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Long-gradient separations coupled with selected reaction monitoring for highly sensitive, large scale targeted protein quantification in a single analysis

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Abstract

Long-gradient separations coupled to tandem MS were recently demonstrated to provide a deep proteome coverage for global proteomics; however, such long-gradient separations have not been explored for targeted proteomics. Herein, we investigate the potential performance of the long-gradient separations coupled with selected reaction monitoring (LG-SRM) for targeted protein quantification. Direct comparison of LG-SRM (5 h gradient) and conventional LC-SRM (45 min gradient) showed that the long-gradient separations significantly reduced background interference levels and provided an 8- to 100-fold improvement in LOQ for target proteins in human female serum. Based on at least one surrogate peptide per protein, an LOQ of 10 ng/mL was achieved for the two spiked proteins in non-depleted human serum. The LG-SRM detection of seven out of eight endogenous plasma proteins expressed at ng/mL or sub-ng/mL levels in clinical patient sera was also demonstrated. A correlation coefficient of >0.99 was observed for the results of LG-SRM and ELISA measurements for prostate-specific antigen (PSA) in selected patient sera. Further enhancement of LG-SRM sensitivity was achieved by applying front-end IgY14 immunoaffinity depletion. Besides improved sensitivity, LG-SRM potentially offers much higher multiplexing capacity than conventional LC-SRM due to an increase in average peak widths (~3-fold) for a 300-min gradient compared to a 45-min gradient. Therefore, LG-SRM holds great potential for bridging the gap between global and targeted proteomics due to its advantages in both sensitivity and multiplexing capacity.

Keywords

long-gradient; targeted quantification; low-abundance protein; human serum; sensitivity; reproducibility

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ASSOCIATED CONTENT

Supporting Information Available

Supplemental tables and figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

INTRODUCTION

Traditionally, accurate and reproducible measurements of protein concentrations in biological samples have primarily relied on antibody-based immunoassays, such as enzyme-linked immuno-sorbant assay (ELISA), because of their sensitivity and throughput. However, analytically validated antibodies are often not available for novel target proteins and *de novo* development of these antibodies is associated with high cost, long development time and high failure rates.^{1–3} Furthermore, immunoassays are inherently limited by their multiplexing capabilities, precluding simultaneous quantification of hundreds of proteins derived from genomics and global proteomics analyses.

Selected reaction monitoring (SRM), also known as multiple reaction monitoring (MRM), has emerged as a promising alternative to antibody-based immunoassays in terms of its relatively good selectivity, sensitivity, and reproducibility (or precision) for measuring hundreds of target proteins simultaneously in highly complex biological samples.^{1,2,4–19} In comparison with immunoassays, SRM offers better specificity and has a greater potential for quantifying protein isoforms²⁰ and posttranslational modifications (PTMs)^{7,21–23} for which good quality antibodies are often not available and/or difficult to generate. However, a major limitation of SRM-based targeted quantification is the lack of sufficient sensitivity for quantification of low-abundance proteins or protein modifications.^{1,6} For example, without immunoaffinity depletion and prefractionation LC-SRM can only detect moderately abundant proteins at the low $\mu\text{g/mL}$ or high ng/mL level in human blood plasma/serum.^{1,2,6,10} Recent advances in sample prefractionation and enrichment strategies^{8,24–28} along with MS instrumentation^{1,29,30} have proven useful for enhancing SRM sensitivity for detecting low-abundance proteins. For example, we recently developed an antibody-free, targeted mass spectrometric approach, termed as PRISM-SRM, for highly sensitive quantification of proteins at low pg/mL levels in human plasma/serum.^{26,31} This strategy capitalizes on high-resolution reversed-phase liquid chromatographic separations for analyte enrichment with significantly reduced co-eluting matrix interferences and ion suppression effects. Intelligent selection (Δ Selection) and multiplexing target fractions, accomplished by on-line SRM monitoring of heavy-isotope labeled internal standards, partially alleviated a general drawback of the fractionation strategies (i.e., the need to analyze many fractions per sample), thus improving overall analytical throughput. However, the front-end sample prefractionation often requires a relatively large amount of starting material and increased instrument analysis time, which is proportional to the number of fractions to be analyzed. In addition, undesired sample losses are often encountered during multi-step sample preparation.

As an alternative to multidimensional sample fractionation strategies, more than a decade ago our group pioneered the use of long and shallow gradient separations with long capillary LC columns (up to 2 m in length) for reducing sample complexity and increasing the number of peptide and protein identifications in global proteomics.^{32–34} The long-gradient separation approach coupled to advanced MS was recently demonstrated to provide nearly complete coverage of the *Escherichia coli* proteome³⁵ and the yeast proteome³⁶ with only minute amounts of samples required (i.e., $\sim 4 \mu\text{g}$ of starting material). Compared to multidimensional fractionations, the long-gradient approach greatly simplifies the shotgun proteomics workflow, minimizes the sample size requirement, and reduces the total analysis time despite the use of long-gradient separation. The long-gradient LC-MS approach has become routinely used in many research groups for achieving deep, highly sensitive proteome coverage and biomarker discovery in global proteomics without prefractionation.^{36–42} However, this benefit of long-gradient LC separations in targeted proteomics has never been explored.

In this work, we investigated the use of long columns and long-gradient separations in combination with selected reaction monitoring (LG-SRM) to achieve highly sensitive, large-scale quantification of hundreds of proteins in a single analysis with minute amounts of starting materials. The sensitivity and multiplexing capacity of LG-SRM for quantifying human serum proteins without prefractionation were evaluated using standard protein spike-in serum samples and clinical prostate patient sera.

EXPERIMENTAL SECTION

Reagents

Bovine carbonic anhydrase and human PSA were purchased from Sigma-Aldrich (St. Louis, MO). Urea, dithiothreitol (DTT), iodoacetamide, ammonium formate, trifluoroacetic acid (TFA) and formic acid were obtained from Sigma (St. Louis, MO). Synthetic peptides labeled with $^{13}\text{C}/^{15}\text{N}$ on C-terminal lysine and arginine for all targeted proteins were from Thermo Scientific (San Jose, CA). Surrogate peptide selection was described in Supporting Information (see Supplemental Methods).

Human Specimens

A human female serum sample was purchased from BioChemed Services (Winchester, VA). Clinical serum samples from prostate cancer patients undergoing PSA screening were provided by the Johns Hopkins Medical Institutions. The use of human serum samples was approved by the Institutional Review Boards of the Pacific Northwest National Laboratory and Johns Hopkins University in accordance with federal regulations.

Target protein spike-in and human serum protein digestion

Bovine carbonic anhydrase and PSA, previously used for evaluation of PRISM-SRM, were spiked into female serum at 0, 1, 2.5, 5, 10, 100, 250, 500, and 1000 ng/mL levels to generate individual samples. The concentrations of target protein stock solutions were determined by the BCA protein assay (Pierce). Each 1 μL aliquot of the serum ($\sim 80 \mu\text{g}$) was diluted 10-fold with 50 mM NH_4HCO_3 (pH 8.0). Protein samples from human serum were digested with the same protocol as previously described.⁴³ Following protein digestion the peptide stock was then diluted to 1 $\mu\text{g}/\mu\text{L}$ with 0.1% formic acid in water and heavy-isotope labeled synthetic peptides were spiked at 0.5 fmol/ μL for bovine carbonic anhydrase and PSA and 5 fmol/ μL (crude peptides) for the other endogenous proteins being monitored. The targeted endogenous serum proteins include epidermal growth factor receptor (EGFR), kallikrein 6 (KLK6), cardiac troponin T (cTnT), myelin basic protein (MBP), matrix metalloproteinase 9 (MMP9), periostin (POSTN), and autotoxin, also known as ectonucleotide pyrophosphatase/phosphodiesterase 2 (ENNP2).

Conventional LC-SRM and LG-SRM analysis

All peptide samples were analyzed by using nanoACQUITY UPLC[®] system (Waters Corporation, Milford, MA) coupled on-line to a TSQ Vantage triple quadrupole mass spectrometer (Thermo Scientific, San Jose, CA). For evaluation of the LG-SRM performance, capillary reversed-phase columns, 75 μm inner diameter (i.d.) $\times$ 25 cm or 150cm length, packed in-house with 3- μm Jupiter C18 bonded particles (Phenomenex, Torrance, CA) were used for regular and long-gradient peptide separations of target protein spike-in samples, respectively. We also packed columns of 50 cm length and 75 μm i.d. with BEH 1.7- μm C18 particles (Waters Corporation, Milford, MA) to further increase the separation resolution and the peak shape, and the 50 cm C18 column packed with smaller particles was used for analyzing peptide mixtures from prostate cancer patient sera. Long-gradient separations with shorter columns was not evaluated because the peak capacity of the long gradient separation was previously demonstrated to be significantly affected by the

column length⁴⁴. For example, the peak capacity for a 10 cm column was less than 50% of that for a 100 cm column in any gradient length.⁴⁴

The optimal column loading was adopted from previous reports without further optimization.^{35,36} Typically, 1 μ L and 4 μ L of tryptic digest sample with a peptide concentration of 1 μ g/ μ L were directly loaded onto 25-cm column for regular gradient time and 50-cm/150-cm column for long gradient time, respectively, without using trap column to avoid dead volume affecting variations in peptide retention time, especially for the long-gradient separations at a lower nL/min flow rate. Peptide separations were performed at mobile phase flow rates of 400 nL/min for 25-cm column and 100 nL/min for 50-cm/150-cm column on the binary pump systems using 0.1% formic acid in water as mobile phase A and 0.1% formic acid in 90% acetonitrile as mobile phase B. The gradient profile is scheduled proportionally as a function of gradient length but with different sample loading time, column washing and equilibrium time. For example, for 45-min gradient time, the binary gradient was 5–15% B in 4 min, 15–25% B in 21 min, 25%–35% B in 11 min, 35–90% B in 9 min; for 300-min gradient time, the gradient profile was 5–15% B in 27 min, 15–25% B in 140 min, 25%–35% B in 73 min, 35–90% B in 60 min. The TSQ Vantage was operated in the same manner as previously described.⁴³ A single scan event was used to monitor all SRM transitions without using scheduling algorithm, 8 SRM transitions per peptide, using the following parameters: Q1 and Q3 unit resolution of 0.7 FWHM, Q2 gas pressure of 1.5 mTorr, scan width of 0.002 m/z and a dwell time of 30 ms for 45-min gradient time and 100 ms for 300-min/600-min gradient time.

Data analysis

SRM data were processed in the same way as previously described.⁴³ Signal-to-noise ratio (S/N) of target peptides was calculated by peak intensity at the apex over the highest background noise in a retention time window of ± 15 s for 45-min gradient and ± 3 min for 300-min gradient. The background noise levels were conservatively estimated by visual inspection of the chromatographic peak regions.²⁶ The LOD and LOQ were defined as the lowest concentration point of each target protein at which the S/N of surrogate peptides was at least 3 and 10, respectively. For conservatively determining the LOQ values, in addition to the requirements of the S/N to equal or be above 10, another criteria was applied: surrogate peptide response over the protein concentration must be within the linear dynamic range. The L/H SRM peak area ratio was used to generate calibration curves and evaluate reproducibility. All calibration and correlation curves were plotted using Microsoft Excel 2010. The RAW data from TSQ Vantage were loaded into Skyline software⁴⁵ to display graphs of extracted ion chromatograms (XICs) of multiple transitions of target proteins monitored.

RESULTS AND DISCUSSION

LG-SRM Protein Quantification in Non-depleted Serum

To evaluate analytical performance of LG-SRM in terms of sensitivity and peak capacity we compared 300-min LG-SRM with typical 45-min LC-SRM for quantification of target proteins at various concentrations in human female serum under optimal column conditions, i.e., loading amount and column flow rate. The gradient profile at 300 min was proportional to that at 45 min but with different loading time and column equilibrium time. It is recognized that for global proteomics an increase of the sample loading amount generally leads to higher numbers of peptide and protein identifications but may decrease peak resolution and deteriorate peak shape due to sample overloading, and peak capacity is not directly correlated with the number of peptide identifications obtained.⁴⁴ Considering that targeted proteomics is aimed at reproducible quantification of endogenous proteins, the

sample loading amount should be lower than the column loading capacity in order to maintain good peak resolution and separation efficiency for reducing background interferences. Long LC column combined with a long and shallow gradient was demonstrated to have higher loading capacity than short columns in conjunction with short gradient. For these reasons, approximately 4 μg and 1 μg of plasma peptides were loaded onto the 150-cm column at 300-min gradient and the 25-cm column at regular 45-min gradient, respectively.^{35,36}

Two proteins, bovine carbonic anhydrase and PSA, previously used for the assessment of PRISM-SRM,²⁶ were spiked into human female serum at different levels to evaluate LG-SRM performance. Following sample processing and protein digestion, the peptide samples were spiked with heavy peptide internal standards prior to LG-SRM measurements. In female serum, ELISA results showed that both the free and total PSA were below the detection limits of the immunoassay (< 0.01 ng/mL), thus the contribution of endogenous PSA concentration could be neglected in the female serum samples.⁸ Two surrogate peptides per protein were selected and for each target peptide four transitions were monitored to achieve maximum selectivity and sensitivity in the SRM assays and the best SRM transition (i.e., with the most intense SRM signal and least co-eluting interference) for each peptide was used to generate the calibration curve and estimate the reproducibility of LG-SRM assay (Table S1).

The linear dynamic range, LOD and LOQ for each surrogate peptide in female serum were evaluated with target protein concentrations ranging from 1 ng/mL to 1000 ng/mL. Assay precision (coefficient of variation, CV) of LG-SRM was evaluated by replicated analyses of samples with the protein concentration at 100 ng/mL in female serum. A side-by-side comparison of the SRM signals of surrogate peptides between LG-SRM and regular LC-SRM were performed to assess the improvement on the SRM sensitivity based on the LOD and LOQ values. Figure 1 shows XICs of transitions monitored for the peptide DFPIANGER derived from bovine carbonic anhydrase at five concentration points using conventional LC-SRM and LG-SRM, respectively. For conventional LC-SRM measurements the signals for light peptide transitions were dominated by co-eluting interferences from 5 to 100 ng/mL (Figure 1A). Clearly long-gradient separation significantly reduces background interference levels and enhances the S/N ratio when compared to conventional LC-SRM measurements (Figure 1B and Figures S2-S3), which led to the LOD and LOQ values down to 5 ng/mL and 10 ng/mL, respectively. The LOQ values obtained from the best transition for each surrogate peptide were in the range of 10 ng/mL to 250 ng/mL (Table 1). The long-gradient separation improved the overall SRM sensitivity by more than 8-fold, and up to ~100-fold depending on the peptide, when compared to conventional LC-SRM analyses (Table 1). The calibration curves of LG-SRM measurements (the best transition for each protein) showed excellent linearity for both target proteins for concentrations ranging from 5 ng/mL to 1000 ng/mL with an average CV of ~7% for LG-SRM triplicate measurements of the protein concentration point at the 100 ng/mL level (Figures 1C, S2-S3 and Table S2). Our results demonstrate that LG-SRM assay enabled quantification of both target proteins at low ng/mL levels in non-depleted human female serum with at least one surrogate peptide. Although we did not use a trap column in this work, we note that there is potential advantages of using a large i.d. trap column (i.e., 200 μm or 500 μm) to provide effective sample clean up and higher loading capacity for peptides.³⁹

Considering that extension of gradient time increases peak capacity, leading to improved sequence coverage in global proteomics, we further compared 600-min vs. 300-min LG-SRM using the same column (3 μm C18, 150 cm $\times$ 75 μm i.d.) for quantification of target proteins at the spiking levels of 5 ng/mL to 1000 ng/mL in human female serum. Compared

to 300-min gradient separation, it is not surprising that better separation was achieved at 600-min gradient with reduced background interference. However, the peak widths were broadened by nearly 1.2-fold for most target peptides and for peptide DFPIANGER, 3-fold increase in the peak width was observed, heavily diluting the SRM signal from the light peptide transitions (Figure S4). The LOQ values at 600-min gradient were in the range of 100 ng/mL to 250 ng/mL, which are higher than those at 300-min gradient. These results suggest that long-gradient separations significantly reduce matrix interference, but also with the trade-off in decreasing the SRM signals due to the peak broadening/dilution. Therefore, to achieve the best SRM sensitivity, a more thorough optimization of many separation parameters such as column loading, column ID and length, C18 particle size, and gradient time will be necessary. In this work, we consider 300-min gradient as a good compromise for achieving high sensitivity and relatively good throughput. This condition was used for targeted mass spectrometric measurements of clinical samples.

Assessment of the Potential Multiplexing Capacity of LG-SRM

To evaluate the potential multiplexing capacity of LG-SRM we manually calculated an average XIC peak width of each heavy peptide across all serial dilution samples using Xcalibur software.³³ The average peak widths at 300-min gradient lie in the range of 1.0 min to 2.0 min, at least 3 times wider than those at regular gradient (Figure 2), however the separation peak capacity for the long column at 300-min gradient time was still estimated to increase by nearly 2-fold when compared to the short column at 45-min gradient time. These results are consistent with previous reports using long-gradient separations for improving the number of unique peptide identification.^{35,37,44} Using the scheduled SRM algorithm a regular LC-SRM can monitor up to 1500 transitions in a single LC-SRM analysis.^{9,12} In principle, the wider peak width allows more transitions to be monitored in a given elution window without affecting the quality of quantification. Therefore, LG-SRM has the multiplexing capacity to monitor nearly 3 times more transitions than regular LC-SRM (i.e., up to 4500 transitions and nearly 400 proteins assuming three transitions per peptide and two surrogate peptides per protein) assuming that the same number of time segments in scheduled SRM will be used. However, the actual maximum number of transitions for the scheduled LG-SRM analysis may be reduced in some degree depending on the overall elution profile of the target peptides and run-to-run reproducibility of peptide retention time. Compared to regular gradient separations, long-gradient separations have more variations in the peptide retention time.³⁹ For example, at 300-min gradient variations in target peptide retention time were in the range of 0.13–3.41% across all serial dilution samples (a median value: 0.56%), whereas for regular 45-min gradient variations were only in the range of 0.11–1.89% with a median value of 0.30%. Improvement in the reproducibility of peptide retention time for the long-gradient separation can be achieved by using column heaters to precisely control and maintain the column temperature for minimizing retention time shifts. We are presently evaluating factors affecting the efficient, precise delivery of mobile phase at the low nL/min flow rate to the long column during the long-gradient separations, such as the mixing efficiency of two mobile phases, variations in the mobile phase delivery by the two nanopumps, and various column flow rates.

Quantification of Endogenous Proteins in Human Sera

We next applied LG-SRM to detect eight endogenous proteins in six serum samples collected from prostate cancer patients using a 50 cm column packed with 1.7 μ m C18 particles. The eight proteins were PSA, EGFR, KLK6, MMP9, POSTN, MBP, cTnT, and ENPP2, all of which were reported with plasma concentrations ranging from pg/mL to ng/mL levels.^{46–52} Approximately 1 μ L of serum (~80 μ g) from each of the six patients was subjected to trypsin digestion followed by LG-SRM measurements. The XICs showed that the LG-SRM assay enables detection and quantification of PSA at low ng/mL levels in

clinical prostate patient sera (Figure S5A). An excellent correlation ($R^2 > 0.99$) was observed between SRM-based assay and ELISA results (Figure S5B and Table S3), which is better than previous reports.^{3,24,28,53,54} This result demonstrated that LG-SRM enabled reproducible quantification of low ng/mL levels of plasma proteins. We also note that the “total PSA” results from LG-SRM were ~3 times higher than that from the ELISA assay (Figure S5C), which is consistent with our previous PRISM-SRM results (up to 60% of the PSA in serum are bound to κ -macroglobulin and therefore not immunoreactive^{28,54}).^{26,55}

The XICs in Figure 3 showed that LG-SRM enabled confident detection and quantification of 6 of the 8 endogenous proteins (PSA, POSTN, MMP9, cTnT, EGFR, and ENPP2) at ng/mL levels in these clinical serum samples (also see Figure S6 and Table S4). KLK6 with reported concentrations at 2.9–6.8 ng/mL in human plasma/serum,⁴⁶ was detected by only one surrogate peptide with the S/N ratio of 7; while for MBP with reported concentrations at 0.67 ng/mL⁵¹ (lower than the LOD of the LG-SRM assay) the SRM signal of endogenous peptides was not detected across all six patient serum samples (Figure S6 and Table S4). These results demonstrate that LG-SRM provides sufficient sensitivity for quantifying endogenous proteins at low ng/mL levels in human plasma/serum. Interestingly, one surrogate peptide, YEINVLR, from the endogenous cTnT, which was reported to have concentrations at 50–100 pg/mL levels in human serum,⁴⁷ was detected by LG-SRM with the S/N ratio of 12 in one prostate cancer patient serum with a PSA concentration at 110.8 ng/mL (see Figure S6 and Table S4). For the other prostate cancer patient sera with lower concentrations of PSA, the SRM signal from the endogenous cTnT was not detected (Figure S7). This observation suggests that plasma cTnT levels are elevated in the particular prostate cancer patient serum with a high PSA level because the reported concentration levels are well below the LOD of the LG-SRM assay. This was confirmed by the back-calculated cTnT concentration at 19.3 ng/mL assuming that the purity of crude heavy peptides is 50%.²⁶ We also note that all seven LG-SRM detected endogenous proteins except POSTN and ENPP2 were reported to be undetectable by coupling MARS Hu-14 immunoaffinity depletion with conventional LC-SRM in a recently reported large-scale quantification of human plasma proteins,⁵⁶ further illustrating the improved sensitivity of LG-SRM when compared to conventional LC-SRM.

To further enhance LG-SRM sensitivity, IgY14 immunoaffinity depletion was incorporated into the workflow to reduce the dynamic range of protein concentrations by removing 14 high-abundance plasma proteins (i.e., ~95% removal of the protein total mass)⁵⁷ and increase target analyte loading onto the analytical column. Direct comparison of the SRM signals of light peptides from endogenous proteins clearly shown that the IgY14 depletion significantly reduced matrix interference and enhanced the S/N ratio by ~4 times for most target peptides (Figures 3, S6 and Table S4), which is consistent with our previous study for comparison of PRISM-SRM with and without the IgY14 depletion.²⁶ Therefore, IgY14-LG-SRM has the potential for providing sufficient sensitivity for quantification of target proteins at the low- to sub-ng/mL level in human plasma/serum. However, we should realize that the IgY14 depletion of high-abundance proteins is often associated with potential loss of target proteins of interest either by nonspecific binding to the depletion column or by forming complexes with the bound high-abundance proteins. For example, after the IgY14 depletion the SRM signal of cTnT was not detected by LG-SRM, whereas when LG-SRM was used alone, we could detect endogenous cTnT protein in some of the prostate patient sera. This strongly suggested that cTnT was partially or fully removed by the IgY14 depletion, which is consistent with a previous report that following the IgY14 depletion only 0.5% of cTnT was recovered from human serum samples.²⁴ Since there is no prior knowledge about whether target proteins are proportionately lost in the depletion process, it is highly desirable to use LG-SRM alone for plasma protein quantification to avoid immunoaffinity depletion. However, in some cases where LG-SRM alone cannot provide sufficient sensitivity for low-

abundance target proteins, immunoaffinity depletion should serve as an effective tool to further reduce sample complexity and enhance the overall LG-SRM sensitivity provided that the targets of interest are not lost in the depletion process.

CONCLUSION

A major constraint for current targeted mass spectrometric approaches is the limited sensitivity and capacity for simultaneous quantification of hundreds of proteins. We demonstrate that long-gradient separations coupled with SRM offer both increased sensitivity and increased multiplexing capacity for large-scale targeted protein quantification. Our results show that LG-SRM enables reliable quantification of plasma proteins at low ng/mL levels in non-depleted human plasma/serum, which is comparable to most low-resolution fractionation-based SRM methods in overall sensitivity. Moreover, LG-SRM has several advantages over fractionation-based SRM in terms of sample throughput (one analysis per sample without multiple fraction runs), minute amounts of starting materials (e.g., ~4 µg), and simple implementation and easy operation. We anticipate broad applications of this simple yet effective approach for highly sensitive quantification of hundreds of cellular low-abundance proteins in systems biology studies, as well as for verifying candidate biomarkers in biofluids.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CV	coefficient of variation
cTnT	cardiac troponin T
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assays
ENNP2	ectonucleotide pyrophosphatase/phosphodiesterase 2
XIC	extracted ion chromatogram
KLK6	kallikrein 6
LOD	limit of detection
LOQ	limit of quantification
LC	liquid chromatography
LG	long-gradient
L/H	peak area ratio of light to heavy peptides
MBP	myelin basic protein

MMP9	matrix metalloproteinase 9
MRM	multiple reaction monitoring
MS	mass spectrometry
POSTN	periostin
PSA	prostate-specific antigen
S/N	signal-to-noise ratio
SRM	selected reaction monitoring

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A **B**

Figure 1.

Sensitivity, reproducibility and accuracy of LG-SRM assay. XICs of transitions monitored for DFPIANGER derived from bovine carbonic anhydrase are shown at various concentrations: (A) Conventional LC-SRM at 45-min gradient time; (B) LG-SRM at 300-min gradient time. DFPIANGER: 509.8/378.7 (*red*), 509.8/756.5 (*blue*). Internal standards were spiked at 0.5 fmol/ μ L. The blue arrows indicate the locations of expected SRM peak apex of light peptides based on the retention time of heavy internal standards. (C) Calibration curve for quantifying bovine carbonic anhydrase.

Figure 2.

Average chromatographic peak width of each isotope labeled internal standard at protein concentrations ranging from 0 ng/mL to 1000 ng/mL (4×2 , where 2 is defined as $0.613h^{33}$ of the corresponding XIC, h = peak height).

Figure 3.

Direct comparison of IgY14-LG-SRM with LG-SRM for XICs of light peptide transitions for six endogenous proteins in a prostate cancer patient serum with a PSA concentration of 110.8 ng/mL measured by ELISA. IVGGWEC_{cam}EK: 539.2/865.4 (red), 539.2/964.4 (blue), 539.2/436.2 (chestnut); LILQNHILK: 546.4/865.5 (red), 546.4/752.4 (purple), 546.4/624.4 (chestnut); AVIDDAFAR: 489.3/807.4 (red), 489.3/694.3 (purple), 489.3/579.3 (chestnut); YEINVLR: 453.8/614.4 (red), 453.8/265.1 (chestnut), 453.8/743.4 (blue); LTQLGTFEDHFLSLQR: 635.7/781.9 (red), 635.7/845.9 (blue); DIEHLTSLDFFR: 498.3/584.3 (red), 498.3/469.3 (chestnut), 498.3/784.4 (blue).

Table 1

Summary of LOD and LOQ of 2 target proteins spiked into female serum by LG-SRM and conventional LC-SRM assays.

Target protein	Surrogate peptide	LOD (ng/mL)		LOQ (ng/mL)	
		LC-SRM	LG-SRM	LC-SRM	LG-SRM
Bovine carbonic anhydrase	DGPLTGYR	500	100	2000	250
	DFPIANGER	250	5	1000	10
Prostate-specific antigen	IVGGWEC _{cm} EK ^a	100	5	1000	100
	LSEPAELTDAVK	250	5	1000	10

^a Cysteine was synthesized as carbamidomethyl cysteine.