Evaluation of Large Scale Quantitative Proteomic Assay Development Using Peptide Affinity-based Mass Spectrometry*

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Stable isotope standards and capture by antipeptide antibodies (SISCAPA) couples affinity enrichment of peptides with stable isotope dilution and detection by multiple reaction monitoring mass spectrometry to provide quantitative measurement of peptides as surrogates for their respective proteins. In this report, we describe a feasibility study to determine the success rate for production of suitable antibodies for SISCAPA assays in order to inform strategies for large-scale assay development. A workflow was designed that included a multiplex immunization strategy in which up to five proteotypic peptides from a single protein target were used to immunize individual rabbits. A total of 403 proteotypic tryptic peptides representing 89 protein targets were used as immunogens. Antipeptide antibody titers were measured by ELISA and 220 antipeptide antibodies representing 89 proteins were chosen for affinity purification. These antibodies were characterized with respect to their performance in SISCAPA-multiple reaction monitoring assays using trypsin-digested human plasma matrix. More than half of the assays generated were capable of detecting the target peptide at concentrations of less than 0.5 fmol/μl in human plasma, corresponding to protein concentrations of less than 100 ng/ml. The strategy of multiplexing five peptide immunogens was successful in generating a working assay for 100% of the targeted proteins in this evaluation study. These results indicate it is feasible for a single laboratory to develop hundreds of assays per year and allow planning for cost-effective generation of SISCAPA assays. Molecular & Cellular Proteomics 10: 10.1074/mcp.M110.005645, 1–10, 2011.

Highly specific and sensitive assays (e.g. immunoassays) are not available for quantifying the vast majority of human proteins, and de novo assay generation is associated with a high cost and long lead time. Consequently, although genomic and proteomic technologies are used to routinely identify many hundreds of candidate biomarkers for a given disease, very few undergo further verification and validation, which require a quantitative assay. This conundrum is likely a major contributing factor to the highly inefficient translation of candidate biomarkers into clinical use (1–3).

Multiple reaction monitoring mass spectrometry (MRM-MS) has been used for decades in clinical reference laboratories for accurate quantitation of small molecules in plasma, such as drug metabolites or metabolites that accumulate as a result of inborn errors of metabolism (4, 5). More recently, MRM-MS has been adapted to measure the concentrations of candidate protein biomarkers in plasma and cell lysates (6–11). To achieve quantitation of proteins, these larger molecules are digested to component peptides using an enzyme such as trypsin. One or more selected peptides whose sequence is unique to the target protein in that species (i.e., “proteotypic” peptides) are then measured as quantitative stoichiometric surrogates for protein concentration in the sample. Hence, coupled to stable isotope dilution methods (i.e. a spiked-in stable isotope labeled peptide standard), MRM can be used to measure concentrations of proteotypic peptides as surrogates for quantification of proteins in complex biological matrices (12, 13). The assays are specific, precise (%CV ≤ 20%) (14), multiplex-able (15), and portable across laboratories and instrument platforms (16). Thus, the MRM-based assay technology has the potential to enable large-scale verification of the hundreds of candidate biomarkers identified in “omic” experiments, thus potentially providing a bridge to clinical validation.

The utility of MRM-based technology to quantify candidate biomarkers in plasma is mitigated by the limits of quantitation of the assays. Without enrichment of the target peptides, MRM-MS is able to measure proteins present in the 100–1000 ng/ml concentration range from small volumes (1–10 μl)
of plasma (10), arguably the most challenging of human bio-
specimens because of the extraordinarily high relative abundance of a small number of proteins that impede detection of all other proteins. For quantification of candidate biomarkers present at lower concentrations in plasma, an enrichment step must be added. For example, previous studies have demonstrated the success of using limited strong cation exchange fractionation (17) or glycopeptide enrichment (18) to analyze low abundance analytes. Alternatively, targeted enrichment can be performed using antipeptide antibodies in SISCAPA assays (“stable isotope standards and capture by antipeptide antibodies”) (19). Coupling SISCAPA to MRM-MS, it is feasible to measure candidate protein biomarkers present in plasma at concentrations of low ng/ml using 10 μl plasma and to the low pg/ml range by increasing the capture volume (20).

SISCAPA has been demonstrated in a variety of platforms and settings (19–25). However, to date SISCAPA has only been implemented in small efforts aimed at producing working assays for a limited number (<10) of targets. These studies have demonstrated the advantages of SISCAPA, including the ability to multiplex measurements and the capability to construct assays where the development of traditional immunoassays is difficult. Missing from these studies is an overall assessment of the technology with respect to the success rates and strategies for antibody development, the time required to implement assays on a large scale and the scalability of the approach to hundreds of target analytes. Such information is critical for gauging the feasibility of generating SISCAPA assays on a much larger scale, for instance in generating assays to hundreds of potential biomarkers or for proteome-wide investigations such as those proposed in a human protein detection and quantitation project (26). Here, we address these issues in the largest SISCAPA effort reported to date, by generating de novo a set of over two hundred individual SISCAPA assays and evaluating their performance. We describe a screening process for identifying working assays, evaluate the overall success rate of the assay pipeline, and discuss the implications for making large sets of SISCAPA assays.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bulk human plasma (K2EDTA plasma, BioReclamation Inc., Clifton, NJ) was obtained from the National Institute of Standards and Technology and stored at −80 °C. Urea, Trizma base, dithiothreitol, iodoacetamide, acetonitrile (liquid chromatography MS grade), water (liquid chromatography MS grade), phosphate buffered saline (PBS), formic acid, and 3-(3-cholamidopropyl) dimethylammonio-1-propane sulfonate (CHAPS) detergent were obtained from Sigma (St. Louis, MO). Bovine trypsin (#T-1426) was used for bulk digestion of plasma was obtained from Sigma.

**Generation of Polyclonal Antibodies**—Rabbit polyclonal antibodies were produced by Epitomics Inc., (Burlingame, CA). Selected tryptic peptide sequences with a cysteine linker at the C terminus (see **supplemental Table 1**) were conjugated to a carrier protein (KLH, keyhole limpet hemocyanin) and used as immunogens for immunization. Two rabbits were immunized and the one yielding the higher antibody titer (based on ELISA) was chosen as the source of polyclonal antiserum. Polyclonal antibodies were affinity purified from 30 ml of antiserum using peptide-agarose beads conjugated with the immunogen peptide. The concentrations of affinity-purified antibodies were determined by Bradford assay.

**Synthetic Peptides**—Synthetic peptides were obtained from GenScript (Piscataway, NJ), MIT Biopolymer Labs (Cambridge, MA), Epitomics (Burlingame, CA), 21st Century Biochemicals (Marlboro, MA), and Thermo Biopolymers (Germany). Peptide sequences were synthesized as unmodified peptides with free N-terminal and C-terminal amino acids. S-carboxymethylated versions of cysteine residues (CAM-C) were used. The purity of the synthetic peptides was >95% as measured by HPLC. For stable isotope labeled peptides, the C-terminal arginine or lysine was labeled with either [13C] or [15N] labeled amino acids. In some cases, labeled peptides were synthesized with [13C6] isoleucine or [15N6] leucine. Although uniformly [13C and 15N] labeled lysine and arginine were the preferred stable isotope label, the choice to use other labels was influenced by price and availability. Peptide stock concentrations were determined by amino acid analysis (AAA) at Dana Farber Cancer Institute (Boston, MA), AAA Service Laboratory (Damasco, OR), or New England Peptide (Gardner, MA).

**Peptide Quality Control**—Peptide quality control analysis was performed using an Agilent 1200 capillary flow system (Agilent, Santa Clara, CA) with a diode array UV detector interfaced to a 4000 QTRAP mass spectrometer (ABSciex, Foster City, CA). The mobile phase A consisted of 0.01% trifluoroacetic acid and 0.05% acetic acid in water and mobile phase B consisted of 90% acetonitrile plus 0.01% trifluoroacetic acid and 0.05% acetic acid in water. Twenty-five picomoles of each peptide were injected onto a 150 × 0.5 mm Zorbax 300 SB C18 column (3.5 μm particle size, Agilent) and eluted at 10 μl/min by the following gradient method: 0% B (0–5 min), 0–50% B (5–55 min), 50–100% B (55–57 min), 100% B (57–61 min), 0% B (61–80 min). 4000 QTrap analysis consisted of one full scan MS followed by one enhanced resolution scan MS followed by three data dependant MS/MS scans with rolling collision energy. Dynamic exclusion parameters included repeat count 2, repeat duration 30 s, exclusion list size 100, and exclusion duration 180 s. MS/MS data were searched by SpectrumMill (Agilent) using a peptide database and “no enzyme” search mode to facilitate detection of truncated forms of the peptides. Variable modifications to methionine (oxidation) and glutamine (pyroglutamic acid) were also included. The data were examined for presence of the correct peptide sequence, for the identification of any impurities, and for analyte retention times. Peptide fragmentation data were assembled in a spectral library using Skyline (27) for MRM method development.

**Plasma Digestion**—Pooled human plasma (100 ml) was thawed and added to 72 ml of solid urea (final conc. 6 M). The mixture was heated to 55 °C for 15 min with shaking. Two milliliters of 1 M Tris (pH 8) and 0.61 g dihydroxyacetone (final concentration 20 mM) were added and then water was added to a final volume of 200 ml. The mixture was incubated at 55 °C for 45 min, then removed from the shaker and allowed to stand at ambient temperature for 15 min. To alkylate the sulfhydryls, 1.85 g of iodoacetamide was added (final concentration 50 mM), and the mixture was stirred briefly and placed in the dark for 30 min under ambient conditions. Tris buffer (900 ml of 10 mM Tris, pH 8) was added and the solution was split into two 500-ml centrifuge tubes. The final pH was adjusted to 8.0 with 6 M sodium hydroxide (NaOH). Bovine trypsin (50 mg) was added to each centrifuge tube, and following rapid mixing the tubes were incubated overnight at 37 °C. The following day, the pH was re-adjusted to pH 8 with 6 M NaOH, another 50 mg of bovine trypsin was added to each tube, and the mixture was further incubated for 4 h at 37 °C. Following incuba-
tion, 0.5 ml of neat formic acid was added to stop the digestion. The digested plasma was desalted using 6-g Oasis HLB cartridges (Waters, Milford, MA) on a vacuum manifold. The cartridges were washed with 40 ml of 75% acetonitrile/0.1% formic acid and equilibrated with 40 ml of 0.1% formic acid. Separate 90 ml amounts of digested plasma were diluted to 250 ml with 0.1% formic acid and loaded onto individual cartridges followed by another 250 ml of 0.1% formic acid for rinsing the tube and cartridge. The bound peptides were eluted from the cartridge with 75% acetonitrile/0.1% formic acid. Finally, the peptide eluates were pooled, aliquotted, and speed-evaporated for storage at −80 °C. Aliquots of plasma digest were resuspended in PBS for use in SISCAPA experiments.

**Peptide Immunoaffinity Enrichment**—Peptide enrichment experiments were performed in 96-well plates (Thermo Kingfishe #97002540). Plasma digest (10 µl) was added to a sample well along with a mixture of all spiked analyte peptides and 1 µg antipeptide antibody for each analyte in the mixture (total 10 µg antibody). The final volume of the sample was adjusted to 100 µl with PBS + 0.03% CHAPS. Once the PBS and CHAPS were added to the sample, the plates were allowed to incubate overnight (~16 h) at 4 °C. A 25 µl suspension of Protein G-coupled magnetic Dynabeads (2.8 µm, #100040, Invitrogen, Carlsbad, CA) beads were washed with PBS + 0.03% CHAPS for 5 min and transferred to each sample well. A KingFisher magnetic particle processor (Thermo Fisher, Waltham, MA) with a PCR magnetic head was used for all bead handling. The beads were transferred to the binding plate (from overnight incubation of antibody) and mixed for 2 hours. 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, the magnetic 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 containing the eluted peptides was covered with adhesive foil and frozen at −80 °C until analysis by mass spectrometry. Passenger peptide determination was performed in the same multiplexed groups of ten by treating the pooled antibodies (washing 1 µg of each antibody (10 µg total) with 5% acetic acid) and analyzing the eluate by liquid chromatography (LC)-MRM-MS.

**Nano-Liquid Chromatography-Mass Spectrometry**—An Eksigent two-dimensional-LC system (Eksigent Technologies, Dublin, CA) equipped with a nano autosampler 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). Ten microliters of the samples were loaded onto a trap column (0.3 mm × 5 mm, PepMap Acclaim C18, LC-Packings, Sunnyvale, CA) for 5 min at 3 µl/min with 3% mobile phase B. For elution, the trap was connected inline with a 0.075 mm × 100 mm PicoFrit (New Objective, Woburn, MA) column packed with 3 µm ReproSil C18-AQ particles (Dr. Maisch, Germany). The LC gradient was delivered at 300 nL/minute and consisted of a linear gradient of mobile phase B developed from 3–40% 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/minute and the analytical column was re-equilibrated at 3% B at 400 nL/minute and consisted of a linear gradient of mobile phase B developed from 3–40% 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/minute and the analytical column was re-equilibrated at 3% B at 400 nL/minute.

The nano-LC system was connected to a hybrid triple quadrupole/ion trap mass spectrometer (4000 QTRAP, ABSciex, Foster City, CA) equipped with a nano electrospray 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 15. Parameters for declustering potential and collision energy were determined in two ways. For a portion of peptides, the parameters were determined empirically by infusing 1 pmol/µl of peptide standard and manually optimizing declustering potential and collision energy. For most peptides, optimum values were taken from a linear regression of previously optimized values in the Skyline software (27). Scheduled MRM transitions were entered to Analyst 1.5 using a retention time window of 90 s and a desired cycle time of 0.5 s, enabling sufficient points across a peak for accurate quantitation. A minimum of three transitions (six total per peptide pair) were recorded for each light and heavy peptide.

**Data Analysis**—MRM data acquired on the 4000 QTRAP were analyzed by MultiQuant v1.1 (ABSciex). Typical integration settings were a smoothing width of 3 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. Reports were generated directly from MultiQuant and compiled in an xml file for review.

**RESULTS**

**Overview**—The purposes of this study were to determine the overall success rate of generating antipeptide antibodies for SISCAPA and to generate data to guide the design of a cost-effective assay development pipeline with a high per-protein success rate. Fig. 1 shows an overview of the study, where proteotypic peptides from a protein list were used to build SISCAPA assays, combining affinity purified polyclonal antibodies with quantitative mass spectrometric methods, and the assays were evaluated by a screening process. Specific questions that we aimed to address were: (a) How many...
peptides from a single protein target can be used to immunize a rabbit and how many antipeptide antibodies need to be affinity-purified for that target to ensure at least one good assay is generated?; (b) Can multiple immunogens be used in the same rabbit to reduce animal costs and use?; (c) What is the average yield of affinity-purified antibody per peptide target, and how many SISCAPA assays will that support?; (d) What is the average and the distribution of assay sensitivities across a large series of analytes? It is important to note that final assay validation and determination of ultimate figures of merit (i.e. limits of detection and quantitation) were not the goals of this study, but rather to evaluate the process as a whole and identify those reagents of sufficient quality to warrant full assay configuration.

Selection of Peptides and Generation of Antipeptide Antibodies—Eighty-nine peptides were selected from a database of proteins previously identified as being potentially relevant to cancer, based on a PubMed mining strategy (28). No effort was made to bias the selection of proteins to those that might be more amenable to assay development. For each protein target, proteotypic peptides were identified for assay development. The peptides were selected according to previously published criteria (12, 19). In brief, the peptides were selected to be proteotypic, be between 8 and 22 amino acids, and have no post-translational modifications or polymorphisms listed in the Swiss-Prot database (to minimize occurrence of variant forms). Peptides containing Cys or Met residues as well as peptides containing dibasic amino acids (e.g. ragged ends KK, KR, and RR) were undesirable (to avoid potential chemical modifications and inconsistencies because of trypsin digestion, respectively); however, for 54 proteins there were a limited number of tryptic peptides available for selection and these amino acids were allowed. In addition to the above criteria, suitable peptides were identified based on empirical or predicted responsiveness during LC-MS/MS, as follows: (a) Where a protein was commercially available, the proteins were subjected to trypsin digestion following by LC-MS/MS analysis, and peptides showing high responsiveness in the mass spectrometer were identified from the empirical data. (b) Where protein was not available, a list of observed peptides was assembled by mining proteomic databases (e.g. PeptideAtlas, GPM). (c) Where no prior empirical data existed for a protein of interest we employed an algorithm (ESP) to computationally predict the best responding peptides (29). Where proteins had greater than five good proteotypic peptides, the ESP score was further employed to rank and select the best peptides.

It is important to note that commonly used antigenicity predictors are based on predicted protein structures, principally surface exposure and structural elements such as beta-turns, and hence are of little use for predicting the ability to generate antibodies to capture the linear epitopes of tryptic peptides for SISCAPA assays (30). We therefore tested a large number of peptides as haptens in order to make generalizable conclusions regarding success rates for assay generation. In this study, 403 unique peptides were selected for assay development, representing up to five proteotypic peptides per protein target. For 64 proteins (72%), we were able to identify five or more peptides meeting the selection criteria with just two proteins (2%) yielding a single eligible peptide. The peptide targets and their representative peptides used for assay development are shown in supplementary Table S1.

To maximize the chances of getting at least one good assay per target protein, multiple proteotypic peptides derived from a single protein were individually conjugated to KLH carriers and used for multiplexed immunization of individual rabbits. Multiplex immunization sets ranging from 1-plex to 5-plex were tested (Fig. 2A and supplementary Table S1), with the majority of proteins (71%) having five peptide-KLH immunogens. Two rabbits were injected per multiplex immunization group, and the rabbit with the higher antibody titer (based on ELISA) was chosen as the source of polyclonal antiserum. To contain costs, two or three antipeptide polyclonal antibodies (with the highest antibody titers) were affinity-purified for the majority (97%) of the 89 protein targets (Figs. 2B, 2C). Fig. 2C summarizes the numbers of proteins targeted in the two-dimensional strategy of using multiple peptide-carrier immunogens and selecting several of the relevant peptides for affinity purification of antibodies. A total of 220 antibodies were affinity purified from ~30 ml of antisera per target and the antibody yields ranged from 0.1 to 40 mg (Fig. 2D), with a median yield of 4.5 mg (supplementary Table S2).

Reagent Quality Control Assessment and Configuration of MRM Assays—In addition to the generation of affinity-purified polyclonal antibodies, light and heavy stable isotope-labeled synthetic peptides were synthesized for configuration of SISCAPA assays. In total, SISCAPA reagents (affinity-purified polyclonal antibodies and light and heavy stable isotope-labeled synthetic peptides) were generated for 220 peptides representing 89 proteins (supplemental Table S2).

Quality control (QC) of the peptides and antibodies is an integral part of the assay development process. Peptide QC was implemented on an LC-UV-MS/MS system to verify purity, to characterize any modifications, and to obtain full MS/MS spectra for sequence confirmation and MRM method development. Two peptides (C’GNLSTC’MLGTYTQDFNK from Calcitonin and C’RPFGGVAR from TNF1B, where C’ denotes carbamidomethylated cysteine) containing N-terminal cysteine residues failed the QC. Mass spectral data indicated cyclization to form a pyro-carbamidomethyl group at the N terminus. N-terminal cysteines have also been shown to undergo oxidation during the alkylation process (either during synthesis or digestion) (31). This suggests N-terminal cysteines should be avoided when possible. A third peptide, GVTNFNVTTVDTK from the chloride intracellular channel protein, was not stable in solution for MRM measurement (data not shown). These three peptide targets were not included in the evaluation.
MS/MS data from the peptides that passed quality control were used to build a spectral library in Skyline (27), a freely available software for MRM analysis. Using the spectral library, a list of the top six y-ion transitions (based on ion abundance) was developed for each peptide. The peptide standards were subsequently analyzed by monitoring the selected transitions in nanoLC-MRM-MS experiments. Data from these standard runs were used to refine the transition list to the top three y-ions, with preference given to y-ions with m/z greater than the precursor. The retention time was also recorded in the standard runs to allow for scheduled MRM method development and to group peptides for multiplexing. Skyline documents containing spectral libraries and MRM transitions are available in the supplemental Materials.

Quality control analysis of affinity-purified antibodies consisted of a confirmation of protein concentration by both OD280 determination and BCA assay and an assessment of “passenger peptide” content. Passenger peptides are “free” or modified peptides bound to the polyclonal antibody as a result of leeching of substrate during the affinity purification process (22, 23). Traditionally, antipeptide antibodies were generated to measure full length proteins using methods tolerant of this interference, but because of the high sensitivity of MRM measurements, any detectable level of peptide coming from the antibody will reduce the overall working concentration range of the assay and make the detection limits difficult to ascertain. Passenger peptides are not an issue for the screening experiments described below because we perform reverse response curves; however, characterizing which antibodies contain passenger peptides will be important for future studies with these reagents. Each antibody was screened for passenger peptide by capturing 1 μg antibody.

**Fig. 2.** Generation of antipeptide antibodies using a multiplexed peptide immunization procedure. A, Distribution across protein targets of the number of peptides that were used for multiplexed immunization. B, Distribution across all proteins of the number of antibodies that were affinity purified. C, Summary of the distribution of number of antibodies that were affinity purified for each immunization multiplex level. D, Distribution of the yields of affinity-purified polyclonal antibodies across all peptides.
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<image of figures A and B>

Fig. 3. An example assay characterization report for the peptide YLAPSGPSGTLK from the protein FSCN1. A, Response curves for each of three monitored transitions. B, Example chromatograms for each transition at two concentrations (0.05 fmol/μL and 0.5 fmol/μL heavy peptide; 10 fmol/μL light peptide). Traces show the chromatograms for the (heavy) stable isotope-labeled peptide (in blue) overlaid on traces of the (light) peptide (in pink).

Evaluation of Large Scale SISCAPA Development

We developed a method for screening the 216 antibodies with respect to SISCAPA capture activity and resultant assay sensitivity in plasma. For each antibody, the target stable isotope-labeled (heavy) peptide was spiked into an undepleted tryptic digest of human plasma at four concentrations (very low, low, medium, high) and the light synthetic peptide was spiked into the plasma digest at a constant concentration (10 fmol/μL). Concentrations were chosen to determine sensitivity at the low end of historical detection limits and to test responses over several orders of magnitude. The “very low” spike level corresponds to ~0.05 fmol/μL or about 1–10 ng/ml protein concentration (assuming complete trypsin digestion and enrichment from 10 μl plasma). The low level corresponds to ~0.5 fmol/μL or the range of 10’s ng/ml protein. Medium level corresponds to ~5 fmol/μL and protein concentrations in the range of low 100’s ng/ml, whereas the high level corresponds to 50 fmol/μL or protein concentrations around 1 μg/ml. Assessing the sensitivity by varying the heavy peptide amount allows for estimation of the detection at each level without interference of the endogenous peptide or passenger peptide. Peptides were multiplexed into groups of ten based on retention time; thus, a total of 22 multiplex groups were created to evaluate the 216 assays. The affinity-purified polyclonal antibodies were used to enrich the spiked peptides from plasma (using the SISCAPA assay format), and the peptide eluates were analyzed by nanoLC-MRM-MS. The peak area ratios of heavy/light peptide were plotted versus the spiked heavy peptide concentration to generate a response curve. The experiments at each concentration point were performed in duplicate.

Data from the assay screening process were collected into a report format. For each peptide-antibody pair, a sheet was generated showing the response curve and example chromatograms for the lowest levels of spiked peptide in the response curves. Fig. 3 shows an example of the report page for the peptide YLAPSGPSGTLK from the Fascin 1 protein. Fig. 3A shows that the response curve was linear for each transition monitored over the four orders of magnitude measured in the experiment. Fig. 3B shows detection of each transition at the lowest spike level (0.05 fmol/μL) and unam-
biguous detection at the next highest level (0.5 fmol/µl). The individual reports for each of the 216 assays are available in the supplemental Materials.

A grading scale (A-F) (Table I) was established to classify each assay according to its sensitivity and response characteristics. To be classified as “grade A,” assays must exhibit detection of more than one transition at the “very low” level and show a linear response across the entire range. To be classified as “grade B,” assays must exhibit robust detection (i.e. more than one transition present) at the “low” spike level. To be classified as “grade C,” assays must exhibit limited detection (usually a single transition) at the “low” level and robust detection (more than one transition) at the “medium” level. To be classified as “grade D,” assays must exhibit detection at the medium or high levels. Finally, grade F assays featured no response detected at any peptide spike level. The distribution of assay sensitivities based on the grading system is plotted in Fig. 4. The range of assay sensitivities is evident from the figure, with the best assays capable of detecting the protein at concentrations in the very low ng/ml range when enriching from 10 µl plasma.

The per-peptide success rate for generating assays can be described based on the grading system. Table I shows a breakdown of the grade classification of the assays. Of the 216 assays tested, 29% received grade A, and 54% received either an A or B grade. Detection at the lowest levels (A’s and B’s) roughly corresponds to detection limits in the 1–100 ng/ml range in plasma when enriching from 10 µl plasma.

Because multiple antibodies were purified for each protein target (Figs. 2B–C), the per-protein success rates were higher than the per-peptide success rates. Table II shows the success rates for generating assays on the protein level. Immunizing with five peptides and purifying antibodies specific for the top 2 (23/29; 79%) or top 3 (32/34; 94%) peptides results in a high success rate for making assays capable of detecting proteins at low abundance (ng/ml in plasma).

**Table I**

Summary of the distribution of assay grades across the series of 216 affinity-purified polyclonal antibodies. Grades were assigned based on detection level of synthetic peptides spiked into 10 µl of plasma. Approximate peptide detection levels are reported in fmol/µl, and the corresponding protein concentration (for an average-sized protein) is reported as ng/ml, assuming complete trypsin digestion.

| Grade | Approximate detection level | Number of assays | Percent of total |
|-------|-----------------------------|------------------|-----------------|
| A     | 0.05 fmol/µl or <10 ng/ml   | 63               | 29%             |
| B     | 0.5 fmol/µl or 10–100 ng/ml | 53               | 25%             |
| C     | 5 fmol/µl or 100 ng/ml      | 46               | 21%             |
| D     | 50 fmol/µl or 1 µg/ml       | 32               | 15%             |
| F     | Not detected                | 22               | 10%             |
| Total |                             | 216              |                 |

**Fig. 4. Distribution of detection levels for graded assays.** Distributions of the detection levels are plotted for each group of graded assays (A–D). Detection levels (plotted on log2 scale) are estimations of assay sensitivity determined from four-point response curves as part of assay characterization. Calculation of protein concentration assumes enrichment from 10 µl plasma and equal stoichiometry between peptide and protein (i.e. complete trypsin digestion).
all 216 heavy stable isotope-labeled standard peptides was analyzed by MRM-MS and absolute intensities were used to rank the peptides. Overall, the relative intensities differed by over three orders of magnitude (Fig. 5A). Looking at the distribution of peptide responsiveness for each SISCAPA assay grade level, we can see a loose upward trend to higher numbers of grade A assays for the best-responding peptides (Fig. 5B). In addition, there were several instances of relatively low-responding peptides that achieved good grades in the SISCAPA assays, indicating that a good antibody can “rescue” poor-performing peptides.

**DISCUSSION**

The present report is the first large-scale study to examine the success rate of generating antipeptide antibodies for SISCAPA assays. Encouragingly, the per-peptide success rate for developing sensitive SISCAPA assays (grades A–B) in this relatively large study was quite high (54%). Indeed, this success rate is comparable to that typically achieved when targeting whole proteins for conventional immunoassays (e.g. immunoprecipitation, ELISA, immunoassays, immunohistochemistry, etc.). The overall success rate for making a sensitive assay (grades A–B) to a target protein was even higher (94% using the optimized multiplexing immunization strategy of five immunogens and three purified antibodies per protein). The sensitivity of the assays was comparable to those produced in other studies employing peptide immunoaffinity enrichment (20, 22, 23). Compared with related techniques, such as enrichment using fractionation of plasma, SISCAPA provides at least comparable detection limits (low ng/ml) with much less sample preparation required. In addition, we previously demonstrated that SISCAPA assays can achieve sensitivities in the low pg/ml range when enriching from larger volumes of plasma (20).

The data presented in this study indicate that hundreds of SISCAPA assays can be generated per lab per year, including: selection of peptides (1 month), synthesis of peptides and generation of antibodies (6 months, including immunization, selection and affinity purification), collection of quality control data and development of methods (2 months), and the preliminary evaluation of response characteristics (2 months). This is encouraging for biomarker verification projects where a large number of highly sensitive assays could be constructed in a reasonable time. Because a large portion of the time is occupied by the development of antibodies, a rolling schedule of immunizations could be outlined, where the delivery of a hundred or more antibodies occurred every 3–4 months. Thus, it is conceivable a small dedicated team could configure up to 600 assays per year. Finally, this study suggests that scaling the scope of work to larger studies (e.g. to a thousand targets or ten thousand targets) would be feasible, depending on the appropriate funding and arrangement of several laboratories and antibody vendors.

**TABLE II**

**Success rates for protein assay development.** The number of successful assays is separated from the number attempted by a slash. Values are reported for each combination of the number of multiplexed peptide immunogens and the number of affinity purified-polyclonal antibodies. (A) Number of protein targets for which grade A and B assays were developed. (B) Number of protein targets for which grades A thru C assays were developed.

| Table 2A | Number of antibodies that were affinity purified |
|----------|-----------------------------------------------|
|          | 1  | 2  | 3  |
| Number of immunogens used per animal | 1/2 | 2/3 |
|                          | 2/3 | 5/8 |
|                          | 5/7 |
|                          | 23/29 |
|                          | 32/34 |
| Table 2B | Number of antibodies that were affinity purified |
|----------|-----------------------------------------------|
|          | 1  | 2  | 3  |
| Number of immunogens used per animal | 1/2 | 2/3 |
|                          | 3/4 | 1/1 |
|                          | 5/8 | 6/7 |
|                          | 26/29 |
|                          | 34/34 |

**Fig. 5.** Distribution of peptide response and relation to assay performance. A, Histogram of peptide response measured by LC-MRM-MS for 216 peptides. Responses vary over three orders of magnitude. B, Distribution of peptide response (i.e. intensity) as a function of assay grade.
In addition, these data make possible cost projections and cost-effective planning of future experiments requiring generation of novel SISCAPA assays. For example, a major cost of generating novel SISCAPA assays is the cost of making the required antipeptide antibodies, particularly for animal handling. Our data demonstrate that this cost can be reduced 80% by multiplexed immunization with at least five peptide immunogens per rabbit. Another significant cost associated with antibody generation is the affinity purification ($500–$1000 per immunogen). Our results demonstrate a high success rate for generating a minimum of one high sensitivity assay (grades A–B) per protein target, whereas only affinity-purifying antibodies for two to three peptide immunogens from that target (Table II).

Finally, this study also demonstrates the range of yields of affinity-purified polyclonal antibodies observed across a large series of immunogens. The median yield was 4.5 mg of antibody from ~30 ml of polyclonal antisera. A single SISCAPA capture in the current format consumes 1 µg of antibody; hence, ~4,500 SISCAPA captures can be performed given the typical antibody yield, providing enough reagent for hundreds to thousands of assays. For biomarker studies, this amount of antibody is sufficient for configuring an assay and doing preliminary verification studies on hundreds of patient samples, without incurring the significant expense ($15–20K) that function in SISCAPA assays have been developed (33, 17). Methods for selecting antipeptide monoclonal antibodies that function in SISCAPA assays have been developed (33, 34), and extending the approach to higher throughput (35) makes the large-scale production of monoclonal reagents a distinct possibility. It remains to be seen whether less expensive, recombinant approaches will be successful for generating high affinity antipeptide antibodies.

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S This article contains supplemental material and supplemental Tables S1 and S2.

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