Multiple Reaction Monitoring-based, Multiplexed, Absolute Quantitation of 45 Proteins in Human Plasma*⑤

Michael A. Kuzyk‡, Derek Smith‡, Juncong Yang‡, Tyra J. Cross‡, Angela M. Jackson‡, Darryl B. Hardie‡, N. Leigh Anderson§, and Christoph H. Borchers‡¶‖

Mass spectrometry-based multiple reaction monitoring (MRM) quantitation of proteins can dramatically impact the discovery and quantitation of biomarkers via rapid, targeted, multiplexed protein expression profiling of clinical samples. A mixture of 45 peptide standards, easily adaptable to common plasma proteomics work flows, was created to permit absolute quantitation of 45 endogenous proteins in human plasma trypsin digests. All experiments were performed on simple tryptic digests of human EDTA-plasma without prior affinity depletion or enrichment. Stable isotope-labeled standard peptides were added immediately following tryptic digestion because addition of stable isotope-labeled standard peptides prior to trypsin digestion was found to generate elevated and unpredictable results. Proteotypic tryptic peptides containing isotopically coded amino acids ($[^{13}C_6]$Arg or $[^{13}C_6]$Lys) were synthesized for all 45 proteins. Peptide purity was assessed by capillary zone electrophoresis, and the peptide quantity was determined by amino acid analysis. For maximum sensitivity and specificity, instrumental parameters were empirically determined to generate the most abundant precursor ions and y ion fragments. Concentrations of individual peptide standards in the mixture were optimized to approximate endogenous concentrations of analytes and to ensure the maximum linear dynamic range of the MRM assays. Excellent linear responses ($r > 0.99$) were obtained for 43 of the 45 proteins with attomole level limits of quantitation (<20% coefficient of variation) for 27 of the 45 proteins. Analytical precision for 44 of the 45 assays varied by <10%. LC-MRM/MS analyses performed on 3 different days on different batches of plasma trypsin digests resulted in coefficients of variation of <20% for 42 of the 45 assays. Concentrations for 39 of the 45 proteins are within a factor of 2 of reported literature values. This mixture of internal standards has many uses and can be applied to the characterization of trypsin digestion kinetics and plasma protein expression profiling because 31 of the 45 proteins are putative biomarkers of cardiovascular disease. *Molecular & Cellular Proteomics 8:1860-1877, 2009.*

MS is capable of sensitive and accurate protein quantitation based on the quantitation of proteolytic peptides as surrogates for the corresponding intact proteins. Over the past 10 years, MS-based protein quantitation based on the analysis of peptides (in other words, based on "bottom-up" proteomics) has had a profound impact on how biological problems can be addressed (1, 2). Although advances in MS instrumentation have contributed to the improvement of MS-based protein quantitation, the use of stable isotopes in quantitative work flows has arguably had the greatest impact in improving the quality and reproducibility of MS-based protein quantitation (3–5).

The ongoing development of untargeted MS-based quantitation work flows has focused on increasingly exhaustive sample prefractionation methods, at both the protein and peptide levels, with the goal of detecting and quantifying entire proteomes (6). Although untargeted MS-based quantitation work flows have their utility, they are costly in terms of lengthy MS data acquisition and analysis times, and as a result, they are often limited to quantifying differences between small sample sets ($n < 10$). To facilitate rapid quantitation of larger, clinically relevant sample sets ($n > 100$) there is a need to both simplify sample preparation and reduce MS analysis time.

Multiple reaction monitoring (MRM)[1] is a tandem MS (MS/MS) scan mode unique to triple quadrupole MS instrumentation that is capable of rapid, sensitive, and specific quantitation of analytes in highly complex sample matrices (7). MRM is a targeted approach that requires knowledge of the molecular weight of an

[1] The abbreviations used are: MRM, multiple reaction monitoring; CE, collision energy; CV, coefficient of variation; CZE, capillary zone electrophoresis; DP, declustering potential; LOQ, limit of quantitation; Q1, quadrupole 1; Q3, quadrupole 3; SIS, stable isotope-labeled standard; XIC, extracted ion chromatogram; iTRAQ, isobaric tags for relative and absolute quantitation.

analyte and its fragmentation behavior under CID. MRM is capable of highly reproducible concentration determination when stable isotope-labeled internal standards are included in work flows and has been used for decades for the quantitation of low molecular mass analytes (<1000 Da) in pharmaceutical, clinical, and environmental applications (7, 8).

The combination of triple quadrupole MS instrumentation with nanoliter flow rate high performance LC and nanoelectrospray ionization provides the necessary sensitivity for detection and quantitation of biological molecules such as peptides in complex samples such as plasma by MRM. When combined with the use of isotopically labeled synthetic peptide standards, MRM analysis is capable of sensitive (attomole level) and absolute determination of peptide concentrations across a wide concentration scale spanning a dynamic range of 10^3–10^4 (1, 9–13).

Several recent studies involving MRM-based analysis of plasma proteins have focused on increasing MRM detection sensitivity by fractionating plasma using either multidimensional liquid chromatography, affinity depletion of high abundance proteins (11, 14, 15), or affinity enrichment of low abundance peptides (16, 17). Anderson and Hunter (14) have shown that LC-MRM/MS analysis is capable of detecting 47 moderate to high abundance proteins in plasma without depletion even though ∼90% of the total protein by weight in trypsin-digested plasma can be attributed to 10 high abundance proteins (18).

Relative abundance of a protein does not preclude its involvement in disease. In fact, 32 of the 47 plasma proteins detected by Anderson and Hunter (14) have been implicated as putative markers for cardiovascular disease. The ability to rapidly quantify proteins in a highly multiplexed manner using MRM and internal standard peptides expands the potential application of MRM quantitation beyond biomarker validation and into the field of biomarker discovery. Targeted, simultaneous quantitation of hundreds of proteins in a single analysis will enable rapid protein expression profiling of large (*n* > 100) clinically relevant sample sets in a manner similar to DNA microarray expression profiling. By allowing researchers to look at *patterns* of expression levels of a large number of proteins in a large number of samples (as opposed to looking at the expression levels of only a single protein), multiplexed MRM-based quantitation will allow the correlation of expression *patterns* with particular diseases. Once these characteristic patterns have been established, physicians will be able to use these protein expression patterns to diagnose diseases in the same way they currently use blood chemistry panels or comprehensive metabolic panels.

When considering the clinical utility of MS-based assays, direct comparisons are often made to ELISA, which is considered the "gold standard" for protein quantitation in clinical samples. Attributes of ELISAs, such as "time to first result" (1–2 h (19)) and the ability to quantify 96 or 384 samples in parallel because of their microtiter plate-based format, are currently difficult to match with MS-based protein assays. However, MRM protein assays may surpass ELISA in the rapid development of clinically useful, multiplexed protein assays. The impact of multiplexed assays in the field of genomics has increased interest in multiplexed quantitation of many proteins in individual clinical samples (19). Development and characterization of MRM-based protein assays using isotopically labeled peptides is rapid and inexpensive compared with the time and cost associated with the generation and characterization of antibodies for ELISA development.

In this study, we describe the creation of a customizable mixture of concentration-balanced stable isotope-labeled standard (SIS) peptides representing an initial panel of 45 human plasma proteins. We used this mixture of SIS peptides to develop a suite of multiplexed, rapid, and reproducible MRM-based assays for expression profiling of these 45 proteins in simple tryptic digests of whole plasma. Additionally we characterized the analytical performance of these MRM peptide assays with respect to their reproducibility, and we demonstrated their utility for absolute protein concentration determination.

Multiplexed MRM quantitation of peptides for protein quantitation has the potential to replace iTRAQ or other isotope label and label-free quantitative proteomics approaches because the approach is much faster than these other methods (30–60 min per analysis compared with 4 days for LC-MALDI-based iTRAQ), has greater reproducibility (CV <5% *versus* iTRAQ CV >20%), and enables absolute quantitation (concentration and copy number *versus* only *x*-fold up- or down-regulated). Additionally MRM-based quantitation with SIS peptides does not "miss" peptides because the SIS peptide must be detected in every sample: this means that if an endogenous peptide is not observed then it is below the limit of detection.

EXPERIMENTAL PROCEDURES

Reagents and Chemicals—All reagents were American Chemical Society (ACS) grade or higher. All solvents used, including water, were LC/MS grade.

Synthesis of Isotopically Labeled Tryptic Peptides—Isotopically labeled peptide standards were synthesized at a 5-μmol scale using Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry with a Protein Technologies Prelude peptide synthesizer (Tucson, AZ). Isotopically labeled amino acids, [$^{13}C_6$]Lys (98% isotopic enrichment) and [$^{13}C_6$]Arg (98% isotopic enrichment), were purchased from Cambridge Isotope Laboratories (Andover, MA) and were conjugated to TentaGel R resin by Rapp Polymere (Tubingen, Germany). Subsequent amino acid residues (100 mM) were double coupled using 20% piperidine as the deprotector and 1*H*-benzotriazolium 1-[bis(dimethylamino)methylene]-5-chloro-,hexafluorophosphate (1-),3-oxide (HCTU) as the activator. The cleavage was performed with 95:2.5:2.5 TFA:water:triisopropylsilane.

Purification of Synthetic Peptides—The cleaved peptides were removed from the synthesizer, and the TFA was evaporated under a stream of nitrogen. Ether was added to precipitate the peptides, and after centrifugation at 3000 × *g* for 5 min, the ether layer was decanted. Peptides were resolubilized in 0.1% TFA and purified by

reversed-phase HPLC (Ultimate 3000, Dionex) while monitoring the peptide elution at 230 nm. The crude peptides were separated using a Vydac C$_{18}$ column (10 × 250 mm, 10-μm resin) with a linear gradient of 0.1% TFA in water (v/v) and 0.085% TFA in 50% acetonitrile (v/v) at a flow rate of 4 ml/min over 60 min. Fractions of interest were spotted onto stainless steel MALDI plates and measured by MALDI-TOF (Applied Biosystems/MDS Sciex, Concord, Ontario, Canada) mass spectrometry. Fractions containing greater than 80% of the target peptide by MALDI-TOF analysis were pooled and lyophilized.

Lyophilized peptides were resolubilized in 30% acetonitrile, 0.1% formic acid, and peptide concentrations were subjected to acid hydrolysis and amino acid analysis. Purity of the HPLC-purified peptides was determined by capillary zone electrophoresis (CZE) using a P/ACE^{TM} MDQ System (Beckman Coulter, Fullerton, CA) equipped with a UV detector monitored at 200 nm. All separations were performed at 25 °C in a bare fused silica capillary (Polymicro Technologies, Phoenix, AZ) of dimensions 50-μm inner diameter × 360-μm outer diameter, 60-cm total length, and 50-cm effective length. The applied voltage was 15 kV, and the background electrolyte was 50 mM sodium phosphate adjusted to pH 2.5 with phosphoric acid. The capillary was rinsed at 20 p.s.i. with 1 M NaOH for 1 min, Millipore-purified water for 1 min, 1 M HCl for 2 min, 0.1 M HCl for 3 min, and finally with background electrolyte (n-butyl glycidyl ether) for 3 min between each separation. Injection of 20 nl of each peptide solution (ranging from 4 to 10 pmol/μl in 30% (v/v) ACN, 0.1% FA) was accomplished by application of 0.5 p.s.i. for 20 s at the inlet end of the capillary. Purity was assessed by peak height for a given concentration of peptide injected relative to signal to noise of the background signal. The limit of quantitation was determined to be ~150 nmol/μl. The absolute concentration of each synthetic SIS peptide was determined by amino acid analysis. These absolute concentrations were adjusted by the percent purity of each synthetic peptide as determined by CZE. This corrects for any possible contribution from incorrect or partial synthesis products.

MRM Q1/Q3 Ion Pair Selection by Nanoinfusion—Isotopically labeled peptides were diluted to 1 pmol/μl (1 mM) in 30% acetonitrile, 0.1% formic acid for infusion at a flow rate of 300 nl/min using a Harvard PicoPlus 11 syringe pump (Harvard Apparatus, Holliston, MA). Infused peptide solutions were analyzed by nanoelectrospray using a 4000 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems/MDS Sciex) equipped with a nanospray ionization source. MS analysis was conducted in the positive ion mode with ion spray voltages in the 1800–2000-V range. The declustering potential was ramped (0–120 V in 2-V increments) during Q1 scans centered on 10-Da-wide mass ranges. MRM scans for optimization of MRM Q1/Q3 ion pairs were conducted with both Q1 and Q3 set to unit resolution (0.6–0.8-Da full width at half-height) while the collision energy was ramped (5–120 V in 2-V increments). An MS operating pressure of 3.5 × 10^{-5} torr was used during all MRM scans.

EDTA-plasma Collection—Plasma was collected from a healthy male donor who fasted for 16 h prior to blood collection. Blood was collected by venous puncture using a 21-gauge BD Vacutainer Multiple Sample Needle (reference number 367213, BD Biosciences). A total of 60 ml of blood was collected into 20 × 3.0-ml BD Vacutainer lavender tubes with 5.4 mg of K$_2$-EDTA (reference number 367856, BD Biosciences). Immediately following blood collection and mixing with the EDTA in the tubes, the samples were centrifuged at 1000 × *g* for 15 min at 22 °C to pellet the cells. Plasma was collected and centrifuged again to remove any remaining cells. The plasma was then divided into 1.0-ml aliquots in sterile cryotubes and immediately frozen at −80 °C for storage. Total elapsed time from collection to storage was 1 h. Plasma analyzed in the experiments described in this study had been stored at −80 °C for 4–8 weeks.

Preparation of Plasma Tryptic Digests and Addition of Internal Standard Peptides—Plasma tryptic digests were prepared by diluting 5 μl of whole plasma (~350 μg of total protein) 1:10 with 25 mM ammonium bicarbonate prior to denaturation with 50 μl of sodium deoxycholate (10% (w/v) in 25 mM ammonium bicarbonate). Denatured plasma samples were reduced for 30 min at 60 °C with 5 mM tris(2-carboxyethyl)phosphine (net 22.5-fold excess over the ~26 mM concentration of protein cysteine in plasma computed from known protein concentrations). Reduced plasma samples were alkylated for 30 min at 37 °C in the dark with 10 mM iodoacetamide (net 50-fold excess over plasma protein cysteine). Ammonium bicarbonate (25 mM) was added to the digests to reduce the sodium deoxycholate concentration to 1% (w/v) and to achieve a 500-μl digest volume containing a 1:100 dilution of plasma upon addition of trypsin. Modified, sequencing grade trypsin (Promega, Madison, WI) was added to the samples at a 20:1 substrate:enzyme ratio. Digestion was carried out for 16 h at 37 °C.

Samples were acidified by adding an equal volume of 1% (v/v) formic acid to stop digestion. For quantitation of unknown samples, a concentration-balanced mixture of 45 isotopically labeled internal standard peptides (0.1% (v/v) formic acid) was added to each plasma digest at a ratio of 1 volume of SIS peptide mixture to 4 volumes of acidified plasma trypsin digest. This came to 50 μl of SIS peptide mixture added per 1-μl equivalent of neat plasma.

Samples were desalted and concentrated prior to MS analysis by solid phase extraction using Waters Oasis reversed-phase 10-mg HLB cartridges (Waters, Milford, MA) following the manufacturer's recommended protocol. Samples were eluted with 200 μl of 50% (v/v) acetonitrile, 0.1% (v/v) formic acid. The eluted samples were frozen and lyophilized to dryness. Prior to LC-MRM/MS analysis, samples were reconstituted in 0.1% formic acid to a concentration of ~1 μg/μl based on an initial plasma protein concentration of 70 mg/ml.

LC-MRM/MS Analysis of Plasma Digests—An Eksigent NanoLC-1Dplus HPLC was used for the injection of desalted plasma digest samples (1 μl) onto reversed-phase capillary columns (75 μm × 15 cm) packed in house using Magic C18AQ (5-μm diameter particles, 100-Å pore size; Michrom, Auburn, CA). A flow rate of 300 nl/min solvent A (2% acetonitrile, 0.1% formic acid) was used for 6 min. Separations were performed using a flow rate of 300 nl/min with a 32-min linear gradient from 0 to 23% solvent B (98% acetonitrile, 0.1% formic acid) followed by a 9-min linear gradient from 23 to 43% solvent B.

An Applied Biosystems/MDS Sciex 4000 QTRAP with a nanoelectrospray ionization source controlled by Analyst 1.5 software (Applied Biosystems) was used for all LC-MRM/MS analyses. All acquisition methods used the following parameters: 1900–2000-V ion spray voltage, a curtain gas setting of 25 p.s.i., a 200 °C interface heater temperature, a collision-activated dissociation pressure at 3.5 × 10^{-5} torr, and Q1 and Q3 set to unit resolution (0.6–0.8-Da full width at half-height).

Spray stability was improved and lifespan of the uncoated fused silica emitter tips (20-μm inner diameter, 10-μm tip; New Objective, Woburn, MA) was improved by use of 3–5-p.s.i. sheath gas and postcolumn, prespray addition of makeup solvent (80% (v/v) isopropanol) at a flow rate of 100 nl/min using a PicoPlus 11 syringe pump (Harvard Apparatus). MRM acquisition methods were constructed using 90 MRM ion pairs with peptide-specific tuned declustering potential (DP), collision energy (CE) voltages, and retention time constraints. A default collision cell exit potential of 23 V was used for all MRM ion pairs, and the scheduled MRM option was used for all data acquisition with a target scan time of 2 s and an 8-min MRM detection window.

MRM Data Analysis—All MRM data were processed using Multi-Quant 1.0 (Applied Biosystems) with the MQL algorithm for peak integration. A 2-min retention time window with "report largest peak"

enabled and a three-point smooth with a peak splitting factor of 2 was used. The default MultiQuant values for noise percentage and base-line subtraction window were used. All data were manually inspected to ensure correct peak detection and accurate integration. Linear regression of all calibration curves was performed using a standard $1/x$ ($x =$ concentration ratio) weighting option to aid in covering a wide dynamic range (20). Raw data files for these analyses are available from the ProteomeCommons Tranche network (http://www.proteomecommons.org/data-downloader.jsp?fileName=O3Hbto5xoHSurD7i5FYQMe6lKix jYxsB8ao8W/gpnNmIxQX2hNxoHXJORjyDg9s5x1mVmm+7+J33b8F aEUBB7MuoGMAAAAAAAAAAUXA==) using hash codes.

RESULTS AND DISCUSSION

Selection and Synthesis of Isotopically Labeled Proteotypic Peptide Standards—Development of protein assays based on proteolytic peptide surrogates requires that the peptides selected are both detectable in every sample and reproducibly observed between sample preparations. Because of differing peptide ionization efficiencies and susceptibilities to CID, selection of a peptide to represent a protein in an MRM assay is a crucial step that dramatically affects the ultimate sensitivity and specificity of an assay. When selecting a peptide, several critical factors must be considered. 1) The peptide amino acid sequence must be unique to the target protein and contain no missed cleavage sites, 2) the peptide should be reproducibly observed in proteolytic digests, and 3) the peptide should not contain amino acids that are susceptible to chemical modification (Cys and Met).

When working with a species for which the genome has been sequenced and widely studied, as it has been for *Homo sapiens*, publicly accessible MS/MS spectra databases such as the Global Proteome Machine and Peptide Atlas are useful for determining which tryptic peptides of proteins are frequently observed by MS/MS analysis (21, 22). The concept of using "proteotypic" peptides for MS-based protein identification and quantitation is gaining wider acceptance, and bioinformatics tools are emerging to assist in their computational prediction (23–25). Additional experiments, however, are still required to verify the utility of these proteotypic peptides for use in MRM assays because public databases of MS/MS spectra often lack information about experimental methods and the MS instrumentation used to obtain these spectra.

We developed an experimental work flow to rapidly select proteotypic peptides that are suitable for use as internal standards with the intention of a creating an expandable mixture of SIS peptides for multiplexed absolute quantitation of proteins by LC-MRM/MS. Stable isotope-labeled derivatives of these peptides were synthesized and were used to develop MRM assays (Fig. 1). Our work flow contains checkpoints that quickly identify proteins with problematic proteotypic peptides (for example, the synthetic peptide is insoluble, the natural MRM signal does not cochromatograph with the SIS peptide, etc.), and these proteins are marked for selection of alternate proteotypic peptides.

For our initial studies, we selected 45 tryptic peptides from a list of moderate to high abundance plasma proteins shown previously to be reproducibly detectable by LC-MRM/MS analysis of whole plasma trypsin digests (14) (Table I). Nearly every one of these 45 proteins has been reported to be a potential marker of a clinically relevant disease (Table I) (18, 26–43). Thirty-one of these 45 proteins have been reported previously to be associated with cardiovascular disease (18).

Isotopically labeled forms (containing either a C-terminal $[^{13}C_6]$Arg or $[^{13}C_6]$Lys residue) of all 45 peptides were synthesized on a micromole scale. The C terminus of each peptide was selected for stable isotope-labeled amino acid incorporation to mass shift the entire y series fragment ions, which is the dominant ion series in CID fragmentation spectra of tryptic peptides (44), of each peptide. Selection of a y series ion as the Q3 fragment ion for each MRM Q1/Q3 ion pair thus ensures that both the Q1 and Q3 m/z values differ from those of the natural peptide. This maximizes the specificity of the MRM signal and eliminates potential cross-talk between the natural and SIS peptide MRM signals.

Proteotypic peptides selected by Anderson and Hunter (14) for apolipoprotein C-III, clusterin, and complement component C9 formed insoluble precipitates following cleavage from synthetic support resins. In cases of problematic peptides such as these, the Global Proteome Machine database of MS/MS spectra was used to select alternative peptides for MRM assay development (21). To enable determination of the *absolute* concentrations of the endogenous proteins by MRM, the concentrations of all of the purified synthetic peptides were determined by amino acid analysis (supplemental results). Postsynthesis peptide purity was assessed by both MALDI-TOF MS analysis and CZE. By CZE, the purity of the peptides was found to range from 77.4 to 98.7% with an average purity of 96.1% (supplemental results). The concentrations of the peptide stock solutions as determined by amino acid analysis were corrected using the percent purities determined by CZE analysis.

Optimized Selection of MRM Q1/Q3 Ion Pairs for Each Peptide—Consensus spectra collected in the Peptide Atlas and the Global Proteome Machine database can be useful for selecting MRM precursor and fragment ion pairs prior to SIS peptide synthesis; however, their utility in selecting the most sensitive MRM precursor/fragment ion pair for a peptide is limited. Creating the most sensitive MRM assay for a peptide requires judicious selection of both precursor ion charge state and fragment ions combined with empirical tuning of MS parameters. Public MS/MS data repositories were acquired with the intent of peptide sequence assignment. This requires fragmentation spectra that represent the entire peptide fragment ion series. The signal intensity of an MRM Q1/Q3 ion pair for a peptide is determined by the combined ionization efficiency of the precursor peptide and how readily it can be dissociated into a *limited* number of fragment ion species. Even sophisticated bioinformatics tools for MRM prediction are currently unable to predict optimal collision energy volt-
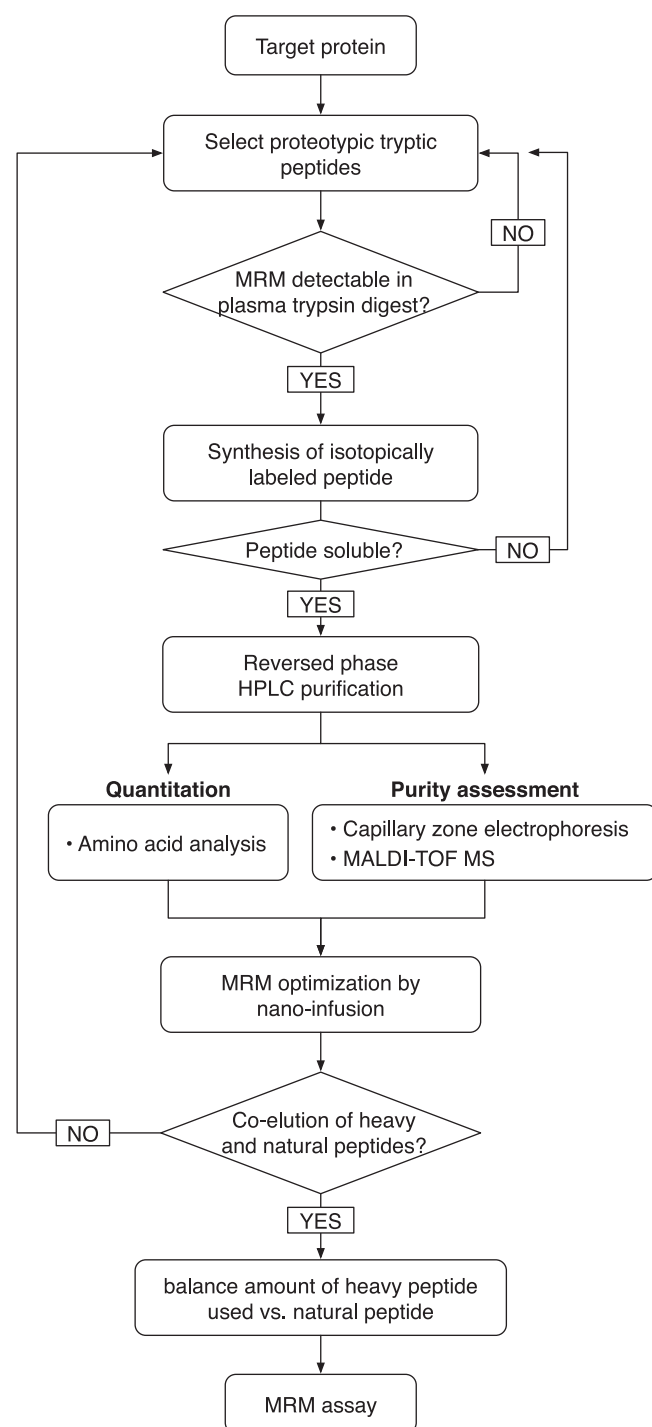
```
                    Target protein
                          │
                          ▼
            Select proteotypic tryptic
                    peptides
                          │
                          ▼                    NO
              MRM detectable in        ─────────►
            plasma trypsin digest?
                          │
                        YES
                          │
                          ▼
            Synthesis of isotopically
                labeled peptide
                          │
                          ▼                    NO
               Peptide soluble?       ─────────►
                          │
                        YES
                          │
                          ▼
               Reversed phase
               HPLC purification
                    │         │
         Quantitation      Purity assessment
                    │         │
          • Amino acid    • Capillary zone electrophoresis
            analysis      • MALDI-TOF MS
                    │         │
                    ▼         ▼
               MRM optimization by
                  nano-infusion
                          │
                          ▼
       NO        Co-elution of heavy
    ◄──────      and natural peptides?
                          │
                        YES
                          │
                          ▼
            balance amount of heavy peptide
              used vs. natural peptide
                          │
                          ▼
                    MRM assay
```

FIG. 1. **Strategy for generation of highly sensitive and specific MRM protein assays.** Isotopically labeled versions of proteotypic, endogenous, tryptic peptides representing each protein (selected from either Anderson and Hunter (14) or the Global Proteome Machine database) were synthesized and purified by reversed-phase HPLC. Concentrations of purified SIS peptides were determined by amino acid analysis, and the peptide concentration of each SIS peptide was corrected by the percent purity as determined by capillary zone electrophoresis. MRM Q1/Q3 ion pairs and parameters were determined empirically. Identities of natural peptide MRM signals

ages without the use of simple formulas that directly correlate collision energy voltages with precursor ion m/z (24, 45).

Because the synthetic SIS peptides ionize and fragment identically to the natural peptides in the mass spectrometer, synthetic peptides can be used to optimize the MS analysis parameters. To ensure that the most sensitive combination of precursor and fragment ions are ultimately selected as the MRM ion pair for each peptide, peptide solutions were infused by nanoelectrospray ionization, and Q1 and MRM scans were used to optimize both the precursor and fragment ion signal intensities, respectively.

First the peptide ionization efficiency was optimized, and the most intense charge state was determined by ramping the DP voltages during the Q1 scans (Fig. 2, a and b). Tuned DP voltages ensure maximum efficiency of ion transfer of the precursor ion into the MS instrument and affect the maximum MRM signal that can be achieved with each peptide. Using the tuned Q1 parameters for each peptide, we next determined which were the dominant CID fragment ions that were generated from each peptide to create an MRM ion pair. To determine the most abundant fragment ions for each peptide, the infused peptides were analyzed using MRM ion pairs containing all possible $[M + H]^+$ and $[M + 2H]^{2+}$ b and y series fragment ions while ramping the collision energy (Fig. 2, c and d). Signal intensities from all MRM Q1/Q3 ion pairs for each peptide were ranked to ensure selection of the most intense precursor and fragment ion pair for MRM-based quantitation. This approach resulted in selection of CE voltages that maximize the generation of each fragment ion species (supplemental results). As a result of these studies, we found that doubly charged y ion fragments were the highest intensity fragment ions for 15 of the 45 peptides (Table I).

There are already several computational methods for the prediction of both proteotypic peptides and their corresponding Q1/Q3 ion pairs for MRM analysis (24, 25). The MIDAS[TM] Workflow Designer (Applied Biosystems) software utility predicts the peptide charge state based on the number of basic residues in a peptide, and commonly uses y ion fragments with m/z values greater than the precursor ion m/z as Q3 masses for the peptide MRM ion pair (46). Collision energy voltages for peptide MRM ion pairs are often predicted using a generic formula (CE $= 0.05 \times$ (precursor m/z) $+ 5$) that directly correlates peptide ion m/z and charge state with the predicted CE voltage (14). To assess the value of empirically selecting the most intense MRM Q1/Q3 ion pair for a peptide, signal intensities obtained with our optimized MRM pairs were compared with the signal intensities obtained with MIDAS-predicted MRM Q1/Q3 ion pairs. Although the same Q3 frag-

---

were confirmed by co-elution with their isotopically labeled forms when analyzed by LC-MRM/MS. Isotopically labeled peptides were titrated to determine the concentration that produced a peak area ratio 1–10 times higher than the natural peptide peak area when added to a plasma tryptic digest.

TABLE I

MRM parameters and analytical precision of MRM assays based on proteotypic tryptic peptides for 45 plasma proteins

Masses listed are for the natural forms all 45 peptides. MRM parameters are sorted by decreasing fragment ion intensity. HC, heavy chain; CVD, cardiovascular disease.

Protein	UniProt accession no.	Peptide sequence	Peptide molecular mass	$Q1^a$	$Q3^b$	DP	CE	Fragment ion	CV^c	-Fold signal increase^d	Putative disease association	Refs.
			Da			V	V		%			
Afamin	P43652	DADPDTFFAK	1125.5	563.8	825.4	60	24	y7	5.4	11.1	Ovarian cancer	30
				563.8	413.2	60	23	y7²⁺	6.1			
				563.8	940.4	60	27	y8	6.9			
Albumin, serum	P02768	LVNEVTEFAK	1148.6	575.3	185.2	70	32	a2	1.4	4.5	CVD	18
				575.3	937.5	70	25	y8	1.4			
				575.3	213.2	70	27	b2	1.1			
α₁-Acid glycoprotein 1	P02763	NWGLSVYADKPETTK	1707.8	570.3	704.9	56	24	y13²⁺	2.2	14.0	CVD, cervical cancers	18, 38
				570.3	273.1	56	30	a2	3.6			
				570.3	301.1	56	25	b2	6.0			
α₁-Antichymotrypsin	P01011	EIGELYLPK	1060.6	531.3	244.2	60	31	y2	4.1	28.2	CVD, Alzheimer disease	18, 27
				531.3	819.5	60	24	y7	3.5			
				531.3	215.1	60	31	a2	2.5			
α₁B-Glycoprotein	P04217	LETPDFQLFK	1236.6	619.3	243.1	65	31	b2	2.0	2.5	Pancreatic, liver and bladder cancers	34, 41, 43
				619.3	894.5	65	28	y7	1.5			
				619.3	215.1	65	35	a2	1.9			
α₂-Antiplasmin	P08697	LGNQEPGGQTALK	1311.7	656.8	771.4	82	33	y8	2.6	1.2	CVD	18
				656.8	542.3	82	31	b5	3.0			
				656.8	900.5	82	33	y9	6.2			
α₂-Macroglobulin	P01023	LLIYAVLPTGDVIGDSAK	1844.0	615.7	586.8	58	17	y12²⁺	2.2	19.1	CVD	18
				615.7	673.4	58	17	b6	1.7			
				615.7	530.3	58	24	y11²⁺	2.9			
Angiotensinogen	P01019	ALQDQLVLVAAK	1267.8	634.9	542.8	73	25	y10²⁺	5.1	3.7	CVD	18
				634.9	956.6	73	29	y9	5.7			
				634.9	289.2	73	29	y3	5.1			
Antithrombin-III	P01008	DDLYVSDAFHK	1308.6	437.2	540.3	47	17	y9²⁺	4.7	7.6	CVD	18
				437.2	483.7	47	18	y8²⁺	4.8			
				437.2	704.3	47	23	y6	6.9			
Apolipoprotein A-I	P02647	ATEHLSTLSEK	1214.6	405.9	572.8	52	19	y10²⁺	1.1	8.4	CVD	18
				405.9	522.3	52	18	y9²⁺	1.7			
				405.9	363.2	52	24	y3	1.4			
Apolipoprotein A-II precursor	P02652	SPELQAEAK	971.5	486.8	443.2	64	26	y8²⁺	5.7	1.6	CVD, prostate cancer	18, 36
				486.8	157.1	64	34	a2	N.D.			
				486.8	788.4	64	25	y7	0.9			
Apolipoprotein A-IV	P06727	SLAPYAQDTQEK	1349.7	675.8	540.3	67	30	y9²⁺	2.7	6.6	CVD	18
				675.8	575.8	67	30	y10²⁺	5.7			
				675.8	1079.5	67	30	y9	4.5			
Apolipoprotein B-100	P04114	FPEVDVLTK	1046.6	524.3	450.8	65	28	y8²⁺	2.9	5.4	Coronary heart disease	40
				524.3	803.5	65	26	y7	4.4			
				524.3	674.4	65	32	y6	8.9			
Apolipoprotein C-I lipoprotein	P02654	TPDVSSALDK	1031.5	516.8	466.2	78	29	y9²⁺	2.5	8.9	CVD	18
				516.8	834.4	78	29	y8	3.0			
				516.8	620.3	78	29	y6	2.3			
Apolipoprotein C-III	P02656	GWVTDGFSSLK	1195.6	598.8	244.1	59	31	b2	1.1	4.5	CVD	18
				598.8	854.4	59	26	y8	1.9			
				598.8	953.5	59	25	y9	2.5			

TABLE I—*continued*

Protein	UniProt accession no.	Peptide sequence	Peptide molecular mass	$Q1^a$	$Q3^b$	DP	CE	Fragment ion	CV^c	-Fold signal increase^d	Putative disease association	Refs.
			Da			*V*	*V*		%			
Apolipoprotein E	P02649	LGPLVEQGR	967.6	484.8	399.7	70	25	$y7^{2+}$	3.6	11.0	CVD	18
				484.8	588.3	70	30	y5	3.8			
				484.8	489.2	70	28	y4	4.3			
β_2-Glycoprotein I	P02749	ATVVYQGER	1021.5	511.8	652.3	65	25	y5	1.6	3.2	CVD	18
				511.8	751.4	65	25	y6	1.6			
				511.8	850.4	65	25	y7	1.9			
Ceruloplasmin	P00450	EYTDASFTNR	1202.5	602.3	624.3	66	29	y5	6.0	1.4	CVD	18
				602.3	911.4	66	29	y4	6.0			
				602.3	695.4	66	32	y6	5.2			
Clusterin	P10909	ELDESLQVAER	1287.6	644.8	375.2	75	30	y3	5.7	2.2	CVD	18
				644.8	802.4	75	33	y7	6.7			
				644.8	602.3	75	31	y5	5.1			
Coagulation factor XIIa HC	P00748	VVGGLVALR	882.6	442.3	685.4	60	21	y7	2.6	15.4	CVD	18
				442.3	171.1	60	25	a2	4.9			
				442.3	199.1	60	21	b2	3.9			
Complement C3	P01024	TGLQEVEVK	1001.5	501.8	731.4	65	25	y6	3.0	1.8	CVD	18
				501.8	603.3	65	26	y5	1.7			
				501.8	422.7	65	25	$y7^{2+}$	6.4			
Complement C4 β chain	P0C0L5	VGDTLNLNLR	1113.6	557.8	629.4	70	31	y5	6.9	1.1	CVD, renal carcinoma	18, 38
				557.8	742.5	70	30	y6	5.4			
				557.8	1015.5	70	32	y9	7.2			
Complement C4 γ chain	P0C0L5	ITQVLHFTK	1085.6	362.9	487.3	88	16	$y8^{2+}$	3.7	5.3	CVD, renal carcinoma	18, 30
				362.9	436.8	88	15	$y7^{2+}$	6.3			
				362.9	215.1	88	18	b2	10.1			
Complement component C9	P02748	TEHYEEQIEAFK	1522.7	508.6	494.3	66	23	y4	11.3	3.6		
				508.6	607.3	66	21	y5	11.8			
				508.6	917.4	66	19	b7	15.6			
Complement factor B	P00751	EELLPAQDIK	1154.6	578.3	671.4	65	25	y6	2.3	18.5		
				578.3	372.2	65	29	b3	3.8			
				578.3	259.1	65	39	b2	5.5			
Complement factor H	P08603	SPDVINGSPISQK	1340.7	671.4	830.4	77	35	y8	7.5	2.4	Acute macular degeneration	32
				671.4	943.5	77	35	y9	6.8			
				671.4	399.2	77	37	b4	7.1			
Fibrinogen α chain	P02671	GSESGIFTNTK	1139.6	570.8	610.3	61	26	y5	2.2	1.5	CVD, oral cancer	18, 26
				570.8	867.5	61	28	y8	3.3			
				570.8	780.4	61	25	y7	3.0			
Fibrinogen β chain	P02675	QGFGNVATNTDGK	1307.6	654.8	706.3	85	34	y7	2.4	1.3	CVD, gastrointestinal stromal tumor	18, 31
				654.8	635.3	85	34	y6	2.1			
				654.8	805.4	85	34	y8	2.7			
Fibrinogen γ chain	P02679	DTVQIHDITGK	1225.6	409.6	505.8	49	17	$y9^{2+}$	2.9	8.1	CVD, Alzheimer disease	18, 35
				409.6	189.1	49	22	a2	6.2			
				409.6	217.1	49	19	b2	8.4			
Gelsolin, isoform 1	P06396	TGAQELLR	886.5	444.3	159.1	70	28	b2	4.9	1.2	Progression to breast carcinoma	42
				444.3	530.3	70	25	y4	5.4			
				444.3	658.4	70	25	y5	6.3			
Haptoglobin β chain	P00738	VGYVSGWGR	979.5	490.8	562.3	61	25	y5	1.7	2.6	CVD	18
				490.8	320.2	61	25	b3	1.7			
				490.8	661.3	61	25	y6	1.9			

TABLE I—*continued*

| Protein	UniProt accession no.	Peptide sequence	Peptide molecular mass (Da)	Q1[a]	Q3[b]	DP	CE	Fragment ion	CV[c] (%)	-Fold signal increase[d]	Putative disease association	Refs.
						V	V					
Hemopexin	P02790	NFPSPVDAAFR	1219.6	610.8	480.3	59	25	$y9^{2+}$	0.8	3.1	CVD	18
				610.8	234.1	59	33	a2	1.1			
				610.8	959.5	59	26	y9	1.3			
Heparin cofactor II	P05546	TLEAQLTPR	1027.6	514.8	814.4	65	25	y7	6.0	7.8	CVD	18
				514.8	685.4	65	27	y6	5.8			
				514.8	373.2	65	25	y3	6.9			
Inter-α-trypsin inhibitor HC	P19827	AAISGENAGLVR	1156.6	579.3	902.5	66	28	y9	3.5	10.3		
				579.3	815.4	66	30	y8	4.2			
				579.3	508.3	66	26	$y10^{2+}$	3.1			
Kininogen-1	P01042	TVGSDTFYSFK	1250.6	626.3	173.1	62	40	a2	2.7	33.0	Colorectal cancer	39
				626.3	1051.5	62	26	y9	2.8			
				626.3	201.1	62	29	b2	2.6			
L-selectin	P14151	AEIEYLEK	993.5	497.8	794.4	64	21	y6	12.4	6.0	CVD	18
				497.8	201.1	64	23	b2	10.1			
				497.8	173.1	64	29	a2	19.6			
Plasma retinol-binding protein	P02753	YWGVASFLQK	1197.6	599.8	849.5	76	28	y8	5.8		Lung cancer	37
				599.8	350.2	76	27	b2	3.7			
				599.8	693.4	76	29	y6	3.7	5.5		
Plasminogen	P00747	LFLEPTR	874.5	438.3	615.3	52	21	y5	2.6	5.5	CVD	18
				438.3	233.2	52	25	a2	3.9			
				438.3	261.2	52	22	b2	3.4			
Prothrombin	P00734	ETAASLLQAGYK	1250.7	626.3	679.4	70	29	y6	5.5	3.9	CVD	18
				626.3	879.5	70	29	y8	7.1			
				626.3	792.5	70	29	y7	4.3			
Serum amyloid P-component	P02743	VGEYSLYIGR	1155.6	578.8	708.4	80	27	y6	6.6	3.2	Renal cancer	38
				578.8	508.3	80	29	y4	7.2			
				578.8	871.5	80	27	y7	8.5			
Transferrin	P02787	EDPQTFYYAVAVVK	1628.8	815.4	693.4	75	34	$y12^{2+}$	1.0	68.7	CVD, early ovarian cancer	33
				815.4	912.5	75	33	y8	1.3			
				815.4	1059.6	75	38	y9	2.3			
Transthyretin	P02766	AADDTWEPFASGK	1393.6	697.8	606.3	70	38	y6	1.9	92.9	Ovarian cancer	38
				697.8	921.5	70	32	y8	2.7			
				697.8	735.4	70	31	y7	4.0			
Vitamin D-binding protein	P02774	THLPEVFLSK	1169.6	390.9	494.3	44	17	y4	3.5	12.7	Prostate cancer	29
				390.9	578.3	44	17	b5	3.6			
				390.9	352.2	44	25	b3	4.8			
Vitronectin	P04004	FEDGVLDPDYPR	1421.7	711.8	875.4	75	31	y7	3.2		CVD	18
				711.8	647.3	75	42	y5	3.6	5.8		
				711.8	762.3	75	32	y6	4.2			
Zinc-α2-glycoprotein	P25311	EIPAWVPFDPAAQITK	1781.9	892.0	1087.6	55	40	y10	3.5	48.1	Prostate cancer	28
				892.0	770.9	55	35	$y14^{2+}$	3.7			
				892.0	696.4	55	34	b6	4.4			

[a] Endogenous peptide precursor m/z.
[b] Fragment ion m/z.
[c] Determined as 1 S.D. proportional to the mean.
[d] Intensity of optimized MRM Q1/Q3 pairs *versus* intensity of *in silico* predicted MRM Q1/Q3 ion pairs using the first y fragment ion m/z that is greater than the precursor ion m/z with a CE voltage calculated using $CE = m/z \times 0.05 + 5$.

a EDPQTFYYAVAVVK

b ATEHLSTLSEK

c E D̄ P̱ Q T F̱Ȳ Ȳ A V A V V K

d A T̄ Ē H L S T Ḻ S̱ E K

FIG. 2. **Peptide solutions were analyzed by infusion.** Nanoelectrospray was used for the selection of the most intense precursor and fragment ions for MRM assay development. Q1 scans with a DP voltage ramp were used to determine the dominant charge state of each ion and its optimal DP voltage. XICs of the double and triple charge states are presented for transferrin (*a*) and apolipoprotein A-I (*b*). MRM scanning with a CE voltage ramp was conducted using Q1/Q3 pairs for all possible singly and doubly charged b and y fragment ions and the most intense Q1 *m/z*. XICs for the most intense MRM Q1/Q3 pairs are presented for transferrin (*c*) and apolipoprotein A-I (*d*). The three most intense MRM pairs identified by nanoinfusion analysis are presented in addition to two MRM pairs (*bold*) containing Q3 fragment ions frequently used when generating MRM Q1/Q3 ion pairs *in silico* using MIDAS Workflow Designer (the b2 ion and one y ion are greater than the precursor). The *vertical dashed lines* represent the calculated CE voltage using a generic formula (CE = (precursor *m/z*) × 0.05 + 5) commonly used during MS/MS analysis and creation of MRM Q1/Q3 ion pairs using MIDAS.
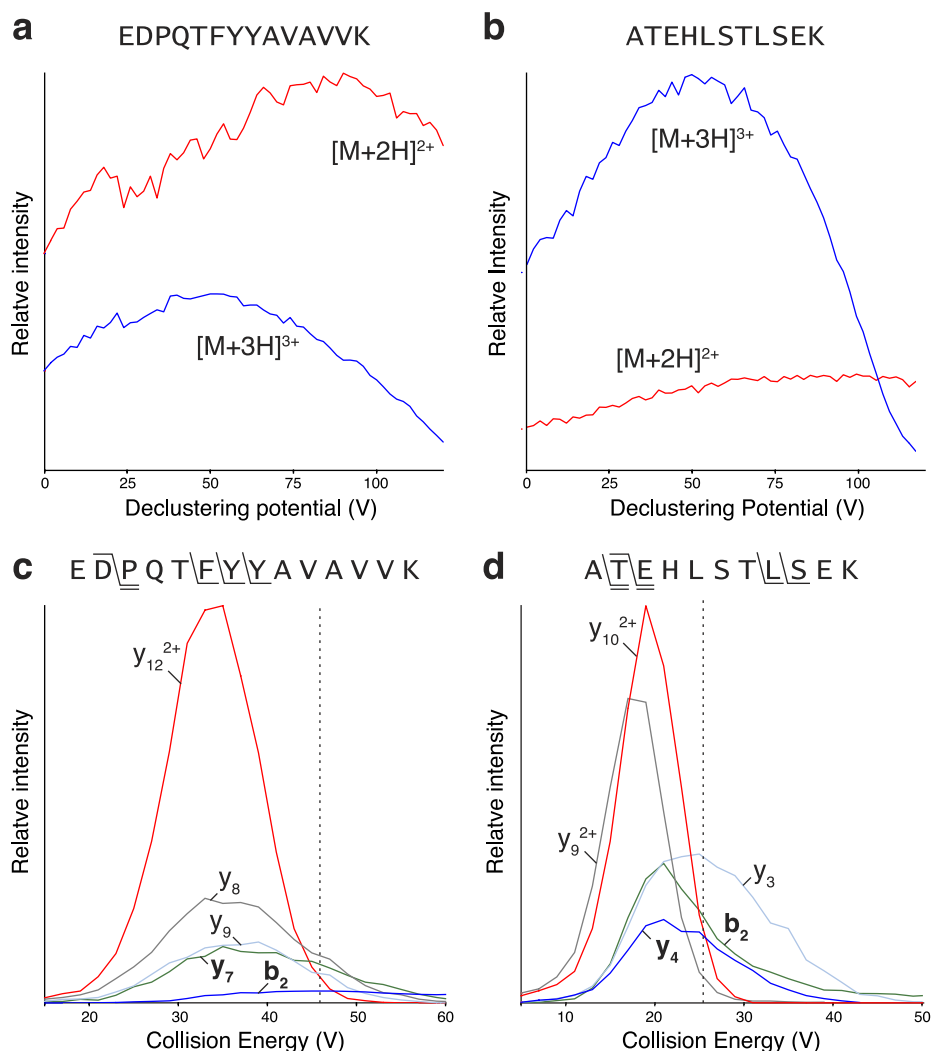
ment ion was predicted by MIDAS for eight of the 45 peptides, the MIDAS-predicted CE and DP values resulted in lower observed signal intensities for these MRM ion pairs. On average, the signal intensity obtained with MRM parameters that were empirically determined by nanoinfusion analysis resulted in an 11.4-fold increase in signal intensity when compared with MRM parameters predicted by MIDAS (Table I).

Although we feel that optimizing the MRM CE voltages yields better results, we analyzed the linear correlation between precursor ion *m/z* and CE voltage for 45 unique peptides. We found that an improved formula for prediction of CE voltages suitable for MRM is defined as CE = 0.043 × (precursor ion *m/z*) + 2.25. This equation fits the data with a linear correlation coefficient (*r*) of 0.79.

Multiplexed LC-MRM/MS Analysis of Trypsin-digested Plasma—Identical MRM parameters can be used for the natural and heavy forms of each peptide taking into account Q1/Q3 mass differences due to stable isotope labels. To select the most sensitive MRM ion pair for each peptide, the three most intense MRM ion pairs determined by nanoinfu-

sion analysis were tested under LC-MRM/MS conditions. LC-MRM/MS analysis of trypsin-digested plasma (1 μg) mixed with all 45 SIS peptides (100 fmol each) confirmed that the relative intensity of the top three MRM ion pairs identified by nanoinfusion was retained under LC-MRM/MS conditions (supplemental results). This analysis also permitted confirmation of the identity of ion signals observed for natural peptides.

Although MRM signals are highly analyte-specific, in a sample as complex as trypsin-digested plasma, multiple peaks generated by non-target ion species can often be observed for an MRM Q1/Q3 ion pair during an LC-MRM/MS analysis. The three most intense MRM ion pairs for each peptide were used to confirm the identity of all 45 natural peptide peaks by co-elution with their SIS peptide standard during an LC-MRM/MS analysis and allowed determination of their corresponding retention times (Fig. 3*a*). The benefit of the increased specificity due to SIS peptide co-elution was dramatically highlighted in the extracted ion chromatograms (XICs) of fibrinogen γ chain and gelsolin (Fig. 3*a*). Nonspecific
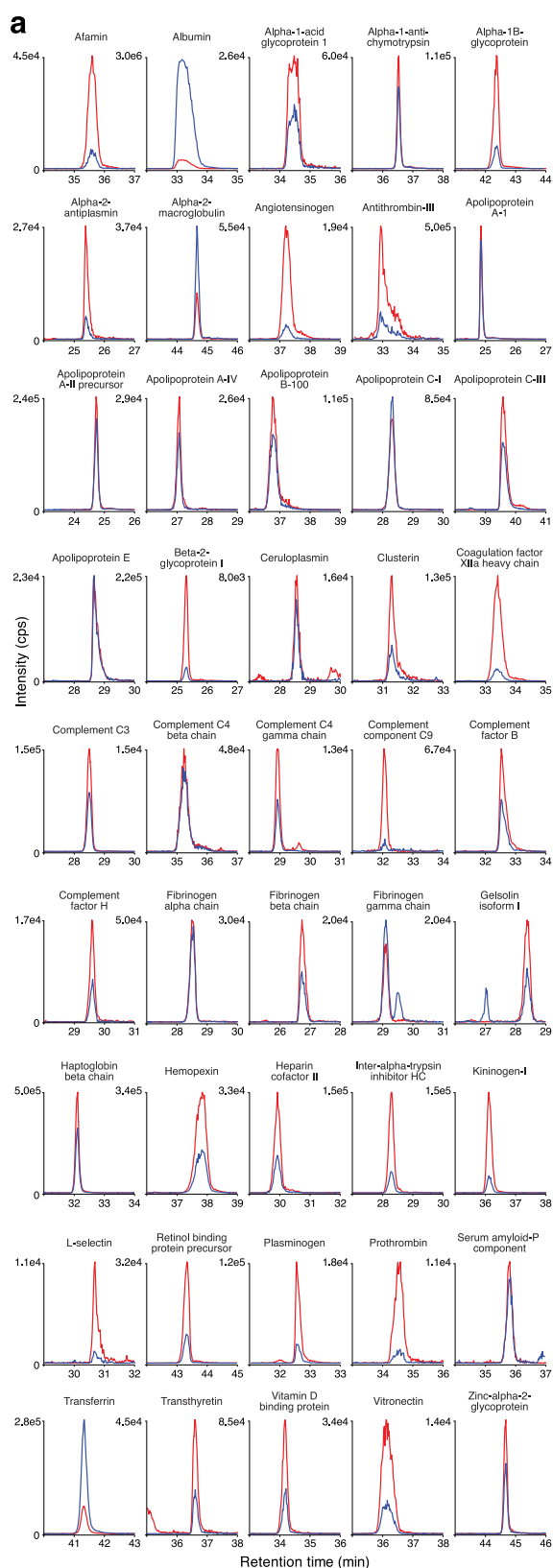
FIG. 3—continued

signals were detected in the XICs of the MRM ion pairs for the natural forms of fibrinogen γ chain and gelsolin, and the two peaks observed for fibrinogen γ chain eluted within 30 s of each other, but only the early peak co-eluted with the authentic fibrinogen γ chain SIS peptide, allowing correct selection of the analyte peak. Analyte retention time certainty permits the use of retention time constraints as a third dimension of analyte specificity for each MRM assay.

Analyte-specific retention times (scheduled MRMs) for MRM ion pairs has only recently been made available to 4000 QTRAP users via a software and MS firmware upgrade. The ability to schedule MRMs based on retention time constraints results in a variable number of MRM Q1/Q3 ion pairs being scanned in each MS cycle, dramatically increases the maximum number of Q1/Q3 pairs that can be monitored per analysis, and also improves the ion statistics collected during the elution profile of a peptide (17).

Specificity of MRM Quantitation—Specificity and analytical precision are key performance characteristics of any assay. With the use of SIS peptides as internal standards, each MRM assay has three analyte-specific criteria: precursor ion *m/z*, fragment ion *m/z*, and retention time. Although we characterized top three MRM ion pairs for each peptide, to maximize the analyte capacity of multiplexed MRM quantitation, we focused on minimizing the number of MRM ion pairs in our analysis method. Careful selection of these criteria approaches absolute specificity of each MRM ion pair for the intended analyte.

Prior to selecting a single representative MRM ion pair for each peptide, the relative response of the three most intense

isotopically labeled peptides. XICs of MRM ion pairs for natural (*blue*) and heavy peptides (*red*) reveal peak areas of heavy peptides within 10-fold of natural peptide levels. *b*, XICs of all 45 MRM protein assays in a single 60-min LC-MRM/MS analysis of 1 μg of plasma tryptic digest spiked with a concentration-balanced mixture of 45 SIS peptide internal standards. MRM ion pair XICs of natural peptides are *blue*, and SIS peptides are *red*. Signal intensity of the natural albumin peptide has been rescaled by a factor of 0.2. *HC*, heavy chain; *cps*, counts/s.

FIG. 3. **Multiplexed MRM quantitation of 45 proteins in a single LC-MRM/MS analysis.** *a*, each MRM assay contains two Q1/Q3 ion pairs to permit discrimination of co-eluting peaks for natural and

MRM Q1/Q3 ion pairs for the natural and SIS peptides as determined by nanoinfusion analysis were compared by LC-MRM/MS analysis of both a plasma digest spiked with all 45 SIS peptides and a mixture of pure SIS peptides alone. This ensured selection of MRM ion pairs that are free of matrix interference from co-eluting ions (supplemental results). To rigorously characterize the linearity and sensitivity of these MRM assays, we chose to maximize the ion statistics collected per peptide by using a single MRM ion pair per peptide. The highest intensity MRM ion pair containing a Q3 y ion fragment free of matrix interference was selected as the ideal ion pair for each peptide (Table I, MRM ion pairs highlighted in bold). The final MRM analysis method was a 60-min analysis containing 90 MRM ion pairs with analyte-specific retention time limits (Fig. 3*b*) (17).

Creation of an SIS Mixture for Multiplexed MRM Analysis of 45 Peptides in Plasma—To achieve reproducible, multiplexed quantitation of 45 peptides in a single LC-MRM/MS analysis, a mixture of all 45 SIS peptides is needed that permits accurate, single step addition of the standard peptides to plasma digests. Quantitation with stable isotope dilution and LC-MRM/MS is linear across a broad 10^3–10^4 concentration range (13), but factors such as sample matrix and SIS peptide isotope impurity often reduce the observed linear dynamic range of individual peptides (15). Because the natural molar concentrations of the 45 plasma proteins to be quantified differ by $>10^4$ (650,000 and 28 pmol/ml for albumin and L-selectin, respectively) (47, 48), an *equimolar* mixture of the SIS peptides would generate high ratios for certain analytes and low ratios for other. This would affect their quantitation accuracy.

Clinical guidelines for creating MS-based assays require that internal standards be added to samples at concentrations close to the mean concentration of the endogenous target analytes or at sufficient levels to permit reproducible measurement (20). Following these criteria, the concentration of each SIS peptide was titrated to determine a concentration that would result in a SIS peptide peak area within a factor of 10 of the endogenous peptide peak area when the SIS peptide was added to a standard plasma trypsin digest. As an example of this concentration balancing, the relative amounts of the highest and lowest abundance peptides, albumin and L-selectin, differed by more than a factor of 100 in the final concentration-balanced SIS peptide mixture. Peak area ratios between 0.1 and 10 (natural:heavy) were obtained for 41 of the 45 peptides when the balanced 45-peptide mixture was added to a trypsin-digested plasma sample and analyzed by LC-MRM/MS (Table II and Fig. 3*a*).

The volume of plasma that should be trypsin-digested and the corresponding amount of SIS peptide mixture required were considered when selecting a final concentration for the SIS peptide mixture. When handling high concentration protein samples like plasma, the error introduced during pipetting is a concern. As a result, 20–50 μl of a 10-fold dilution of

plasma is routinely used for the tryptic digestion, although only 14 nl of neat plasma ($\sim$1 μg of total protein) is required per LC-MRM/MS analysis. The final concentration of the balanced SIS peptide mixture was adjusted so that a 50-μl volume of the mixture was added to each microliter of neat plasma.

The SIS peptide mixture can be added to plasma samples either pre- or post-tryptic digestion. It is normally best to add internal standards as early in a sample preparation work flow as possible to compensate for any variation, like nonspecific degradation, during sample preparation. However, when the SIS peptides were added early in the work flow prior to digestion, we found that the peak area ratios (light:heavy) were elevated when compared with those obtained with post-digestion addition of SIS peptides (data not shown). The degree of the peak area ratio increase varied depending on the peptide and was not predictable. These ratio increases suggest that internal standard peptides are either being degraded or chemically modified over the course of the digestion. Depending on how rapidly peptides are liberated from their parent protein by trypsin, an internal standard peptide may be exposed to degradation effects to which the natural form is not subjected. To avoid this source of variation, in all subsequent experiments the SIS peptide mixture was added to the plasma tryptic digests postdigestion, *i.e.* during digest acidification and prior to desalting and concentration by solid phase extraction, so that any losses incurred during sample handling would still equally affect both natural and heavy peptides. Postdigestion addition of standards also requires less of the SIS peptide mixture because only as much sample as is required for LC-MRM/MS analysis needs to be prepared.

Reproducibility of MRM Quantitation with SIS Peptides—To characterize the effects on the analytical precision of the MRM measurements when the natural peptide peak areas were normalized against SIS peptide peak areas, we conducted replicate analyses of a standard plasma digest sample. This data set was used to evaluate how using peak area *ratios*, instead of analyte peak areas alone, affects measurement reproducibility (Fig. 4). Two aliquots from a plasma tryptic digest were spiked with either an equimolar mixture or a *concentration-balanced* mixture of all 45 SIS peptides. Each spiked plasma sample was analyzed by LC-MRM/MS 12 times with blank solvent injections between sample injections. Measurement of analyte peak areas over the course of all 24 LC-MRM/MS analyses resulted in $>$10% variation for all 45 analytes with peak area CVs of $>$20% for 39 of the 45 peptides (Fig. 4*a*).

Normalizing the natural peptide peak areas against the peak areas of co-eluting SIS peptides should improve the analytical precision by accounting for subtle interanalysis variations in ionization efficiency (20). By normalizing the natural peptide peak areas to the peak areas of the co-eluting reference SIS peptides, peak area differences between analyses can be effectively removed. Analytical precision

TABLE II

MRM assay linearity, sensitivity, and technical reproducibility

L, light; H, heavy; HC, heavy chain.

Protein[a]	Fragment ion[b]	Average peak area ratio (L:H)	Linearity[c]			LOQ[d]	Assay reproducibility	
			Analyte response (slope)	Correlation coefficient (r)	Linear concentration range		Plasma concentration	CV[e]
						amol	*μM*	*%*
Afamin	y7	0.156	1.035	0.999	6,250	16	0.629	7.3
Albumin, serum	y8	13.4	1.067	0.999	31,250	4,388	1101	6.1
α_1-Acid glycoprotein 1	y13^{2+}	0.474	1.059	0.998	1.250	625	4.44	18.8
α_1-Antichymotrypsin	y7	0.676	1.085	0.998	1,250	179	5.27	6.5
α_{1B}-Glycoprotein	y7	0.172	1.603	0.999	250	2,718	3.13	15.2
α_2-Antiplasmin	y8	0.263	0.997	0.999	31,250	59	2.20	8.1
α_2-Macroglobulin	y12^{2+}	2.858	1.133	0.993	10	168,014	272	15.0
Angiotensinogen	y10^{2+}	0.090	1.034	0.997	1,250	207	1.68	7.4
Antithrombin-III	y9^{2+}	0.164	0.961	0.996	250	3,012	3.77	13.9
Apolipoprotein A-I	y9^{2+}	0.826	0.980	1.000	1,250	1,773	68.8	7.3
Apolipoprotein A-II precursor	y8^{2+}	0.714	0.990	1.000	1,250	6,263	56.8	9.5
Apolipoprotein A-IV	y9^{2+}	0.627	0.998	0.998	1,250	62	2.68	6.0
Apolipoprotein B-100	y8^{2+}	0.752	1.123	0.998	250	2,087	3.14	5.7
Apolipoprotein C-I lipoprotein	y9^{2+}	0.90	1.033	0.999	6,250	219	8.1	6.1
Apolipoprotein C-III	y8	0.562	1.065	0.991	50	1,704	10.6	16.2
Apolipoprotein E	y7^{2+}	1.078	0.961	0.999	6,250	15	0.865	7.5
β_2-Glycoprotein I	y6	0.125	1.004	1.000	6,250	106	5.16	6.9
Ceruloplasmin	y5	0.759	1.079	0.989	250	2,086	3.08	14.2
Clusterin	y5	0.353	1.093	0.993	250	1,856	2.62	14.3
Coagulation factor XIIa HC	y7	0.120	1.081	0.998	250	293	0.415	5.2
Complement C3	y6	0.524	1.030	0.999	31,250	562	22.6	6.9
Complement C4 β chain	y5	0.658	1.037	0.995	250	368	2.41	13.3
Complement C4 γ chain	y8^{2+}	0.379	1.008	0.998	250	1,544	3.33	8.3
Complement component C9	y4	0.067	0.842	0.971	50	211	0.259	19.7
Complement factor B	y6	0.552	1.077	0.998	1,250	1,064	2.21	7.0
Complement factor H	y8	0.261	1.050	0.995	1,250	224	0.95	11.0
Fibrinogen α chain	y5	0.998	1.017	1.000	31,250	82	21.0	4.8
Fibrinogen β chain	y7	0.700	1.006	0.999	6,250	293	15.1	4.6
Fibrinogen γ chain	y9^{2+}	1.36	1.124	0.994	250	678	1.42	15.1
Gelsolin, isoform 1	y4	0.369	0.906	0.998	50	4,146	1.63	7.9
Haptoglobin β chain	y5	0.561	1.073	0.999	31,250	104	21.1	5.1
Hemopexin	y9^{2+}	0.475	1.074	1.000	1,250	2,706	18.9	10.3
Heparin cofactor II	y7	0.489	1.024	0.997	6,250	203	1.89	8.5
Inter-α-trypsin inhibitor HC	y9	0.244	1.019	0.999	6,250	17	0.979	8.0
Kininogen-1	y9	0.171	1.046	0.999	1,250	516	3.94	9.4
L-selectin	y6	0.094	1.046	0.984	50	33	0.0760	17.8
Plasma retinol-binding protein	y6	0.299	0.982	0.999	125	388	2.67	24.6
Plasminogen	y5	0.257	1.065	0.997	1,250	232	1.88	10.5
Prothrombin	y8	0.166	1.120	0.999	250	771	1.482	7.6
Serum amyloid P-component	y6	0.490	0.993	0.998	25	1,274	1.89	23.0
Transferrin	y12^{2+}	3.29	0.978	0.998	125	25,919	235	13.0
Transthyretin	y6	0.279	1.115	0.999	250	10,222	11.9	8.5
Vitamin D-binding protein	y4	0.319	0.951	0.997	50	1,390	0.84	59.6
Vitronectin	y5	0.231	1.037	0.999	1,250	568	4.12	13.3
Zinc-α_2-glycoprotein	y14^{2+}	0.313	0.968	0.996	125	288	3.16	11.6

[a] Concentrations of bold proteins were within previously reported clinical concentrations, whereas concentrations of italicized proteins were within 2-fold of literature values.

[b] MRM ion pair Q3 fragment ion used to characterize assay linearity and calculate protein concentration.

[c] Linear regression with 1/*x* weighting.

[d] Limit of quantitation defined as the number of attomoles of peptide on column per analysis.

[e] Determined as 1 S.D. proportional to the mean.

was dramatically improved when peak area ratios were used to compare data between replicate LC-MRM/MS analyses (Fig. 4, *b* and *c*).

An unanticipated benefit of balancing the SIS peptide concentrations, which had been primarily performed to maximize the linear range of the assays, was a further reduction of analytical variation between analyses (Fig. 4*c*). When a *concentration-balanced* mixture of SIS peptides was spiked into plasma tryptic digests, MRM assays for 29 of the peptides had CVs of <4%, whereas only one MRM assay (for complement compo-
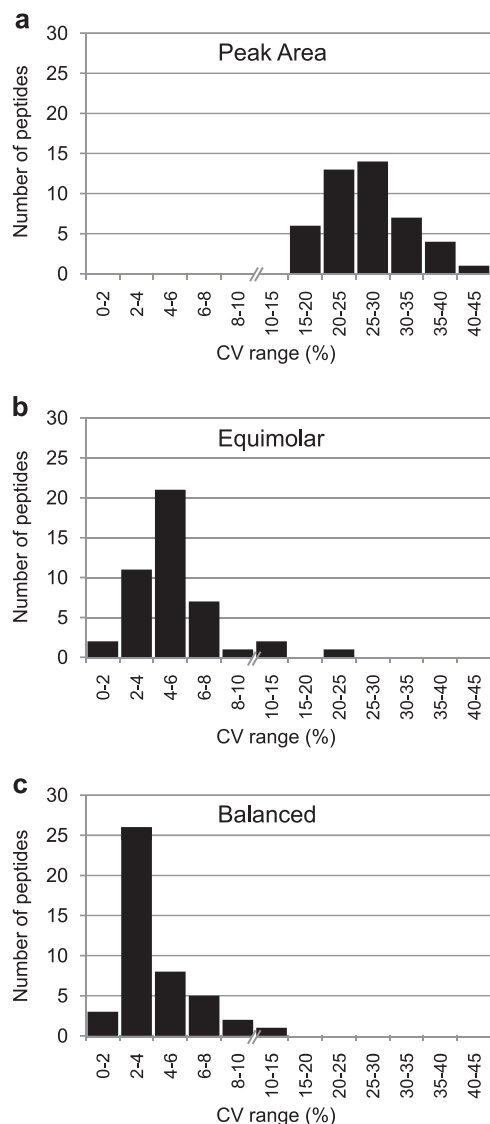
Fig. 4. **Analytical reproducibility of MRM-based quantitation.** CV frequencies of 45 peptide assays using raw peak areas (*a*), peak area ratios normalized to an equimolar SIS peptide mixture (*b*), and peak area ratios normalized to a concentration-balanced SIS peptide mixture (*c*) are shown.

nent C9) had a CV >10%. When *equimolar* amounts of each SIS peptide were added to the same plasma digest, only 13 MRM assays had CVs of <4%, whereas three assays had CVs of >10% with a maximum variation of 20.2% observed for vitamin D-binding protein. We attribute the additional reduction in percent CV that resulted from the use of concentration-balanced SIS peptides to the increased signal intensity and the improvement in signal quality for 18 of the SIS peptides whose concentrations had been increased to match the areas from the natural peptides. All subsequent MRM quantitation was conducted using the concentration-balanced 45-SIS peptide mixture.

To compare the analytical precision obtained with each of the top three most intense MRM ion pairs for each peptide, a plasma tryptic digest spiked with the *concentration-balanced* mixture of SIS peptides was analyzed 18 times by LC-MRM/MS. Although the lowest percent CVs did not always correlate with the highest intensity fragment ion, the reproducibility of the three MRM ion pairs did not vary greatly for each MRM peptide (Table I). For 41 of the MRM peptide assays, the difference in percent CV observed between MRM ion pairs was <5%. Increased variation correlated with decreasing fragment ion intensity. The largest range of variation between MRM ion pairs was <10% and correlated with low intensity fragment ions for lower concentration analytes (Table I).

Linear Response of MRM Quantitation—To ensure that analyte concentrations routinely encountered when quantifying unknown samples are measured within the linear response range for that analyte, the assays must be validated over a wider concentration range (49). Assay attributes such as linearity of response, limit of quantitation (LOQ), and interassay variation also need to be characterized to validate an assay (49). These parameters allow comparison with established assay technologies and are of particular interest when considering the potential translation of MRM-based protein assays into clinically relevant applications.

The standard method for characterizing assay linearity and LOQ would be to add standard proteins to a constant amount of blank sample matrix and internal standards. However, when assaying endogenous plasma proteins, preparation of a blank sample matrix devoid of all 45 endogenous proteins is not feasible. Similar difficulties were encountered when developing an MRM-based assay for alcohol dehydrogenase, which is endogenously expressed in liver (50). In such cases, a dilution series of pure internal standards is sometimes used to characterize assay linearity, but this likely over-represents the true linear range of the assay due to the absence of interfering components from the sample matrix (9, 51). We therefore used an alternative method, similar to that of Janecki *et al.* (50), to determine linear range. We diluted a tryptic digest of our standard plasma sample to generate a range of endogenous analyte concentrations spanning a 31,250-fold concentration range. These various analyte concentrations were combined with a constant amount of the concentration-balanced 45-SIS peptide mixture. Five replicate LC-MRM/MS analyses of each sample dilution were performed in the order from most dilute to most concentrated with two blank solvent injections between the different sample concentrations. To optimize ion statistics and sensitivity, a single MRM ion pair was used for each peptide (fragment ion noted in Table II).

Linear regression analysis was performed on the observed peak area ratios (natural:heavy) *versus* concentration ratios to generate calibration curves for each peptide (Fig. 5 and supplemental results). To calculate concentration ratios, we first needed to estimate the concentrations of all 45 natural peptides in our standard plasma sample because their actual concentrations are unknown. To accomplish this, a single concentration point was measured by LC-MRM/MS five
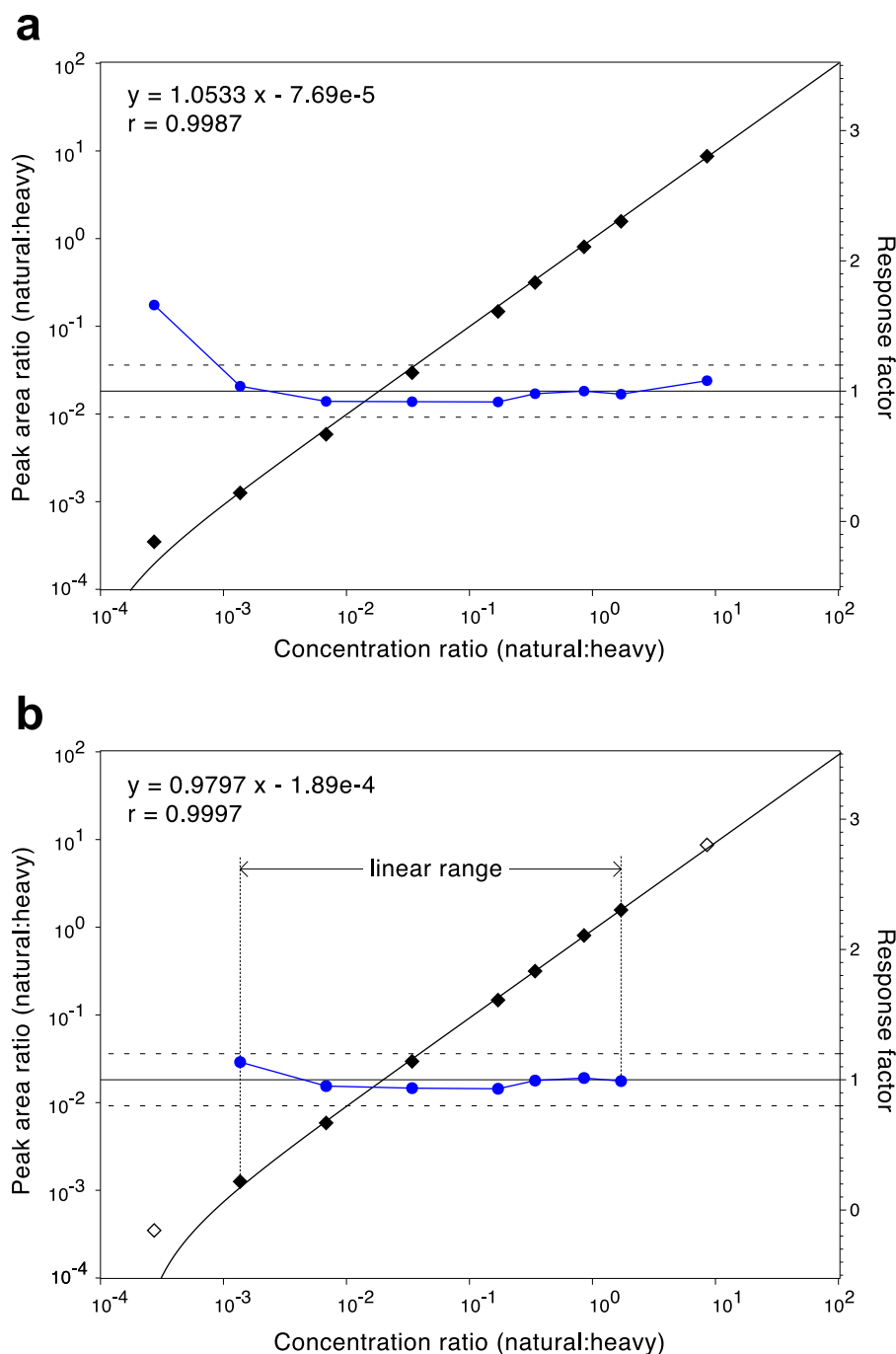
a

$y = 1.0533\ x - 7.69e\text{-}5$
$r = 0.9987$

b

$y = 0.9797\ x - 1.89e\text{-}4$
$r = 0.9997$

linear range

FIG. 5. **Calibration curves and response factor plot for apolipoprotein A-I.** *a*, linear regression analysis ($1/x$ weighted) and response factor plot (*blue line*) for apoA-I using all analyte concentrations. *b*, final linear regression and response factor plot for apoA-I illustrating the linear dynamic range of the assay when analyte concentrations that respond non-linearly are excluded (◇).

times, and the concentration of each natural peptide was calculated from the average ratio compared with the area of its SIS peptide, which is at a known concentration (Table II). Then as we create a dilution series for the standard curves, we can measure how the observed peak area ratios change in response to changes in the peptide concentrations.

To avoid over-representing the degree of linearity in our calibration curves, we combined linear regression analysis with response factor plots to identify analyte concentrations that did not respond in a linear manner (49). Ideally response

factor plots should have a near zero slope. Peak area ratios were sequentially excluded from the linear regression analysis until individual response factors for all data points included in the regression analysis fell within 80–120% of the average analyte response (slope) (Fig. 5*b*) (49). This approach ensures that the best linear fit is achieved, thereby improving quantitation accuracy and accurately representing the linear concentration range of each peptide assay.

Using this approach to define the assay linear range, 24 of the MRM peptide assays yielded linear responses over a

>1000-fold concentration range (Table II). Using response factor plots to define the assay linear range also resulted in strong linear correlations ($r > 0.99$) for 42 of the 45 assays with average response factors (slopes) of 0.9–1.1 for 38 of the 45 assays (Table II). Biologically concentrations of these 45 plasma proteins typically vary by only a factor of 2–10 (48). Therefore, routine quantitation of these 45 proteins in unknown samples is performed using calibration curves that represent a narrower 30-fold concentration range. Linear concentration ranges over concentration ranges of >30 were obtained for 43 of the 45 peptide assays. By validating the linearity of these assays over a much wider concentration range, we can ensure that any biological concentrations encountered will fall well within the linear portion of the calibration curve.

Limit of Quantitation by MRM—The LOQ for an assay is an important measure of performance as it defines the lowest analyte quantity that can be accurately measured, and it is the true measure of the sensitivity of an assay. A signal-to-noise ratio of >10 is frequently used to define LOQ (15, 51). However, because MRM is an MS/MS scan mode, background noise is extremely low, and there is no practical way to accurately measure co-eluting noise in an MRM Q1/Q3 ion pair channel of interest. Therefore, it is advantageous to empirically determine the LOQ of an assay, defined as the lowest analyte concentration that can be measured with <20% CV (49). Five replicate LC-MRM/MS analyses of each analyte concentration in the dilution series used to define the assay linear range were used to define LOQs for each assay.

When considered together, assay linearity, linear range, and LOQ can help to identify problematic proteotypic peptides. One such problematic peptide is LLIYAVLPTGDVIGDSAK for α_2-macroglobulin. Although α_2-macroglobulin was measured with excellent analytical reproducibility (2.5% CV), the current assay had a poor linear dynamic range and an LOQ only 5-fold lower than the concentration normally encountered during routine quantitation of unknowns (Table II). No interference from co-eluting ion species was detected when the relative response of multiple MRM ion pairs was compared for analysis of this peptide in solvent alone *versus* in a sample matrix (supplemental results), and this peptide was well resolved as a symmetrical peak using our chromatographic conditions (Fig. 3*a*). These results suggest that an alternative proteotypic peptide should be selected for further development of an α_2-macroglobulin MRM assay.

Emphasizing the sensitivity of these MRM assays, LOQs for 27 of the peptides were found to be in the attomole range per analysis for a peptide on column (Table II). To place the LOQ of each peptide in context, it is important to take into account how the LOQ for an analyte relates to the natural biological concentration of the protein. Even a 4.3-fmol LOQ for albumin is still >2000 times lower than the natural mean concentration of albumin, a concentration difference far greater than what would be naturally encountered (48).

Reproducibility and Accuracy of MRM Quantitation—When characterizing assay reproducibility, it is important to determine the amount of technical variation introduced during sample preparation to estimate how well an assay can detect changes in analyte concentration. Analysis of replicate preparations of a standard sample can be used to assess the interassay reproducibility, or ruggedness, of an assay. An analytical assay can be further "stress-tested" by measuring the variation observed when these standard samples are prepared over the span of multiple days (49). To measure the interassay reproducibility of these 45 MRM peptide assays, three analytical runs containing 11 separate tryptic digests of a standard plasma sample were performed on different days. All samples were analyzed using the top three MRM ion pairs for each peptide (Table I) to determine whether reproducible concentration determination is achievable with a single MRM ion pair. Analyte response was measured for each day using one of the 11 plasma digests to create a six-point calibration curve that spanned a 30-fold range of concentration ratios. To create these calibration curves, the concentration of the SIS peptides was held constant while the amount of total digest was varied over a 30-fold concentration range. The highest and lowest concentration ratios used for these calibration curves contained 3-fold higher and 10-fold lower concentrations of natural peptide than used in unknown samples. Concentrations of the natural peptides in the standard sample were estimated using the calibration curve from the first analytical run as described above.

Analyte protein concentrations were calculated as the average of 30 measurements across all three analytical runs. Each analytical run consisted of a six-point calibration curve and 10 manually prepared plasma trypsin digests, and each run was performed and analyzed on a different day. When measured across three separate analytical runs, concentrations of 25 of the proteins had CVs of <10%, whereas 42 of the assays exhibited clinically useful CVs of <20% (Table II). These results demonstrate the highly reproducible nature of MRM protein quantitation and highlight its potential for clinical applications. Interassay variation should be further reduced by automation of sample preparation. Protein concentrations, determined from MRM assays based on a single peptide and multiple MRM Q1/Q3 ion pairs for each protein, were found to agree with previously reported clinical ranges for 23 of the proteins (Table II and supplemental results) (47, 48). An additional 16 measured protein concentrations were within a factor of 2 of literature values. Excellent agreement of concentrations calculated using different MRM ion pairs was obtained even when low mass a2 and b2 fragment ions, which should statistically be more susceptible to nonspecific interferences, were used. MRM ion pairs exhibiting interference were readily identified by changes in both their signal intensity relative to the other two MRM ion pairs and either an increase or a decrease in the calculated protein concentration (supplemental results). All protein concentrations were calculated

with less than 30% technical variation except for vitamin D-binding protein (Table II). Closer examination of the vitamin D-binding protein results revealed that the signal obtained from the MRM ion pair used for quantitation (390.9/493.3) was attenuated in approximately half of the samples. The vitamin D-binding protein MRM ion pairs containing b3 and b5 ions did not exhibit this behavior and gave higher calculated concentrations that were just below clinically reported concentrations and only differed from each other by only 6.4% with CVs of 5.7 and 5.6%, respectively (supplemental results).

Fibrinogen α, β, and γ should be present at equimolar concentrations in plasma (48). Our MRM assays for fibrinogen α and β resulted in concentrations that fall within clinically reported ranges, whereas the concentration measured for fibrinogen γ was >10-fold lower than expected (Table II). This could be explained by incomplete tryptic digestion of fibrinogen γ. We are currently using our mixture of SIS peptides to characterize the kinetics of trypsin digestion for each of these 45 proteins. Preliminary data indicate that the levels of the fibrinogen α and β proteotypic peptides that we are measuring plateau within 2 h during trypsin digestion, whereas the fibrinogen γ peptide increases linearly in concentration over 22 h (data not shown). These results indicate that this particular fibrinogen γ peptide is located within a region of the protein that is resistant to tryptic digestion and that an alternate proteotypic peptide should be selected for development of a fibrinogen γ MRM assay. Such studies will assist in improving the overall accuracy of multiplexed protein quantitation achievable through the use of this balanced mixture of SIS peptides. Furthermore the speed of LC-MRM/MS analysis, combined with the application of this balanced mixture of SIS peptides, should be extremely valuable for revealing previously undetectable patterns of protein expression characteristic of specific diseases.

Conclusion—We have described the creation of an optimized mixture of 45 SIS peptides that we used to develop rapid, specific, and sensitive MRM assays for a panel of 45 human plasma proteins. Requiring minimal sample preparation, these MRM protein assays were reproducible and linear across a broad range of concentrations. This panel of isotopically labeled peptides should be easily expandable to include additional proteins. This would make this assay very useful for the discovery of biomarkers through expression profiling. A recent review of plasma protein concentrations by Hortin *et al.* (48) lists an additional 100 plasma proteins whose mean molar concentrations exceed that of L-selectin, which is the lowest concentration analyte of our 45-protein panel (14). These additional 100 plasma proteins should be directly detectable in tryptic plasma digests by LC-MRM/MS analysis and represent logical candidates for addition to our SIS peptide mixture. Ultimately this initial panel of characterized MRM assays for 45 human plasma proteins could be expanded into an assay capable of rapid, multiplexed profiling of more than 100 plasma proteins.

Accurate determination of analyte concentration is an essential characteristic of any assay for clinical use. Plasma protein concentrations from a single donor sample measured by MRM quantitation using a single tryptic peptide per protein resulted in 23 measured protein concentrations that were within reported clinical ranges (Table II) (48). Concentrations of an additional 16 proteins were within a factor of 2 of reported concentration ranges. Incomplete tryptic digestion of parent proteins can result in lower measured protein concentrations, whereas interference from co-eluting ion species can result in elevated measurements of protein concentration. Using our new MRM-based assay, accurate concentrations were rapidly obtained for 23 proteins using a single peptide plus an isotopically labeled internal standard peptide. However, to increase the accuracy of the concentration measurements of the remaining 22 proteins and to improve resistance of these assays to interferences, development of assays using additional proteotypic peptides for these proteins will likely be necessary.

When adapting these 45 MRM assays to third party laboratories, it would be prudent to verify that each peptide MRM assay is free of interferences by initially comparing the relative signal intensities of multiple MRM ion pairs for each peptide in addition to redetermination of both the analytical and technical reproducibility of each assay. Implementation of MRM-based protein assays for clinical applications and additional assay characteristics, such as interlaboratory accuracy and the effect of changing chromatographic conditions and MS instrumentation on reproducibility, will require validation and verification. Triple quadrupole LC-MS/MS instrumentation is already well established in hospital laboratories and is presently used to quantify several metabolites in clinical blood, serum, and urine samples (20). In addition to continued assay verification and validation, automated sample preparation work flows are needed to adapt these MRM-based protein assays to the clinical environment. An additional benefit of developing these MRM assays using trypsin-digested whole plasma samples is that sample preparation is relatively simple and highly amenable to automation using liquid-handling robotics.

Previous work by Kronkvist *et al.* (52) has shown that use of liquid-handling robotics to handle plasma samples and dispense internal standards can reduce relative standard deviations to as low as 0.2%, which could further reduce interassay variation for this panel of 45 MRM protein assays. Recent advances in ultrahigh pressure liquid chromatography could reduce analysis times by as much as 3-fold when combined with MRM analysis (53). MRM-based assays using isotopically labeled internal standard peptides permit absolute specificity with three analyte-specific criteria (retention time, precursor, and fragment ion *m/z*) that is not attainable with assays that depend on indirect colorimetric, fluorescent, or radioactivity readouts. In addition to multiplexed protein

quantitation, MRM-based assays are also capable of quantifying non-synonymous small nucleotide polymorphisms, post-translational modifications, and alternate protein splice variants.

REFERENCES

1. Ackermann, B. L., and Berna, M. J. (2007) Coupling immunoaffinity techniques with MS for quantitative analysis of low-abundance protein biomarkers. *Expert Rev. Proteomics* **4,** 175–186
2. Ong, S. E., and Mann, M. (2005) Mass spectrometry-based proteomics turns quantitative. *Nat. Chem. Biol.* **1,** 252–262
3. Aggarwal, K., Choe, L. H., and Lee, K. H. (2005) Quantitative analysis of protein expression using amine-specific isobaric tags in Escherichia coli cells expressing rhsA elements. *Proteomics* **5,** 2297–2308
4. Aebersold, R., and Mann, M. (2003) Mass spectrometry-based proteomics. *Nature* **422,** 198–207
5. Ong, S. E., Foster, L. J., and Mann, M. (2003) Mass spectrometric-based approaches in quantitative proteomics. *Methods* **29,** 124–130
6. Wei, J., Sun, J., Yu, W., Jones, A., Oeller, P., Keller, M., Woodnutt, G., and Short, J. M. (2005) Global proteome discovery using an online three-dimensional LC-MS/MS. *J. Proteome Res.* **4,** 801–808
7. Kondrat, R. W., McClusky, G. A., and Cooks, R. G. (1978) Multiple reaction monitoring in mass spectrometry/mass spectrometry for direct analysis of complex mixtures. *Anal. Chem.* **50,** 2017–2021
8. Gergov, M., Ojanperä, I., and Vuori, E. (2003) Simultaneous screening for 238 drugs in blood by liquid chromatography-ion spray tandem mass spectrometry with multiple-reaction monitoring. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **795,** 41–53
9. Kamiie, J., Ohtsuki, S., Iwase, R., Ohmine, K., Katsukura, Y., Yanai, K., Sekine, Y., Uchida, Y., Ito, S., and Terasaki, T. (2008) Quantitative atlas of membrane transporter proteins: development and application of a highly sensitive simultaneous LC/MS/MS method combined with novel in-silico peptide selection criteria. *Pharm. Res.* **25,** 1469–1483
10. Barnidge, D. R., Goodmanson, M. K., Klee, G. G., and Muddiman, D. C. (2004) Absolute quantification of the model biomarker prostate-specific antigen in serum by LC-Ms/MS using protein cleavage and isotope dilution mass spectrometry. *J. Proteome Res.* **3,** 644–652
11. Kuhn, E., Wu, J., Karl, J., Liao, H., Zolg, W., and Guild, B. (2004) Quantification of C-reactive protein in the serum of patients with rheumatoid arthritis using multiple reaction monitoring mass spectrometry and 13C-labeled peptide standards. *Proteomics* **4,** 1175–1186
12. Zhang, F., Bartels, M. J., and Stott, W. T. (2004) Quantitation of human glutathione S-transferases in complex matrices by liquid chromatography/tandem mass spectrometry with signature peptides. *Rapid Commun. Mass Spectrom.* **18,** 491–498
13. Kirkpatrick, D. S., Gerber, S. A., and Gygi, S. P. (2005) The absolute quantification strategy: a general procedure for the quantification of proteins and post-translational modifications. *Methods* **35,** 265–273
14. Anderson, L., and Hunter, C. L. (2006) Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins. *Mol. Cell. Proteomics* **5,** 573–588
15. Keshishian, H., Addona, T., Burgess, M., Kuhn, E., and Carr, S. A. (2007) Quantitative, multiplexed assays for low abundance proteins in plasma by targeted mass spectrometry and stable isotope dilution. *Mol. Cell. Proteomics* **6,** 2212–2229
16. Anderson, N. L., Anderson, N. G., Haines, L. R., Hardie, D. B., Olafson, R. W., and Pearson, T. W. (2004) Mass spectrometric quantitation of peptides and proteins using stable isotope standards and capture by anti-peptide antibodies (SISCAPA). *J. Proteome Res.* **3,** 235–244
17. Stahl-Zeng, J., Lange, V., Ossola, R., Eckhardt, K., Krek, W., Aebersold, R., and Domon, B. (2007) High sensitivity detection of plasma proteins by multiple reaction monitoring of N-glycosites. *Mol. Cell. Proteomics* **6,** 1809–1817
18. Anderson, L. (2005) Candidate-based proteomics in the search for biomarkers of cardiovascular disease. *J. Physiol.* **563,** 23–60
19. Kingsmore, S. F. (2006) Multiplexed protein measurement: technologies and applications of protein and antibody arrays. *Nat. Rev. Drug Discov.* **5,** 310–320
20. Chace, D. H., Barr, J. R., Duncan, M. W., Matern, D., Morris, M. R., Palmer-Toy, D. E., Rockwood, A. L., Siuzdak, G., Urbani, A., Yergey, A. L., and Chan, Y. M. (2006) *Mass Spectrometry in the Clinical Laboratory: General Principles and Guidance; Approved Guideline*, Clinical and Laboratory Standards Institute, Wayne, PA
21. Craig, R., Cortens, J. P., and Beavis, R. C. (2004) Open source system for analyzing, validating, and storing protein identification data. *J. Proteome Res.* **3,** 1234–1242
22. Deutsch, E. W., Lam, H., and Aebersold, R. (2008) PeptideAtlas: a resource for target selection for emerging targeted proteomics workflows. *EMBO Rep.* **9,** 429–434
23. Craig, R., Cortens, J. P., and Beavis, R. C. (2005) The use of proteotypic peptide libraries for protein identification. *Rapid Commun. Mass Spectrom.* **19,** 1844–1850
24. Mallick, P., Schirle, M., Chen, S. S., Flory, M. R., Lee, H., Martin, D., Ranish, J., Raught, B., Schmitt, R., Werner, T., Kuster, B., and Aebersold, R. (2007) Computational prediction of proteotypic peptides for quantitative proteomics. *Nat. Biotechnol.* **25,** 125–131
25. Sanders, W. S., Bridges, S. M., McCarthy, F. M., Nanduri, B., and Burgess, S. C. (2007) Prediction of peptides observable by mass spectrometry applied at the experimental set level. *BMC Bioinformatics* **8,** Suppl. 7, S23
26. Cheng, A. J., Chen, L. C., Chien, K. Y., Chen, Y. J., Chang, J. T., Wang, H. M., Liao, C. T., and Chen, I. H. (2005) Oral cancer plasma tumor marker identified with bead-based affinity-fractionated proteomic technology. *Clin. Chem.* **51,** 2236–2244
27. DeKosky, S. T., Ikonomovic, M. D., Wang, X., Farlow, M., Wisniewski, S., Lopez, O. L., Becker, J. T., Saxton, J., Klunk, W. E., Sweet, R., Kaufer, D. I., and Kamboh, M. I. (2003) Plasma and cerebrospinal fluid alpha1-antichymotrypsin levels in Alzheimer's disease: correlation with cognitive impairment. *Ann. Neurol.* **53,** 81–90
28. Hale, L. P., Price, D. T., Sanchez, L. M., Demark-Wahnefried, W., and Madden, J. F. (2001) Zinc alpha-2-glycoprotein is expressed by malignant prostatic epithelium and may serve as a potential serum marker for prostate cancer. *Clin. Cancer Res.* **7,** 846–853
29. Hlavaty, J. J., Partin, A. W., Shue, M. J., Mangold, L. A., Derby, J., Javier, T., Kelley, S., Stieg, A., Briggman, J. V., Hass, G. M., and Wu, Y. J. (2003) Identification and preliminary clinical evaluation of a 50.8-kDa serum marker for prostate cancer. *Urology* **61,** 1261–1265
30. Jackson, D., Craven, R. A., Hutson, R. C., Graze, I., Lueth, P., Tonge, R. P., Hartley, J. L., Nickson, J. A., Rayner, S. J., Johnston, C., Dieplinger, B., Hubalek, M., Wilkinson, N., Perren, T. J., Kehoe, S., Hall, G. D., Daxenbichler, G., Dieplinger, H., Selby, P. J., and Banks, R. E. (2007) Proteomic profiling identifies afamin as a potential biomarker for ovarian cancer. *Clin. Cancer Res.* **13,** 7370–7379
31. Kang, H. J., Koh, K. H., Yang, E., You, K. T., Kim, H. J., Paik, Y. K., and Kim, H. (2006) Differentially expressed proteins in gastrointestinal stromal tumors with KIT and PDGFRA mutations. *Proteomics* **6,** 1151–1157
32. Klein, R. J., Zeiss, C., Chew, E. Y., Tsai, J. Y., Sackler, R. S., Haynes, C., Henning, A. K., SanGiovanni, J. P., Mane, S. M., Mayne, S. T., Bracken, M. B., Ferris, F. L., Ott, J., Barnstable, C., and Hoh, J. (2005) Comple-

ment factor H polymorphism in age-related macular degeneration. *Science* **308,** 385–389

33. Kozak, K. R., Su, F., Whitelegge, J. P., Faull, K., Reddy, S., and Farias-Eisner, R. (2005) Characterization of serum biomarkers for detection of early stage ovarian cancer. *Proteomics* **5,** 4589–4596

34. Kreunin, P., Zhao, J., Rosser, C., Urquidi, V., Lubman, D. M., and Goodison, S. (2007) Bladder cancer associated glycoprotein signatures revealed by urinary proteomic profiling. *J. Proteome Res.* **6,** 2631–2639

35. Lee, J. W., Namkoong, H., Kim, H. K., Kim, S., Hwang, D. W., Na, H. R., Ha, S. A., Kim, J. R., and Kim, J. W. (2007) Fibrinogen gamma-A chain precursor in CSF: a candidate biomarker for Alzheimer's disease. *BMC Neurol.* **7,** 14

36. Malik, G., Ward, M. D., Gupta, S. K., Trosset, M. W., Grizzle, W. E., Adam, B. L., Diaz, J. I., and Semmes, O. J. (2005) Serum levels of an isoform of apolipoprotein A-II as a potential marker for prostate cancer. *Clin. Cancer Res.* **11,** 1073–1085

37. Patz, E. F., Jr., Campa, M. J., Gottlin, E. B., Kusmartseva, I., Guan, X. R., and Herndon, J. E., 2nd (2007) Panel of serum biomarkers for the diagnosis of lung cancer. *J. Clin. Oncol.* **25,** 5578–5583

38. Polanski, M., and Anderson, N. L. (2006) A list of candidate cancer biomarkers for targeted proteomics. *Biomark. Insights* **1,** 1–48

39. Qiu, Y., Patwa, T. H., Xu, L., Shedden, K., Misek, D. E., Tuck, M., Jin, G., Ruffin, M. T., Turgeon, D. K., Synal, S., Bresalier, R., Marcon, N., Brenner, D. E., and Lubman, D. M. (2008) Plasma glycoprotein profiling for colorectal cancer biomarker identification by lectin glycoarray and lectin blot. *J. Proteome Res.* **7,** 1693–1703

40. Talmud, P. J., Hawe, E., Miller, G. J., and Humphries, S. E. (2002) Nonfasting apolipoprotein B and triglyceride levels as a useful predictor of coronary heart disease risk in middle-aged UK men. *Arterioscler. Thromb. Vasc. Biol.* **22,** 1918–1923

41. Tian, M., Cui, Y. Z., Song, G. H., Zong, M. J., Zhou, X. Y., Chen, Y., and Han, J. X. (2008) Proteomic analysis identifies MMP-9, DJ-1 and A1BG as overexpressed proteins in pancreatic juice from pancreatic ductal adenocarcinoma patients. *BMC Cancer* **8,** 241

42. Winston, J. S., Asch, H. L., Zhang, P. J., Edge, S. B., Hyland, A., and Asch, B. B. (2001) Downregulation of gelsolin correlates with the progression to breast carcinoma. *Breast Cancer Res. Treat.* **65,** 11–21

43. Yoon, S. Y., Kim, J. M., Oh, J. H., Jeon, Y. J., Lee, D. S., Kim, J. H., Choi, J. Y., Ahn, B. M., Kim, S., Yoo, H. S., Kim, Y. S., and Kim, N. S. (2006) Gene expression profiling of human HBV- and/or HCV-associated hep-

atocellular carcinoma cells using expressed sequence tags. *Int. J. Oncol.* **29,** 315–327

44. Tabb, D. L., Huang, Y., Wysocki, V. H., and Yates, J. R., 3rd (2004) Influence of basic residue content on fragment ion peak intensities in low-energy collision-induced dissociation spectra of peptides. *Anal. Chem.* **76,** 1243–1248

45. Cox, D. M., Zhong, F., Du, M., Duchoslav, E., Sakuma, T., and McDermott, J. C. (2005) Multiple reaction monitoring as a method for identifying protein posttranslational modifications. *J. Biomol. Tech.* **16,** 83–90

46. Unwin, R. D., Griffiths, J. R., Leverentz, M. K., Grallert, A., Hagan, I. M., and Whetton, A. D. (2005) Multiple reaction monitoring to identify sites of protein phosphorylation with high sensitivity. *Mol. Cell. Proteomics* **4,** 1134–1144

47. Haught, W. H., Mansour, M., Rothlein, R., Kishimoto, T. K., Mainolfi, E. A., Hendricks, J. B., Hendricks, C., and Mehta, J. L. (1996) Alterations in circulating intercellular adhesion molecule-1 and L-selectin: further evidence for chronic inflammation in ischemic heart disease. *Am. Heart J.* **132,** 1–8

48. Hortin, G. L., Sviridov, D., and Anderson, N. L. (2008) High-abundance polypeptides of the human plasma proteome comprising the top 4 logs of polypeptide abundance. *Clin. Chem.* **54,** 1608–1616

49. Green, J. M. (1996) A practical guide to analytical method validation. *Anal. Chem.* **68,** 305A–309A

50. Janecki, D. J., Bemis, K. G., Tegeler, T. J., Sanghani, P. C., Zhai, L., Hurley, T. D., Bosron, W. F., and Wang, M. (2007) A multiple reaction monitoring method for absolute quantitation of the human liver alcohol dehydrogenase ADH1C1 isoenzyme. *Anal. Biochem.* **369,** 18–26

51. Lin, S., Shaler, T. A., and Becker, C. H. (2006) Quantification of intermediate-abundance proteins in serum by multiple reaction monitoring mass spectrometry in a single-quadrupole ion trap. *Anal. Chem.* **78,** 5762–5767

52. Kronkvist, K., Gustavsson, M., Wendel, A. K., and Jaegfeldt, H. (1998) Automated sample preparation for the determination of budesonide in plasma samples by liquid chromatography and tandem mass spectrometry. *J. Chromatogr. A* **823,** 401–409

53. Yu, K., Little, D., Plumb, R., and Smith, B. (2006) High-throughput quantification for a drug mixture in rat plasma-a comparison of Ultra Performance™ liquid chromatography/tandem mass spectrometry with high-performance liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **20,** 544–552