An Automated and Multiplexed Method for High Throughput Peptide Immunoaffinity Enrichment and Multiple Reaction Monitoring Mass Spectrometry-based Quantification of Protein Biomarkers*[S]

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There is an urgent need for quantitative assays in verifying and validating the large numbers of protein biomarker candidates produced in modern "-omics" experiments. Stable isotope standards with capture by anti-peptide antibodies (SISCAPA) has shown tremendous potential to meet this need by combining peptide immunoaffinity enrichment with quantitative mass spectrometry. In this study, we describe three significant advances to the SISCAPA technique. First, we develop a method for an automated magnetic bead-based platform capable of high throughput processing. Second, we implement the automated method in a multiplexed SISCAPA assay (nine targets in one assay) and assess the performance characteristics of the multiplexed assay. Using the automated, multiplexed platform, we demonstrate detection limits in the physiologically relevant ng/ml range (from 10 μl of plasma) with sufficient precision (median coefficient of variation, 12.6%) for quantifying biomarkers. Third, we demonstrate that enrichment of peptides from larger volumes of plasma (1 ml) can extend the limits of detection to the low pg/ml range of protein concentration. The method is generally applicable to any protein or biological specimen of interest and holds great promise for analyzing large numbers of biomarker candidates. ***Molecular & Cellular Proteomics 9:184–196, 2010.***

The current gold standard for quantifying protein biomarkers is the ELISA. A well functioning ELISA can be run at high throughput and has excellent sensitivity; however, the cost associated with development is very high, the lead time is very long, and the failure rate can be high. In addition, sandwich immunoassays are subject to potential interference from endogenous antibodies (1). Unfortunately, there are no quantitative assays available for the majority of biomarker candidates, and a considerable investment is required to generate assays *de novo*, creating a bottleneck in the biomarker pipeline (2, 3).

A technique that has shown potential for bridging the gap between discovery and validation of biomarkers is stable isotope standards with capture by anti-peptide antibodies (SISCAPA)[1] (4) coupled to multiple reaction monitoring (MRM) MS. SISCAPA has several advantages over other immunoassays in that the mass spectrometer provides excellent specificity for the analyte of interest; the sample (including endogenous immunoglobulins) is digested to peptides, avoiding potential interference from endogenous antibodies; and precise, relative quantification is possible via the use of an internal standard. Additionally, although it is very difficult to combine multiple analytes into one assay (*i.e.* multiplex) using ELISAs, SISCAPA assays can in theory be highly multiplexed as many analytes can be measured from a single enrichment step. To date, individual SISCAPA assays have been successfully configured to a number of analytes (4–9), and up to three peptides have been enriched simultaneously (7, 8). In this study, we sought to advance the utility of SISCAPA for testing large numbers of biomarker candidates in large numbers of patient samples by automating the method to improve throughput and performance, testing the performance of multiplexing analytes, and improving sensitivity.

EXPERIMENTAL PROCEDURES

Materials—Stable isotope peptide standards were obtained from Sigma as the absolute quantification paired reagents, including purification by HPLC and quantification by amino acid analysis. The stable isotope label (^{13}C,^{15}N) was incorporated at the lysine or arginine position, resulting in a mass shift of +8 or +10 Da, respectively. Dynabeads® Protein G magnetic beads were obtained from Invitrogen. An ELISA kit for osteopontin (product number DY441) was obtained from R&D Systems (Minneapolis, MN). Solvents and chemical reagents were obtained from Fisher.

Generation of Anti-peptide Antibodies—Tryptic peptide sequences with a C-terminal linker (Gly-Ser-Gly-Cys) were conjugated to a carrier

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[1] The abbreviations used are: SISCAPA, stable isotope standards with capture by anti-peptide antibodies; MRM, multiple reaction monitoring; LOD, limit of detection; LOQ, limit of quantification; CV, coefficient of variation; OPN, osteopontin.

protein (keyhole limpet hemocyanin) and used as antigens for immunization. Two rabbits were immunized, and one rabbit with higher antibody titer (based on ELISA) was chosen as the source of polyclonal antiserum. Polyclonal antibodies were affinity-purified on peptide-agarose conjugates. The concentration of purified antibody was determined by Bradford assay.

Plasma Digestion—A pool of mouse plasma obtained from Sigma (catalog number P9275) was used as a matrix for immunoaffinity enrichment experiments. 9 M urea, 300 mM Tris, pH 8.0, and 500 mM DTT solutions were added to a pool of 5 ml of plasma (for individual sample digestions, 10-μl aliquots were used) for a final concentration of 6 M urea and 20 mM DTT. The plasma was incubated for 30 min at 37 °C, and a 500 mM iodoacetamide solution was added for a final concentration of 40 mM iodoacetamide and incubated for another 30 min at room temperature in the dark. Before addition of trypsin, the urea concentration in plasma was diluted with 100 mM Tris, pH 8.0 to a final concentration of 0.55 M urea. Sigma trypsin (L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated, catalog number T1426) was prepared at 1 μg/μl in 100 mM Tris, added to plasma with gentle mixing to achieve a 1:50 enzyme/substrate ratio, and incubated at 37 °C for 16 h. To quench the trypsin activity after digestion, concentrated formic acid was added for a final concentration of 1% (v/v). The plasma digest was desalted on a Supelco DSC-18 column. The cartridge was conditioned with 3 × 10 ml of 0.1% formic acid in 80% acetonitrile and equilibrated with 4 × 10 ml of 0.1% formic acid in water. The plasma digest was applied to the cartridge at a low flow rate to ensure maximum binding. The cartridge was washed with 0.1% formic acid in water four times. The digest was eluted with 10 ml of 0.1% formic acid in 80% acetonitrile two times. The plasma digest was dried by vacuum centrifugation and resuspended in PBS to the original plasma volume. The pH of the digest was adjusted to pH 7.4 using 2 M Tris, pH 9.0 prior to peptide immunoaffinity enrichment experiments.

Antigen Capture and Elution—Enrichment experiments were performed in 96-well plates. For the capture experiment, 10 μl of plasma digest was added to a sample well along with analyte peptides and 1 μg of anti-peptide antibody. The final volume of the sample was adjusted to 100 μl with PBS + 0.03% CHAPS. For multiplexed experiments, 1 μg of anti-peptide antibody was added for each analyte in the mixture (total, 9 μg of antibody). Once the antibody was added to the sample, the plates were allowed to incubate overnight (~16 h) at 4 °C. A KingFisher (Thermo Fisher, Waltham, MA) magnetic particle processor with a PCR magnetic head was used for all bead handling associated with peptide immunoaffinity enrichment experiments (see supplemental information for a detailed description of the automation method). 25 μl of Protein G-immobilized magnetic beads were loaded per well of a 96-well plate and washed with PBS + 0.03% CHAPS for 5 min. The beads were transferred to the binding plate (from overnight incubation of antibody) and mixed for 1 h. The beads were passed to the next position for a 1-min wash in PBS buffer + 0.03% CHAPS. The wash step was repeated in the next two positions for a total of three washes. For the final wash, the PBS was diluted 1:10 to reduce the salt concentration. Finally, beads were moved to the elution plate containing 13 μl of 5% acetic acid with 0.03% CHAPS and were incubated for 5 min. The elution plate was covered with adhesive foil and frozen at −80 °C until analysis by mass spectrometry.

Nanoliquid Chromatography-Mass Spectrometry—An Eksigent 2DLC system (Eksigent Technologies, Dublin, CA) equipped with a nanoautosampler was used for liquid chromatography. Solvents used were water, 0.1% formic acid (mobile phase A) and 90% acetonitrile, 0.1% formic acid (mobile phase B). Samples were loaded onto a trap column (0.3 × 5 mm, LC Packings PepMap Acclaim C_{18}) for 5 min at 3 μl/min with 3% mobile phase B. For elution, the trap was connected in line with a 0.075 × 100-mm PicoFrit (New Objective, Woburn, MA) column packed with 3-μm ReproSil C18-AQ particles (Dr. Maisch). The LC gradient was delivered at 300 nl/min and consisted of a linear gradient of mobile phase B developed from 3 to 40% B in 10 min. At the end of the run, the trap column was back-flushed with 3% mobile phase B for 5 min at a flow rate of 3 μl/min. The nano-LC system was connected to a hybrid triple quadrupole/ion trap mass spectrometer (4000 QTRAP, Applied Biosystems, Foster City, CA) equipped with a nanoelectrospray interface operated in the positive ion mode. Typical instrument settings included a spray voltage of 2.3 kV, an ion source temperature of 150 °C, a GS1 (nebulizer gas) setting of 12, and curtain gas setting of 15. Optimum transitions and parameters for declustering potential, entrance potential, collision energy, and cell exit potential were determined for each peptide by infusion of a solution of 1 pmol/μl peptide (see supplemental Table 1). Three transitions for each peptide were selected for monitoring. Retention times for the peptides were determined by analyzing a standard solution. Scheduled MRM transitions were inputted to Analyst 1.5 using a retention time window of 80 s and a desired cycle time of 0.5 s (minimum dwell time, 10 ms), enabling sufficient points across a peak for accurate quantitation.

Data Analysis—MRM data acquired on the 4000 QTRAP were analyzed by MultiQuant v1.1 (Applied Biosystems). Typical integration settings were a smoothing width of three points and a peak splitting factor of 2. Peak integrations were reviewed manually, and transitions from analyte peptides were confirmed by the same retention times of the light synthetic peptides and heavy stable isotope-labeled peptides. Linear regression was performed in MultiQuant on the peak area ratio *versus* spiked peptide mole ratio using $1/x^2$ weighting to construct response curves. The most abundant transition was selected as the "quantifier" transition to be used in quantitative and statistical analyses. The limits of detection (LODs) and limits of quantification (LOQs) were calculated from the response of a triplicate analysis of a blank sample (light peptide + endogenous peptide signal only, no heavy isotope-labeled peptide added). The LOD was determined by the average response of the heavy peptide in the blank plus 3 times the standard deviation of the noise in the blank (determined by integrating the detector response at the time of expected peptide elution). Similarly, the LOQ was defined as the average response plus 10 times the standard deviation of the noise in the blank. Percent coefficient of variation is expressed as the percent relative standard deviation (standard deviation divided by the mean). Peptide concentration was calculated using a single point calibration (*i.e.* response relative to spiked internal standard), assuring the measured ratio was within the linear range determined by the response curve.

RESULTS

Selection of Peptides—We sought to partially automate the current SISCAPA protocol, to assess the effects of multiplexing on SISCAPA assay performance, and to assess whether capturing from larger plasma volumes results in improved sensitivity of SISCAPA assays. We targeted 15 proteins from our previous murine biomarker discovery efforts (5). Proteotypic peptides for each protein were selected based on several criteria, including uniqueness to the protein of interest, reasonable ionization efficiency and fragmentation based on empirical MS/MS data, and no known sites for post-translational modifications. (Peptide antigenicity based on *protein* properties was not considered because secondary protein structure is less likely to affect the choice of a peptide in SISCAPA as enrichment is performed on digested proteins.)

TABLE I

Protein targets, their respective proteotypic peptides, and performance of individual and multiplexed SISCAPA assays

Recovery is expressed as a percentage. S.D. refers to one standard deviation of the mean. NA, not applicable.

Description	Peptide	Individual assay		Multiplex assay	
		Recovery	S.D.	Recovery	S.D.
		%		%	
Calumenin	SFDQLTPEESK	51.0	3.0	55.5	3.3
Disulfide isomerase	VEGFPTIYFAPSGDK	84.0	7.1	67.0	3.1
Fibulin-2	IGPAPAFAGDTISLTITK	13.2	3.4	25.8	1.5
Hypoxia up-regulated	LYQPEYQEVSTEEQR	75.0	37.9	63.4	6.9
Legumain	DYTGEDVTPENFLAVLR	58.5	9.1	62.0	4.2
L-plastin	YTLNILEDIGGGQK	22.0	18.3	51.2	15.1
Osteopontin	GDSLAYGLR	76.4	10.8	66.0	5.9
Plectin-8	AGTLSITEFADMLSGNAGGFR	21.9	1.8	21.9	10.2
Tumor protein D52	LGISSLQEFK	7.0	0.9	8.6	0.6
Ewing sarcoma	GDATVSYEDPPTAK	0.0	0.0	NA	NA
Ezrin	SQEQLAAELAEYTAK	0.1	0.0	NA	NA
Fibrinogen	YLQEIYNSNNQK	0.3	0.1	NA	NA
K-casein	GEKNDIVYDEQR	0.5	0.3	NA	NA
Nucleobindin	AATADLEQYDR	0.1	0.0	NA	NA
Tenascin C	VPGDQTSTTIR	0.1	0.0	NA	NA

The proteins and their respective peptides are shown in Table I. For each of the 15 peptides in Table I, light and heavy stable isotope-labeled standards were synthesized, and affinity-purified polyclonal anti-peptide antibodies were generated. The synthetic peptides were used to select transitions and optimize MS parameters for MRM experiments (see supplemental Table 1). The three most abundant transitions were chosen for each peptide with preference given to y-ions to ensure that the heavy peptide MRM transitions contained the isotope label ([^{13}C,^{15}N]lysine or [^{13}C,^{15}N]arginine).

Implementation of Automated Bead Handling Process—We have previously reported on the use of magnetic beads for peptide immunoaffinity enrichment (10), raising the possibility of a higher throughput, automated SISCAPA protocol. In addition, magnetic beads are an attractive platform for the SISCAPA enrichment because many samples can be processed in parallel. To enable analysis of a large number of samples with good repeatability, we sought to automate the sample handling during the enrichment process using a KingFisher magnetic particle processor to perform all the bead handling steps of the protocol. Among other applications, the KingFisher has been used for protein and peptide profiling in serum (11) and phosphopeptide enrichment (12). Our automation protocol is described in detail under "Experimental Procedures." Briefly, we used seven plates to accomplish several steps, including a preliminary washing of the beads in PBS, incubation and mixing of the beads with the sample, washing of the bead-antibody-peptide complex, elution of the peptide for MS analysis, and removal of the beads from the elution buffer plate. The addition of CHAPS detergent to buffers in the bead handling process was observed to facilitate dispersal of the beads and help mixing. Samples were processed in 96-well plates for high throughput parallel sample processing.

Estimation of Recovery Efficiency of Peptide Immunoaffinity Enrichment—Peptide recovery following SISCAPA capture provides a metric for optimizing and evaluating changes to the overall protocol. In addition, the recovery gives an initial assessment of the potential performance of the assay before embarking on full characterization of the assay (*e.g.* a full calibration curve). We use the peptide recovery to accomplish two goals: 1) compare the relative recoveries of each analyte and 2) compare the results of the individual assays with those from a multiplexed SISCAPA experiment.

A work flow was designed to estimate the relative recovery of each peptide following immunoaffinity enrichment (see supplemental Fig. 1 for a flow diagram of the experiment). The recovery was determined by measuring the relative amount of peptide before and after the enrichment process. A known amount of light synthetic peptide was spiked into a digest of plasma along with 100 fmol of heavy stable isotope-labeled standard. The sample was diluted and analyzed by MRM mass spectrometry to determine the amount of light peptide in the sample relative to the spiked internal standard. To a separate aliquot of plasma digest, the light peptide was spiked at the same concentration, and the sample was processed by immunoaffinity enrichment (without the addition of the heavy peptide standard). The heavy peptide standard was added to the sample following the immunoaffinity enrichment, and the ratio of light/heavy peptide was determined by MRM. Finally, the relative ratio of light/heavy peptide following the enrichment process was compared with the ratio before enrichment to estimate the recovery efficiency.

First, recovery was measured for each peptide in an individual assay (Table I). Overall, recoveries ranged from less than 1% to over 80%. The six antibodies yielding recovery less than 7% were deemed to be non-working. For three of the targets for which the antibody did not work (Ewing sarcoma (GDATVSYEDPPTAK), ezrin (SQEQLAAELAEYTAK),

and nucleobindin (AATADLEQYDR)), the affinity-purified polyclonal antibodies from a second rabbit were evaluated for capture efficiency. These antibodies also did not produce a detectable amount of peptide in the eluate, suggesting that some peptides have poor antigenicity. Those assays that showed recovery greater than 7% (nine in total) were carried forward to a multiplexed assay. Note that in the multiplexed assay an equivalent amount of antibody per peptide was added (1 μg per target; 9 μg total), and the amount of magnetic beads added was increased by the same factor ($\times$9) compared with the individual assays. This was done to ensure the same concentration ratios between the two experiments.

As shown in Table I, recoveries of disulfide isomerase (VEGFPTIYFAPSGDK), hypoxia up-regulated (LYQPEYQEVSTEEQR), and osteopontin (GDSLAYGLR) went down slightly with multiplexing (average 18%), whereas the recoveries of fibulin-2 (IGPAPAFAGDTISLTITK) and L-plastin (YTLNILEDIGGGQK) went up with multiplexing (average 115%), and the recoveries of calumenin (SFDQLTPEESK), legumain (DYTGEDVTPENFLAVLR), plectin-8 (AGTLSITEFADMLSGNAGGFR), and tumor protein D52 (LGISSLQEFK) were the same whether multiplexed or individual. Hence, where the highest sensitivity assays are required (*i.e.* when measuring analytes near the LOQ), further optimization of individual assays may be necessary to optimize assay sensitivity, and it may be beneficial to multiplex assays associated with similar optimization parameters (*e.g.* capture volume, capture and elution buffer, etc.) Nonetheless, based on the data in Table I, we were satisfied that overall the recovery efficiencies for the 9-plex panel was sufficient for further assay characterization as described below.

Performance Characteristics of Multiplexed SISCAPA Measurements—To determine the performance characteristics of the automated, multiplexed method, we assessed the linear range, limits of detection and quantification, and precision for each analyte by constructing a response curve in a pooled reference mouse plasma matrix. Some analytes were expected to yield a response due to endogenous protein in the sample prohibiting direct determination of the lower detection limits and complete linear range; therefore, we spiked in the heavy stable isotope-labeled synthetic peptide at varying concentrations (0.064–1000 fmol) to be used as the analyte while spiking in light synthetic peptide at the same concentration in each sample (100 fmol) to be used as internal standard. This allowed for response curves to be generated to characterize the lower limits of detection/quantitation and the full linear range of response in the matrix of interest by eliminating peptide interference from the endogenous peptides. Subsequent calculations of peptide concentration were based on a single point calibration (*i.e.* relative to the internal standard), assuring that the measured ratio was within the linear range. This assumes the responses of light and heavy synthetic peptides are equivalent. The enrichment process was performed in triplicate for each concentration, and the entire curve was performed on three independent days to evaluate repeatability.

The KingFisher platform was used to automate processing of the samples in 96-well plate format prior to analysis by LC-MRM mass spectrometry. For each sample, nine peptides were spiked into the plasma digest and enriched using the anti-peptide antibodies. The peak area ratio of heavy stable isotope-labeled peptide to light synthetic peptide was plotted to obtain a response curve. For each curve, the average concentration was calculated from repeats across three separate days and plotted for each analyte. The response curves for the multiplexed assay are shown in Fig. 1*a* as a plot of the (back-calculated) measured concentration of each analyte as a function of the expected peptide concentration based on the spiked peptide level (response curves for the individual analytes are plotted in supplemental Fig. 2; note that each of the three transitions per analyte generated very similar curves, indicating no matrix interference and good specificity using the selected transitions). For reference, Fig. 1*b* shows the response curves for the same analytes converted to protein concentration (in ng/ml) in plasma based on the peptide amount and molecular mass of the protein, assuming there is no loss to incomplete digestion.

Overall, the dynamic range of response was about 3–4 orders of magnitude with excellent linearity ($R^2 > 0.99$). Parameters for the linear regression of each analyte are provided in supplemental Fig. 2. The precision (expressed as percent coefficient of variation, %CV) for the replicate captures from all 3 days is presented in Table II. Overall, the median CV was 12.6%. Of all the interday data, 84% of the values had a measured CV lower than 25%. Generally, the variation appeared to be highest near the LOQ. For example, the %CV for calumenin (SFDQLTPEESK) and legumain (DYTGEDVTPENFLAVLR) tends to be higher at lower concentrations.

LODs and LOQs were defined as the mean plus three standard deviations and 10 standard deviations, respectively, of the response measured in the blank runs (internal standard peptide added to plasma and processed by SISCAPA). Table III shows the calculated LOD and LOQ for each analyte enriched from 10 μl of plasma. Overall, the values were excellent for most analytes, permitting quantification in the pg/ml range of peptide concentration, corresponding to the ng/ml range of protein concentrations (assuming complete trypsin digestion). Comparison of these levels with the performance characteristics reported in Table II shows acceptable precision determined at or near the LOQ, empirically validating the LOQ for most analytes. Indeed, some analytes show good precision ($<$25%) at lower levels than that determined statistically to be the LOQ. For example, the LOQ for calumenin (SFDQLTPEESK) was calculated to be 2.4 ng/ml protein but was measured with acceptable performance at 1.2 ng/ml. The LOD and LOQ levels correspond to femtomole to subfemtomole levels of peptide enriched from plasma and loaded on column,

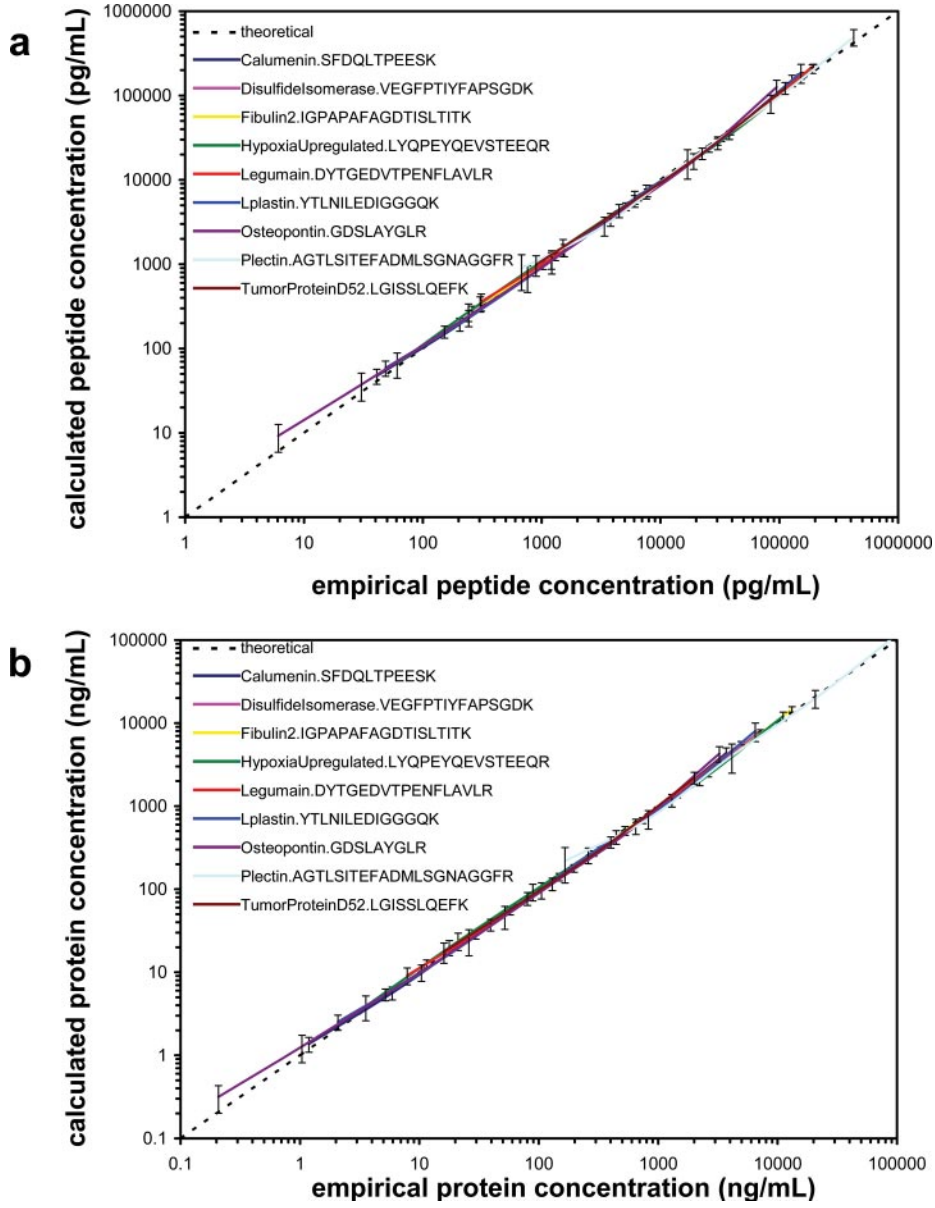

FIG. 1. **Response curves for multiplexed SISCAPA assay.** The ion signals for varying amounts of heavy stable isotope-labeled peptide were measured relative to the signal of the light peptide channel. *a*, the peptide concentrations are calculated from the response curve and plotted *versus* the expected peptide concentration based on the level of peptide added to the sample. *b*, protein concentrations are plotted based on the molar equivalents of the peptides determined in *a* and hence do not adjust for trypsin digestion efficiency, which could be an issue if the peptide were being released from the endogenous protein *versus* spiked-in as in this experiment. Each point was measured in triplicate on three separate days; *error bars* are ±1 S.D.

consistent with the detection capabilities of the mass spectrometer and the measured recoveries of the enrichment process. Visual inspection of the MRM extracted ion chromatograms confirm the detection limits (see supplemental Fig. 3).

To compare the sensitivity of SISCAPA enrichment with direct measurement, we spiked peptides into undepleted plasma (diluted 1:170) at a range of concentrations and directly measured their response relative to the internal standard peptide by MRM (see supplemental Fig. 4 for response curves). Table IV shows the LODs and LOQs determined directly in plasma. In general, LODs in plasma without enrichment were on the order of hundreds of ng/ml to μg/ml protein. By comparison with the values determined for peptide immunoaffinity enrichment (see Table III), the SISCAPA process offers a sensitivity improvement on the order of 100–1000-fold with a single step enrichment. Overall, the SISCAPA response curves and improvement over direct MRM measurement demonstrate that the immunoaffinity enrichment can be effectively multiplexed to isolate a number of peptide targets of interest with highly sensitive quantification capabilities and sufficient analytical precision for reproducible relative quantitation suitable for screening biomarker candidates (3).

Improvement in Limits of Detection by Enrichment from Large Sample Volumes—One potential advantage of the SISCAPA assay is the ability to enrich peptides from a large volume of plasma, theoretically lowering detection limits. To evaluate the enrichment of peptides from a large sample volume, we performed SISCAPA using peptides spiked into 1

TABLE II

Variability (reported as %CV) for multiplexed SISCAPA assay performed on 3 separate days in 10 μl of plasma

Peptide concentration (in pg/ml) refers to the spiked peptide level. Protein concentration (in ng/ml) represents the amount of protein that corresponds to a given spike level of the proteotypic peptide, assuming 100% recovery (*e.g.* complete trypsin digestion). The total %CV values are calculated using all measurements; *n* refers to the number of independent replicates.

Analyte	Peptide concentration	Protein concentration	%CV				
			Day 1	Day 2	Day 3	Total	*n*
	ng/ml	*ng/ml*					
Calumenin, SFDQLTPEESK	0.04	1.2	13.1	24.3	22.0	23.4	9
	0.2	5.9	17.1	25.6	5.9	16.0	9
	1.0	29.7	4.1	8.6	14.0	9.9	9
	5.1	148	0.8	8.7	11.6	11.3	8
	25.6	741	10.1	4.2	8.3	10.7	9
	128	3,706	6.4	1.5	0.7	15.3	9
Disulfide isomerase A4, VEGFPTIYFAPSGDK	0.3	11.5	12.3	10.4	14.3	15.5	9
	1.3	57.6	3.5	7.9	19.6	13.8	9
	6.5	288	1.9	3.8	14.1	8.5	9
	32.5	1,439	1.6	1.3	9.8	5.0	9
	163	7,197	3.7	2.3	3.0	3.8	9
Fibulin-2, IGPAPAFAGDTISLTITK	0.2	21.1	12.9	77.8	28.8	39.9	9
	1.2	105	5.8	24.4	13.1	17.1	9
	6.1	527	10.1	28.8	17.9	20.8	9
	30.3	2,636	15.2	1.9	6.7	10.0	9
	151	13,182	12.4	5.9	3.3	7.8	9
Hypoxia up-regulated, LYQPEYQEVSTEEQR	0.1	3.6	37.0	3.7	52.2	55.8	9
	0.3	17.8	17.7	39.3	18.6	36.5	9
	1.5	89.0	8.1	12.6	13.1	14.9	9
	7.6	445	9.7	11.0	5.6	13.4	8
	38.0	2,224	18.8	4.0	8.1	13.6	9
	190	11,121	12.7	2.5	2.4	7.5	9
Legumain, DYTGEDVTPENFLAVLR	0.3	7.9	14.4	9.0	20.3	16.4	9
	1.6	39.5	13.1	17.9	14.4	14.8	9
	7.8	197	4.6	4.2	11.9	8.3	9
	38.8	987	0.7	4.0	9.0	5.4	9
	194	4,937	8.8	0.3	0.9	4.9	9
L-plastin, YTLNILEDIGGGQK	0.05	2.1	4.2	14.2	15.5	16.0	9
	0.2	10.4	33.0	16.9	14.8	24.0	9
	1.2	52.0	23.3	34.8	16.8	33.5	9
	6.1	260	30.4	15.7	11.8	18.1	9
	30.4	1,300	24.3	2.6	17.3	26.0	9
	152	6,500	41.2	1.3	7.1	24.3	9
Osteopontin, GDSLAYGLR	0.03	1.9	15.9	31.2	6.5	25.9	9
	0.2	9.6	9.4	10.0	13.3	11.9	9
	0.8	48.0	1.3	11.0	16.1	11.4	9
	3.8	240	4.5	35.3	10.9	24.2	9
	19.0	1,200	2.4	1.5	10.6	37.2	9
	95.1	6,000	4.1	0.5	2.1	31.2	9
Plectin, AGTLSITEFADMLSGNAGGFR	0.7	166	74.3	21.4	55.5	68.9	9
	3.4	828	36.2	16.7	19.4	27.0	9
	16.9	4,139	69.1	26.9	20.3	52.5	9
	84.6	20,693	55.2	16.2	23.9	36.3	9
	423	103,466	39.5	15.7	19.3	25.2	9
Tumor protein D52, LGISSLQEFK	0.2	3.2	22.9	18.0	15.0	22.5	9
	0.9	16.0	24.1	45.8	19.0	31.1	9
	4.5	80.2	16.7	22.3	2.9	15.0	9
	22.4	401	27.6	1.0	9.1	15.0	9
	112	2,006	31.1	1.0	3.2	15.6	9

ml of plasma. The experiment was performed in a manner similar to that above (where peptides were spiked into 10 μl of plasma) except the concentration of peptides is lower by a factor of 100 because of the larger plasma volume. Fig. 2 shows the response curves for duplicate process replicates of enrichment from 1 ml of plasma using the multiplexed assay. Fig. 2*a* shows the response curve for measured peptide concentrations, and Fig. 2*b* plots the equivalent protein concentrations based on the molar amount of peptide (assuming no losses during trypsin digestion). Performance characteristics

TABLE III

Limits of detection and quantitation for multiplexed assay performed on 10 μl of plasma

For each peptide, "fmol" corresponds to the amount in the sample, "pM" is the molar concentration, peptide concentrations are reported in pg/ml, and protein concentrations (in ng/ml) represent the amount of protein that corresponds to a given spike level of the proteotypic peptide, assuming 100% recovery (*e.g.* complete trypsin digestion).

Description	Peptide	Protein molecular weight	LOD				LOQ			
			fmol	pM	Peptide (pg/ml)	Protein (ng/ml)	fmol	pM	Peptide (pg/ml)	Protein (ng/ml)
Calumenin	SFDQLTPEESK	37,064	0.3	25	32.1	0.9	0.7	66	84.5	2.4
Disulfide isomerase	VEGFPTIYFAPSGDK	71,973	0.3	29	46.4	2.1	0.5	48	78.2	3.5
Fibulin-2	IGPAPAFAGDTISLTITK	131,818	0.3	29	44.6	3.9	0.7	69	103.7	9.0
Hypoxia up-regulated	LYQPEYQEVSTEEQR	111,209	0.5	53	100	5.9	1.3	133	251.9	14.8
Legumain	DYTGEDVTPENFLAVLR	49,373	0.6	64	124	3.1	1.5	150	289.9	7.4
L-plastin	YTLNILEDIGGGQK	65,000	0.2	16	24.6	1.1	0.4	43	65.6	2.8
Osteopontin	GDSLAYGLR	60,000	0.1	10	9.2	0.6	0.3	27	26.1	1.6
Plectin-8	AGTLSITEFADMLSGNAGGFR	517,328	2.0	196	207	101	5.2	520	550.4	269
Tumor protein D52	LGISSLQEFK	20,059	3.7	374	419	7.5	10.4	1,038	1,163	20.8

TABLE IV

Limits of detection and quantitation for multiplexed assay measured directly in plasma with no peptide enrichment

Peptide concentrations are reported in ng/ml, and protein concentrations (in μg/ml) represent the amount of protein that corresponds to a given level of the proteotypic peptide, assuming 100% recovery (*e.g.* complete trypsin digestion). The column "SISCAPA improvement" reports the difference in LOD determined by direct measurement (no enrichment) and enrichment from 10 μl of plasma (*i.e.* ratio of enriched LOD/direct LOD).

Description	Peptide	Protein molecular weight	LOD		LOQ		SISCAPA improvement
			Peptide	Protein	Peptide	Protein	
			ng/ml	*μg/ml*	*ng/ml*	*μg/ml*	
Calumenin	SFDQLTPEESK	37,064	20.1	0.6	67.1	1.9	626
Disulfide isomerase	VEGFPTIYFAPSGDK	71,973	175	7.7	583	25.8	3,769
Fibulin-2	IGPAPAFAGDTISLTITK	131,818	90.9	7.9	303	26.4	2,039
Hypoxia up-regulated	LYQPEYQEVSTEEQR	111,209	132	7.8	443	25.9	1,315
Legumain	DYTGEDVTPENFLAVLR	49,373	321	8.2	1,072	27.3	2,597
L-plastin	YTLNILEDIGGGQK	65,000	236	10.1	789	33.7	9,608
Osteopontin	GDSLAYGLR	60,000	3.6	0.2	12.1	0.8	391
Plectin-8	AGTLSITEFADMLSGNAGGFR	517,328	97.2	23.8	324	79.3	470
Tumor protein D52	LGISSLQEFK	20,059	164	2.9	548	9.8	391

of the enrichment from 1 ml of plasma were very similar to those found in the 10-μl plasma response curves. The linear range of the assay was over 3 orders of magnitude and extends well below the 1 ng/ml protein level. Table V reports the LODs and LOQs for each protein/peptide. There was significant improvement in the overall sensitivity of the method by enriching from larger volumes as limits of quantitation extend to the tens of pg/ml protein level for most targets. Several targets show 100-fold lower LOQs (*e.g.* L-plastin (YTLNILEDIGGGQK), osteopontin (GDSLAYGLR), and plectin (AGTLSITEFADMLSGNAGGFR), consistent with the difference in enriching the peptide from 10 and 1000 μl of plasma. The improvement was in the range of 20–60-fold for other targets (*e.g.* calumenin (SFDQLTPEESK), disulfide isomerase (VEGFPTIYFAPSGDK), and tumor protein D52 (LGISSLQEFK), indicating that an increase in ion suppression or binding interference from nonspecifically bound components in the plasma may require an optimization of binding and washing protocols for enrichment from large volumes. Overall, the significant improvement in detection and quantitation limits demonstrates the effectiveness of enriching from large volumes as one means to improve the sensitivity of SISCAPA.

Table VI shows the measures of precision (%CV) for the 1-ml plasma SISCAPA experiment. The variation of the measurements is consistent with captures from 10 μl as the CV over the range of concentrations determined from the duplicate measurements had a median of 9%. Similar to enrichments from 10 μl, the precision is generally lower at the lowest measured concentrations. In summary, we were successful in demonstrating the effectiveness of using immunoaffinity enrichment from a larger volume of plasma to obtain very low limits of detection and quantitation (pg/ml) while maintaining the performance of the assay.

Evaluation of Trypsin Digestion—An important step of the SISCAPA process is proteolytic digestion. The reproducibility of the digestion process is important to ensure release of the monitor peptide in a consistent manner. To estimate the reproducibility of the entire SISCAPA process, including trypsin digestion, we used the multiplexed method to determine the concentration of *endogenous* peptides in 10 μl of the standard plasma sample following three independent trypsin diges-

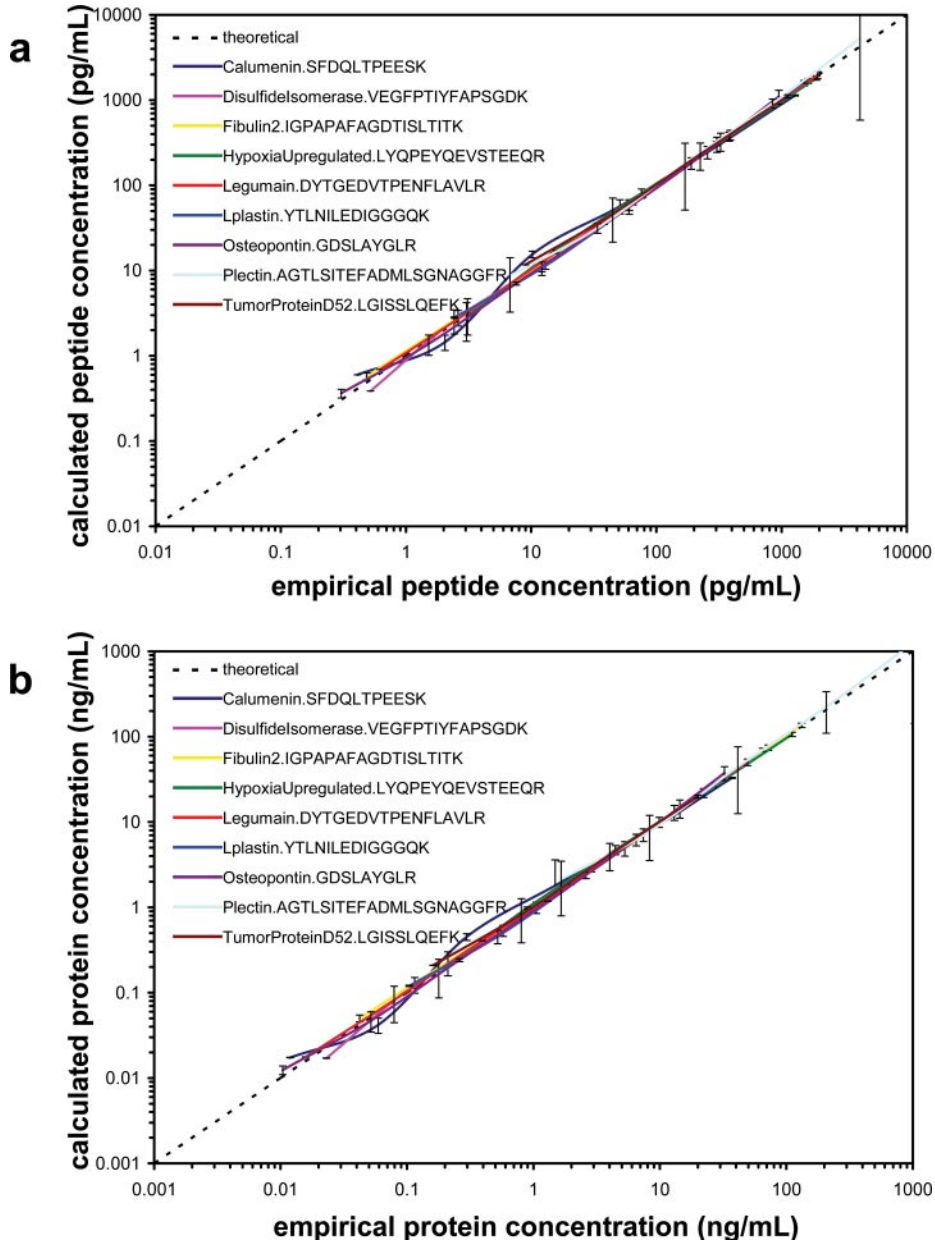

FIG. 2. **Improving overall sensitivity through enrichment from large volume of plasma.** Response curves for the multiplexed SISCAPA assay measured in 1 ml of plasma are shown. The ion signals for varying amounts of heavy stable isotope-labeled peptide were measured relative to the signal of the light peptide channel. *a*, the concentration of each peptide is calculated based on the response curve and plotted *versus* the expected peptide concentration based on the level of peptide added to the sample. *b*, protein concentrations are plotted based on the molar equivalents of the peptides determined in *a* and hence do not adjust for trypsin digestion efficiency, which could be an issue if the peptide were being released from the endogenous protein *versus* spiked-in as in this experiment. Each point was measured in duplicate; *error bars* are the range of measured values.

tions. Because in this experiment we are measuring peptides released from the *endogenous proteins* by trypsin digestion, variation in the efficiency of digestion of these proteins would increase variability in our SISCAPA-MRM measurements across multiple trypsin digestions. Individual aliquots of plasma were digested, and the endogenous peptides were enriched using the multiplexed SISCAPA assay. 50 fmol of each heavy stable isotope-labeled peptide was spiked into the plasma as a standard, and the peak area ratio was determined by MRM mass spectrometry. Seven of the nine targets showed high enough endogenous levels to be quantifiable in the sample by SISCAPA-MRM. Table VII shows the average measured peptide and protein concentrations from the three complete process replicates. Protein concentration is calculated by multiplying the measured peak area ratio by the amount of internal standard peptide (50 fmol) and converting to protein concentration, assuming there are no losses in the digestion process. Overall, the variation in the assay including digestion had a median CV of 15.9%. This does not significantly deviate from that found in the response curves (12.6%), indicating that the digestion procedure does not contribute significantly to measurement error for these proteins.

Without protein standards for each analyte, it is difficult to determine whether the peptide is quantitatively released from the protein during digestion (*i.e.* the completeness of digestion). However, we can compare the peptide-based SISCAPA measurement with the protein-based ELISA measurement (where available). A commercial ELISA was available for the

TABLE V

Limits of detection and quantitation for multiplexed assay performed on 1 ml of plasma

For each peptide, "fmol" corresponds to the amount in the sample, "pM" is the molar concentration, peptide concentrations are reported in pg/ml, and protein concentrations (in ng/ml) represent the amount of protein that corresponds to a given spike level of the proteotypic peptide, assuming 100% recovery (*e.g.* complete trypsin digestion).

Description	Peptide	Protein molecular weight	LOD				LOQ			
			fmol	pM	Peptide (pg/ml)	Protein (pg/ml)	fmol	pM	Peptide (pg/ml)	Protein (pg/ml)
Calumenin	SFDQLTPEESK	37,064	0.6	0.6	0.8	22.2	1.3	1.3	1.6	47.6
Disulfide isomerase	VEGFPTIYFAPSGDK	71,973	0.4	0.4	0.7	31.5	0.9	0.9	1.5	66.4
Fibulin-2	IGPAPAFAGDTISLTITK	131,818	0.3	0.3	0.5	43.7	0.9	0.9	1.3	116.8
Hypoxia up-regulated	LYQPEYQEVSTEEQR	111,209	0.2	0.2	0.3	18.7	0.4	0.4	0.8	44.3
Legumain	DYTGEDVTPENFLAVLR	49,373	0.2	0.2	0.3	8.6	0.5	0.5	0.9	22.2
L-plastin	YTLNILEDIGGGQK	65,000	0.2	0.2	0.3	11.5	0.4	0.4	0.6	24.6
Osteopontin	GDSLAYGLR	60,000	0.1	0.1	0.1	3.6	0.1	0.1	0.1	7.2
Plectin-8	AGTLSITEFADMLSGNAGGFR	517,328	2.0	2.0	4.2	1,026	3.8	3.8	8.1	1,973
Tumor protein D52	LGISSLQEFK	20,059	24	24	27	479	59	59	66.3	1,186

osteopontin (OPN) protein. We used both ELISA and SISCAPA-MRM to measure endogenous levels of OPN protein in identical aliquots of plasma using the same procedure for evaluating the trypsin digestion; by ELISA, the concentration of OPN was 30.0 ± 1.7 ng/ml in general agreement with the SISCAPA-MRM analysis of the same sample (11.0 ± 2.9 ng/ml OPN). The lower level measured in SISCAPA may indicate that the digestion, although reproducible, is not 100% complete.

DISCUSSION

The ability to make highly multiplexed, quantitative measurements of mRNA or DNA copy number levels on a genome-wide scale (*i.e.* through nucleic acid arrays) has had a major impact on biology (13, 14), and it is reasonable to expect that having highly multiplexed, quantitative assays for a large suite of human protein forms would have an equal or greater impact (15). Currently, the ELISA is the gold standard for making quantitative protein measurements, but ELISAs are not available for the majority of human proteins in part due to the high cost, long lead time, and high failure rate for generating such assays. Although early in its development, SISCAPA-MRM is emerging as an attractive technology for highly multiplexed, quantitative measurements of human peptides and hence proteins. This is in part due to the shorter lead time, lower costs, and potentially high multiplex capacity of SISCAPA compared with ELISA as discussed below.

The relative ease of reagent procurement is an attractive feature of SISCAPA-MRM assays when compared with ELISAs. Internal standards can be chemically synthesized with relative ease, including the incorporation of multiple isotopic labels. In contrast, standards for ELISAs are generally purified recombinant proteins, the generation and characterization of which are far more costly and time-consuming.

The most costly and rate-limiting step for development of a novel SISCAPA assay is generation of an anti-peptide antibody. In this work, we attempted to develop SISCAPA-MRM assays to 15 targets using affinity-purified rabbit polyclonal antibodies. It took 6 months from immunization to affinity-purified antibody, and the success rate (defined as an associated capture efficiency >10%) was 60% per peptide (9 of 15). For the 40% of peptides that did not work (6 of 15), it is possible that the antibody does not have sufficient affinity for the peptide, that the off-rate of the antibody is too short to retain the target during sample handling, that there is cross-reactivity to high abundance peptides in the plasma that compete with the desired peptide for binding, or that the antibody recognized cross-linking moieties used in immunization and affinity purification. Of course it is also possible that the peptide was not immunogenic. "Antigenicity predictors" are often applied to select peptide immunogens when one seeks to generate an antibody that recognizes the native *protein* form; in this application, it is advantageous to select peptides in regions of the protein that are solvent-accessible (located in loops connecting secondary structure motifs or near N and C termini), which is the approach of most antigenicity predictors. However, the SISCAPA application is different in that we seek to generate antibodies that recognize linear peptide epitopes, not native proteins. Hence, commonly used antigenicity predictors may not be of use. In subsequent studies,[2] we have found that multiplexing the antibody generation process by immunizing animals with multiple peptides per protein target improves the success rate of generating an assay for every desired protein target; we are currently optimizing this process to maximize the number of immunogens that can be multiplexed while avoiding immunodominance (where one strong antigen dominates the serological response). We also note that only a single antibody per peptide is required for a SISCAPA assay, whereas two antibodies that are active under similar conditions and that do not sterically interfere with each other are required for a classical sandwich ELISA, resulting in higher costs and lead time for ELISA development compared with SISCAPA.

[2] J. R. Whiteaker, L. Zhao, E. Kuhn, M. Pope, T. Pearson, L. Anderson, S. A. Carr, and A. G. Paulovich, unpublished data.

TABLE VI

Variability (reported as %CV) for multiplexed SISCAPA assay performed on 1 ml of plasma

Peptide concentration (in pg/ml) refers to the spiked peptide level. Protein concentration (in pg/ml) represents the amount of protein that corresponds to a given spike level of the proteotypic peptide, assuming 100% recovery (*e.g.* complete trypsin digestion). The total %CV values are calculated using all measurements; all measurements are performed in duplicate.

Analyte	Peptide concentration	Protein concentration	%CV	*n*
	pg/ml	*pg/ml*		
Calumenin, SFDQLTPEESK	2.0	59.3	18.5	2
	10.2	297	6.6	2
	51.2	1,483	14.3	2
	256	7,413	12.5	2
	1,280	37,064	1.1	2
Disulfide isomerase A4, VEGFPTIYFAPSGDK	0.5	23.0	4.9	2
	2.6	115	14.2	2
	13.0	576	10.1	2
	65.1	2,879	3.9	2
	325	14,395	16.4	2
	1,627	71,973	4.9	2
Fibulin-2, IGPAPAFAGDTISLTITK	2.4	211	21.1	2
	12.1	1,055	10.3	2
	60.5	5,273	13.1	2
	303	26,364	3.4	2
	1,513	131,818	3.9	2
Hypoxia up-regulated, LYQPEYQEVSTEEQR	3.0	178	34.2	2
	15.2	890	2.3	2
	76.0	4,448	8.9	2
	380	22,242	8.5	2
	1,899	111,209	4.2	2
Legumain, DYTGEDVTPENFLAVLR	3.1	79.0	16.7	2
	15.5	395	0.2	2
	77.6	1,975	0.3	2
	388	9,875	9.1	2
	1,939	49,373	6.2	2
L-plastin, YTLNILEDIGGGQK	2.4	104	1.0	2
	12.2	520	11.9	2
	60.8	2,600	6.1	2
	304	13,000	13.3	2
	1,520	65,000	2.7	2
Osteopontin, GDSLAYGLR	0.3	19.2	0.5	2
	1.5	96.0	3.2	2
	7.6	480	7.0	2
	38.0	2,400	2.2	2
	190	12,000	6.8	2
	951	60,000	0.5	2
Plectin, AGTLSITEFADMLSGNAGGFR	3.4	828	40.9	2
	16.9	4,139	69.7	2
	84.6	20,693	46.9	2
	423	103,466	67.4	2
	2,115	517,328	58.4	2
Tumor protein D52, LGISSLQEFK	9.0	160	10.8	2
	44.8	802	32.2	2
	224	4,012	21.1	2
	1,121	20,059	2.3	2

A well characterized ELISA can be configured to run ~1000 samples in a period of about 2 days. The current SISCAPA protocol requires separate overnight digestion and incubation steps prior to mass spectrometric analysis, resulting in an overall sample processing time of about 2 days. In our study, we sought to improve throughput by using a KingFisher magnetic particle processor to perform all the bead handling steps of the protocol. Automating sample handling improves

TABLE VII

Peptide concentration (pg/ml) measured by SISCAPA in standard mouse plasma sample following three independent process replicates (trypsin digestion followed by multiplexed SISCAPA enrichment and quantitation by mass spectrometry)

Protein concentration (in ng/ml) represents the amount of protein that corresponds to the measured peptide level, assuming 100% recovery (*e.g.* complete trypsin digestion).

Description	Peptide	Protein molecular weight	Peptide	Protein	%CV
			pg/ml	*ng/ml*	
Calumenin	SFDQLTPEESK	37,064	204	5.9	35.3
Disulfide isomerase	VEGFPTIYFAPSGDK	71,973	115	5.1	23.5
Fibulin-2	IGPAPAFAGDTISLTITK	131,818	978	85.2	15.9
Hypoxia up-regulated	LYQPEYQEVSTEEQR	111,209	2,504	147	10.5
Legumain	DYTGEDVTPENFLAVLR	49,373	1,644	41.9	6.6
L-plastin	YTLNILEDIGGGQK	65,000	6,333	271	15.2
Osteopontin	GDSLAYGLR	60,000	175	11.0	19.9

throughput while preserving or even improving assay precision. The partially automated assays showed good precision (median CV, 12.6%), and automating the sample handling dramatically improved the processing throughput. With the automated method, it is possible to process 96 samples (one plate) in about 2.5 h following the overnight incubation period, making the number of samples that can be *processed* (*i.e.* prepared for analysis) on par with ELISAs. Performing the same operations manually would likely take 3–4 times longer. In addition, automation results in overall less handling time between incubation and elution (compared with manual processing), allowing for the use of antibodies with shorter off-rates. Recent work has indicated that advances in digestion techniques will dramatically shorten the time required for proteolysis (16–21). Additionally, it may be possible to shorten the incubation period for selected targets. Further development is underway to automate additional steps in the process, including serial dilutions for calibration curves and trypsin digestion, and optimize these steps to shorten the total analysis time.

Although a large number of samples can be processed, the limiting factor in *analyzing* these samples is the LC-MS. Thus, further improvements in sample throughput can be expected by optimizing the LC-MS protocol or using multiple mass spectrometers. Because the SISCAPA enrichment process results in a relatively clean sample, ion suppression effects expected from the background matrix components are minimized. Therefore, we were able to run a relatively short 10-min LC gradient analysis with trap column loading for shortened LC-MS analysis time (total run times about 45 min) and improved throughput (about 25 samples/day). New techniques in the area of nano-LC separations, such as chip-based separations (22) and fast monolithic columns (23, 24), are expected to make the technique even more suitable for high throughput. Alternatively, adapting the SISCAPA technique to quantitative MALDI mass spectrometry (25) has the potential for greatly increasing the sample throughput.

Ultimately, assays will need to be multiplexed so that large panels of protein measurements can be made in a single pass. The ability to schedule MRMs based on analyte-specific retention times results in a variable number of MRM transitions being scanned in each MS cycle and dramatically increases the maximum number of transitions (and thus peptides) that can be monitored per analysis; this also improves the sampling rate of the elution profile of a peptide. Hence, for SISCAPA-MRM, multiplexability of the assay is less limited by the mass spectrometer as detection of a large number of analytes is possible without degradation of sensitivity or specificity (26–28). Rather, for SISCAPA, the amount of beads required for the assay is currently the limiting factor for larger multiplexed groups. In this study, we demonstrate successful multiplexing of nine assays using 25 μl of beads in a small elution volume (13 μl). Larger volumes of beads make mixing problematic and increase the likelihood for nonspecific binding, contributing to increased background signal and limiting sensitivity. Changes in bead geometry (*e.g.* smaller diameter and improved binding capacity), currently under development, will enable larger surface areas per volume and potentially allow capture of more analyte. Development of a bead surface with decreased activity for nonspecific binding of peptide components in the sample may also improve detection limits by decreasing the background signal.

Another potentially significant advantage to using SISCAPA, compared with ELISA, is the exquisite specificity of the mass spectrometer. Variation in response can arise between multiple vendors' ELISAs to a common target due to recognition of different epitopes on the target protein (29). SISCAPA overcomes this problem by analyzing a specific peptide sequence; signals from other peptides are not observed. Other interferences in ELISA include the presence of autoantibodies for the target protein, the presence of anti-reagent antibodies prohibiting capture of the analyte, and other nonspecific binding contributing to the signal response (30). SISCAPA overcomes these limitations because trypsin digestion reduces any endogenous autoantibody or anti-reagent antibody interference. Furthermore, once detected, any non-analyte signal (*i.e.* matrix interference) in the mass spectrometer can be

eliminated by choosing another (unique) transition for the targeted peptide.

Finally, the imperfect nature of trypsin digestion, for which no current standards exist, is a source of error in using proteotypic peptides as stoichiometric surrogates of protein abundance. Incomplete tryptic digestion of parent proteins can result in underestimation of protein concentrations. One possibility is to use stable isotope-labeled *proteins* as standards in immunoaffinity enrichment coupled to quantitative mass spectrometry (31–33); however, this is also imperfect because post-translational modifications affecting trypsin digestion in the biospecimen under analysis may not be present in the recombinant protein standards.

Perhaps more critical than the completion of trypsin digestion is the reproducibility of digestion because precise relative quantification could be achieved if the digestion were incomplete, yet reproducible. Our studies indicate that the reproducibility of trypsin digestion is within the variation of the assay on the whole for the seven targets in this study with measurable endogenous levels. However, the efficiency of trypsin digestion is likely to vary considerably among proteins (19–21); hence, for each analyte, it will be critical to take measures to minimize the effects of variable digestion and to detect it when it occurs. For example, in selecting proteotypic peptides from empirical MS/MS data, one might avoid peptides that were also identified as partial cleavage products (where possible), which may indicate some inherent difficulty with trypsin-mediated release. This would allow one to minimize variation due to trypsin digestion. This approach will likely not be adequate to identify all partial digestion products (*i.e.* some peptides will be too large to analyze by mass spectrometry); thus, additionally monitoring $\geq$2 proteotypic peptides from each protein would provide independent confirmation of the stoichiometry (equivalent to having multiple enzyme-linked immunosorbent assays with different antibody pairs), serving to control for the possibility of incomplete digestion or subsequent losses. Discordant results between the two or more peptides from a given protein would indicate differences in trypsin digestion efficiency (*e.g.* perhaps due to a nearby post-translational modification), protein cleavage, or alternative splicing.

In summary, SISCAPA offers an attractive alternative to ELISA for building large numbers of quantitative assays. The performance demonstrated here is suitable for use in verifying biomarker candidates, for example, as a bridging technology to ELISA. In addition, the numerous benefits described above would make a well configured and well characterized SISCAPA assay useful in large validation studies.

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S The on-line version of this article (available at http://www.mcponline.org) contains supplemental Table 1 and Figs. 1–4.

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