Sequential Multiplexed Analyte Quantification Using Peptide Immunoaffinity Enrichment Coupled to Mass Spectrometry*

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Peptide immunoaffinity enrichment coupled to selected reaction monitoring (SRM) mass spectrometry (immuno-SRM) has emerged as a technology with great potential for quantitative proteomic assays. One advantage over traditional immunoassays is the tremendous potential for concurrent quantification of multiple analytes from a given sample (i.e., multiplex analysis). We sought to explore the capacity of the immuno-SRM technique for analyzing large numbers of analytes by evaluating the multiplex capabilities and demonstrating the sequential analysis of groups of peptides from a single sample. To evaluate multiplex analysis, immuno-SRM assays were arranged in groups of 10, 20, 30, 40, and 50 peptides using a common set of reagents. The multiplex immuno-SRM assays were used to measure synthetic peptides added to plasma covering several orders of magnitude concentration. Measurements made in large multiplex groups were highly correlated ($r^2 \geq 0.98$) and featured good agreement (bias $\leq 1\%$) compared with single-plex assays or a 10-plex configuration. The ability to sequentially enrich sets of analyte peptides was demonstrated by enriching groups of 10 peptides from a plasma sample in a sequential fashion. The data show good agreement (bias $\leq 1.5\%$) and similar reproducibility regardless of enrichment order. These significant advancements demonstrate the utility of immuno-SRM for analyzing large numbers of analytes, such as in large biomarker verification experiments or in pathway-based targeted analysis. Molecular & Cellular Proteomics 11: 10.1074/mcp.M111.015347, 1–10, 2012.

There is growing interest in the development of multiplex protein assays for a broad array of clinical or biological studies, including large scale biomarker candidate verification studies (1), systems analyses of targeted biological pathways or networks, high content proteomic surveys, and validation of genomic discoveries (2). Concurrent quantification of multiple protein analytes is highly desirable in these applications to minimize sample consumption, minimize sample handling, minimize per analyte assay costs, and maximize throughput (3, 4).

Uniplex ELISAs are the mainstay for quantifying individual proteins in clinical samples. However, configuration of highly multiplex, specific, quantitative assays using the ELISA (or other antibody-based platforms) is extraordinarily difficult, in large part because of loss of specificity from cross-reactivity of antibodies (5). Indeed, the majority of multiplex ELISA assays target <10 analytes (a few select commercial assays have achieved a few tens of concurrent measurements). Furthermore, the challenges of optimizing the assay format for each protein, selecting common dilution factors, and establishing robust quality control algorithms are substantial (3). Recent technologies, such as proximity ligation assays, have made progress in improving the sensitivity and specificity in multiplex ELISA (6).

Selected reaction monitoring mass spectrometry (SRM-MS)† performed on triple quadrupole mass spectrometers has emerged as an analytically robust technology that, when coupled to stable isotope dilution, enables precise, specific quantification of proteins using proteotypic peptides as stoichiometric surrogates (7–9). The molecular specificity of SRM is conferred by three orthogonal physicochemical properties of each peptide: its mass, retention properties on HPLC, and production of a set of fragment ions (usually two to five ions) of specific mass. The assays are portable across laboratories and instrument platforms (10). Current instrumentation and software allow for scheduling of the transitions being monitored (based on peptide retention times on the HPLC column), theoretically enabling hundreds of peptide analytes to be quantified in a single LC-MS run (11). Thus far, a 47-plex SRM-MS assay has been characterized for measuring the major plasma proteins, yielding a dynamic range of 4.5 orders of magnitude in a single experiment (12, 13). Without enrichment of the target peptides, typical lower limits of quantification of SRM-based assays for quantifying proteins in plasma range from 100 to 1000 ng protein/ml (12). For proteins pres-

† The abbreviations used are: SRM, selected reaction monitoring; SISCAPA, stable isotope standards and capture by anti-peptide antibodies; CV, coefficient of variation.
ent at lower concentrations, enrichment steps are required, such as abundant protein depletion combined with strong cation exchange fractionation of peptides (14) or glycopeptide enrichment (15).

Stable isotope standards and capture by anti-peptide antibodies (SISCAPA) (16) is an alternative method for targeted enrichment. SISCAPA uses anti-peptide antibodies to enrich peptides of interest from a clinical or biological sample prior to SRM-MS analysis; a good antibody can increase the sensitivity of the assay by >10^5, which in many cases is necessary and sufficient to render the assay sensitive enough for making measurements in clinical or biological specimens. Coupling SISCAPA to SRM-MS, it is possible to generate immuno-SRM assays that are precise (CV < 20%) (17–20), specific (21), and highly sensitive with lower limits of quantification in the low pg protein/ml range from 1 ml of plasma or low ng protein/ml from <50 μl of plasma (22).

Given the high potential for multiplexing SRM measurements on the triple quadrupole instrument and the difficulty of multiplexing immunoassays using conventional platforms, it is of great interest to determine the capacity for multiplexing immuno-SRM assays. Previous reports have demonstrated the successful multiplexing of three and nine analytes (18, 19, 22) in immuno-SRM assays, but the magnetic bead platform of SISCAPA (23) has the potential to multiplex much higher numbers. In addition, the magnetic bead platform has the potential to increase analyte capacity by repeatedly capturing different sets of analytes from the same sample (24). We recently described a pipeline for large scale production of immuno-SRM assays (25), allowing sufficient content to be generated to test higher levels of multiplexing in these scenarios using the immuno-SRM assay technology.

The purpose of this study was to evaluate the success rate for de novo configuration of multiplex (10-20-, 30-, 40-, and 50-plex) immuno-SRM assays. We hypothesized that achieving high levels of multiplexing with immuno-SRM would be far easier than using traditional immunoassay formats because of three theoretical advantages of the immuno-SRM assay technology: (i) the specificity afforded by using the mass spectrometer as the detector (which compensates for off target binding to the antibodies), (ii) the ability to detect and avoid matrix interferences (via selection of appropriate transitions), and (iii) the elimination of interferences from heterophile or auto-antibodies (via the trypsin digestion process).

**EXPERIMENTAL PROCEDURES**

**Materials**—Bulk pooled human plasma (K2EDTA plasma, BioReclamation no. HMPL EDTA2) collected in BD vacutainers was purchased from Bioreclamation and delivered frozen and stored at −80 °C. Urea, Trizma base, DTT, iodoacetamide, formic acid, and CHAPS were obtained from Sigma. Acetonitrile (LC-MS grade), water (LC-MS grade), and PBS were obtained from Fisher. Bovine trypsin (T-1426) used for bulk digestion of plasma was obtained from Sigma.

**Antibodies**—Rabbit polyclonal and monoclonal antibodies were produced by Epitomics Inc. (Burlingame, CA), as described previously (25, 26). Briefly, peptide sequences were conjugated to a carrier protein (keyhole limpet hemocyanin) via a C-terminal cysteine linker and used as immunogens in rabbits. 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-Carbamidomethylated versions of cysteine residues 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-labeled or 15C- and 15N-labeled atoms depending on the supplier. Uniformly 13C- and 15N-labeled lysine and arginine were the preferred stable isotope label. Peptide stock concentrations were determined by amino acid analysis (AAA) at Dana Farber Cancer Institute (Boston, MA), AAA Service Laboratory (Damascus, OR), or New England Peptide (Gardner, MA).

**Plasma Digestion**—A bulk preparation of plasma was done to provide a background matrix for the experiments. The procedure for preparation of the plasma has been previously described in detail (25). Briefly, pooled human plasma (100 ml) was denatured in 72 g of solid urea (final concentration, 6 M) at 55 °C for 15 min. The proteins were reduced by adding 2 ml of 1 M Tris buffer (pH 8) and 0.6 g DTT (final concentration, 20 mM). The total volume was adjusted to 200 ml with water and incubated at 55 °C for 45 min. Alkylation of the sulfhydryls was done by the addition of 1.85 g of iodoacetamide (final concentration, 50 mM) in the dark for 30 min. Tris buffer (900 ml of 10 mM Tris, pH 8) was added, and the solution was split into two 500-ml tubes, the final pH was adjusted to 8.0 with 6 M NaOH. Bovine trypsin (50 mg) was added at an approximate enzyme-to-substrate ratio of 1:50, and after rapid mixing the tubes were incubated overnight at 37 °C. A second addition of bovine trypsin (50 mg) was added and incubated for 4 h at 37 °C. Following incubation, neat formic acid (final concentration, ~0.2% v/v) was added to stop the digestion. The digested plasma was desalted using 6-g Oasis HLB cartridges (Waters, Milford, MA) on a vacuum manifold and speed evaporated for storage at −80 °C. Aliquots of plasma digest were resuspended in PBS for use in SISCAPA experiments.

**Peptide Immunofinity Enrichment**—Peptide enrichment experiments were performed in 96-well plates (catalog number 97002540; Thermo KingFisher). Plasma digest containing the approximate equivalent of 1 μl of original plasma was added to a sample well along with a mixture of all spiked analyte peptides. 100 fmol of each light synthetic peptide and varied amounts of heavy stable isotope-labeled peptide (0.06, 0.4, 2.3, 14, 83, and 500 fmol) were added to the samples by serial dilution. For each group of multiplex analysis, 1 μg of antibody and 1 μl of Dynabeads MyOne protein G-coated beads (1-μm diameter) were added for each target. Thus, the maximum multiplex level used 50 μg of antibodies with 50 μl of beads. The final volume of the sample was adjusted to 100 μl with PBS + 0.03% CHAPS. The plates with plasma, peptides, antibodies, and beads were allowed to incubate overnight (~16 h) at 4 °C on a rotating tumbler (Labquake, Albany, NY). A KingFisher magnetic particle processor (Thermo Fisher, Waltham, MA) with a PCR magnetic head was used for all bead handling. The beads in the overnight incubation plate were briefly mixed for 5 min and then 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 (catalog...
number HSP901; Bio-Rad) containing 26 µl of 5% acetic acid with 0.03% CHAPS and incubated for 5 min, and the beads were removed. The eluted peptides were transferred to a clean 96-well plate, covered with adhesive foil, and frozen at −80 °C until analysis by mass spectrometry.

For sequential enrichment, once the beads were removed from the initial overnight incubation plate (by the KingFisher), the next set of beads and antibodies was immediately added (to the incubation plate), and the plate was placed on a tumbler at 4 °C for overnight incubation. The addition of the sequential set of antibodies and beads slightly increased the total volume. Wash and elution steps were the same as described above. The process was repeated in an identical manner for each sequential capture.

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. The solvents used were water with 0.1% formic acid (mobile phase A) and 90% acetonitrile with 0.1% formic acid (mobile phase B). Ten microliters of the sample was loaded onto a trap column (0.3 mm × 5 mm; PepMap Acclaim C18, LC Packings, Sunnyvale, CA) for 3 min at 10 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. Albin Maisch, Ammerbuch, Germany). The LC gradient was delivered at 300 nl/min and consisted of a linear gradient of mobile phase A) and 90% acetonitrile with 0.1% formic acid (mobile phase B). Ten microliters of the sample was loaded onto a trap column (0.3 mm × 5 mm; PepMap Acclaim C18, LC Packings, Sunnyvale, CA) for 3 min at 10 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. Albin Maisch, Ammerbuch, Germany). The LC gradient was delivered at 300 nl/min and consisted of a linear gradient of mobile phase B developed from 3–40% B in 20 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, and the analytical column was re-equilibrated at 3% B for 20 min at 400 nl/min. The nano-LC system was connected to a hybrid triple quadrupole/ion trap mass spectrometer (4000 QTRAP; AB SCIEX, 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 a previous study (25), scheduled SRM transitions were determined in a previous study (25), scheduled SRM transitions were configured using Skyline (27) and entered in Analyst 1.5 using a retention time window of 90 s and a desired cycle time of 0.5 s. The actual cycle time depends on the number of concurrent transitions (see supplemental Table 1). The most intense transition for each peptide was used as a "quantifier," to be used in analyses. The other transitions were used as "qualifiers" and were required to have the same retention time. Statistical analyses were performed using R (version 2.9). Correlation plots were constructed by determining the average peak area ratios for each peptide at each concentration level. Then the average peak area ratio was compared pairwise between plex levels (using either the single-plex individual assays or the 10-plex level as a reference value) for all peptides. To evaluate whether there was systematic bias in multiplexing, the median value from replicate measurements at each plex level and at each concentration level were treated as responses, and the multiplexing group factors and concentration blocks (six total) were treated as predictors. The p values for testing whether the multiplexing group factors were significant were calculated using a multiple linear regression model.

RESULTS

The goal of this study was to test the capability of peptide immunoaffinity enrichment and mass spectrometry to quantify larger numbers of analytes in a single sample by exploring two areas: (i) evaluating the potential for high levels of multiplexing (i.e. concurrent analysis of multiple analytes) in a single assay and (ii) providing proof-of-principle demonstration of the sequential enrichment of analytes from a given sample. Fifty immuno-SRM assays targeting prototypic peptides were selected from a group of over 200 assays previously described (25). The only criteria applied to the selection of assays from the larger group were that over 5 mg of antibody was available and that the antibody concentration was >0.8 mg/ml. In addition, five immuno-SRM assays using monoclonal antibodies were used for evaluation of individual assays compared with multiplexing. Supplemental Table 2 contains a list of peptides, their respective proteins, and selected properties.

Evaluation of Multiplexing—Previous experiments indicated that multiplexing immuno-SRM assays in a 10-plex did not change the recovery compared with individual assays (21). To further examine the performance of individual versus multiplexed assays, we compared the response of five assays configured as individual assays with the response when combined with a large number (total 50) of other assays. A set of samples was prepared by serially diluting heavy stable isotope-labeled peptides in plasma keeping the light peptide at a constant concentration. The samples were constructed in this manner to provide a set of measurements over a wide concentration range free from interference of any endogenous peptide analyte in the plasma sample. They were not used to generate assay calibration curves; rather the measured peak area ratios (heavy to light) were used for comparing the single plex to the multiplexed assays. Comparisons of the responses in individual versus multiplexed assays for the five peptides are shown in supplemental Fig. 2. Overall, the measurements show significant overlap (p values > 0.05; see supplemental Table 3) for each peptide, indicating that the assays are readily multiplexed and obtain equivalent performance as a single assay or when grouped together.

To further compare the performance using high levels of multiplexing, a large number of assays were combined at various plex levels, and the results were compared across a series of samples covering several orders of magnitude of peptide concentration. Fig. 1A provides an overview of the multiplexing study. Fifty assays were initially divided into five groups of 10-plex assays selected to maximize the distribution of retention times of the peptides in the LC-MS system within a multiplex group (and thus to avoid elution of all peptides at the same time within a group). From these 10-plex groups, higher order multiplex assays were constructed by combining the groups (as depicted in Fig. 1A). Similar to what is described above, a set of samples was prepared by serially
ratios overlap each other, demonstrating excellent agreement. The measured peak area ratios (heavy to light), intensities, and precision (coefficient of variation) were used to evaluate the performance of the assays regardless of the plex level of analysis. At the lowest concentration points, it was possible to observe detection in one multiplex group but not another (for example, see the lowest point in Fig. 2B, where detection only occurred in the 40-plex assay). This is because the response at that concentration is near the peak area threshold. Overall, these results indicate that equivalent results can be expected for the vast majority of these assays regardless of the multiplex level.

The reproducibility of measuring the peptides in multiplex groups is also summarized in Fig. 2C as box plots showing the distribution of CVs for all peptides calculated across all concentration points. The median CV ranged from 10 to 16%, values typical of those achieved in previous studies using smaller multiplexed groups (22).

Multiplex Correlation and Agreement of Measurements—We next examined the overall correlation and agreement of measurements. For the five assays measured as individual and multiplexed assays, the measured peak area ratios were compared in a correlation plot (Fig. 3A). The peak area ratio values were highly correlated (slope = 1.01, $r^2$ = 0.98), reaffirming the ability to multiplex analytes. For the highly multiplexed study, measured peak area ratios were compared pairwise using the 10-plex results as a reference value. Fig. 3C shows the correlation of data points from all peptides detected. Linear regression between each set of data shows good correlation between the measurements (the correlation coefficient, $r^2$ = 0.98–0.99; see Fig. 3C). Furthermore, the slopes of the linear regression lines range from 0.96 to 0.97, indicating good agreement among the measurements. The ratio of measured values for the 50-plex assays compared with individual single-plex assays was plotted in Fig. 3E, showing good agreement. The median bias was 3%, with 79% of the multiplexed measured peak area ratios within 20% of the individual assay values (96% of values were within 2-fold). High multiplex levels (20-, 30-, 40-, and 50-) were also compared with values obtained by the 10-plex assays (Fig. 3F). Overall there was very good agreement in the peak area ratios between each higher plex level compared with the 10-plex measurements. The median difference for each multiplex group was within 1% of the 10-plex values (the numerical values for the box plots are available in supplemental Table 4). These differences were well within the relative error typical for the technique (22) and consistent with the differences found in comparing the multiplexed and individual assays (above). The percentage of data points measured at high plexes within 20% of the values measured in the 10-plex were 71, 71, 66, and 66 (for the 20-, 30-, 40-, and 50-plex groups, respectively), and 97% of the data were within a factor of 2.

The largest deviation in agreement occurred at the lowest concentration values, where there is a greater variation associated with the measurements (see supplemental Fig. 4 for a differences plotted at each concentration level). Measurements at the lowest concentrations are also most affected by diluting heavy stable isotope-labeled synthetic peptides in digested plasma containing a constant amount of light synthetic peptide (28). Again, the resulting data were not used as assay calibration curves; rather, the measured peak area ratios (heavy to light), intensities, and precision (coefficient of variation) were used to evaluate the performance of the assays at the various multiplex levels.

The response of 50 peptides was measured across a range of peptide concentrations in a series of plex levels (10-, 20-, 30-, 40-, and 50-plex). One peptide had low signal intensity (peak area < 500) and was eliminated from further analysis, and another peptide did not have an adequate number of observations for analysis (i.e., it was only detected at a single level). The response for each of the 48 remaining peptides was plotted for each multiplex level over the range of peptide concentrations (supplemental Fig. 3). In comparing the response in different multiplex groups for the 48 peptides measured, a single peptide was significantly different in the highly multiplexed assay ($p < 0.05$; supplemental Table 3). Two representative peptides are shown in Fig. 2: VLDETLAR from the keratin protein and DWRPAITIK from ubiquitin-conjugating enzyme. The log peak ratio measured at each concentration point was plotted versus the log concentration ratio based on the known amount of spiked peptides. The insets in Fig. 2 show the peak area ratios versus concentration ratios in linear space. The values for the peaks measured in each plex level are separated by color. The measured peak area ratios overlap each other, demonstrating excellent agreement.
any increase in noise levels associated with a greater amount of background. One analyte showed evidence of an increase in background binding with increasing multiplexing level by the addition of an interfering peak in the chromatogram (supplemental Fig. 5). This suggests that further optimization of the binding and washing conditions to reduce or eliminate nonspecific background binding for highly multiplexed groups may benefit the performance of the assays.

It is conceivable that the peak area ratio is conserved at higher plex levels, but there is a change in signal intensity. A loss in intensity would result in a corresponding loss in sensitivity; thus, we also sought to compare the absolute signal intensities when multiplexing. Because absolute signal intensity has larger variation compared with peak area ratios (there was an average 29% variation in absolute peak area for the measurements in this study), differences in peak area may not be informative for small changes in sensitivity; however, it is still useful for assessing relatively large differences. A good correlation was found when comparing the absolute intensities measured for individual assays versus multiplexed (Fig. 3B), as well as the highly multiplexed assays measured at each plex level (Fig. 3D). As expected, the absolute measured intensity was not as precise as a peak area ratio using an internal standard peptide, and there was slightly more spread in the correlation coefficients ($r^2$ values ranging between 0.88 and 0.97). We also examined the ratio of peak areas measured in the 50-plex assays over those determined from individual single-plex assays (Fig. 3E) and the ratio of absolute peak areas in multiplexed assays compared with the 10-plex assays (Fig. 3G). The median bias for these also showed good agreement (28, 0, −7, −10, and −8% for the 1-, 20-, 30-, 40-, and 50-plexes, respectively), indicating there was not a large change in sensitivity when multiplexing. It is interesting to note that we observed a slight overall increase in intensity when comparing the 50-plex versus a single-plex assay.

Sequential Analysis of Multiplex Assays—Another manner to increase the number of analytes measured in a single sample is to utilize the assays in a sequential fashion. To demonstrate the potential for sequential, multiplex enrichment of analytes from a single sample, 30 peptide analytes were selected from the same groups used in the multiplex experiment described above (Fig. 1B, groups A, B, and C). The 30 peptides (along with stable isotope standards) were spiked into digested plasma at a single concentration, subsequently enriched by SISCAPA in their respective 10-plex groups sequentially (Fig. 1B), and analyzed by SRM-MS. The sequence was varied (i.e., ABC, BCA, CAB) such that each 10-plex group was tested in all three SISCAPA capture positions (i.e., first, 20-plex (red circles), 30-plex (maroon triangle), 40-plex (black diamond), and 50-plex (green circle)) data points were plotted. C, distribution of measured CVs at each plex level. The black line indicates the median, the inner quartiles are represented by boxes, and the whiskers show 95% of the data.

Fig. 2. Responses at each level of multiplexing. A and B, example responses plotted at each plex level for two peptides, VLDELTLAR (A) and DWRPATIK (B). Light and heavy stable isotope-labeled peptides were added to the digested equivalent of 1 μl of neat plasma. Log$_{10}$ peak area ratio was plotted versus log$_{10}$ concentration ratio of spiked peptides. The insets plot the same responses on a linear scale. The error bars show the range of measurements. 10-plex (blue squares), 20-plex (red circles), 30-plex (maroon triangle), 40-plex (black diamond), and 50-plex (green circle) data points were plotted. C, distribution of measured CVs at each plex level. The black line indicates the median, the inner quartiles are represented by boxes, and the whiskers show 95% of the data.
second, or third to be captured), and each sequence was performed in triplicate. It was hypothesized that the performance of the assays (measured peak area ratios, intensities, and precision) would not depend on capture order.

The peak area ratios (heavy/light) for each peptide in the first enrichment position from a given sample was compared with the analyses from the second and third enrichment positions by taking the ratio of the measurements. Fig. 4A shows the ratio from comparison of each capture position, revealing agreement between values in the first capture and subsequent capture orders. The median bias for peptides measured in the second serial capture differed from values obtained in the first capture by 1%. The values in the third serial capture also differed from the first by 1% and from the second by 1.5%. These values were well within reported relative accuracy of the method, indicating successful implementation of a sequential analysis strategy.

To test whether sequential captures affected the reproducibility of the measurements, we examined the precisions of measurements in sequential captures compared with the first capture from the sample. As shown in Fig. 4B, assay precision was preserved with sequential capture. The median assay CVs for positions 1 through 3 were 8.5, 4.9, and 6.3%, respectively.

Finally, to test whether sequential captures affected the recovery of the analyte peptides, we examined the absolute intensities measured in sequential captures compared with the first capture from the sample (Fig. 4C). The mean bias of the measurements shows a difference of 16 and 20% for the second and third serial captures, respectively, compared with the first capture. The third serial capture differs from the second by only 2%. This indicates that there is no appreciable peptide loss occurring between enrichments, allowing for sequential analysis without a significant decrease in intensity (maintaining assay sensitivity).

**DISCUSSION**

In this manuscript, we demonstrate the capability of peptide immunoaffinity enrichment coupled to SRM mass spectrometry to analyze a large number of analytes in a single multiplex assay. Of the 48 peptides assessed, 47 met significance, a 98% success rate. This dramatically extends the demonstrated capabilities of the immuno-SRM assay format over previous studies using 3-plex or 9-plex arrangements (18, 19, 22) and exceeds what has been generally achievable with conventional immunoassays. The overall performance of the immuno-SRM assay is maintained regardless of the size of the multiplex group.

The present work provides an initial demonstration of the multiplexing capabilities. Further work must be done to validate such a highly multiplexed method in comparison with individual assays. For example, the comparison of highly characterized quality control samples in conjunction with calibration curves could be used to compare validated assays, as might be done in the context of a clinical assay. Such a study would be an important application to formal biomarker validation studies.

The results presented herein suggest that higher levels of multiplexing are possible. Multiplexing on the mass spectrometer is only limited by the number of SRM transitions that can be reliably monitored in a given time, a number that depends on many variables that can be adjusted to achieve the desired level (e.g. retention time, chromatography reproducibility, scheduling windows, gradient length, dwell time). Quantitative SRM assays for a large number of peptides have been demonstrated (12, 13), and recent advances show that thousands of transitions can be monitored in a given run (29).

The greatest limitation to multiplexing several hundred assays using immuno-SRM, given the current configuration, is the volume of magnetic beads that can be handled practically and reproducibly. Currently, the most difficult step is eluting peptides from a large volume of beads into a small elution volume (to concentrate the target peptides). There are possible approaches for overcoming this limitation. First, the use of capture reagents with very high affinities would reduce the amount of antibody required per target, making it possible to get a higher number of targets for the same amount of beads. Second, generating beads with higher antibody binding capacity would reduce the volume of beads required per analyte. Third, the elution step could be performed into a large volume (compatible with the volume of magnetic beads used) and loaded onto a chromatographic trapping column or subsequently concentrated prior to mass spectrometry analysis.

**Fig. 3. Correlation and agreement among multiplexed groups.** A, the correlation of \( \log_{10} \) peak area ratio measurements made at 50-plex versus those made in individual assays (slope = 1.01, \( r^2 = 0.98 \)). B, the correlation of \( \log_{10} \) absolute intensity for the same assays (slope = 0.91, \( r^2 = 0.97 \)). C and D, the correlation of measurements made at high multiplex levels (e.g. 20-, 30-, 40-, and 50-plex) versus those made in a 10-plex assay were plotted for \( \log_{10} \) peak area ratio (C) and \( \log_{10} \) absolute intensity (D). The lines represent the least squares linear regression through the data, and the corresponding slope (\( m \)) and Pearson correlation coefficient squared (\( r^2 \)) are displayed. For all peptides, the correlation of measurements made in 20-plex (red), 30-plex (orange), 40-plex (green), and 50-plex (blue) assays with the 10-plex assays were plotted. E, the ratio of peak area ratio and absolute peak area for individual (single-plex) assays compared with the same peptides measured as part of a 50-plex assay. F, the ratio of measured peak area ratio values for 20-, 30-, 40-, and 50-plex assays compared with the same peptides in a 10-plex assay were plotted to assess any gross changes in sensitivity. A ratio of 1 indicates perfect agreement. In the box plots, the black line indicates the median, the inner quartiles are represented by boxes, and the whiskers show 95% of the data.
An additional limitation to achieving very high levels of multiplexing appears to be an increase in background binding of high abundance matrix components, likely on the surface of the stationary media (in this case, magnetic particles coated with protein G and antibody). These nonspecifically bound matrix components can decrease the signal-to-noise ratio of the target analytes and can also result in interferences (as seen in one of the peptides in this study; see supplemental Fig. 5). Further optimization of the enrichment process, including wash steps, precapture depletion of interfering proteins, and evaluation of various nonfouling bead surfaces may improve this aspect. Despite the increase in background, the immuno-SRM assay described here has a decided advantage over multiplexing in traditional immunoassays (e.g. ELISA). In ELISA, an increase in multiplexing decreases the specificity of the assays because of cross-reactivity of the antibodies. In contrast, with immuno-SRM assays, matrix interferences are either destroyed by trypsin digestion (e.g. auto- and heterophile antibodies) or can be detected and avoided by leveraging the high specificity of the mass spectrometer (e.g. selection of a transition free of background interference, as exemplified by the analyte transitions shown in supplemental Fig. 5).

We previously demonstrated that enriching peptides from larger volumes of plasma (e.g. 1000 μl) extends immuno-SRM assay lower limits of quantification to the pg protein/ml range (22). In contrast to the clinical setting, where an accepted biomarker can merit the use of large sample volumes, a biomarker verification study consuming large volumes of clinical plasma samples for measurement of one or a small number of analytes is not cost effective and is only justified for very highly credentialed candidate biomarkers or for answers to critical questions. However, the combination of high assay multiplex levels coupled to the capability to analyze groups of peptides sequentially from the same plasma sample renders the use of larger plasma volumes a viable option for a larger number of biomarker studies. Indeed, it is feasible to quantify over 100 (and perhaps hundreds) of analytes from a single sample, offering several advantages over existing techniques.

Multiplexing offers considerable cost savings. Once an assay has been developed, the primary per sample costs of an immuno-SRM assay are the reagents associated with processing the sample (e.g. trypsin, magnetic beads, synthetic peptides) and the mass spectrometer time for running the

![Figure 4](image_url)

Fig. 4. Agreement of measurements made sequentially from the same sample. A, the ratio of peak area ratio for each multiplexed set of 10 peptides measured sequentially from the same sample.

In each replicate, the ratio of peak area ratio for each multiplexed set of 10 peptides measured sequentially from the same sample is shown.

x axis labels indicate the relative comparison of enrichment order (for example, pos 2..1 refers to the difference between position 2 and position 1 in enrichment order). B, the CV for three replicate measurements for each SISCAPA position. C, the difference in log₁₀ intensity for each peptide at each capture position. A difference of zero indicates perfect agreement. For each plot, the bold black line indicates the median, the inner quartiles are represented by boxes, and the whiskers show 95% of the data. pos, SISCAPA position.
sample. As the multiplexing level increases, the same amount of time (and the same amount of most of the reagents) is invested in the sample; thus the cost per analyte decreases substantially. Finally, the ability to tailor large multiplex panels of sensitive and specific assays to a given set of proteins enables a broad array of clinical or biological studies, including large scale biomarker candidate verification studies and systems analyses of targeted biological pathways or networks.

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