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Platform for Establishing Interlaboratory Reproducibility of Selected Reaction Monitoring-Based Mass Spectrometry Peptide Assays

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Mass spectrometry (MS) is an attractive alternative to quantification of proteins by immunoassays, particularly for protein biomarkers of clinical relevance. Reliable quantification requires that the MS-based assays are robust, selective, and reproducible. Thus, the development of standardized protocols is essential to introduce MS into clinical research laboratories. The aim of this study was to establish a complete workflow for assessing the transferability and reproducibility of selected reaction monitoring (SRM) assays between clinical research laboratories. Four independent laboratories in North America, using identical triple-quadrupole mass spectrometers (Quantum Ultra, Thermo), were provided with standard protocols and instrumentation settings to analyze unknown samples and internal standards in a digested plasma matrix to quantify 51 peptides from 39 human proteins using a multiplexed SRM assay. The interlaboratory coefficient of variation (CV) was less than 10% for 25 of 39 peptides quantified (12 peptides were not quantified based upon hydrophobicity) and exhibited CVs less than 20% for the remaining peptides. In this report, we demonstrate that previously developed research platforms for SRM assays can be improved and optimized for deployment in clinical research environments.

Keywords: SRM • MRM • mass spectrometry • proteomics

Introduction

Over the years, mass spectrometry (MS) has emerged as a powerful analytical technique for the identification of unknown compounds, determination of molecular weights, elucidation of molecular structures, and quantification of a wide variety of analytes.[1,2] The versatility of the instrumentation is demonstrated in the vast array of molecules that can be detected by MS, including trace elements, small molecules such as steroids, vitamins and related metabolites, and drugs for therapeutic drug monitoring and toxicology. The analysis of such molecules by MS is currently routine in a large number of clinical diagnostic laboratories with results applied to patient care[3,4] In recent years, another emerging area of MS has been the identification and quantification of large molecules in complex mixtures, such as proteins in biological fluids.[5−7] While protein quantification has been largely dominated by immunoassays in clinical laboratories, targeted selected reaction monitoring (SRM) assays for protein biomarkers are increasingly being applied in the research laboratory setting.[6] An advantage of SRM-based assays is that a specific analyte detection reagent (e.g., antibody) is not necessarily required to measure the protein of interest, therefore saving time and expense in development and validation. Also, SRM-based assays can be used in a multiplexed format to measure dozens of molecules, and are a powerful technique for the measurement and quantification of analytes with post-translational isoform disease-specific alterations. MS is also the only technique that can deliver the specificity required to detect isoforms associated with protein sequence microheterogeneity and many clinically relevant variants.[8,9] Such assays could potentially be used for disease diagnosis and prognosis, and they are typically robust and selective, even in complex matrices.[10,11] High-abundance proteins present in biological samples at concentrations of 1 ug/mL or higher have been successfully assayed previously by SRM in complex matrices such as tissues and plasma.[12,13] However, the published methods require sample preparation such as albumin depletion and nanoflow LC, which will be difficult to deploy in a clinical setting due to low throughput, lack of robustness, need for operator expertise and economic considerations. Many low-abundance proteins are still not amenable to SRM analysis without up-front purification or enrichment. Nevertheless, the application of MS for protein quantification affords compelling advantages such high ana-

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lytical selectivity allowing the precise quantification of individual protein isoforms difficult to distinguish with traditional immunoassays as well as moderate (but constantly improving) sensitivity and the ability to monitor multiple analytes in a single assay at relatively low reagent cost.

The development of clinically useful protein and peptide SRM assays can be challenging for a variety of reasons. First, appropriate "proteotypic" peptides must be selected to serve as surrogates for the intact proteins of interest. Second, sensitive and robust MS/MS transitions (peptide precursor to product ion fragmentations) of target peptides need to be identified. Third, the liquid chromatography (LC) and MS parameters require optimization and refinement, and this process is typically time-consuming and labor intensive if performed manually. Multiplexed assays present an additional challenge because multiple sets of proteins/peptides are monitored during a single assay. This requires that all of the above parameters are coordinated and scheduled to optimize signal-to-noise.[12] In most cases, the initial SRM assay optimization is accomplished using synthetic peptides in a matrix that is much less complex than the real clinical sample matrix, for example, plasma or serum. Therefore, the choice of peptides, transitions and the optimized LC retention time and MS instrument parameters may change considerably depending on the interferences caused by the ultimate clinical sample matrix. Ultimately, the broad applicability of SRM assays in clinical research environments will depend on the method reproducibility across laboratories. Also, precision and accuracy must conform to the standards already set for other clinical assays, including immunoassays.[14−16]

In general, two major approaches to protein quantification by SRM have been reported: (i) approaches that require relatively complex sample preparation including albumin depletion and fractionation in order to enrich a handful of low-abundance proteins prior to LC-SRM, coupled to analysis of a relatively small number of samples;[17−22] or (ii) methods that have assayed medium-to-high abundance proteins in unfractionated digests of biological fluids, coupled to analysis of relatively larger number of samples.[11,13,23] In the first approach, enrichment strategies such as immunoaffinity purification, depletion of high-abundance proteins, or strong-cation exchange chromatography, facilitated quantification of low-abundance proteins such as cytokines,[24,25] prostate-specific antigen (PSA),[18,20,21] carcinoembryonic antigen (CEA),[26] and thyroglobulin.[27] These proteins were measured in plasma or serum with limits of quantification (LOQ) in the low ng/mL ranges. Improvements in the sensitivity and selectivity of LC−ESI−MS instrumentation spurred the interest and development of SRM-based assays for medium-to-high-abundance proteins in biological fluids. As a result, a large interlaboratory study was initiated to evaluate the performance of different LC−MS platforms in proteomics laboratories.[12] Although the above-mentioned studies were developed, in many cases, for biomarker verification in a proteomics research setting, there is clearly a need for MS-based assays for routine use in clinical laboratories due to their high specificity for clinically relevant protein isoforms.[9,28] However, in their current form, most SRM assay protocols for proteins and peptides are tedious, resource- and time-consuming and thus, may not be very practical for large studies requiring high-throughput or routine use.

For peptide and protein quantification by MS to make its way into routine use in clinical research laboratories, standardized methods for assay development and verification must be identified and deployed across different laboratories by different operators. In this regard, the only other published study on inter laboratory reproducibility of SRM assays[12] utilized nanoflow which introduced instability of the LC configuration and deterioration of the LC columns causing shifts in peptide retention times and peak broadening or tailing. These factors limit the broad applicability of the developed assay protocols in clinical research laboratories, where operators will not necessarily be experts in mass spectrometry, thoughput will be higher and where the cost per assay is a key parameter. This report presents a robust, high-flow platform and workflow that streamlines the steps involved in developing SRM assays and facilitates rapid deployment in clinical research laboratories due to its simplicity.

The workflow presented in this report provides for automated, iterative optimization in the actual biological matrix in a high-flow, high throughput mode. Initially, the selection of proteotypic peptides is derived from experimental protein identification data or *in-silico* digestion of the target protein sequence. Next, empirical (derived from LC−MS/MS data) and heuristic (predicted algorithmically) approaches are applied to define the initial set of transitions. Further steps incorporate data acquisition using the initially defined set of transitions with subsequent assessment of data quality to eliminate less-than-optimal transitions and confirm the next set. Sequential iterations are maintained in the algorithm memory, allowing fully automated optimization. Once the optimal peptides and transitions are finalized, instrument parameters are adjusted to provide the most favorable signal-to-noise ratio (above the calculated LOQ). The described workflow also allows for fully automated testing of multiple instrument parameters, including collision energy and LC scheduling to provide maximum dwell times for increased detection sensitivity of individual peptides.

Even when an SRM assay has been fully optimized in one laboratory, it typically requires an operator with a high level of expertise in mass spectrometry, (especially when using nanoflow), so it may be time-consuming to transfer the assay to another laboratory while maintaining reproducible and consistent results.[12] Our objectives in this study were: (i) To outline a complete, high-flow platform for highly multiplexed peptide SRM assay development, (ii) Deployment of the platform across multiple laboratories engaged in clinical research and (iii) Development of QC parameters and examination of platform reproducibility across all the laboratories in a rapid time frame, that is, less than 6 months. The workflow presented herein describes specific steps and protocols for the standardization of instrument and chromatographic parameters that can be easily interchanged and incorporated in different laboratories. As part of the process, a standard test sample was prepared consisting of 51 heavy isotope-labeled synthetic peptides. These peptides were derived from 39 human proteins that are typically found in human plasma in relatively high abundance.[29,11] This test sample was used at each step of the standardization process to establish and monitor instrument and chromatographic performance. The resulting method and instrument platform was implemented in four different clinical research laboratories, and high SRM assay reproducibility was successfully demonstrated in a relatively short period of time, (4 months). Finally, in this report we demonstrate that previously developed research platforms for SRM assays[12] can be improved and optimized for deployment in clinical research environments. We believe that the described protocols will be useful to those laboratories developing and validating SRM

assays for proteins and for sharing and implementing such assays in routine practices for clinical research.

Materials and Methods

Plasma Samples. Human venous plasma samples (right arm) were collected using a Vacutainer Safety-Lok Blood collection set (21 g 3/4" × 12") (Becton Dickinson) into K2EDTA plasma collection tubes (Part Number 366643, 16 × 100 mm^2) (Becton Dickinson, NJ), discarding a partial first tube. Tubes were inverted 12 times and immediately centrifuged at 2800 rcf for 30 min. Avoiding 4 mm from the RBC at the bottom, plasma was pipetted out into a 15 mL Falcon tube and gently inverted to mix. Aliquots of 1.1 mL and 300 μL were placed in 1.5 mL Axygen microcentrifuge tubes (MCT-150) (Thermo Fisher Scientific) and placed into a labeled and dated cardboard sample box. Boxes of plasma were then placed into the −80 °C freezer for storage.

Trypsin Digestion, Reduction/Alkylation and Desalting of Plasma Samples. Plasma samples (25 μL) were thawed on ice and mixed with 100 μL of 8 M Guanidine HCl, 150 mM Tris-HCl, 10 mM DTT, pH 8.5. Samples were incubated at 37 °C for 1 h. The denatured samples were then alkylated with 45 mM iodoacetic acid (500 mM stock concentration in 1 M ammonium bicarbonate) in the dark for 1 h at room temperature. Residual alkylation agent was then reacted with 15 mM DTT. Samples were then diluted with 25 mM Tris-HCl, 5 mM $CaCl_2$ to 2.5 mL, then added to a glass vial of trypsin (Pierce, 20 μg, in 250 μL of 25 mM acetic acid). Samples were allowed to digest overnight (24 h) at 37 °C. Digested samples were acidified with TFA, pH <3 to quench the reaction and then desalted with HyperSep−96 C18 solid phase extraction media (Thermo Fisher Scientific). Briefly, the HyperSep C18 resin was conditioned before use with n-Propanol, then 0.25% TFA (v/v) in water. Samples were then loaded on the resin and washed with 0.25% TFA/water (v/v) and eluted with 75% (v/v) acetonitrile in 0.1% (v/v) formic acid. Finally, samples were dried *via* high-vacuum centrifugation before analysis by MS.

High Resolution LC−MS/MS for Protein Identification. High resolution LC−MS/MS analysis was carried out on an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific). Samples in 5% (v/v) acetonitrile, 0.1% (v/v) formic acid were injected onto a 75 μm × 25 cm fused silica capillary column packed with Hypersil Gold C18AQ 5 μm media (Thermo Fisher Scientific). LC separation was accomplished with a 250 μL/min gradient of 5% (v/v) acetonitrile, 0.1% (v/v) formic acid to 30% (v/v) acetonitrile, 0.1% (v/v) formic acid over the course of 180 min with a total analysis time of 240 min. The LTQ-Orbitrap was operated in a top 5 data-dependent configuration at 60K resolving power for a full scan, with monoisotopic precursor selection enabled, and +1 (singly charged) and unassigned charge states rejected. The analysis was carried out with CID and HCD fragmentation modes.

Liquid Chromatography Selected Reaction Monitoring (LC-SRM) Assays. All SRM assays were developed on a TSQ Quantum Ultra triple quadrupole mass spectrometer, Surveyor MS pump, Micro Autosampler and an IonMax Source equipped with a low flow metal needle (Thermo Fisher Scientific), flow rate 160−200 μL/min. Reverse phase separations were carried out on a 1 mm ×150 mm Hypersil Gold 3 μm C18 particle. Solvent A was LC−MS grade water with 0.2% (v/v) formic acid, and solvent B was LC-MS grade acetonitrile with 0.2% (v/v) formic acid (Optima grade reagents, Thermo Fisher Scientific). The HPLC was plumbed using 1/32 red peek tubing. The instrument divert valve was switched to waste before and after the peptides eluted in order to keep the source free of excess salts and debris. Pinpoint software (Thermo Fisher Scientific) was used for developing SRM assays. The software algorithm facilitates selection of proteotypic peptides based on the identification data and prediction of optimal fragment ions for SRM assay design, instrument method development, automatic peptide identity confirmation and quantitative data processing. Pinpoint software is available at Thermo Fisher Scientific website at www.thermo.com/pinpoint. Polytyrosine-1,3,6 calibrant was obtained from CS Bio Company, Menlo Park, CA, product Number CS0272S. Glucagon was obtained from Sigma Chemical Co, St Louis, MO, product number G2044−25MG.

The TSQ Ultra was run in unit resolution with Q1 and Q3 set to 0.7 fwhm. The instrument operating software was Xcalibur 2.0.7 SP1 and TSQ 2.2.0. Nonscheduled methods were run with a cycle time of 0.8 s. Scheduled methods were run with a cycle time of 1 s. Cycle times were optimized to ensure a minimum of 12 scans across each peak.

Light and Heavy Labeled Peptides. Light and isotopically heavy labeled versions (incorporating ^{13}C- and ^{15}N-labeled arginine or lysine) of each target peptide were synthesized (Thermo Fisher Scientific, Ulm Germany). Heavy peptides had identical sequences to the light peptides, but the C-terminal lysine or arginine was fully labeled (>98.5%) with ^{13}C and ^{15}N. The high purity (>97%) of these peptides enabled confident characterization of their ionization, elution and fragmentation and therefore, facilitated optimization of the SRM assay. A collection of 51 peptides were chosen and synthesized based upon the following criteria:

1. All derived from relatively high abundance proteins in human blood.
2. Hydrophobicity spanning a broad range (see Supplementary Table S6, Supporting Information).
3. No cysteines.
4. No glycosylation motifs.
5. Length between 7−20 aa.
6. Good ionization and fragmentation behavior observed in previous discovery LC−MS/MS experiments.

All of these peptides had been identified in previous projects and some previously published.[9,29] However, SRM parameters for a single multiplexed assay including all the peptides together had not previously been optimized or published.

Step-by-Step Method for Instrument and LC Setup Across Four Laboratories. Step 1: TSQ Ultra Calibration. All four laboratories had a TSQ Ultra triple quadrupole equipped with an ESI probe and a 32 gauge metal needle. Calibration was performed in positive mode using polytyrosine-1,3,6 calibrant (CS Bio Company Product Number CS0272S). The TSQ Quantum Ultra Getting Started guide, available on all TSQ Ultra desktop computers, provided complete instructions for calibration. Each laboratory produced calibration reports, which were placed on a central ftp site and reviewed before proceeding to the next step.

Step 2: Tuning on a Single Peptide (LLVVYPWTQR from *β*-Globin). Each laboratory received a tryptic peptide, LLVVYPWTQR, representing human β-globin at a concentration of 5pmol/μL. A solution of 50% ACN/50% water at 2.5pmol/μL was prepared. Next, the peptide was infused into the instrument using a "T" junction with a flow of 160 μL/min of 50% ACN 0.2% FA/50% water 0.2% FA from the LC pump. During infusion ESI probe conditions were set to the following values:

probe position: C; spray voltage: 4000 V; sheath gas: 30; aux gas: 3; capillary temperature: 300 °C.

Once stable signal for the +2 charged peptide (precursor m/z: 637.938) was obtained in full scan mode, the top 8 transitions for the peptide were automatically chosen based upon intensity and cross-checked against theoretical b and y fragment ions. Collision energy and tube lens values were also automatically optimized for each transition using the TSQ Tune software. Each lab produced tune reports, which were placed on a central ftp site and reviewed by a TSQ expert before proceeding to the next step.

Step 3: LC−MS of LLVVYPWTQR (Light) and LLVVYPWTQ*R (Heavy Labeled Synthetic Peptide). The top 6 SRM values and parameters acquired from tuning the single light LLVVYPWTQR peptide were imported into an instrument method (Supplementary Table S1, Supporting Information). Corresponding heavy isotope labeled peptide LLVVYPWTQ*R transitions (doubly charged precursor m/z: 642.938) were also included in the instrument method, using the same parameters as those acquired from tuning the equivalent light peptide transitions. All transitions used in the final instrument method were verified as real peptide fragments using Pinpoint software (Supplementary Table S1, Supporting Information).

The LC front end instrumentation included a Surveyor LC pump and Surveyor autosampler with a 150 mm by 1 mm Hypersil Gold 3 μm column heated to 50 °C. Supplementary Table S2 (Supporting Information) describes the LC method. Solvent A was water with 0.2% FA and Solvent B was ACN with 0.2% FA. The TSQ Ultra divert valve allowed flow from the pump to be diverted to waste 2 min before and after the peptide eluted.

All participating laboratories received 10fmol/μL solutions of both the Light and Heavy peptide versions of LLVVYPWTQR in a solution of 97% water, 3% ACN, 0.2% FA, and 200 μg/mL glucagon. In previous experiments at BRIMS (Biomarker Research Initiatives in Mass Spectrometry), (data not shown), it was found that glucagon stabilizes formulations of hydrophobic peptides, preventing loss of peptide from adsorption to plastics and significantly improves %CV. Ten microliter full loop injections were made with a Surveyor AS using a 10 μL PEEK loop. Each laboratory analyzed the sample in triplicate and placed the RAW files on a central ftp site. Data from each lab were reviewed with Pinpoint software in order to verify peak shape, peak intensity and %CV before proceeding to the next step. The criteria for passing step 3 was a peak area response of at least 1×10^6 and %CV of less than 15% for triplicate runs. The peak area response was based on summed transitions. Supplementary Table S5 (Supporting Information) displays the response and precision for LLVVYPWTQR across all laboratories. In addition, peak widths were evaluated and had to meet the requirement of less than 20 s. All laboratories had peak widths ranging from 14−18 s. The %CV and peak widths for the corresponding heavy isotopically labeled peptide were similar to the light peptide.

Step 4: Neat Standard Curve and Unknowns for LLVVYPWTQR. Four 96-well plates were prepared at BRIMS and analyzed in the 4 laboratories. Each plate included samples corresponding to several points on a calibration curve and two unknown concentrations. The points on the curve represented 0, 1, 10, 100, 500, 1000, and 2000 fmol of light peptide (LLVVYPWTQR), and the two unknown samples contained 20 and 200 fmol of the light peptide. Each point and unknown were analyzed in triplicate, and all samples contained 100 fmol of the heavy peptide LLVVYPWTQ*R spiked in as an internal standard. All values represented final amounts loaded on column. All samples were reconstituted in a solution of 97% water, 3% ACN, 0.2% FA and 200 μg/mL of glucagon. The chromatography from Step 3 was used. RAW files from each lab were placed on a central ftp site and reviewed and processed with Pinpoint software.

Step 5: Standard Curve for LLVVYPWTQR in Plasma Matrix. Four 96-well plates were prepared at BRIMS and then analyzed in each laboratory. Each plate included wells corresponding to points on a calibration curve in a background of digested plasma matrix and 4 unspiked plasma sample replicates. The points on the curve corresponded to 0.5, 1, 10, 100, 500, 1000, and 2000 fmol of Light peptide (LLVVYPWTQR). Each point and test plasma sample were analyzed in triplicate, and all samples contained 100 fmol of the heavy peptide LLVVYPWTQ*R spiked in as an internal standard. Three micrograms of digested plasma matrix were used in the calibration curves. All values represent final amounts loaded on column. The amount of endogenous Light peptide (LLVVYPWTQR) representing human β-globin was determined from the y-intercept of the calibration curve. Final plasma sample values were adjusted to incorporate the endogenous amount of light peptide present in the background of plasma matrix. For this correction, the y-intercept calculated from the calibration curve is used to calculate the endogenous amount, which is then used to correct the total amount of the light peptide and regenerate the new linear equation. Calibration curve point samples and test plasma samples were reconstituted in a solution of 97% water, 3% ACN, 0.2% FA and 200 μg/mL of glucagon. The chromatography from Step 3 was used. RAW files from each laboratory were placed on a central ftp site and reviewed and processed with Pinpoint software.

Step 6: Neat Standard Curve and Unknowns for 51 Peptides. A stock solution of 51 heavy peptides was made up in 97% water, 3% ACN, 0.2% FA at a concentration of 80 fmol/μL for each peptide. Four 96-well plates were prepared at BRIMS and then used in each laboratory. Each plate included samples corresponding to points on a calibration curve and one unknown. The points on the curve included 0, 100, 200, 400, and 800 fmol of the 51 peptide mix. Each sample contained 250 fmol of light peptide LLVVYPWTQR as an internal standard. Initially, in order to obtain the retention times of the peptides, all 4 participating laboratories implemented the same unscheduled instrument method in triplicate. This unscheduled method used SRM transitions and parameters that were previously optimized with Pinpoint software. The unscheduled RAW files were placed on a central ftp site, and each laboratory then received a customized scheduled method produced by Pinpoint. Once the scheduled method was received, the calibration curve was measured, and the unknowns were quantified. All values represent final amounts loaded on column. Each sample was reconstituted in 97% water, 3% ACN, 0.2% FA. The LC method shown in Supplementary Table S3 (Supporting Information) was used. The TSQ Ultra divert valve allowed flow to be diverted to waste before and after peptides eluted.

Step 7: Standard Curve and Unknowns in Matrix for 51 Peptides. Four 96 well plates were prepared at BRIMS and then analyzed in each laboratory. Each plate included samples corresponding to points on a calibration curve and two unknowns, all in a background of 3 μg of digested plasma matrix. The points on the curve included 100, 200, 400, and 800 fmol of the 51 peptide mix, and two unknown samples

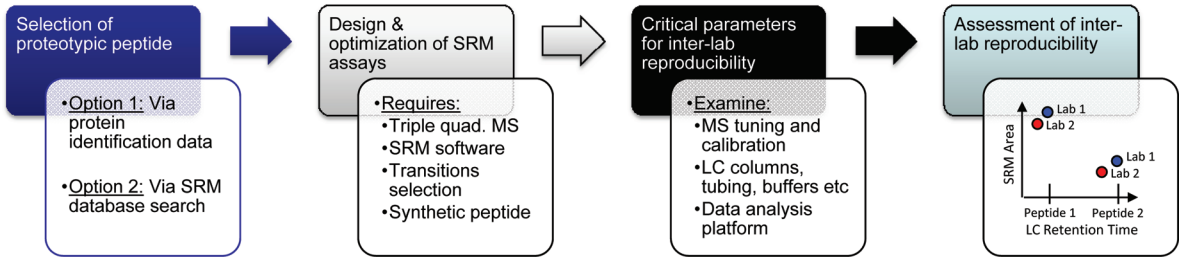

Figure 1. Workflow for the development and optimization of SRM assays. (i) Proteotypic peptides are selected based on either protein identification data or searching of publicly available SRM databases such as MRM Atlas (www.mrmatlas.org) or GPM Proteomics Database (http://mrm.thegpm.org). (ii) Multiplexed SRM assays are designed using specialized software (e.g., Pinpoint), which also facilitates further iterative optimization of the initial set of SRM parameters. (iii) Critical LC−MS parameters for interlaboratory reproducibility are set by an initial MS operator to ensure near identical performance of an LC−MS platform. (iv) The finalized set of SRM and LC−MS parameters, internal standards, and reagents are provided to each laboratory. To exclude any bias in analysis of SRM assay results, centralized data analysis may also be required.

contained 80 and 400 fmol of the peptide mix. As each sample was spiked in plasma matrix, the endogenous amount of the light peptide (LLVVYPWTQR) representing human β-globin was used as an internal standard. Initially, in order to obtain the retention times of the peptides, all laboratories implemented the same unscheduled instrument method in triplicate. This unscheduled method used SRM transitions and parameters that were previously optimized with Pinpoint software. The unscheduled RAW files were placed on a central ftp site, and each laboratory then received a customized scheduled method produced by Pinpoint. Once the scheduled method was received, the calibration curve was measured and the two unknowns were quantified. All values represent final amounts loaded on column. Each sample was reconstituted in 97% water, 3% ACN, 0.2%FA. The LC method in Step 6 (Supplementary Table S3, Supporting Information) was used.

Step 8: 51 Peptides in Glucagon Solution. Finally, four 96 well plates were prepared at BRIMS and then analyzed in each laboratory. Each plate included samples corresponding to replicates of the 51 heavy peptides prepared in a solution of 97% water, 3% ACN, 0.2% FA and 200 μg/mL glucagon (Sigma G2044−25MG). Each sample contained 400 fmol of the 51 heavy peptide mixture and 70 fmol of light peptide (LLVVYPWTQR) as an internal standard. Initially, in order to obtain the retention times of the peptides, all laboratories implemented the same unscheduled instrument method in triplicate. This unscheduled method used SRM transitions and parameters that were previously optimized with Pinpoint software. The unscheduled RAW files were placed on a central ftp site, and each laboratory then received a customized scheduled method produced by Pinpoint. Once the scheduled method was received, samples were analyzed in duplicate. All values represent final amounts loaded on column. The LC method in Step 6 (Supplementary Table S3, Supporting Information) was used.

CV Calculations were performed as follows: All samples were run in triplicate. CV's for final amounts in unknown samples were compared across the 4 laboratories by grouping the samples from the first run, second run and third run. A CV could then be assigned to each batch across the samples from the four laboratories.

Results

Workflow for Development of SRM Assays. To achieve excellent interlaboratory reproducibility of peptide SRM assays, it is critical to have an optimized SRM assay, as well as mass spectrometry instruments that have been tuned in a similar fashion. The challenging steps in the development of such SRM assays include (i) selection of proteotypic peptides, (ii) selection of sensitive and robust transitions for the target peptides, (iii) optimization of LC and MS parameters and (iv) multiplexed analysis of dozens of peptides with a single SRM assay. Specialized software packages, such as Pinpoint, facilitate *ab initio* prediction and optimization of many of these parameters, although it is still difficult to predict, prior to the experiment, which peptide will be efficiently ionized and fragmented, and thus will provide the most stable and most intense signal. Currently, proteotypic peptides can be selected empirically based on LC−MS/MS identification data or by searching the publicly available databases (Figure 1).

Laboratories that have access to mass spectrometry instrumentation for the identification of proteins (such as ESI-LTQ-Orbitrap or ESI-qTOF) can quickly and efficiently translate identification data into multiplexed SRM assays for a triple quadrupole mass spectrometer. Such laboratories have the advantage of developing SRM assays for unique proteins that have never been identified or analyzed. Laboratories equipped with only a triple quadrupole mass spectrometer (e.g., many clinical diagnostic laboratories) can start SRM assay development with a database search in order to select proteotypic peptides for proteins of interest. Fortunately, in recent years, a vast array of discovery data have been made available in public databases such as MRM Atlas[30,31] (www.mrmatlas.org) or GPM Proteomics Database[32] (http://mrm.thegpm.org). The disadvantage of such an approach is the relative lack of high-quality data for low and medium abundance proteins; thus, only high-abundance proteins will be amenable to SRM assay development.

Once proteotypic peptides are selected, the optimization of SRM transitions and LC−MS parameters can be accomplished with Pinpoint software. As a result, optimized parameters can be distributed to participating laboratories and used to quantify target proteins in a standardized fashion (Figure 1).

Parameters for Interlaboratory Reproducibility of SRM Assays. A variety of SRM assays were monitored across four different laboratories with identical LC−MS-triple-quadrupole platforms. Supplementary Figure S1A and B (Supporting Information) shows the mass calibration and gain calibration outputs obtained by following standard calibration methods. The linearity of the curves and comparable values of the gain setting among the four laboratories show successful calibration. Following this, a single peptide was infused into the mass

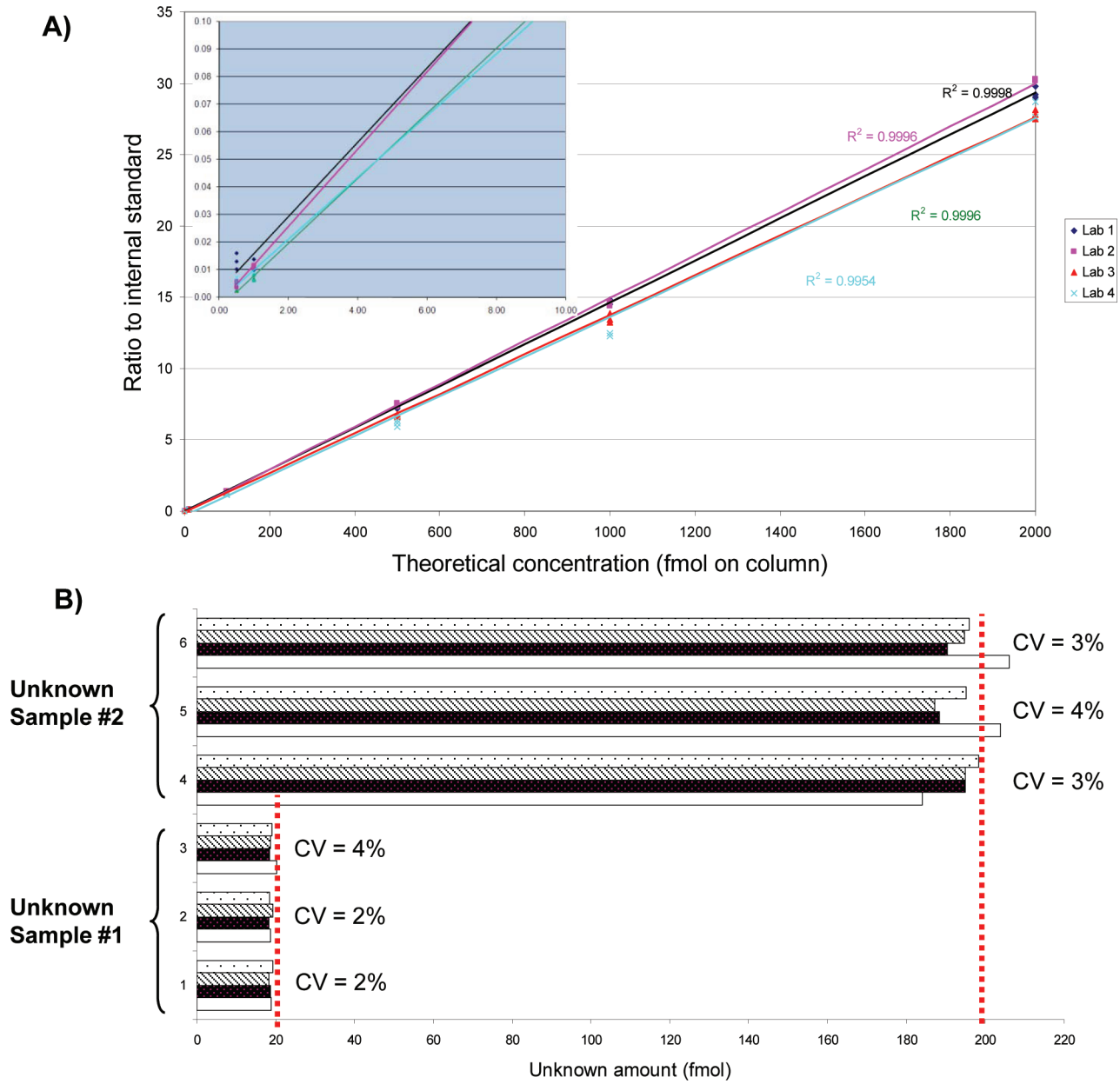

Figure 2. Analysis of one peptide in a simple matrix across 4 laboratories. (A) Standard curve for the peptide LLVVYPWTQR spiked at five different concentrations in simple matrix. Four standard curves generated with 1/*x* weighting, representing each participating laboratory, were used to compute the amount of the unknown samples. (B) Absolute amount and CVs calculated for the peptide LLVVYPWTQR in six samples (representing 2 samples prepared in triplicate) on four instruments using standard curve in simple matrix. In all six cases, the CV was <5%. Each laboratory is represented by a bar.

spectrometer by the four participating laboratories. Supplementary Table S4 (Supporting Information) shows the optimal collision energies obtained by performing a breakdown curve measurement for the top two transitions selected by each of the four laboratories. The collision energy values across the laboratories were very similar, showing similar peptide fragmentation behavior, and thus suggesting that an SRM assay optimized at one laboratory could be transferred, as is, to another laboratory without the need for reoptimization.

Next, a single peptide was analyzed to compare the retention times and signal values across the four laboratories. We limited our focus to a single peptide in order to simplify the process and provide a starting point for benchmarking LC performance. In general, the reproducibility of retention time values and signal values within replicates for each laboratory were very good (typical for high-flow methods). Within multiple runs in a single laboratory peak drift was 6 s or less. Based upon this performance, and a peak width of 14−18 s, we chose retention time windows of 50 s for scheduling. The retention time CV between laboratories was ca 10%, and the signal values (area under the curve) varied by approximately 35%. This variability emphasized the need for an internal standard to normalize for differences in injection volumes, spray differences, and most importantly, instrument sensitivity. The heavy peptide LLVVYPWTQ*R was selected as an internal standard, and a multipoint standard curve (as specified in the Methods section) was generated in each of the four laboratories. In our experience, multipoint calibration curves are more robust than single-point relative quantification, when doing quantification over 3 orders of magnitude. Moreover, single-point relative quantification is

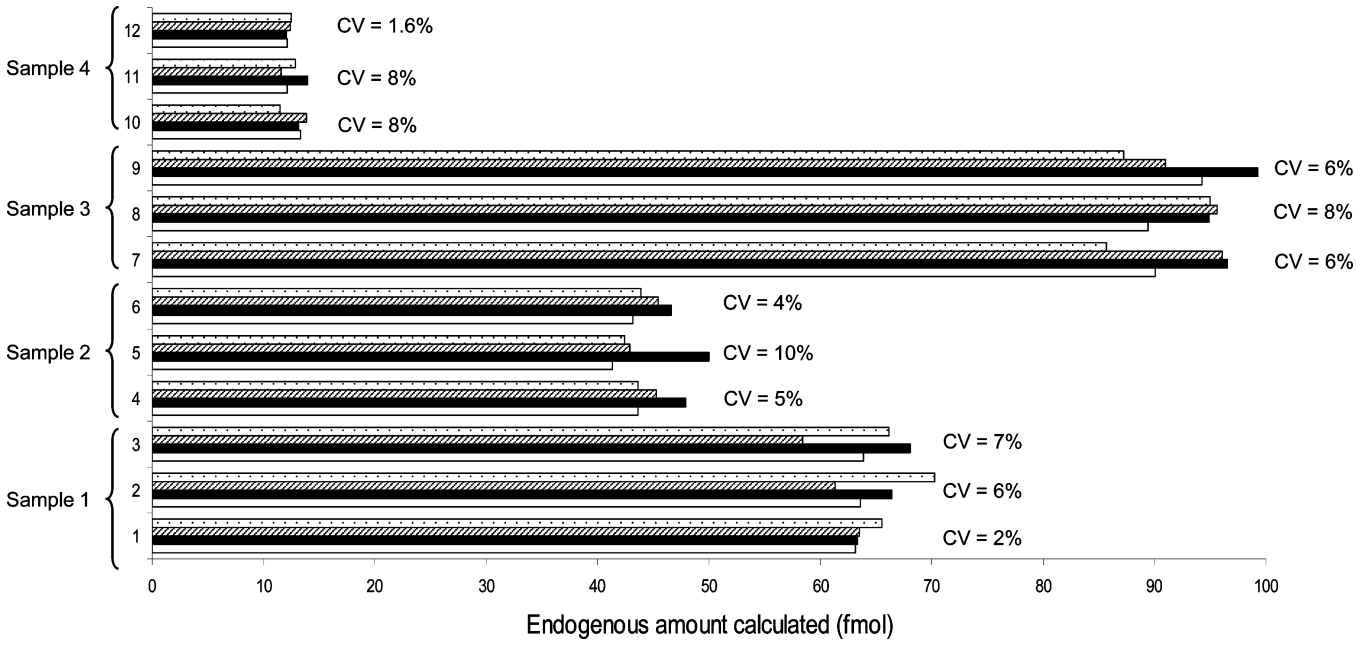

Figure 3. Endogenous amount (fmol) and CVs calculated for the peptide LLVVYPWTQR in four triplicate plasma test samples analyzed on four instruments using standard curves generated in a plasma matrix. The resulting interlab CVs were ≤10% in all 12 cases. When compared to the simple matrix, the CVs were slightly higher, likely due to the higher complexity and increased interference of the matrix. Each laboratory is represented by a bar.

heavily dependent on equal response of light and heavy peptides for equimolar amounts and extrapolation of this response to other points on the curve 2−3 orders of magnitude higher or lower. Equal response is not necessarily always the case for light and heavy versions of the same peptide.[2,3] Creating a standard curve by varying the amount of light peptide in a constant amount of heavy peptide, (thereby creating a multipoint curve), solves this problem. Figure 2A displays the resulting four standard curves, generated with $1/x$ weighting. All four curves had high r-square values ($r^2 = 0.99$) based upon a linear fit, and these curves were used to compute the peptide amounts in the unknown samples (for all other peptide curves, see Supplementary Figures S2−S40, Supporting Information). All transitions were summed and included in the calculations. The square of the correlation coefficient is an accepted method for calculating goodness of fit for the standard curve in clinical research laboratories.[33] Figure 2B plots the unknown amount calculated for six samples (two unknowns in triplicate) across the four laboratories with the corresponding interlaboratory CV. In all six cases, the CV was <5%. The same process was repeated in a plasma matrix where four test plasma samples were used as unknowns and analyzed in triplicate by all laboratories. Figure 3 plots the calculated endogenous amounts in plasma for all 4 samples (in triplicate) and the resulting interlaboratory CVs were <10% in all 12 cases. The accuracy across the different laboratories is given by the standard deviations of the unknown sample amounts (Figure 2B) When compared to the neat matrix, the CVs were slightly higher, which is not unexpected due to the increased sample complexity resulting from higher numbers and abundances of interfering protein components.

Interlaboratory SRM Assay for 51 Peptides. To test the reproducibility of a more complex sample, a mixture of 51 synthetic peptides was analyzed in all laboratories. Transitions for the 51 peptides were initially optimized at BRIMS, resulting in a single method containing 204 transitions (4 transitions per peptide, Supplementary Table S5, Supporting Information). Once the initial unscheduled method was generated, it was implemented in all laboratories, and the observed retention times were used to create a scheduled method (for each laboratory) with an LC retention time window of ±0.5 min the observed time for each peptide. This optimization provided a 7−15 fold increase in dwell time, depending on the number of peptides coeluting at any given retention time. Using this scheduled method, standard curves were generated in the four laboratories, and the amounts of unknown samples were calculated. However, the 51 peptide mixture presented a challenge due to varying retention characteristics. For example, in the mixture 6 peptides were very hydrophilic, therefore eluting very early and not all were identified using the method. Another 6 peptides were very hydrophobic and consequently eluted very late (see Supplementary Table S6 for peptide hydrophobicity values, Supporting Information). The 6 very hydrophobic peptides were bound irreversibly to plastic, leading to a loss of signal and, in some cases, a complete absence of signal. As a result, for our calculations, we chose to exclude these 12 highly hydrophilic and hydrophobic peptides from the interlaboratory comparison.

Figure 4 presents the interlaboratory CV distribution of the absolute amount calculated for each of the remaining 39 peptides under 3 different conditions (simple matrix, complex matrix with 400 fmol of spiked peptides, and a complex matrix with 80 fmol of spiked peptides). Relatively high concentrations for the spiked in peptides were chosen for several reasons: (i) The point of this study was to focus on reproducibility and method robustness, not sensitivity. (ii) We chose peptides from human proteins that are typically found in high abundance, such as apolipoproteins. A number of these peptides were previously detected in a recent publication,[28] and in that study the LOQ ranged from 1−5 fmol. Typically, these proteins are found in blood in μm/L to mm/L quantities. (iii) The goals for this project focused on standardization of methods useful in a

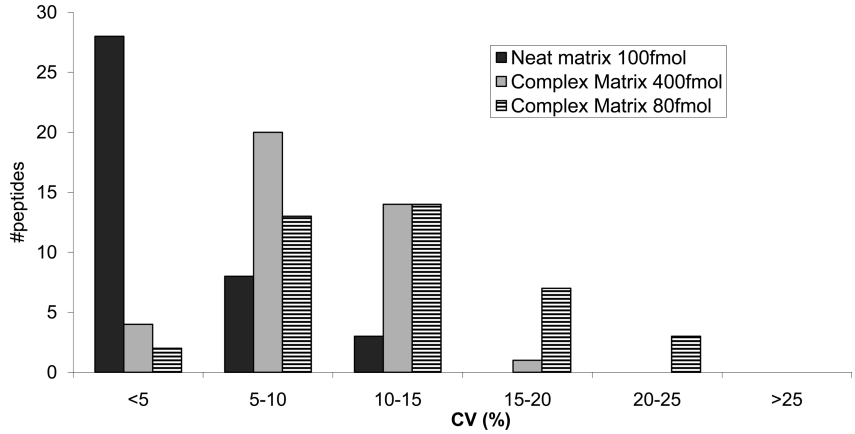

Figure 4. CV distribution of absolute amounts calculated for 39 peptides in four different laboratories using 3 different sample matrix conditions: simple matrix, complex matrix with spiked peptides at 400 fmol and complex matrix with peptides spiked at 80 fmol. In the simple matrix, ~27/39 peptides had CVs < 5% and the remaining peptides had CVs < 10−15%. In the complex matrix with 400 fmol spiked peptides, ~25/39 peptides had CVs < 5−10% and the remaining peptides had CVs < 15−20%. Finally, in the complex matrix with 80 fmol spiked peptides, the vast majority of peptides had CVs < 10−15% with a few displaying CVs < 20−25%.

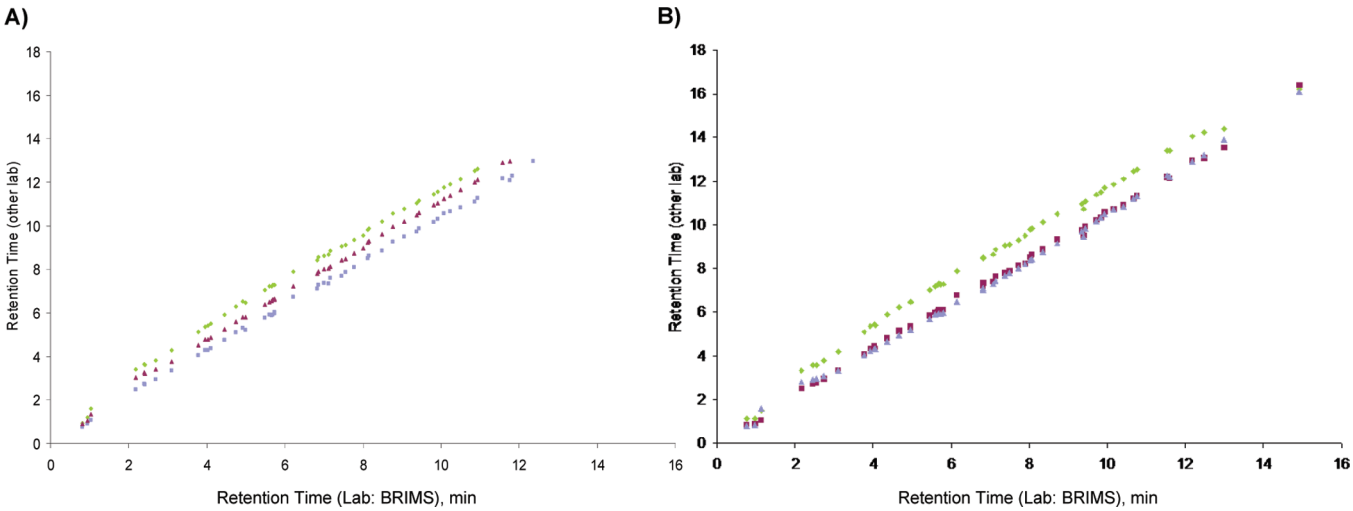

Figure 5. Retention time reproducibility of 51 peptides spiked in (A) matrix and (B) glucagon solution across four laboratories. Glucagon, a highly hydrophobic peptide, binds to the available plastic surfaces and thus prevents the loss of the other hydrophobic peptides. In the presence of a high concentration of glucagon (200 mg/mL) (B), all the hydrophobic peptides were recovered in all the laboratories (retention time >12 min), when compared to the no-glucagon (A) experiment. The *x*-axis represents the retention time observed at BRIMS, and the *y*-axis is the retention time observed in the other 3 laboratories (each color represents a different laboratory).

clinical research environment, not a biomarker research environment. As such, we focused on the development of a high-flow, (as opposed to nanoflow), very robust LC platform that could be implemented by clinical research laboratories that were not necessarily experts in mass spectrometry.

Initially, we compared the resulting precursor-to-fragment ion ratios observed in complex matrix to the ion ratios observed in the simple matrix experiment, in order to ensure that the calculations were not affected by matrix interferences. Also, to increase throughput we chose to use a single peptide as an internal standard for all 39 peptides (instead of internal standards corresponding to each of the 39 peptides). This was possible due to the high spray stability of the instrument and method. Specifically, we selected an endogenous human peptide as the internal standard for normalization of all peptides (the synthetic version of this peptide was spiked into the simple matrix experiment).

In general, for all 39 peptides, the interlaboratory CV was <15% in both the simple matrix and the complex matrix. The gray bars in Figure 4, representing the complex matrix with 400 fmol of spiked peptides, illustrate that all but one peptide had CV < 15%, with 62% of peptides CV < 10%. The striped bars, representing the complex matrix with 80 fmol of spiked peptides, show that 75% of the peptides had interlaboratory CVs < 15%. The higher CVs observed in these two experiments (versus the experiment without matrix) were limited to only 2 of the peptides and are not unexpected due to the increased complexity of the background matrix which introduces interferences. All the laboratories generated CV's that were well within the parameters required for many approved Laboratory Developed Molecular Assays.[32]

A plot of the observed retention times in the four laboratories for the peptides spiked into the plasma matrix is shown in Figure 5A. The *x*-axis represents the retention time observed at BRIMS, and the *y*-axis is the retention time observed in the other 3 laboratories (each color represents a different lab). The linearity between the different laboratories serves to highlight the highly reproducible LC system across the four laboratories.

The difference in the y-intercepts of the three linear curves is due to the different dead volumes in the LC setups in the different laboratories (slightly different lengths of LC tubing). This variability highlights the importance of developing a clearly defined SOP (standard operating procedure) for achieving clinical reproducibility. Notably, the 6 very hydrophilic peptides are clustered in the lower left corner of the plot and the highly hydrophobic peptides are clustered in the upper right corner of the plot. The missing points in the upper right-hand corner of the plots emphasize that the hydrophobic peptides were not consistently observed by all laboratories. This may be due to irreversible binding to plastic surfaces. In order to test this hypothesis, we analyzed the samples in a matrix containing high concentrations of glucagon, a highly hydrophobic peptide. The *a priori* hypothesis was that the glucagon would bind to the available plastic surfaces and prevent the loss of the other hydrophobic peptides (pageing effect). The retention times for the peptides in a matrix containing glucagon (Step 8) are presented in Figure 5B and it is clear that all hydrophobic peptides were recovered in all laboratories, when compared to the no-glucagon experiment (Figure 5A).

Discussion

The advantages of mass spectrometry as a protein or peptide analytical platform include the potential for high sensitivity and sequence specificity, simultaneous analysis of multiple analytes in a single experiment, fewer reagent requirements (lower cost), and a relatively short assay development time. All of these attributes qualify MS as a compelling alternative for analysis of proteins versus immunoassays, particularly for novel protein biomarkers of clinical relevance. In addition, with the evolving concept of personalized medicine, the need for analysis of panels of biomarkers will emerge, and MS is an ideal platform for such multiplexed assays. Historically, the disadvantages of MS platforms for protein quantification in clinical laboratories have been associated with relatively poor reproducibility, lack of standards for performance assessment and inter- and intralaboratory comparability, the need for manual sample processing, availability and cost of internal standards, the need for expert operators and the high cost of the LC and MS instruments.[28,34,35]

In this study, we describe a very robust, semiautomated, standardized workflow including all steps involved in developing an SRM assay for proteins (peptides), and demonstrate the feasibility of transferring method parameters across four laboratories with different operators in widely dispersed geographical locations. Initially, we chose to optimize the SRM method at a central site, in order to develop the simple workflow rapidly. Now that the workflow has fully matured, it has been implemented at all sites without any central site supervision required. The iterative SRM assay optimization process is fully automated, very fast (on the order of minutes), and easily deployable.

We demonstrated successful high-flow SRM assays with simultaneous analysis of 39 peptides in simple and complex matrices across four different laboratories with CVs, for the most part, <10−15%. In addition, by monitoring 4 transitions per peptide, we were able to demonstrate high reproducibility of the assays. This study also highlights the ease with which sample preparation can be performed for accurate quantification of medium-to-high abundance peptides. The current study was limited to a single platform in order to standardize all components. However, now that methods are developed, it should possible to translate the standardized assays to other platforms.

There are several key parameters that must be properly controlled in order to ensure optimal SRM inter laboratory reproducibility. First, each triple quadrupole instrument must be properly calibrated to ensure optimal instrument performance. Next, once the LC method and hardware have been optimized, a quality control standard must be chosen to serve as the benchmark for MS and LC performance. For this project, a synthetic peptide, LLVVYPWTQR was selected and based upon the data presented in Table S1, this peptide standard provided a useful guide for setting platform precision and response parameters across the 4 laboratories. This peptide standard ensured that LC and MS performance within each lab and across the four laboratories was within the acceptable parameters given in the Methods (peak area response of at least 1×10^6 and %CV of less than 15% for triplicate runs). Other parameters that must be monitored include MS ion source conditions (including the cleanliness of the ion transfer tube) and LC column pressures. In general, once a proper SOP has been implemented across all participating laboratories, most problems that arise tend to be associated with minor LC and autosampler issues that are typically easily addressed. However, in order to ensure operational reproducibility, instrument QA/QC determination including calibration and tuning should be implemented on a regular basis.[2,3]

Some of the important features to bear in mind regarding SRM assay development include: (i) the appropriate selection of proteotypic peptides based on identification/discovery data, (ii) the exclusion of redundant peptide sequences and (iii) the careful selection of peptides based upon the hydrophobicity index (filtering out very hydrophobic and hydrophilic peptides, Supplementary Table S6, Supporting Information). One of the goals of this study was to understand and establish the selection criteria for "good" peptides. Therefore, as shown in Supplementary Table S6 (Supporting Information) and described in the Methods, we selected peptides across a broad range of hydrophobicity and other characterisitics. The determination of which peptides might not behave in an optimal way is highly useful information for effective assay development. For example, the 12 peptides that eluted too early or too late under our assay conditions are not good candidates for a routine assay. We also report a novel solution for recovery of hydrophobic peptides in the Methods, (addition of glucagon), that can lead to more reproducible retention on column.

Last, the selection of MS/MS transitions, filtering out nonspecific transitions in the appropriate matrix of interest, and optimization and refinement of LC and MS parameters such as collision energy, are crucial steps in developing an SRM method. The advantages of mining previously obtained or spectral library discovery LC−MS/MS data are that the SRM assays can be developed quickly, unique proteins can be identified and the need for synthetic peptides is diminished. For this approach, an appropriate MS instrument suitable for discovery experiments is needed. The use of optimized software (Pinpoint) was instrumental in developing the multiplexed SRM assay in this study because it provided a semiautomated vehicle for LC−MS/MS data mining and optimization of all the relevant SRM parameters described above.

If protein analysis in biological fluids based on MS-centered assays is to make its way into the clinical setting, efficient SRM assay development and standardization with excellent inter-

laboratory reproducibility will be required, given the increasing demand for standardization in clinical laboratories. In this regard, our work demonstrates that if a streamlined protocol is used, along with appropriate software and internal standards, this goal can be achieved in a timely fashion and with excellent reproducibility across different laboratories. Based on these results, our next phase of research will involve further optimization and standardization of our methodology and procedures for interlaboratory measurements of real-world important and novel clinical analytes in biological fluids.

Abbreviations: LC, liquid chromatography; MS/MS, tandem mass spectrometry; SPE, solid phase extraction; ESI, electrospray ionization; ACN, acetonitrile; FA, formic acid; *m*/*z*, mass-to- charge ratio; SRM, selected reaction monitoring; LOD, limit of detection; LOQ, limit of quantitation; RBC, red blood cell; SOP, standard operating procedure.

Supporting Information Available: Figure S1. Calibration output from the four instruments. (A) Graphs plotting Peak Width vs Q1 Resolution. (B) Gain curves for the four instrument ESI sources. Figures S3−S40. Analysis of peptides in a simple matrix across 4 laboratories. Table S1. Instrument method for the top 6 SRM transitions and parameters for the light (precursor *m*/*z* 637.938) and heavy (precursor *m*/*z* 642.398) peptide, LLVVYPWTQR. Table S2. LC-gradient used for analysis of peptide LLVVYPWTQR. Solvent A was water with 0.2% FA and solvent B was ACN with 0.2% FA. Table S3. LC-gradient used for analysis of 51 peptides. Solvent A was water with 0.2% FA and solvent B was ACN with 0.2% FA. Table S4. Optimal collision energies obtained by all 4 laboratories for the top two SRM transitions by tuning the synthetic peptide LLVVYPWTQR. Table S5. Transitions for the 51 peptides investigated in this study. A single method containing 204 transitions, representing 4 transitions per peptide, was developed. Table S6. Hydrophobicity values for all peptides and transitions. This material is available free of charge via the Internet at http://pubs.acs.org.

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