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Determination of the concentration range for 267 proteins from 21 lots of commercial human plasma using highly multiplexed multiple reaction monitoring mass spectrometry†

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Multiple reaction monitoring (MRM) is a key tool for biomarker validation and the translation of potential biomarkers into the clinic. To demonstrate the applicability of MRM towards achieving this goal, we set out to determine the concentration ranges of 267 plasma proteins, including 61 FDA-approved/LDT developed biomarkers, in 21 commercial human plasma lots, as well as to assess accuracy and precision. Each target protein was quantified by calculating the area ratio of the endogenous tryptic target peptide to its stable isotope-labelled internal standard equivalent and compared to a standard curve. This highly multiplexed approach utilized a standard-flow UHPLC system linked to a triple quadrupole. All samples were analyzed across three separate days and assessed for robustness and accuracy. The standard curves and quality control samples showed excellent performance, with >93% of standards and QCs meeting the acceptance criteria. A total of 248 proteins were able to be quantified in at least one sample on at least one of the three days, with 111 proteins being quantified in all 21 samples on all three days. The protein concentrations across all proteins covered six orders of magnitude. Furthermore, excellent three-day precision was demonstrated with 86% of CVs falling below 15%. Overall, the protein concentration differences ranged from 1.1-fold for metalloproteinase inhibitor 2, to 69-fold for serum amyloid A-1/A-2.

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Introduction

Over the past few decades, mass spectrometry-based proteomics has become an invaluable tool for unraveling the molecular blueprint of biology and disease, and to begin to understand their underlying complexity. Bottom-up discovery proteomics approaches using high resolution mass spectrometers in an untargeted fashion are commonly used to assess disease-associated proteins for up- or down-regulation, or for modifications such as post-translational modifications (PTMs).[1] The identified proteins have the potential to be used as biomarkers for a variety of applications – including disease prognosis, diagnosis, and status, as companion diagnostics to determine eligibility for certain treatments, or for drug monitoring. After a potential biomarker or set of potential biomarkers has been identified, the biomarker candidates are transferred to a clinically compatible assay platform, followed by analytical and clinical validation. This transfer is required to overcome the limitations of the discovery platforms which include difficulty in standardizing measurements, the complexity of the workflow and data analysis which requires significant expertise, and low sample throughput.

The most common methodologies for biomarker validation and routine diagnostic use in clinical laboratories are the enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry (IHC).[2]

Although robust, sensitive, amenable to automation, relatively easy to implement and perform once developed and validated, ELISAs rely heavily on the specificity of the antibody.

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Cross-reactivity of the antibodies with non-target antigens can result in protein overestimation and antigen recognition can be affected by unexpected or unknown PTM patterns that can result in an underestimation of protein expression.[3] Moreover, ELISAs are susceptible to the Hook effect, which results in the underestimation of protein concentrations in samples with high protein concentrations.[4] In addition, interferences by auto-antibodies have been reported.[5]

A powerful mass spectrometry-based technique, very suitable for studies designed to validate protein biomarkers, is multiple reaction monitoring (MRM), also called selected reaction monitoring (SRM). This is due to its high multiplexing capability (*i.e.*, 10s to hundreds of proteins per run), its accuracy and precision when using stable isotope-labelled standard (SIS) peptides, its linear responses over several orders of magnitude, as well as low sample consumption. Furthermore, MRM achieves nearly absolute specificity due to the combination of peptide-specific retention time, fragment ion ratios, and mass-to-charge (m/z) information.[6,7]

MRM has been used for protein quantitation in various sample backgrounds, including human plasma,[8] mouse plasma and tissues,[9,10] and dried blood spots,[11] among others. In addition to targeting non-modified peptides, MRM has been successfully applied to quantifying modified peptides either directly, *e.g.* phosphorylated[12] or glycosylated[13] peptides, or after enzymatic removal of these modifications.[14,15] Moreover, the enrichment of peptides prior to MRM analysis (immuno-MRM) can be used to increase sensitivity, if necessary.[16,17]

An important requirement for biomarker validation studies is the ability to achieve reproducible results between sample batches and between laboratories, and this has been demonstrated in many multi-laboratory studies using MRM.[18–20] The use of standardized procedures and reagents, however, is required to achieve high reproducibility, which highlights the importance of commercially available reagent kits so that the research community can achieve transferable results. Our goal, therefore, was to address the need for standardization by developing easy-to-use MRM assay kits[21] which had been rigorously validated following the Clinical Proteomic Tumor Analysis Consortium (CPTAC) guidelines.[22]

To expand upon previous work which resulted in a MRM panel for the quantitation of 76 human plasma proteins,[21] the University of Victoria – Genome BC Proteomics Centre increased the number of protein targets to 267. Each of the 267 target proteins is quantified based on a unique tryptic peptide utilizing synthetic light and isotope-labelled standard peptides. The proteins cover a wide range of potential disease biomarkers, including neurodegenerative disease, renal disease, cancer, eye disease, bone disease, blood disorders, pregnancy complications, diabetes and autoimmune disorders among others. Of the 267 protein targets, 61 are FDA-approved protein biomarkers[23] and 67 are putative biomarkers for cardiovascular disease.[24] Upon rigorous assay validation and interference screening following CPTAC guidelines, the MRM assays were turned into a kit and used for this study.

The kit consists of two lyophilized peptide mixtures, preweighed trypsin, and bovine serum albumin (BSA), as well as information on retention times, transitions, and optimized MS parameters for the following instruments: Agilent 6490/6495, Sciex 6500 Q-TRAP, and Thermo Q-Exactive. The first peptide mixture consists of 267 light (natural isotopic abundance, NAT) peptides that had been characterized for purity and concentration by capillary zone electrophoresis (CZE) and amino acid analysis (AAA). These peptides have been extensively screened for interferences during assay validation and are used to generate calibration curves with digested BSA as the background matrix. BSA background as a surrogate matrix for the generation of calibration curves has previously been shown to result in comparable accuracies as calibration curves generated in plasma.[25] The second peptide mixture consists of the analogous 267 SIS peptides. These are spiked into both the plasma samples and the samples used to generate the calibration curve, and act as normalizers for ionization efficiency differences as well as any chromatographic variations between samples.

Here we show the human plasma panel performance as determined by quantifying target proteins from 21 lots of commercial human plasma from BioIVT (formerly known as Bioreclamation IVT).

Experimental

Materials

Reagents and labware. Phosphatase buffered saline (PBS) tablets, trizma pre-set crystals (pH 8.0), urea, dithiothreitol (DTT), and iodoacetamide (IAA) were purchased from Sigma Aldrich. Deep-well plates (1.1 mL) were purchased from AXYGEN. Protein LoBind tubes and LoBind 96-well PCR plates were purchased from Eppendorf. Oasis HLB µElution plates (2 mg sorbent per well, 30 µm particle size) were purchased from Waters. Ultrapure water was obtained with a Milli-Q Direct 8 water purification system. Formic acid (FA), methanol (MeOH) and acetonitrile (ACN) were purchased from Fisher Scientific. Eppendorf protein LoBind tubes were used for preparing the serial dilutions of the NAT mixture, and Falcon 15 mL conical tubes (Corning) were used for the preparation of the SIS mixture.

PeptiQuant 267-protein human plasma MRM panel. MRM Proteomics Inc.'s PeptiQuant™ 267-protein human plasma MRM assay kits contain light and heavy peptide mixes, as well as trypsin and BSA. The synthetic proteotypic peptides contained in the two mixtures (sequences shown in ESI Table 1†) serve as molecular surrogates for the 267 human plasma proteins as described previously, following strict rules and criteria.[26] Each protein is quantified by a single tryptic peptide to maximize the number of proteins quantifiable in a single run. The peptides were carefully selected using PeptidePicker[27] to ensure protein-specific uniqueness and lack of modifications based on The Universal Protein Resource (UniProt).[28] In cases where peptide variants have been documented within their sequences, the canonical sequence was selected unless specified. Similarly, when protein isoforms were noted, peptide

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sequences present in all isoforms were preferentially selected. When no peptide sequence present in all isoforms was found to meet all criteria, a peptide sequence found in the majority of isoforms was picked and the isoforms noted. Proteotypic peptides found in more than one plasma protein are noted. While the best possible peptides were selected for each protein, it should be kept in mind that nonetheless, in rare cases, gene mutations and/or PTMs could affect the trypsin cleavage efficiency. Each of the peptides was characterized for purity and accurate concentration by CZE and AAA, respectively. Furthermore, the synthetic peptides were tested for detectability when spiked into human plasma, and the ionization conditions were optimized empirically. Peptides were validated for use in LC/MRM-MS experiments, including establishing the limit of detection, linear range (lower limit of quantitation – LLOQ, and upper limit of quantitation – ULOQ), precision, and interferences, all in accordance with the CPTAC guidelines for assay development,[22] which are available on the CPTAC assay portal website.[29]

Human plasma. Commercially available human plasma samples from 21 different lots were purchased from BioIVT. All the lots of human plasma analyzed were from whole blood donations. As shown in ESI Table 2,† ten of the samples were obtained from individual female donors, ten were from individual male donors, and one, BRH1447353, was a pooled human plasma sample. None of the samples had any indication of the health or disease state of the subjects other than testing negative for HIV 1/2 AB and HCV AB, and being non-reactive for HBSAG, HIV-1 RNA, HCV RNA, HBV DNA, and STS, all in accordance with FDA regulations.

Repeatability assessment. The 21 human plasma samples were thawed once and divided into three aliquots, followed by storage at −80 °C until the day of analysis. The samples were prepared on three different days with their own calibration curves and quality control samples (QCs) to determine the repeatability of the approach.

Digestion of human plasma and BSA surrogate matrix. The 21 different plasma lots and the BSA surrogate matrix were proteolytically cleaved with trypsin. Briefly, 10 μL of either BSA at 10 mg mL^{-1} or human plasma were denatured and reduced at pH 8 by addition of a urea/DTT/TrisHCl buffer at final concentrations of 6 M urea, 13 mM DTT, and 200 mM TrisHCl, followed by incubation at 37 °C for 30 min. After denaturation and reduction, proteins were alkylated by adding IAA to a final concentration of 40 mM and incubating at room temperature (RT) in the dark for 30 min. After the alkylation step, TPCK-treated trypsin (Worthington) was added at a 20 : 1 (protein to enzyme, w/w) ratio, and samples were incubated overnight (18 hours) at 37 °C for proteolytic cleavage. The next day, the samples were acidified to a final concentration of 1.0% FA (pH ≤ 2) to quench the digestion reaction, leading to a final peptide mixture with an estimated concentration of 1 μg μL^{-1}. Samples were kept on ice until analyzed on the same day.

Reference standard and QC preparation. A digested 10 mg mL^{-1} BSA-in-PBS-buffer surrogate matrix was used to prepare standards and QC samples. The lyophilized NAT peptide mix, previously balanced to the LLOQ of each peptide, was dissolved in 260 μL of 30% ACN/0.1% FA to give a final concentration of 100× LLOQ per μL. This NAT peptide mixture was serially diluted with 30% ACN/0.1%FA to yield eight concentrations: 100×, 40×, 16×, 4×, 2×, 0.5×, 0.25× and 0.1× LLOQ per μL. The QC samples were prepared by diluting the 100× LLOQ per μL NAT peptide mix to give final concentrations of 0.4× (QC-A), 5× (QC-B), and 50× (QC-C) LLOQ per μL. Three replicates per QC concentration were prepared and analyzed along with each batch of samples.

Solid-phase extraction and SIS addition. An aliquot consisting of 55 μL of each plasma digest sample was transferred to a well in an Eppendorf LoBind skirted PCR plate, along with 55 μL of the surrogate matrix (10 mg mL^{-1} BSA) for each QC sample and standard. The SIS peptide mixture was solubilized in 220 μL of 30% ACN/0.1% FA, transferred to a 15 mL Falcon tube, and then diluted to 10× LLOQ per μL with 0.1% FA. The eight-point standard curve was prepared by combining the surrogate matrix, SIS peptide mixture, and the level-specific light peptide mixture at a ratio of 1 : 1 : 1 (v/v/v). All plasma and QC samples were spiked with the same amount of the SIS peptide mixture as the standards. Plasma samples, QC samples, and standards were then concentrated by solid-phase extraction (SPE) using an Oasis HLB μElution plate. Briefly, the SPE plate was conditioned with 600 μL MeOH, equilibrated with 600 μL of 0.1% aqueous FA followed by loading the plasma sample digests, QC samples, and standards. The wells were washed three times with 600 μL of H_2O, and the bound peptides were eluted with 55 μL of 70% ACN/0.1% FA. After the SPE step, the concentrated eluate was evaporated using a speed vacuum concentrator and then stored at −80 °C. Plasma samples, standards and QC samples were then resolubilized and analyzed on the Agilent 6495B.

LC separation and MS analysis. Samples were solubilized with aqueous 0.1% FA to give a final peptide mix concentration of 1 μg μL^{-1} for LC/MRM-MS analysis. Of each of the rehydrated plasma digests, QCs and standards, 10 μL were injected and separated with a Zorbax Eclipse Plus RP-UHPLC column (2.1 × 150 mm, 1.8 μm particle diameter; Agilent), contained within an Agilent 1290 Infinity system and maintained at 50 °C. The performance of UHPLC and nano-HPLC for MRM-based quantitation of putative plasma biomarker proteins has been previously investigated.[30] UHPLC was found to be the better choice, when sufficient amount of sample can be obtained. In this case, UHPLC was found to provide overall higher sensitivity for the majority of peptides tested, and more stable retention times. Thus, for our purposes, peptide separations were achieved at UHPLC flow rates of 0.4 mL min^{-1} over a 60 min run, *via* a multi-step LC gradient. The aqueous mobile phase was composed of 0.1% FA in LC-MS grade water and the organic mobile phase of 0.1% FA in LC-MS grade ACN. The gradient was set up to start at 2% organic mobile phase, increase to 7% at 2 min, to 30% at 50 min, 45% at 53 min, 80% at 53.5 min and hold at 80% until 55.5 min, go back to 2% at 56 min, and then hold at 2% until 60 min. A post-gradient column re-equilibration of 4 min was used after the analysis of each plasma sample, QC sample, and standard.

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MS analysis was performed on an Agilent 6495B triple quadrupole instrument operated in the positive ion mode. MRM data were acquired at 3.5 kV and 300 V capillary voltage and nozzle voltage, respectively. The sheath gas flow was set to 11 L min^{-1} at a temperature of 250 °C, and the drying gas flow was set to 15 L min^{-1} at a temperature of 150 °C, with the nebulizer gas pressure at 30 psi. The collision cell accelerator voltage was set to 5 V, and unit mass resolution was used in the first and third quadrupole mass analyzers. The high energy dynode (HED) multiplier was set to −20 kV for improved ion detection efficiency and signal-to-noise ratios. A single transition per peptide target was monitored over 700 ms cycles and 90 s detection windows were used for the quantitative analysis.

Data analysis. Skyline Quantitative Analysis software (version 19.1.0.193, University of Washington)[31] was used to visually examine the resulting LC/MRM-MS data. The chromatographic peaks for the NAT and SIS peptides in the plasma samples, calibration curves and QCs were assessed manually for shape and accurate integration. Calibration curves were generated using $1/x^2$-weighted linear regression and were used to calculate the peptide concentrations in the samples as fmol per μL of plasma (see Fig. 1). Further data analysis and visualization was performed using R, including the generation of volcano plots and the heatmap with hierarchal clustering.

Standard and quality control acceptance criteria. The standards and QC samples were examined, and either accepted or rejected based on a set of rules and criteria. Standards and QC samples were acceptable if the values fell within ±20% of the theoretical concentration. A standard curve was deemed to be acceptable if the back-calculated concentrations of at least 5 out of the 8 standards were found to be within ±20% of the theoretical concentration at each point, including the LLOQ. Similarly, at least 66% of all QC samples were required to fall within ±20% of the theoretical concentration. An experiment was deemed to be successful if at least 90% of the peptide calibration curves were acceptable and passed criteria.

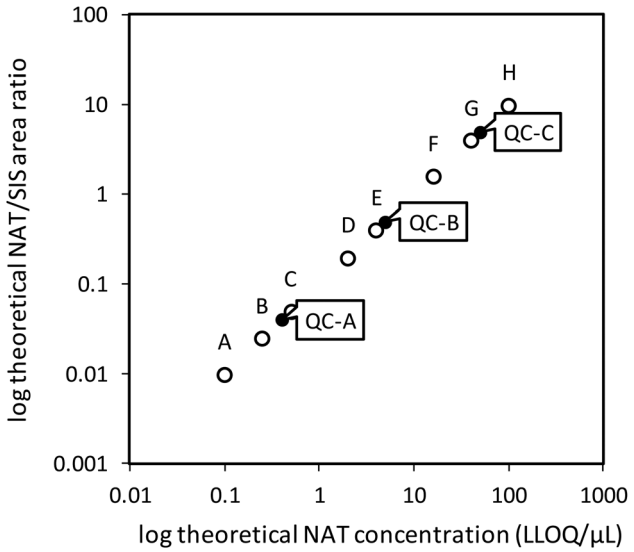

Fig. 1 Overview of theoretical concentrations for calibration curve points and quality control samples.

Results and discussion

Performance of calibration curves and QCs

Three sets of standard curves were generated to determine the precision and accuracy in the standards used for the calibration curve, the QC samples, and the 21 different plasma lots. The calibration curves and QC samples were evaluated following the acceptance criteria described in the previous section (at least 5/8 calibration curve standards, and at least 66% of all QC samples were within ±20% of the theoretical concentration, *i.e.* the expected concentration based on our calculations). As presented in Table 1, more than 93% of all standards and QC samples acquired during the three-day study met the target criteria.

The standard curves for all 267 peptides met the criteria shown above and were within ±20% of the theoretical value for a minimum of 5 out of 8 standard levels. As can be seen in Fig. 2, the majority of the three sets of standard curves were generated with 8 out of 8 standard points, followed by 7, 6, and 5 points, demonstrating the linear performance of the selected peptides for MRM analysis over several orders of magnitude and giving high confidence in the protein quantitation results.

As shown in Fig. 3, all three QC levels performed as expected and fell within acceptable limits for the majority of proteins. The few rejected QCs were either outside the linear range (OR) or outside the acceptable limits (OAL). Thus, in a curve where standards A (1× LLOQ) and B (2.5× LLOQ) were rejected, QC-A (4× LLOQ) was automatically rejected and denoted "OR". The lowest point on curve (LPOC), or LLOQ for that particular curve, became standard C (5×). In this case, the curve itself was deemed to be acceptable if the other two QC samples (QC-B and QC-C) passed the acceptance criteria. The OAL annotation was used for QC samples that fell outside the ±20% acceptance criterion, and fewer than 1% of the QC samples were OAL. This suggests proper performance of the calibration curve and QC samples for most peptides, thus providing high confidence in the quantitative results of the plasma samples analyzed.

Protein quantitation in 21 lots of commercial human plasma

Quantitation of all 267 proteins was attempted for all 21 sample lots (10 male, 10 female, 1 pooled sample; see ESI Table 2†) using the three standard curves prepared on days 1

Table 1 Overview of the percentages of curve standards and QC samples meeting acceptance criteria for analysis days 1 to 3

Day	Accepted curve standards (%)	Accepted QC samples (%)
1	94.4%	95.0%
2	95.5%	93.9%
3	96.5%	94.1%

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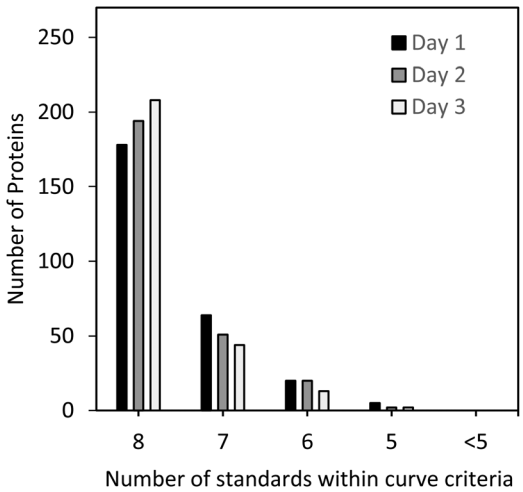

Fig. 2 Number of proteins with 8, 7, 6, 5, and <5 points (out of 8 points per standard curve) meeting the acceptance criteria for each protein measured on days 1 to 3. A standard curve was deemed to be acceptable if the back-calculated concentrations of at least 5 out of 8 standards were found to be within ±20% of the theoretical concentration at each point, including the LLOQ.

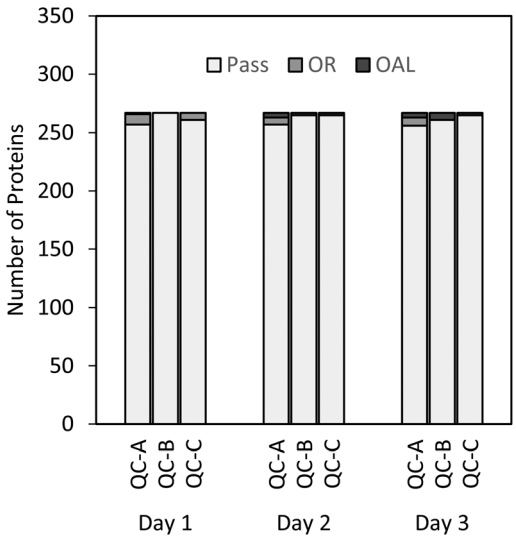

Fig. 3 Number of protein QCs meeting the criteria for each of the 267 proteins measured on days 1 to 3. OR = out of linear range; OAL = out of acceptable limits; Pass = at least one of the three QC samples at each level was within ±20% of the theoretical concentration.

to 3. All standard curves were generated with five to eight standards, as shown in Fig. 2. Each standard curve possessed an LLOQ and a ULOQ, as determined by the lowest and highest passing standards, respectively. In total, 248 proteins were quantifiable (within the linear range) in at least one sample on at least one day, and 157 proteins were quantifiable in at least one sample on all three days. A total of 111 proteins were quantifiable in all 21 samples on all three days. Notably, no protein in any sample was found to be above the ULOQ, and 110 proteins were below the LLOQ in all samples. Sixty of these proteins are thought to be associated with diseases such as neuropathy, lung cancer, epilepsy, rheumatoid arthritis, cardiomyopathy, among others, according to UniProt.[28] Hence, despite not all proteins being quantifiable across all samples, their analysis is still relevant, for instance when assessing different diseases which might result in up-regulation of some of the proteins.

The number of proteins quantified between different samples and between different days of analysis was found to be very consistent (see Fig. 4), with a median of 145 proteins quantified, and a standard deviation of 15.8. Furthermore, the overlap of the quantifiable proteins per sample was considerable with on average 84% of the proteins being quantified across all three days (see ESI Fig. 1†). Interestingly, two samples (BRH1447341, day 3, and BRH1447347, day 1), showed elevated numbers of proteins quantified on one of the three days. A possible explanation for this phenomenon could be a slight heterogeneity in the sample aliquots, for example, caused by plasma clots. This is supported by the observation that the QC samples and standard curves across all three days are very similar, thus ruling out LLOQ shifts of the calibration curves between days as the cause of the elevated numbers of proteins. This highlights the importance of adhering to strict sample preparation protocols to ensure as little variation as possible.

Overall, the data demonstrated the high reproducibility of the MRM approach for highly multiplexed protein quantitation.

Fig. 5 shows the distribution of % CVs calculated for all the proteins that were quantifiable in the plasma samples on each of the three days (a total of 2793 CVs were calculated for the 21 samples analyzed). More than 86% of the CVs were below 15% CV, with the majority falling below 10% CV. Furthermore, 6% of the CVs were between 15–20%, 4.6% were between 20–30%, and only 3.4% of the CVs were >30%. This shows the quality and precision of the selected peptides and demonstrates their suitability for reliable measurements and quantitative analysis in a variety of samples, which could easily be transferred to a clinical setting.

Concentration range

Fig. 6A shows the average protein concentrations for each protein that could be quantified in three replicate analyses in at least one of the 21 plasma samples. The concentration ranges across all plasma samples were very similar, and covered approximately six orders of magnitude, from ~1 fmol μL^{-1} for P-selectin, to 747 pmol μL^{-1} for human serum albumin, which falls within the normal accepted concentration range for serum albumin.[32] Furthermore, the concentrations for the proteins quantified in all 21 samples across all three days (111 proteins) were very comparable, ranging from 4.13 fmol μL^{-1} to 747 pmol μL^{-1}. This demonstrates the suitability of MRM for quantitation of proteins that vary in concentration by many orders of magnitude.

Plasma protein concentration measurements using traditional ELISA methods *versus* modern LC-MS/MS techniques have been compared previously. Strong correlation was found between the

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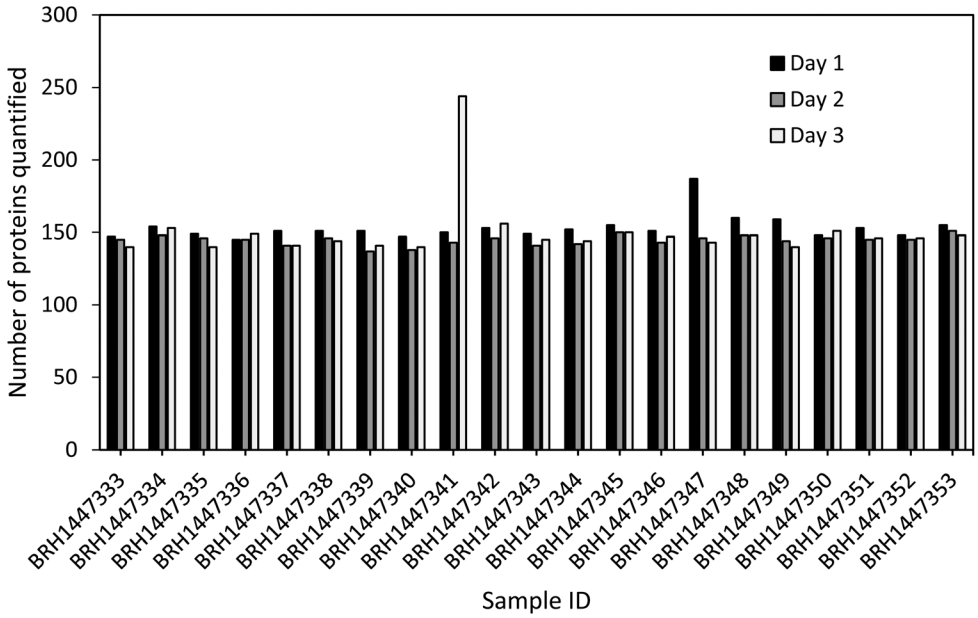

Fig. 4 Number of proteins with quantifiable endogenous concentrations as well as standard curves and QC samples within the acceptance criteria in 21 plasma samples across three days.

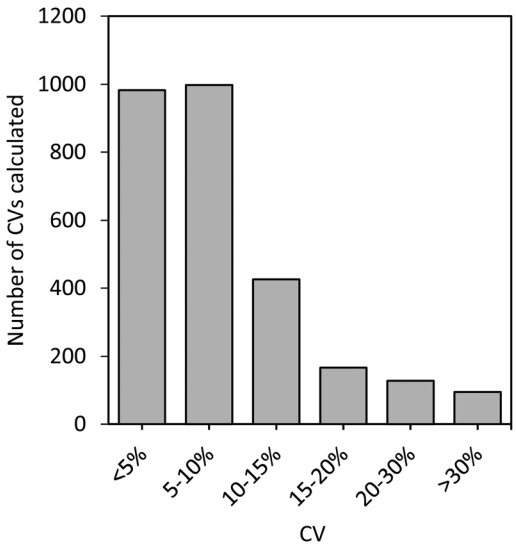

Fig. 5 Distribution of % CVs calculated for all sample protein concentrations determined on all three days.

two methods when measuring plasma PCSK9 in 30 human plasma samples.[33] Although discrepancies between ELISA and LC-MS/MS have been documented in the past, further investigation has revealed that the strong dependence of ELISAs on the antibody specificity can become a hinderance. For example, the discrepancy found between ELISA and LC-MS/MS assay results on a PEGylated scaffold protein in post-dose monkey samples was shown to be due to ELISA measuring only the active circulating drug (target-binding), whereas the LC-MS/MS method measured the total circulating drug.[34] The bias observed in ELISA results supports that LC-MS/MS could in many instances reveal a more accurate picture, as reviewed previously.[35] Thus, we believe that a direct comparison between ELISA and our LC-MRM-MS methods would be of limited value.

Of the 267 proteins, 144 proteins were quantified in at least 5 of the 21 samples, and differences in protein concentrations were determined (Fig. 6B). Of these 144 proteins, 48 are FDA-approved/laboratory developed test (LDT) biomarkers, as listed by Anderson *et al.*[23] Depending on the protein, these concentration ranges were as low as 1.1-fold for metalloproteinase inhibitor 2, to 69-fold for serum amyloid A-1/A-2 (see ESI Table 3† for details). The highest fold-difference observed for these FDA-approved biomarkers was 60-fold for C-reactive protein. The data show that although most protein abundances between samples are greatly conserved, some proteins show significant differences. For instance, C-reactive protein and serum amyloid A, both recognized as acute phase proteins, are known to increase in concentration by several hundred-fold or more in response to inflammation.[36,37] The differences in protein abundances between samples highlight the importance of, ideally, establishing patient-specific baseline measurements in preventative medicine and disease monitoring rather than establishing a baseline for a cohort of patients. Furthermore, it highlights the suitability of MRM-MS based methods for their multiplexing capabilities across multiple orders of magnitude.

Data assessment for statistically significant differences

The protein concentrations determined for all samples were further assessed for statistically significant differences between male and female, as well as ethnicity (Hispanic, Black and Caucasian). It is important to note that only one sample was from a Caucasian donor.

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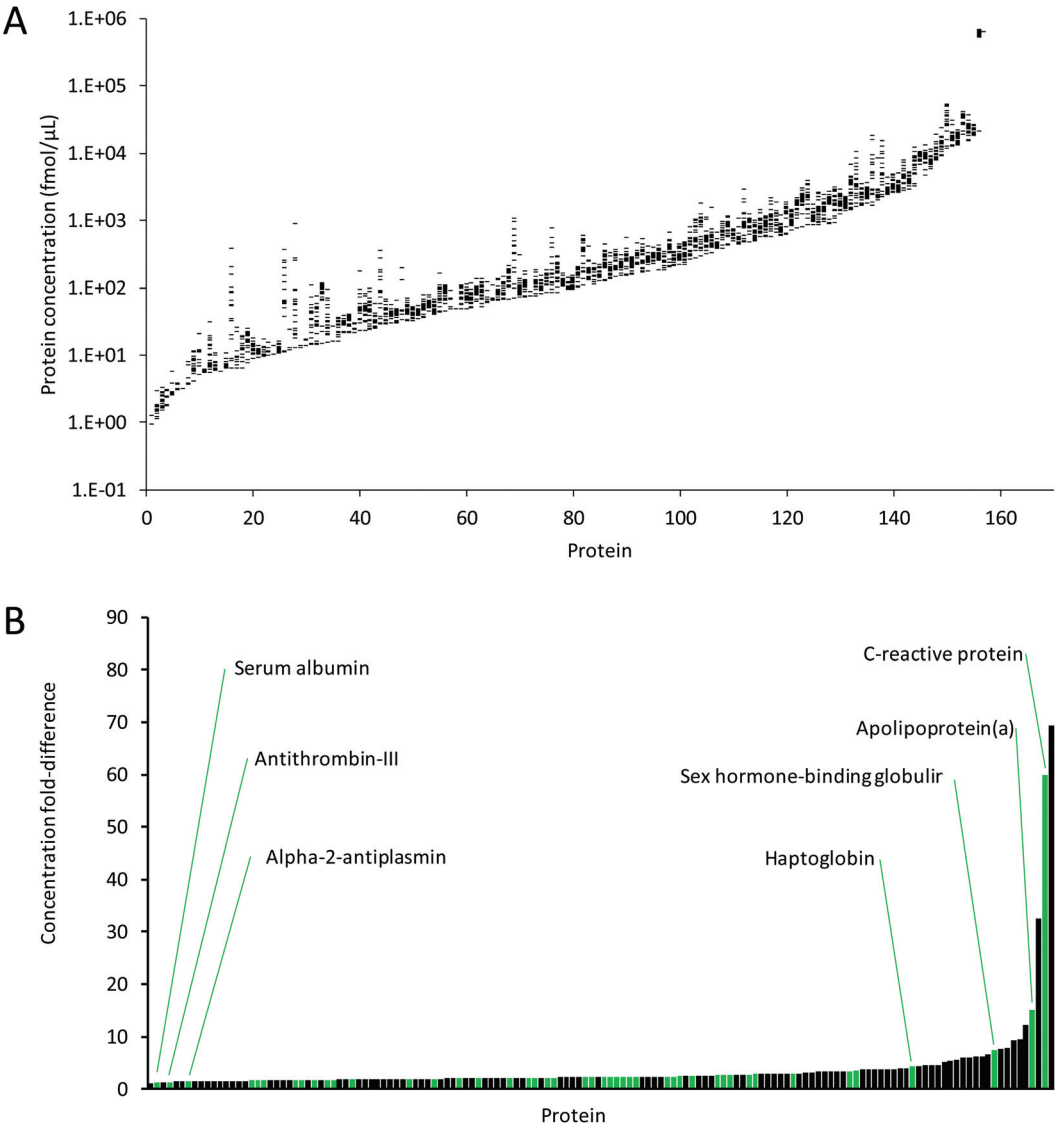

Fig. 6 (A) Ranges of average concentrations for the proteins quantified across three days in the 21 plasma samples. Proteins are sorted by minimum concentration across all samples. (B) Concentration ranges (*i.e.* fold-differences) between samples for proteins with quantifiable protein concentrations in at least 5 samples (144 proteins). Green bars show the FDA-approved and LDT biomarkers, as listed by Anderson *et al.*[23]

Upon statistical analysis, the concentrations for the majority of proteins showed no significant differences between Black and Hispanic groups (Fig. 7A). All but four of the values were within the ±0.5-fold change. The hemoglobin subunit alpha (P69905), Apolipoprotein A-IV (P06727) and Apolipoprotein C-III (P02656) concentrations were found to be significantly higher in the Hispanic group than in the Black subject, while the immunoglobulin heavy constant gamma 1 (P01857) was significantly lower in the Hispanic group than in the Black subject.

The plasma concentrations for hemoglobin subunit alpha (P69905) were found to be comparable between female and male in the samples from Black subjects. In contrast, the samples from male subjects with a Hispanic background showed 3-fold higher average levels than the female Hispanic samples, and approximately 6-fold higher average levels than the samples from Black subjects (Fig. 7B). Furthermore, male subjects showed slightly elevated concentrations compared to female subjects in both the Hispanic and Black groups for Apolipoprotein A-IV (P06727; Fig. 7C) and Apolipoprotein C-III (P02656; Fig. 7D). Immunoglobulin heavy constant gamma 1 (P01857), however, was found to have a higher concentration in Black subjects compared to Hispanic subjects (Fig. 7E). It is important to note, however, that the spread of P01857 concentrations in all groups was wide, which reflects a natural spread of IgG and the function of the immune system. Here, as we do not have any additional information on the medical history of these samples, it is hard to reach any definitive conclusion about why P01857 is elevated in one group and not another. Nonetheless, this demonstrates the ability of the assay to

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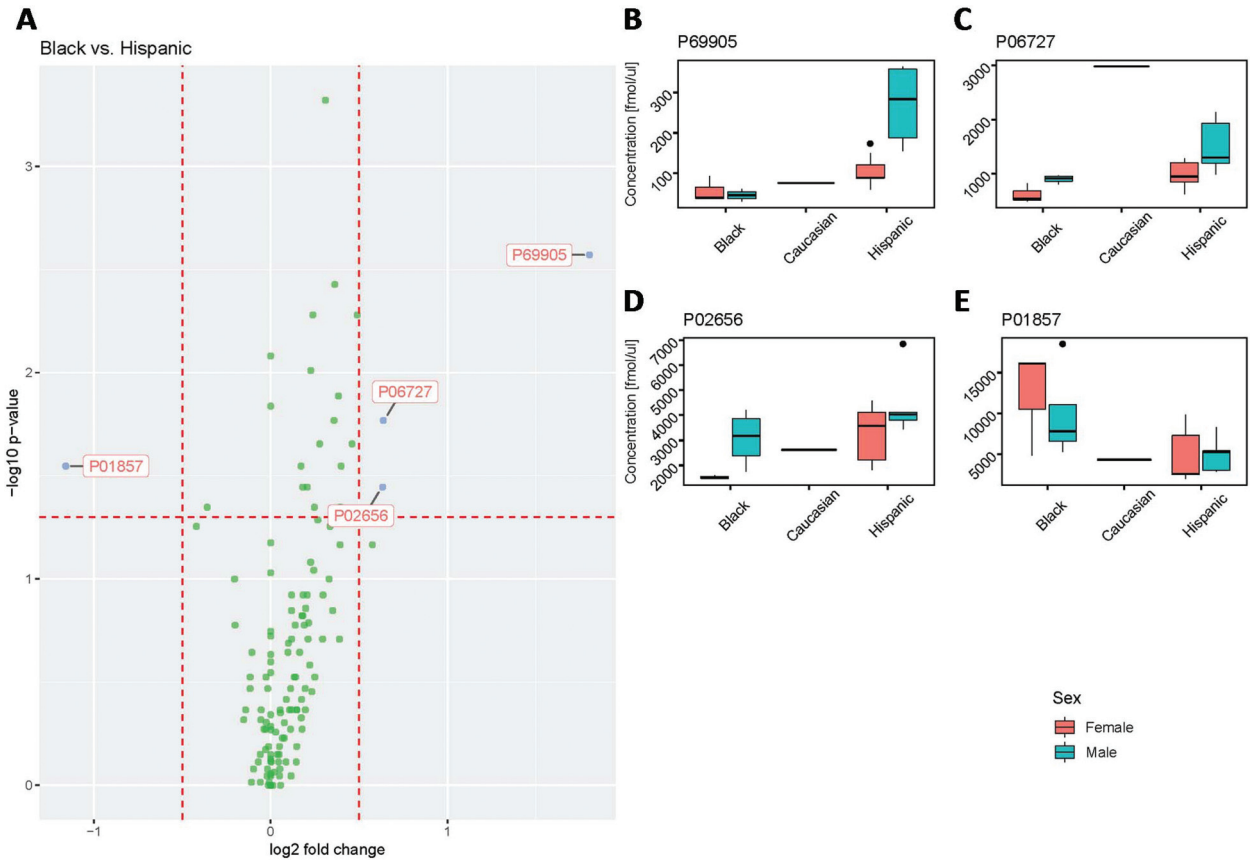

Fig. 7 Assessment of Black *vs.* Hispanic subject groups for statistical differences in proteins quantified across all plasma samples. (A) Volcano plot. (B–E) Box plots for protein concentrations determined for (B) hemoglobin subunit alpha (P69905), (C) Apolipoprotein A-IV (P06727), (D) Apolipoprotein C-III (P02656), and (E) immunoglobulin heavy constant gamma 1 (P01857).

capture and measure the elevation of immunoglobulin heavy constant gamma 1 levels, which could potentially be used to monitor the function of the immune system.

A similar trend was seen when assessing the protein concentration differences between male and female groups (Fig. 8).

While the majority of concentration values showed no statistically significant differences (Fig. 8A), the proteins CD5 antigen-like (O43866; Fig. 8B), sex hormone-binding globulin (P04278; Fig. 8C), fibronectin (P02751; Fig. 8D), and immunoglobulin heavy constant mu (P01871; Fig. 8G and H) were significantly higher in females than in males. This observation for immunoglobulin heavy constant mu agrees with previously reported data by Oyeyinka *et al.*[38]

Interestingly, the two different tryptic peptides (VSVFVPPR and GFPSVLR) used as surrogates to quantify immunoglobulin heavy constant mu (P01871), showed the same fold difference, demonstrating the validity of this approach to assess quantitative differences between samples. Despite the fact that both peptides were used to quantify the same protein and showed a statistically significant difference between female and male samples, the absolute concentration values between the two peptides differed by a factor of approximately 1.5, suggesting different release efficiencies and/or degradation behaviour of these peptides.[39] The excellent correlation of the quantitative results (with an R^2 value of 0.997, see ESI Fig. 2†), however, indicates that the concentrations determined are highly reproducible and therefore valid for protein quantitation. Furthermore, the concentrations of the proteins Apolipoprotein A-IV (P06727; Fig. 8E) and Apolipoprotein D (P05090; Fig. 8F) were significantly lower in females than in males.

Additionally, hierarchical clustering showed no obvious clusters (Fig. 9 and ESI "Gaither_Popp_Borchers_Figure_9_data.csv"†), further supporting the lack of statistically significant differences in the majority of protein concentrations between samples, although sub-clusters for male and female within the Black and Hispanic groups were observed.

Taken together, the data suggest that there are no significant concentration differences in all of the human plasma samples analyzed, supporting the idea that determining protein concentration references ranges for groups of patients, such as patients considered "healthy", is a valid approach. Previous studies using subsets of these assays on samples from cancer and control patients demonstrated the ability to capture a protein signature reflecting the two groups.[40]

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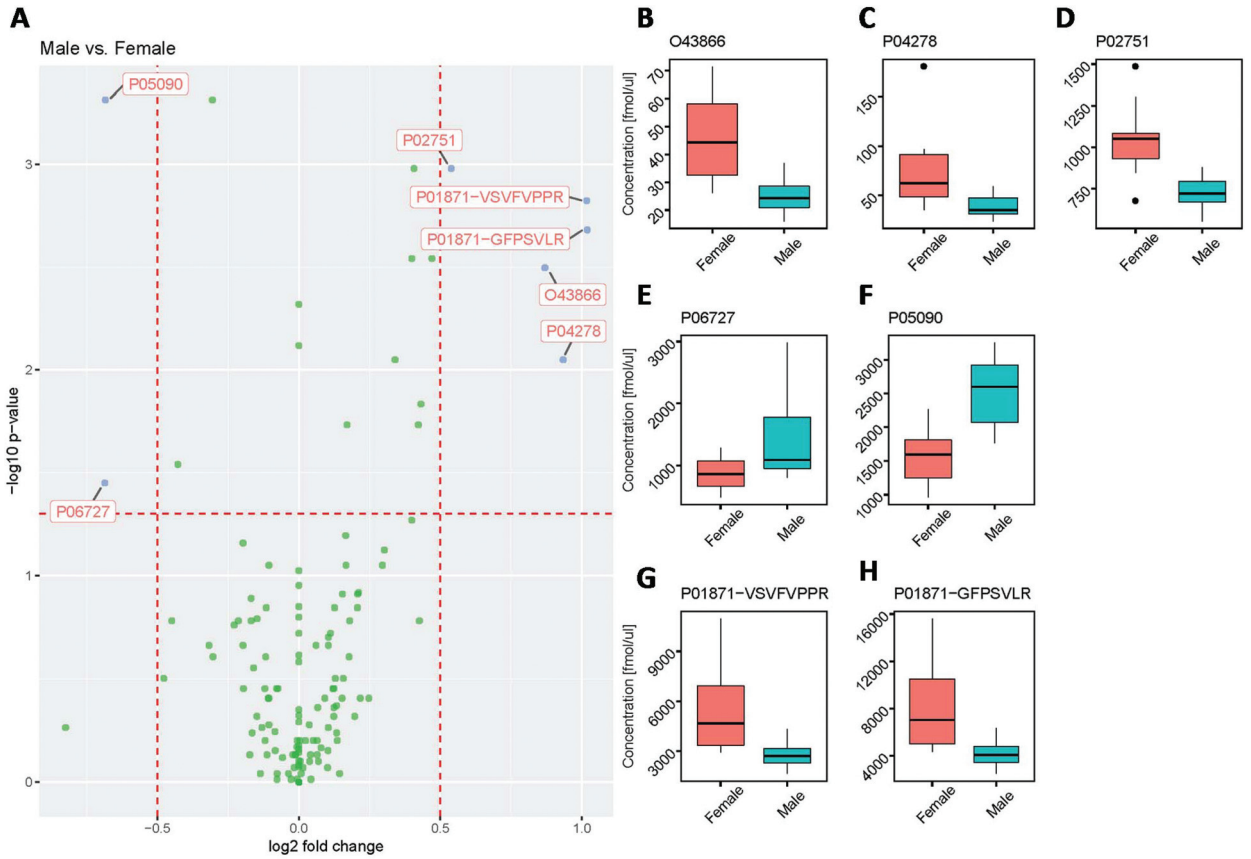

Fig. 8 Assessment of male *vs.* female groups for statistical differences in proteins quantified across all plasma samples. (A) Volcano plot. (B–H) Box plots for protein concentrations determined for (B) CD5 antigen-like (O43866), (C) sex hormone-binding globulin (P04278), (D) fibronectin (P02751), (E) Apolipoprotein A-IV (P06727), (F) Apolipoprotein D (P05090), and the tryptic peptides (G) GFPSVLR and (H) VSVFVPPR from immunoglobulin heavy constant mu (P01871).

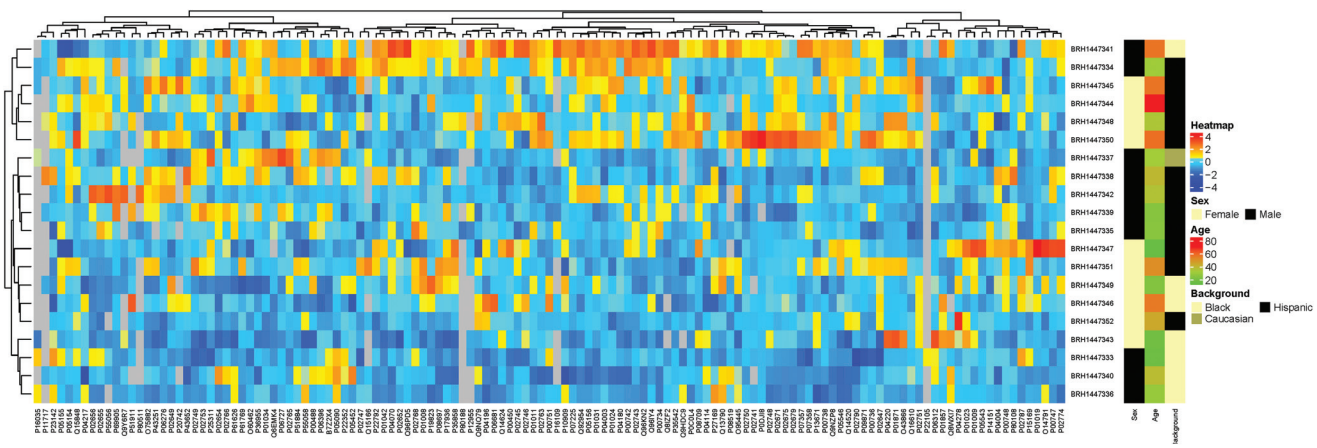

Fig. 9 Hierarchical clustering results for the patient samples analyzed, using ethnic background and sex as groups to be compared.

Conclusion

The PeptiQuant 267-protein MRM kit has been successfully used for determining the protein concentrations across six orders of magnitude in 21 commercially available human plasma samples in a highly reproducible and accurate fashion using stable isotope-labelled standard peptides. We believe this approach is well-suited to advance the biomarker discovery efforts to the next stage, biomarker validation, to finally bring more biomarkers into the clinic. Additionally, the poten-

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tial for differentiating diseased state *vs*. healthy state with peptide surrogates with mutated sequences as observed in a diseased state remains a possibility for the future of MRM-based clinical diagnostics and a project we look forward to exploring in the future.

Conflicts of interest

CHB is the CSO of MRM Proteomics, Inc. CG and RP are staff members of MRM Proteomics, Inc.

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