Antibody-free, targeted mass-spectrometric approach for quantification of proteins at low picogram per milliliter levels in human plasma/serum

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Edited* by Ronald W. Davis, Stanford Genome Technology Center, Palo Alto, CA, and approved August 9, 2012 (received for review March 13, 2012)

Sensitive detection of low-abundance proteins in complex biological samples has typically been achieved by immunoassays that use antibodies specific to target proteins; however, de novo development of antibodies is associated with high costs, long development lead times, and high failure rates. To address these challenges, we developed an antibody-free strategy that involves PRISM (high-pressure, high-resolution separations coupled with intelligent selection and multiplexing) for sensitive selected reaction monitoring (SRM)-based targeted protein quantification. The strategy capitalizes on high-resolution reversed-phase liquid chromatographic separations for analyte enrichment, intelligent selection of target fractions via on-line SRM monitoring of internal standards, and fraction multiplexing before nano-liquid chromatography-SRM quantification. Application of this strategy to human plasma/serum demonstrated accurate and reproducible quantification of proteins at concentrations in the 50–100 pg/mL range, which represents a major advance in the sensitivity of targeted protein quantification without the need for specific-affinity reagents. Application to a set of clinical serum samples illustrated an excellent correlation between the results obtained from the PRISM-SRM assay and those from clinical immunoassay for the prostate-specific antigen level.

biomarker verification | systems biology | targeted proteomics

Select reaction monitoring (SRM), also known as multiple reaction monitoring (MRM), has recently emerged as a promising technology (1–16) for high-throughput mass spectrometry (MS)-based quantification of targeted proteins in biological and clinical specimens (e.g., tumor tissues). SRM has demonstrated relatively good selectivity, reproducibility (or precision), and sensitivity for a range of multiplexed protein assays (2, 4–8, 11, 17, 18) and has potential for quantifying protein isoforms (19) and posttranslational modifications (PTMs) (3, 20–22) for which good quality antibodies often do not exist. Nevertheless, a major limitation of current SRM technology is insufficient sensitivity for detecting low-abundance proteins present at sub-nanogram per milliliter levels in human blood plasma or serum and extremely low-abundance proteins in cells or tissues. Without sample prefractionation, liquid chromatography (LC)-SRM measurements have been limited to only moderately abundant proteins in human plasma present in the low microgram per milliliter range (2, 6, 8).

More recently, the combination of immunoaffinity depletion and fractionation by strong cation exchange (SCX) chromatography (4, 23), along with advances in MS sensitivity (24), has extended SRM quantification of plasma proteins to low nanogram per milliliter levels (4, 23). SISCAPA (stable isotope standards and capture by anti-peptide antibodies) coupled with SRM demonstrated quantification of target proteins in the same range, using as little as 10 μ L of human plasma (8, 25–27). SISCAPA assays have some distinct advantages over conventional immunoassays in terms of assay development and specificity, and

offer sensitivity for low nanogram per milliliter detection of plasma proteins (8, 10). However, antibody reagents with sufficient specificity for the target proteins or their surrogate peptides are generally not available, and development of such reagents is expensive and requires a long lead time.

To tackle this challenge, we developed a strategy involving antibody-free PRISM (high-pressure, high-resolution separations with intelligent selection and multiplexing) that effectively enriches target peptides for conventional LC-SRM analysis. Application of the strategy demonstrated greatly reduced background interferences and allowed for high-sensitivity SRM quantification of proteins in human plasma/serum. Herein, we show that this technique can reliably quantify low-abundance proteins at sub-nanogram per milliliter levels in plasma/serum, including prostate-specific antigen (PSA) in clinical serum samples, and with high correlation with results from ELISA.

Results

Sensitivity and Reproducibility of PRISM-SRM Assays. The concept of PRISM is depicted in Fig. 1A. High-resolution reversed-phase capillary LC (cLC) with pH 10 mobile phase is used to separate and fractionate the peptide sample into 96 fractions. High-pH reversed-phase cLC fractionation provides higher resolution and more reproducible separations than widely used SCX, as well as final enriched samples that are compatible with downstream LC-MS analyses without the need for additional cleanup (28). The approach also addresses a general drawback of fractionation strategies (i.e., the need to analyze many fractions per sample, thus limiting overall analytical throughput) by introducing the concept of intelligent selection (iSelection) and multiplexing of target fractions for subsequent LC-SRM analysis. iSelection of target fractions is accomplished by on-line SRM monitoring of the heavy-isotope-labeled synthetic peptide internal standards during the first-dimension separation. The accurate elution profiles of the internal standards allow precise determination of the locations of target peptides in the 96-well plate, thus allowing the selection of the most informative target fractions for downstream nano-LC-SRM measurements (Fig. 1 and *SI Appendix, Fig. S12*). While the on-line SRM monitoring is the most accurate strategy for locating the target peptide fractions, it is also practically feasible to use other alternative strategies to pinpoint the target fractions, such as off-line determination of the locations of target fractions and relying on the reproducibility of

Author contributions: T.S. and W.-J.Q. designed research; T.S., T.L.F., X.S., A.A.S., M.H., and F.X. performed research; R.Z., S.W., J.-S.K., N.J., R.J.M., L.P.-T., J.K., and R.D.S. contributed new reagents/analytic tools; T.S. and X.S. analyzed data; and T.S., K.D.R., T.L., K.T., D.G.C., R.D.S., and W.-J.Q. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1204366109/-DCSupplemental.

Fig. 1. Schematic diagram of the PRISM-SRM workflow. (A) PRISM workflow. Peptide sample ($\sim 20 \mu\text{g}$) spiked with internal standard (IS) heavy peptides was injected and separated by a high-resolution reversed-phase cLC system using high-pH mobile phases. The eluent from the cLC column at a flow rate of $3.3 \mu\text{L}/\text{min}$ was split into two flowing streams via a Tee union (the split ratio of flow rates is 1:10): a small fraction (9%) of the column eluent went to a triple quadrupole mass spectrometer for on-line SRM monitoring IS peptides; a large fraction (91%) of the column eluent was automatically collected every minute into a 96-well plate during a $\sim 100\text{-min}$ LC run. The specific target peptide fractions were either selected based on the same elution times of IS being monitored by the on-line SRM (*i*Selection) or multiplexed. For example, 96 fractions collected along with the first-dimension LC separation can be multiplexed into 12 fractions by combining 8 fractions from the first-dimension LC separation into 1 fraction for downstream LC-SRM analyses, such as pooling fractions 2, 14, 26, 38, 50, 62, 74, and 86 (marked in red color) into one sample (#2) for the second-dimension LC-SRM. (B) Conventional LC-SRM workflow. Following *i*Selection, a target peptide fraction was either directly subjected to nano-LC-SRM with $4 \mu\text{L}$ of sample per injection ($\sim 45 \text{ ng}$ of peptides on the nano-LC column) or multiplexed with other target fractions with a final volume of $20 \mu\text{L}$ before nano-LC-SRM analysis.

HPLC to locate target fractions from subsequent experiments. Furthermore, a limited number of target fractions eluted at different times during the first-dimension separation can be multiplexed before nano-LC-SRM (Fig. 1) to enhance the overall sample throughput. This multiplexing strategy is based on the observation that the high- and low-pH reversed-phase LC are partially orthogonal (28). Fractions eluted at early, middle, and late retention times have little overlap in their elution profiles of the second-dimension LC separation and, thus, can be effectively combined before LC-SRM (28).

The sensitivity and reproducibility of PRISM-SRM assays for plasma protein detection were evaluated by comparing SRM signals of surrogate peptides with those obtained in standard LC-SRM analyses of the same samples. IgY14 immunoaffinity depletion was applied as an initial step to remove 14 high-abundance plasma proteins (29, 30). Tryptic digests of four target proteins (bovine carbonic anhydrase, bovine β -lactoglobulin, *Escherichia coli* β -galactosidase, and PSA) were spiked into IgY14-depleted human female plasma digest (*SI Appendix, Fig. S1*) at protein concentrations ranging from 50 pg/mL to 100 ng/mL .

Following the PRISM workflow (Fig. 1A), $\sim 20 \mu\text{g}$ of plasma digest was loaded onto the cLC column for fractionation, and the eluent was split into two streams (split ratio of flow rates is 1:10). The smaller portion was used for on-line SRM monitoring, and the rest was automatically transferred onto a 96-well plate at a rate of one minute per fraction over a $\sim 100\text{-min}$ LC separation (Materials and Methods). Following *i*Selection, a given fraction of interest was either directly analyzed using LC-SRM (Fig. 1B) or multiplexed with other target peptide fractions and then analyzed using LC-SRM. Sample size for direct LC-SRM analysis (Fig. 1B) without PRISM was $1 \mu\text{g}$ of total peptides. SRM sensitivity was assessed based on limit of detection

(LOD) and limit of quantification (LOQ) values (*SI Appendix, SI Methods*).

Fig. 2A depicts extracted ion chromatograms (XICs) of transitions monitored for peptide DFPIANGER derived from bovine carbonic anhydrase at various concentrations with and without application of PRISM. Note that PRISM significantly reduces background interference levels and enhances signal-to-noise ratios (S/N) for analytes at 50 pg/mL and 1 ng/mL levels. LOQ values obtained from the best transition for each surrogate peptide from the four proteins demonstrate that PRISM improves SRM sensitivity by nearly 200-fold for six of the eight peptides (Table 1 and *SI Appendix, Tables S2 and S3*). The other two peptides (DGPLTGTYR and VDEDQPFPAVPK) showed 20- and 5-fold LOQ improvements, respectively, primarily attributable to interference from coeluting species (*SI Appendix, Figs. S2.1, S2.2, S6.1, and S6.2*).

Because of the partial orthogonality between the first-dimension high-pH and the second-dimension low-pH reversed-phase LC, we anticipated that four to eight fractions from different elution times could be multiplexed (28) without significantly affecting the second-dimension separation (*SI Appendix, Fig. S10*). To test this potential of multiplexing fractions for higher throughput analysis, following *i*Selection, six fractions including all of the eight target peptides were pooled and concentrated to a final volume of $\sim 20 \mu\text{L}$, i.e., the same volume as an original single fraction. A single LC-SRM analysis for the pooled peptide fractions produced similar SRM signals to those obtained from individual peptide fractions, as reflected by the signals of both the light and heavy peptides at two representative concentration points (50 pg/mL and 1 ng/mL) (Fig. 2B). Noted exceptions were the two most hydrophobic peptides, VYVEELKPTPEGDLE-ILLOK from bovine β -lactoglobulin and LWSAEIPNLYR from

Fig. 2 Sensitivity, multiplexing capability, reproducibility, and accuracy of PRISM-SRM assays at the peptide level. (A) XICs of transitions monitored for DFPIANGER derived from bovine carbonic anhydrase at various concentrations. IgY14 only: 509.8/378.7 (red), 509.8/546.3 (blue); IgY14 plus PRISM: 509.8/378.7 (red), 509.8/756.5 (blue), 509.8/546.3 (purple). The black arrowheads mark the peak retention times of the heavy internal standards. The blue arrows indicate the locations of SRM peaks of light peptides based on the retention time of heavy internal standards. (B) XICs of transitions monitored for two peptides representing two target proteins (bovine carbonic anhydrase and PSA) at a concentration of 50 pg/mL and 1 ng/mL levels, where all eight target peptide fractions were multiplexed before LC-SRM analysis. Internal standards were spiked at 2 fmol/ μL . DFPIANGER: 509.8/378.7 (red), 509.8/546.3 (blue); LSEPAELTDAVK: 636.8/943.5 (red), 636.8/775.5 (brown), 636.8/846.5 (purple), 636.8/472.3 (chestnut). (C) Calibration curves for quantifying bovine carbonic anhydrase and PSA. (D) Correlation curves between calculated and expected concentrations for bovine carbonic anhydrase and PSA. DFPIANGER and LSEPAELTDAVK were peptides derived from bovine carbonic anhydrase and PSA, respectively. Inset plots show the details of the low concentration range.

E. coli β -galactosidase, where the SRM signal dropped ~ 3 - and ~ 100 -fold, respectively, for pooled samples (*SI Appendix, Fig. S10*). This drop most likely resulted from hydrophobic peptide losses in pooling and sample lyophilization steps. The loss of hydrophobic peptides may be alleviated by adding isopropanol water mixture rather than water solvent into the 96-well plate.

Alternatively, selection of highly hydrophobic peptides should be avoided during assay development.

Fraction multiplexing was further assessed by spiking 42 nonhuman target peptides (21 light and 21 heavy peptides) spiked into a nondepleted human serum sample. All target peptides were spiked at a 5 attomol/ μL level ($\sim \text{ng/mL}$ level of target proteins).

Table 1. Summary of the LOD and LOQ of four target proteins in female plasma with IgY14 only and IgY14-PRISM

Proteins	Surrogate peptides	Fractionation strategies	LOD, ng/mL	LOQ, ng/mL
Bovine carbonic anhydrase	DGPLTGYR	IgY14 only	5	10
	DFPIANGER	IgY14 plus PRISM	0.05	0.5
Bovine β -lactoglobulin	VLVLDTDYKK	IgY14 only	5	10
	VYVEELKPTPEGDLEILLQK	IgY14 plus PRISM	<0.05	0.05
		IgY14 only	5	5
<i>E. coli</i> β -galactosidase	VDEDQFPAPVK	IgY14 plus PRISM	<0.05	<0.05
	LWSAEIPNLYR	IgY14 only	10	25
		IgY14 plus PRISM	0.5	5
PSA	IVGGWECamCEK*	IgY14 only	10	100
		IgY14 plus PRISM	0.05	0.1
	LSEPAELTDAVK	IgY14 only	5	10
		IgY14 plus PRISM	<0.05	<0.05

*Cysteine was synthesized as carbamidomethyl cysteine.

Two levels of fraction multiplexing were assessed, with all 96 fractions being concatenated into 12 or 6 final fractions regardless whether a fraction contains target peptides. It was observed that the multiplexing of 96 into 12 fractions still provided similar PRISM-SRM sensitivity for detecting target proteins at the levels close to the LOQ compared with the SRM measurements of each individual peptide fraction (*SI Appendix, Fig. S11 and Table S4*). The sensitivity was significantly reduced when multiplexed into a total of six final fractions because of the increased sample complexity and background interference levels. The general rule of thumb for fraction multiplexing is to combine individual fractions with relative large differences in their LC retention times, so that their elution profiles during the second-dimension LC separation will have minimum overlap. The overall results demonstrated the potential of the *i*Selection and multiplexing strategy to enhance overall PRISM-SRM throughput, which is comparable to and, in some cases, even better than that of conventional LC-SRM with limited SCX fractionation (4, 23).

The calibration curves of PRISM-SRM assays for the four target proteins demonstrated excellent linearity over the concentration range of 50 pg/mL to 100 ng/mL (Fig. 2C and *SI Appendix, Figs. S2–S9*). The reproducibility of PRISM-SRM is significantly better than that of regular SRM based on the coefficient of variation (CV) values from triplicate measurements, most likely because of improved S/N (*SI Appendix, Tables S2 and S3*). PRISM-SRM reproducibility was further evaluated by analyzing three processing replicates (1 ng/mL for each target protein in IgY14-depleted plasma). The average CV across all of the processing replicates was 5.5%, which illustrates the high precision for quantifying low-abundance proteins in human plasma (*SI Appendix, Table S3*).

Although SRM assays do not provide accurate absolute quantification of target protein unless calibrated against protein standards (to account for the issues associated with digestion efficiency and sample losses), the exact amount of light surrogate peptides derived from target proteins can be determined based on the ratio of peak areas of light to heavy peptides (L/H) when heavy synthetic peptides (internal standards) are spiked at known concentrations. These values can be used to estimate target protein concentrations to evaluate the recovery and accuracy of SRM assays (4, 8, 31). We calculated protein concentrations for the four target proteins based on the L/H area ratio of the best transition. All four proteins except *E. coli* β -galactosidase displayed a good correlation between calculated and expected protein concentrations with an approximate 1:1 molar ratio (Fig. 2D and *SI Appendix, Table S3 and Fig. S5.4*), which illustrates the high accuracy achievable using PRISM-SRM assays. However, calculated concentrations for *E. coli* β -galactosidase deviated significantly from expected concentrations below 1 ng/mL, most likely because of substantial background interferences (*SI Appendix, Fig. S7.4 and Table S3*).

Detection of PSA in Female Serum Spiked at the Protein Level. Assay sensitivity was further evaluated by measuring PSA spiked at known concentrations into human female serum before IgY14 depletion at the protein level, which allowed us to account for possible bias introduced by peptide-level spiking. The PSA peptide LSEPAELTDAVK was readily detected in all female serum samples that were spiked with PSA at 50 pg/mL or higher concentrations (Fig. 3). These results further illustrate the high sensitivity of the PRISM-SRM assay. At 300 pg/mL or higher concentrations, SRM signals for a lower intensity transition (636.8²⁺/846.5⁺ from LSEPAELTDAVK) were also clearly observed (Fig. 3A and *SI Appendix, Fig. S15*). To evaluate both reproducibility and protein/peptide recovery, we prepared three processing replicate samples, and each processed sample was analyzed with three technical replicates. The median CV across all concentrations was ~10%, indicating good reproducibility during sample processing and fractionation (*SI Appendix, Table S6*). The calibration curve from the best PSA peptide transition demonstrated excellent linearity over a concentration range of

Fig. 3. Sensitivity, reproducibility, and accuracy of PRISM-SRM assays at the protein level. (A) XICs of transitions monitored for LSEPAELTDAVK derived from PSA with IgY14 depletion coupled with PRISM along with internal standard at 2 fmol/μL. LSEPAELTDAVK: 636.8/943.5 (red), 636.8/846.5 (blue). (B) Calibration curve for quantifying PSA in female serum. (C) Correlation curve between calculated and expected concentrations for PSA in female serum. Inset plots show the details of the low concentration range.

0.3–10.0 ng/mL (Fig. 3B). However, the surrogate peptide response over the PSA concentrations becomes nonlinear at concentrations <0.3 ng/mL and then reaches a plateau, which can be attributed to background interference in the SRM measurement. This background likely arises from either light-peptide impurities in the heavy-synthetic-peptide standard or endogenous PSA in the female serum.

Having demonstrated the high accuracy of PRISM-SRM for quantifying PSA in female plasma at the peptide level (nearly 1:1 correlation between calculated and expected PSA concentrations) (Fig. 2D), we were able to estimate PSA recovery during sample processing at the protein level. Protein-level PSA recovery following IgY14 depletion, trypsin digestion, and sample cleanup was ~13%, which was determined by dividing the L/H area ratio at the protein level by that at the peptide level at a PSA concentration of 10 ng/mL (*SI Appendix, SI Methods*). This recovery is consistent with the measured recovery of PSA protein in human plasma flowing through an IgY12-depletion column (4). Using the PSA protein recovery during sample processing and the L/H ratio from the PRISM-SRM measurement, we calculated PSA concentrations at the protein level (*SI Appendix, SI Methods*); these calculated concentrations, again, correlated well with the expected PSA concentrations (Fig. 3C). The measured PSA concentrations were further compared with results from ELISA measurements (*SI Appendix, Tables S7 and S8*). The L/H area ratio correlated well ($r^2 = 0.9973$) with the PSA concentrations determined by ELISA (*SI Appendix, Fig. S15.4 and Table S8*). These findings illustrate the robustness of the PRISM-SRM assay in terms of high accuracy and precision for quantifying PSA concentrations in serum extending to 50–100 pg/mL.

LC-SRM Analysis. Following *i*Selection and multiplexing of target peptide fractions, the final fraction of interest was subjected to LC-SRM analysis. All peptide fractions were analyzed using a nanoACQUITY UPLC system coupled on-line to a TSQ Vantage triple quadrupole mass spectrometer (Thermo Scientific), with details described in *SI Appendix, SI Methods*. Briefly, solvents consisted of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in 90% acetonitrile (vol/vol, mobile phase B). Peptide separations were performed at a flow rate of 400 nL/min, using an ACQUITY UPLC BEH 1.7- μ m C18 column (75 μ m internal diameter $\times$ 25 cm; Waters).

Data Analysis. SRM data acquired on the TSQ Vantage were analyzed using Xcalibur 2.0.7 (Thermo Scientific). The two most abundant transitions for each peptide were used for quantification (*SI Appendix, Table S1*). The RAW data from TSQ Vantage were loaded into Skyline software (15) to create high-resolution figures of XICs of multiple transitions monitored for target

proteins. Detailed criteria for peak detection and integration and LOD and LOQ determination were described in *SI Appendix, SI Methods*.

ACKNOWLEDGMENTS. We thank Drs. Lori Sokoll and Daniel Chan at Johns Hopkins University for providing the clinical patient serum samples and corresponding immunoassay results and Dr. Arul Chinnaiyan at the University of Michigan for providing the sequences of TMPRSS2-ERG fusion constructs. Portions of this work were supported by National Institutes of Health (NIH) Director's New Innovator Award Program DP2OD006668; NCI Early Detection Research Network Interagency Agreement Y01-CN-05013-29; NIH Grants 8P41 GM103493, 5P41 RR018522, CA111244, and U24-CA-160019; and a US Department of Energy (DOE) Early Career Research award. The experimental work described herein was performed in the Environmental Molecular Sciences Laboratory, a national scientific user facility sponsored by DOE/Biological and Environmental Research (BER) and located at Pacific Northwest National Laboratory, which is operated by Battelle Memorial Institute for the DOE under Contract DE-AC05-76RL0 1830.

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