemental Information – Protocols** for a detailed description of the required materials, procedures, and platform-dependent conditions required to effectively perform the standardization experiments. To further assist in the standardization process, a performance quality guide is also provided in the protocol package.

*Chemicals and Reagents*

All chemicals and reagents were obtained at the highest grade available from commercial vendors, and were stored according to suppliers' recommendations (*e.g.*, dithiothreitol and iodoacetamide were stored at 4ºC). K2·EDTA-treated human plasma was acquired from Bioreclamation (catalog no. HMPLEDTA2; Westbury, NY, USA) and was stored at -20°C until used. This sample represents a pooled sample from 30 healthy, race- and gender-matched human donors between the ages of 18 and 50. Since the plasma is pre-screened for only a few viruses (*e.g.*, hepatitis B and C, HIV, syphilis), it must be handled according to Biosafety Level 2 guidelines. TPCK-treated trypsin was obtained from Worthington (Lakewood, NJ, USA). LC mobile phases and proteomic standards were prepared from LC/MS grade solvents (*e.g.*, acetonitrile, methanol) from Sigma-Aldrich (St. Louis, MO, USA).

*Proteotypic Peptides – Selection and Synthesis*

 An initial panel of 43 high-to-moderate abundance plasma proteins, spanning a 4 order--of-magnitude range in concentration (from 41 mg/mL for albumin to 1.6 µg/mL for L-selectin) was selected for monitoring with the standardization kits. As described previously [20], these proteins were selected since they can be detected routinely without the need for depletion, enrichment, or 2-D LC fractionation. One proteotypic peptide was chosen to act as the molecular surrogate of each corresponding plasma protein. These peptides followed the selection rules for MRM assay development [21], and have been previously observed to be free from synthesis and chromatography issues [20, 22, 23]

 For preparation and deployment of the kit, larger quantities of SIS tryptic peptides were synthesized in-house using standard Fmoc (9-fluorenylmethoxycarbonyl) chemistry [23] and then purified and characterized, as explained previously [22]. Briefly, this involved incorporating stable 13C and/or 15N isotopes (both at >98% isotopic enrichment; Cambridge Isotope Laboratories; Andover, MA, USA) on the C-terminal arginine or lysine residue. After synthesis, purification was performed in-house using high performance liquid chromatography (HPLC) and checked with matrix-assisted laser desorption/ionization-time-of-flight-MS (MALDI-TOF-MS) analysis. The purities were then verified by capillary zone electrophoresis (CZE) at the University of British Columbia (Vancouver, BC, Canada), while the "absolute" peptide concentrations were determined by amino acid analysis (AAA) at the Hospital for Sick Children (Toronto, ON, Canada). The composition and purity information from AAA and CZE analyses are later used to correct for the presence of partial or incorrect synthesis products in order to obtain more accurate protein quantitation determinations.

*Preparation of Standard Samples*

The plasma tryptic peptide digests were prepared as described in our previous standardization manuscript [24], with the exception of the digestion conditions. Briefly, 10x diluted human plasma (24 µL raw) was denatured with 10% sodium deoxycholate (1% final), reduced with 0.05 M tris(2-carboxyethyl)phosphine (5 mM final), alkylated with 100 mM iodoacetamide (10 mM final), and quenched with 100 mM dithiothreitol (10 mM final; all prepared in 25 mM ammonium bicarbonate). The first two steps were conducted simultaneously, for 30 min at 60ºC, while the alkylation and quenching steps were performed separately at 37ºC for 30 min each. The proteolytic digestion was achieved with Worthington TPCK-treated trypsin (0.9 mg/mL, prepared in 25 mM ammonium bicarbonate), which was added at a 10:1 substrate:enzyme (S:E) ratio. This condition was first evaluated against our previous digestion conditions (2 separate additions of substrate (offset by 4 h), each at 20:1 S:E ratio, 9 h total incubation, 37ºC) [24] and an alternate (1 substrate addition, 20:1 ratio, 16 h, 37ºC) digestion procedure for a comparison of efficiency and reproducibility. After a 16 h incubation at 37ºC, digestion was stopped by the addition of a chilled SIS peptide mixture which contained SIS peptide concentrations ranging from 250 fmol/µL in 0.1% aqueous formic acid (FA) for standard G to 0.025 fmol/µL in 0.1% FA for standard A (dilution ratio from standard G: 1:2:5:2:5:10:10) followed by a chilled 1% aqueous FA solution. The addition of FA to the SIS-peptide spiked plasma digest dropped the pH to below 3, which facilitated precipitation of the deoxycholate surfactant. After centrifugation (12,000 x g for 10 min), the supernatant was removed for subsequent solid phase extraction (10 mg Oasis HLB cartridge; Waters, Milford, MA, USA). The eluted samples were then frozen, lyophilized, and resolubilized in 0.1% aqueous FA for LC/MRM-MS. The final concentrations were 1 µg digest/µL and 10 fmol SIS peptide/µL (initial plasma concentration estimated to be 70 mg/mL).

*LC/MS Platforms*

 The following six LC/MS platforms were investigated in this study:

1. 1290 Infinity UHPLC system interfaced to a 6490 triple quadrupole mass spectrometer (both Agilent Technologies; Santa Clara, CA, USA),
2. UltiMate 3000 LC system (Dionex; Sunnyvale CA, USA) interfaced to a QTRAP 4000 hybrid triple quadrupole/linear ion trap mass spectrometer (AB/Sciex; Concord, ON, Canada),
3. NanoLC-1D Plus HPLC system (Eksigent Technologies; Dublin, CA, USA) interfaced to a QTRAP 4000 hybrid triple quadrupole/linear ion trap mass spectrometer (AB/Sciex),
4. nanoAcquity UPLC system interfaced to a Xevo TQ triple quadrupole mass spectrometer (both Waters; Milford, MA, USA),
5. UltiMate 3000 LC system (Dionex) interfaced to a TSQ Vantage triple quadrupole mass spectrometer (Thermo Scientific; San Jose, CA, USA), and
6. UltiMate 3000 LC system (Dionex) interfaced to a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Scientific).

In general, the instrument platforms consisted of a reversed-phase liquid chromatography (RPLC) system (typically 2.1 mm or 75 µm x 15 cm) coupled on-line to a triple-quadrupole mass spectrometer *via* a positive electrospray ionization (ESI) source. The first 3 platforms were in-house, while the remaining 3 were located in external laboratories. Note that platform 1 was used to evaluate the revised digestion strategies and to test the SIS peptide stability aliquots.

Column injection volumes were typically 10 and 1 µL on the standard-flow and nano-flow LC systems, respectively. This equates to an on-column sample loading of 10 µg of plasma digest and a variable amount of SIS peptide (100 fmol in the interference screening studies, but 0.1 to 1000 fmol in the quantitation studies) in the standard-flow measurements. The loading amounts of the natural (endogenous, NAT) and SIS peptides in the nano-flow experiments were a factor of 10 less. The 10 µg column load was previously determined to be optimum for the Zorbax RRHD Eclipse Plus C18 column (150 x 2.1 mm, 1.8 μm particles [25, 26], which was used for all standard-flow systems. In contrast, 1 μg on the RPLC columns is the common load limit for nanoLC columns [27]. The endogenous and exogenous peptides were separated by C18 RPLC at 0.4 mL/min (standard-flow) or 300 nL/min (nano-flow) over a 30-236 min run. This was facilitated by a multi-step gradient from 3-90% mobile phase B (typical composition: 0.1% FA in 90% ACN). The autosampler was maintained at 4ºC, while the standard-flow UHPLC columns were held in a 50ºC thermostatted compartment. To minimize carryover, an injection of mobile phase A (composition: 0.1% FA) was run between each concentration level on all instrument platforms, while a blank solvent injection was run between each injection in the nanoLC/MRM-MS runs.

 The peptide eluate was analyzed by MRM or pseudo MRM using a combination of general MS parameters, such as 1.5-4.8 kV capillary voltage and unit resolution (0.7 Da full-width-at-half-maximum) in the quadrupoles, and peptide-specific or transition-specific parameters. The latter involved empirical tuning of such variables as collision energy (CE), declustering potential, cone voltage (CV), and retention time. The CV parameter in the Xevo TQ instrument, for instance, was optimized by injecting and RPLC separating groups of SIS peptides (our “parameter tuning kits”) into the mass spectrometer, where +2 to +4 precursors were gated while the CV was ramped from 20 to 70 V in 5 V increments. Similarly, CE values were optimized through direct infusion of the SIS mixtures into the mass spectrometer. Various combinations of doubly- and triply-charged precursor ions with b- and y-product ions were acquired over the mass range from *m/z* 300 to 1400. The precursor/product MRM ion pairs were scanned from *ca.* 5 to 53 V with a 20 ms dwell time/transition. The 3-5 precursor/product ion transitions that yielded the highest ion abundance were selected as targets for interference screening. In the MRM analysis, 1-3 transitions were monitored for each interference-free peptide, with the concentrations being determined from the quantifier ion only.

*Stability Analyses*

The long-term stability of the 43 peptides was determined by storing lyophilized aliquots of a freshly prepared balanced SIS peptide mixture at ambient temperature and -80ºC. At defined time intervals, two aliquots were resolubilized in 0.1% aqueous FA to give a final concentration of 10 fmol/µL), and then measured by standard-flow LC/MRM-MS analysis on the 6490. In the assay, a single MRM transition was monitored for each peptide target. Each peptide's stability was determined by first normalizing the average SIS response (n = 3) against the sum of the average SIS peptide responses at a given time point, and then further normalizing that value against the average response ratio that was measured on day 0. The values were then multiplied by 100 to obtain the percent original abundance.

In the trypsin stability study, aliquots of acidified TPCK-treated trypsin (final concentration: 0.9 mg/mL in 10 mM HCl) were lyophilized and stored at ambient temperature and -80ºC. At defined time intervals, two aliquots were resolubilized in the company's proprietary assay buffer and analyzed in triplicate according to the procedure specified in the trypsin activity assay kit (GenWay Biotech; San Diego, CA, USA). Sample measurements were performed on a Lab Systems Multiskan EX plate reader (Thermo Scientific) at 405 nm. The trypsin activity calculation used the time difference between 2 readings (0 and 15 min), the amount of p-nitroaniline (p-NA) generated over the incubation period, the volume of sample added to the reaction well, and a dilution factor.

*Interference Screening*

From the curated list of optimized MRM transitions, the 3-5 most intense precursor/product ion pairs for each target peptide were selected for interference screening. As described previously [24, 26], screening was performed under matrix-free (SIS peptides in 0.1% aqueous FA) and matrix-containing (SIS peptides in digested blood plasma) conditions, with duplicate LC/MRM-MS analyses run for each condition. The response ratios of each peptide’s SIS transition in buffer, SIS transition in plasma, and NAT transition in plasma, relative to the transition with the highest response, was calculated. From the individual response ratios, the average could be calculated within and between each group (*i.e.*, SIS in buffer, SIS in plasma, and NAT in plasma**)**. For a peptide to be considered interference-free, 2 of the 3 transitions monitored must have an average coefficient of variation (CV) below 25%, while their SIS and NAT transitions must exhibit identical chromatographic behavior (in terms of retention time and peak shape). Ultimately, the transition that produced the highest average relative ratio for each of the interference-free peptides was selected as the representative transition and was used for quantitation.

*Data Analysis*

Data was analyzed with vendor-specific software (*i.e.*, MassHunter Quantitative Analysis, Agilent; MultiQuant, AB/Sciex; PinPoint, Thermo Scientific) or freeware (Skyline, MacCoss Labs, Seattle, WA, USA). Regardless of the type of software, all peaks were first inspected manually to ensure correct peak detection and accurate integration. Standard curves consisted of 7 concentration levels (spanning a 10,000 fold range) and were generated with a linear regression weighting of 1/x2. To be used for the calibration curve, the replicates within a given level had to be both precise (average CV <20%) and accurate (average 80-120%), as recommended by the US FDA guidelines to industry [28]. The accuracy is determined from the quotient of the expected to the actual SIS peptide concentration (corrected with the data from the AAA and CZE analyses), with the expected concentration being calculated from the linear regression equation. Ultimately, a minimum of 3 levels must "pass" in order to generate a curve suitable for protein quantitation. From the final curves, assay attributes (*e.g.*, precision, lower limit of quantitation, dynamic range, protein concentration) can be extracted, as described previously [24]. The protein concentrations, for instance, were determined from the average of the experimentally-determined NAT concentrations (the products of the NAT/SIS response and the corrected SIS peptide concentrations) across the qualified levels. The protein molecular weights (obtained from ExPASy’s Compute pI/MW tool) were used to convert from fmol/µL to ng/mL.

**RESULTS AND DISCUSSION**

 A targeted proteomic approach involving bottom-up MRM-MS with isotopically labeled internal standards is rapidly emerging as a favourable quantitative approach for verifying and validating the clinical utility of candidate disease protein biomarkers in human biofluids. Blood plasma is the preferred sample source for these investigations since its collection is fairly non-invasive and it has the potential to provide a detailed record of the physiological status of an individual through comprehensive quantitative proteomic analyses. Despite the gain in popularity of plasma proteomics, the MS community continues to lack plasma proteomic standards for performance assessment. To address that deficiency, we previously developed two standardization kits for evaluating a sample preparation procedure and/or the LC/MS platform in MRM-based plasma proteomic analyses [24]. Despite the promising results, these earlier findings [24] indicated that the sample preparation process for kit #2 needed to be revised and that the utility of these kits had to be verified on additional LC/MRM-MS platforms before making them available to the wider proteomics community. To that end, we have refined the standardization kits and generated reference values on six LC/MS/MS platforms. The results of these preliminary studies, in addition to the kit component stabilities, are discussed below.

*Reference Kits Defined*

 Supplied in kit #1 (the “LC/MS performance kit for quantitative plasma proteomics”) are 7 samples (labeled standard A to G) of a plasma tryptic digest that have been spiked with a 10,000-fold concentration range of a SIS peptide mixture which has been concentration-balanced to reflect the concentrations of the endogenous peptides. The standards are lyophilized and ready for LC/MRM-MS analysis upon solubilization with an equal volume of 0.1% FA. Kit #2 (the “workflow performance kit for quantitative plasma proteomics”) contains 3 raw materials (namely plasma, trypsin, and a balanced SIS peptide mixture) that are to be used for the preparation of 7 standards (labeled A to G), which, alike kit #1, have constant endogenous and variable exogenous peptide amounts (see **Figure 1** for an overview of the kit workflows). The plasma is supplied as a solution, while the trypsin and SIS-peptide mixture are lyophilates (all at -80ºC). After preparing a plasma tryptic digest, the internal standard mixture (diluted serially from the 250 fmol/µL concentration provided; see **Table 9** of **Supplemental Information – Protocols** for details) is added to 7 vials and the resulting mixtures are desalted before LC/MRM-MS analysis.

*Refinement of Digestion Protocol*

 In our previous standardization manuscript [24], plasma proteolytic digests were optimized for maximum efficiency and reduced cost. The ideal conditions involved enzymatically digesting the denatured plasma proteins for 9 h at 37ºC, with 2 separate doses of TPCK-treated trypsin (Worthington), each at a 20:1 substrate:enzyme ratio, added 4 hours apart. Unexpectedly, these conditions were found to provide comparable reproducibility and efficiency to the often-used, but more expensive, sequencing grade trypsin (Promega) in technical/analytical replicate analyses (n = 3). Despite its merits, however, this procedure was found to be impractical during intra-laboratory testing because it compressed the sample preparation step into a single, 13 h day. The duration and S:E ratio used in proteolytic digestion was therefore revised to provide a more practical solution. This was evaluated in replicate analyses of three digestion conditions. The average relative responses (*i.e.*, NAT/SIS) of each peptide was then calculated and compared by arranging the peptides into 3 classification groups (rapidly digestible, moderately digestible, and proteolytically resistant) according to their digestion efficiency (see **Figure 2** for the digestion comparison results). Interestingly, our classification groups were similar to those reported previously [29], despite using a different grade of trypsin. For instance, albumin and complement C3 were found to be rapidly digested, clusterin and complement factor H had an intermediate range of digestion efficiency, while apolipoprotein C-III and prothrombin were slow to digest under the employed conditions. Our results revealed that nearly optimal digestion efficiency could be achieved with a single 10:1 addition of TPCK-treated trypsin, followed by overnight incubation at 37°C. While comparable efficiencies were obtained, it should be noted that the digestion efficiency is not as important a criterion as digestion reproducibility. Since the new conditions maintained the high reproducibility observed previously (3.5% vs. 3.2% CV) and retained the low preparation cost, the new digestion procedure was considered suitable for kit evaluation *via* intra- and inter-laboratory testing.

*Stability Evaluations of Kit Components*

The SIS peptides are key components of the kits and must remain stable for ultimate accuracy in protein quantitation. In general, the chemical stability of peptides is highly dependent on amino acid sequence and is negatively affected by freeze-thaw cycles. To assess the long-term stability (1.5 years) of the SIS peptide mixture that is spiked into the plasma tryptic digest, aliquots were removed at defined intervals and measured by standard-flow LC/MRM-MS. After 2.5 months of storage, 31 of the 43 peptides were found to be stable at a storage temperature of -80ºC, since their abundances were within 80-120% of their original peptide abundance (see **Figure 3**). The average abundance of the 43 peptides was 105%, with 2 peptides measuring <80% of its original abundance and 10 peptides measuring >120%. Similar overall trends were observed with the sample storage at ambient temperature, but with different peptide instabilities (data not shown). For instance, alpha-2-macroglobulin was found to maintain only 7% of its original peptide abundance after 2.5 month storage at ambient temperature, but, in contrast, had a normalized SIS response of 106% when stored at -80ºC over the same time period. For added confirmation of the peptide stabilities, AAA and CZE will be periodically performed. Ultimately, only those peptides that remain within 80-120% of its original peptide abundance (as measured by MRM-MS), within 10% of its original composition (as measured by AAA), and within 5% of their original peptide purities (as measured by CZE) will be considered stable for inclusion in the final target panel. In the meantime, however, the entire 43 peptide panel must be interrogated for each kit being evaluated and on each platform being investigated (see **Table 1** of the **Supplemental Information – Protocols** for details on the protein/peptide panel).

Two other key kit components are the plasma and the trypsin. The current lot of plasma is stable for approximately 2 years at -20ºC and is listed to expire in August 2014. At that time, the reference values will need to be regenerated using a new lot of human plasma that is pooled from the same types of healthy donors as is presently used. With regard to the trypsin, we are currently investigating the long-term stability of storing acidified trypsin as a lyophilate then recovering the enzymatic activity with a neutral assay buffer at defined intervals. This p-NA based colorimetric study should reveal the optimal storage temperature and the time frame over which such aliquots are effective. Although the study is currently ongoing, the results after 1 month of storage are promising and reveal that the trypsin activity can best be recovered without loss of efficiency when a -80ºC storage temperature is used (data not shown). Our preliminary findings support the idea of preparing standard peptide samples from lyophilized, acidified trypsin aliquots.

*Interference Screening*

 After empirically tuning several instrumental parameters for improved sensitivity, the precursor/product MRM ion pairs for the 43 target peptides were screened for chemical and electronic interference. This is particularly necessary when human plasma serves as the matrix due to its sample complexity (*ca.* 10,000 proteins, 10 order of magnitude concentration range, 6 order of magnitude molecular weight range) [30]. Its complexity is further increased upon digestion, wherein thousands of proteins are divided into hundreds of thousands, possibly millions, of peptides. Our interference evaluations were conducted by monitoring the SIS and NAT peptide forms in the presence and absence of human plasma. The resulting signals were compared, the variability between buffer and plasma was calculated, and the transitions within each peptide were ranked. Overall, the results revealed that the nano-flow platforms yielded an average of 5 fewer interference-free peptides than the standard-flow platforms (36 vs. 41; see **Tables 4** to **8** in **Supplemental Information - Protocols** for the platform-specific transitions of the interference-free peptides and **Supplemental Table 1** for a list of the interference-free peptides for each platform). This could be explained by the inferior separation capacity and broader peak shapes observed in the nano-flow measurements. In comparing the interference-free peptide lists across instrument platforms, it is evident that 6 peptides were rejected from a single platform, 4 peptides from two platforms, 4 peptides from 3 platforms, and 1 peptide (NWGLSVYADKPETTK from alpha-1-acidglycoprotein 1) from 5 platforms. Although 28 matching peptides passed our interference screening on all 6 of the LC/MRM-MS platforms investigated, the entire interference-free peptide panel (ranged from 34 to 42 peptides) was targeted in the quantitative assays.

 Once the reference values have been generated and verified on a multitude of LC/MRM-MS platforms and the component stabilities are finalized, a consistent panel of interference-free, stable, peptides will be assembled for method/platform standardization. The interference exercise described above need only be repeated if: (i) the lot of plasma is changed, (ii) the LC gradient is modified, or (iii) a new instrument platform is evaluated. Since the plasma and LC/MS parameters are supplied with the kits, the peptides designated as interference-free are likely to remain interference-free in their implementation on platforms where reference values have previously been determined, which removes a common source of variability. In implementation, the user is required only to schedule the peptide retention times (using SIS in buffer followed by SIS in plasma with standard E) prior to quantitating the target protein panel. This scheduling process will account for any differences associated with peptide elution (related to differences in dead volumes or capillary tubing), which will enable the target peptides to be quantitatively assessed with high precision and accuracy, provided that the user adheres to the required and recommended settings (see section 4 in **Supplemental Information – Protocols** for specific guidelines).

*Reference Value Generation and Verification*

Generating curves from the MRM or pseudo MRM analysis of 7 standard samples enables quantitative information to be extracted for QC assessment. Pertinent information includes the signal and retention time variability, lower limit of quantitation, dynamic range, coefficient of determination, and plasma protein concentration. These metrics help reveal the performance of the sample preparation process and the LC/MS platform, both of which are key components of reproducibility and potential sources of inter-laboratory variability. With the exception of the protein concentration, however, the remaining assay attributes are platform dependent and cannot be compared across dissimilar platforms for accuracy assessment. Since in this study, the 6 instrument platforms tested were different, a comparison of the plasma protein concentrations, as determined on each instrument, will reveal the inter-platform accuracy and reproducibility of the assay.

 Standard samples for both kits were prepared in-house and first analyzed on 3 different LC/MS platforms – 6490 with standard-flow LC, QTRAP 4000 with standard-flow LC, and QTRAP 4000 with nanoLC. The samples contained a variable amount of the balanced SIS peptide mixture and a constant amount of the plasma digest, to keep the matrix effect constant. Due to our strict qualification criteria, not all interference-free peptides were quantified. This eliminated a maximum of 4 interference-free peptides (alpha-2-macroglobulin, antithrombin-III, apolipoprotein C-III, and serum amyloid P-component) across these 3 platforms, which left 31 to 42 peptides available for quantitation, depending on the platform. There were 2 contributing factors to their removal: (i) poor accuracy in determining the expected peptide concentration (alpha-2-macroglobulin on the QTRAP 4000 under standard-flow and nano-flow LC/MRM-MS) and (ii) poor proteolytic digestion (antithrombin-III, apolipoprotein C-III, and serum amyloid P-component) due to structural or biological reasons. Nonetheless, for a consistent protein panel, nearly equivalent concentrations were obtained, as illustrated by the log-log plots in **Figures 4a** and **4b**. This implies that procedural or instrumental errors were absent in the preparation and processing of these samples. If such errors were present, they would have manifested themselves as incorrect SIS/NAT ratios between concentration levels (which denotes a sample preparation issue) or a lack of precision in the signal or retention time of the analytical replicates (which signifies a LC issue), among other interpretable anomalies. For added confirmation, the inter-day reproducibility of the reference values should be determined. Based on our previously demonstrated high reproducibility in quantitation [22, 25, 31], similar concentrations should be obtained. Nonetheless, studies on the long-term reproducibility of these measurements are currently being performed.

 To evaluate the effectiveness of these kits for use in the field, the standardization kits were prepared then distributed to 2 geographically distant locations (the Mass Spectrometry Laboratory within the Institute of Biochemistry and Biophysics at the Polish Academy of Sciences, Poland, and the Leibniz-Institut für Analytische Wissenschaften, Germany) for unsupervised implementation. There, reference values were obtained for two LC/MS platforms (TSQ Vantage and Q Exactive, both interfaced to a nanoLC system) using kit #1 at one site, and for the Xevo TQ instrument (with nanoLC system) using kit #2 at another site.

At the first site, similar plasma protein concentrations were determined between the two investigated platforms (coefficient of determination: 0.94, regression line slope: 0.96). These concentrations also compared well with those determined from the 6490 platform (see **Figures 4c**,**d** for the log-log plots). However, three outliers were apparent in **Figure 4d** (specifically fibrinogen alpha chain, heparin cofactor II, and apolipoprotein A-IV). These values were not replicated on the TSQ Vantage, when the same sample was processed, which suggests that the results from the Q Exactive were erroneous. Replicate platform analysis could probably help to elucidate the cause. Nonetheless, these findings, although preliminary, are promising overall and were not altogether unexpected considering that the 7 standards are normalized by NAT and that the supplied standards only had to be rehydrated prior to analysis. It did, however, reveal that the peptide mixtures were not adversely affected by the shipment and that the instrument platform was free from common nanoLC/MS issues, such as column deterioration or ESI spray instability, over the course of the replicate analyses, which is exactly what kit #1 was designed to assess.

Of greater importance is the data collected at the second site since it involved preparing the peptide standards for bottom-up LC/MRM-MS starting from the raw materials. Despite the different user and platform of analysis, similar concentrations were obtained for a consistent set of plasma proteins (see **Figure 4e** for a comparison of the quantitative results from the QTRAP 4000 and Xevo TQ instruments, both operated under nanoLC/MRM-MS conditions). The two visually apparent outliers in this log-log plot correspond to vitamin D binding protein (log-log deviation: 0.60) and complement factor H (log-log deviation: 0.92). A contributing factor could be peptide instability, which is supported by our ongoing stability evaluations. As mentioned previously, unstable peptides will ultimately be removed in the final panel, and their removal should improve the overall accuracy of quantitation, provided that procedural errors or instrument deficits are absent. Based on the results generated at these two sites, the absence of such errors appears likely.

 The quantified proteins were finally compared across the 6 investigated platforms for accuracy. This amounted to a total of 22 plasma proteins, with concentrations spanning a range from 20 mg/mL (for albumin) to 433 ng/mL (for complement factor B), for this lot of plasma. While these concentrations were 2-fold lower, on average, than those reported previously with a different lot of pooled plasma donors (see Percy et al. [24]) and may not correlate well with literature values [32], the results were nonetheless reproducible within the evaluated panel (using a consistent lot of plasma, but dissimilar instrument platforms), as illustrated in **Figure 5** (see **Supplemental Table 2** for the raw concentrations). In fact, the variability was <8% CV for the log concentrations of the 22 plasma proteins. It is this high reproducibility of quantitation that is more valuable than the absolute accuracy of the numbers compared to the literature. A few outliers were, however, visually apparent in the radar plot shown in **Figure 5** (specifically fibrinogen alpha chain, fibrinogen beta chain, heparin cofactor II, and vitamin D-binding protein). Further examination of the protein concentrations revealed some interesting findings. For instance, discrepancies in the NAT and SIS responses were found between the Xevo TQ analysis and our in-house measurements. Although the ratios of SIS and NAT between concentration levels correlated appreciably to those expected (1:2:5:2:5:10:10 for SIS from standard G, 1:1:1:1:1:1:1 for NAT), the absolute responses for SIS were approximately 3-fold higher, while NAT values varied depending on the peptide (*e.g.*, NAT responses of fibrinogen alpha chain and fibrinogen beta chain were 2.6-fold higher and 9-fold lower, respectively, in the Xevo TQ analyses compared to the 6490). The deviations could be related to sample preparation, for example, due to incorrect SIS spiking amounts and/or variable denaturation/digestion efficiencies. Nonetheless, these preliminary findings are promising and support continued evaluation on similar and dissimilar LC/MS platforms through inter-site and inter-day analyses. These results also point out how necessary this standardization -- and the use of standardization kits -- is. Further use of these kits should allow the source of any discrepancies to be determined and corrected, and should also allow the determination of accurate plasma protein concentrations, as well as other performance metrics, paving the way toward global standardization.

**CONCLUSIONS AND FUTURE CONSIDERATIONS**

 Despite the demand for high inter-laboratory precision and accuracy in MRM-based quantitative plasma proteomics, no reference standards are currently available. Standardization is necessary to achieve a high degree of method transferability and inter-laboratory reproducibility, and is critical if such assays are to eventually be deployed in a clinical setting. To that end, we had previously developed two standardization kits for evaluating all phases of the MRM-with-SIS-peptide method, either independently or in combination, for the quantitation of 40 proteins in undepleted human plasma [24]. In this current study, we refined the kits to overcome the impractical nature of the sample preparation process for kit #2, evaluated the stabilities of a few key kit components, and applied these kits to a total of 6 LC/MRM-MS platforms (3 internal and 3 external), after parameter optimization and interference screening. The concentration determinations were highly reproducible. In fact, for the same panel of 22 plasma proteins, equivalent concentrations were obtained with high precision (CV <8% in log concentration) regardless of the kit, operator, and instrument. Optimal quantitative performance requires strict adherence to the sample preparation SOPs and the recommended/required guidelines for the LC/MRM-MS analysis. Inter-laboratory evaluations on similar and dissimilar platforms are currently in progress for verification of not only the concentrations, but also the platform-dependent performance metrics, such as limit of quantitation and robustness. Routine testing will eventually enable "expectation ranges" to be set for each platform, which will alert the user to any error or deficiency that would need immediate attention prior to proceeding. The refined kits provide a more practical solution in terms of sample preparation, and extend the scope of application from 1 platform to 6 platforms, which makes their use a more viable option for standardization of MRM-based quantitative plasma proteomics.

 In addition to their intended use for absolute quantitation, it is possible that these reference kits could also be used evaluate *relative* quantitative methods (*e.g.*, iTRAQ, label-free with ion profiling, etc.) using NAT/SIS ratios similar to those described above. A kit that would allow the objective cross-platform evaluation of the accuracy and reliability of different relative quantitation methods would greatly assist in biomarker discovery studies. This additional use for our reference kit will be explored in a future study.

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**Figure Legends**

**Figure 1**. Overview of the reference kit workflows. Peptide standards that have been spiked with a SIS peptide mixture are provided in kit #1 for direct LC/MRM-MS analysis. In kit #2, these standards are to be prepared using 3 supplied components (*i.e.*, plasma, trypsin, and SIS peptide mixture), in addition to other general laboratory consumables, within a bottom-up MRM-with-SIS-peptide approach.

**Figure 2.**  Digestion efficiency comparison of 43 peptides using different incubation times and substrate:enzyme ratios. Each bar in the histogram represents the average relative responses (NAT/SIS) for each classification group, normalized against the average relative response for the rapidly digested protein group using the original optimized conditions. Standard deviations are shown by error bars.

**Figure 3.** Stability of the SIS peptides after 2.5 months of storage. The abundances of each peptide were calculated from the average response ratio measured at a given time point, normalized to the average response ratio at day 0 (each at n = 3).

**Figure 4.** Comparison of concentrations determined for a congruent panel of plasma proteins on 2 different LC/MRM-MS platforms. Concentrations of **a)** 38 proteins, **b)** 31 proteins, **c)** 37 proteins, **d)** 32 proteins, and **e)** 25 proteins were quantitated on two standard-flow LC/MRM-MS platforms in **a)**, a standard-flow and nano-flow LC/MRM-MS platform using the mass spectrometer in **b)**, a standard-flow and nano-flow LC/MRM-MS platform using different mass spectrometers in **c)** and **d),** and two different nano-flow LC/MRM-MS platforms in **e)**. The concentrations, in ng/mL, are plotted as common logarithms.

**Figure 5.** Comparison of concentrations determined for a congruent panel of 22 human plasma proteins on 6 different LC/MRM-MS platforms. The concentrations, in ng/mL, are plotted as logarithms and arranged according to protein.

**Figures**

**Figure 1**

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**Figure 2**

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**Figure 3**



**Figure 4**

**a)**

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**b)**



**c)**



**d)**



**e)**

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**Figure 5**

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