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Antibody-coupled magnetic beads can be re-used in immuno-MRM assays to reduce cost and extend antibody supply

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Abstract

Immuno-affinity enrichment of peptides coupled to targeted, multiple reaction monitoring-mass spectrometry (immuno-MRM) enables precise quantification of peptides. Affinity-purified polyclonal antibodies are routinely used as affinity reagents in immuno-MRM assays, but they are not renewable, limiting the number of experiments that can be performed. In this report, we describe a workflow to regenerate anti-peptide polyclonal antibodies coupled to magnetic beads for enrichments in multiplex immuno-MRM assays. A multiplexed panel of 44 antibodies (targeting 60 peptides) is used to show that peptide analytes can be effectively stripped off of antibodies using acid washing without compromising assay performance. The performance of the multiplexed panel (determined by correlation, agreement, and precision of reused assays) is reproducible (R^2 between 0.81–0.99) and consistent (median CVs 8–15%) for at least ten times of washing and re-use. Application of this workflow to immuno-MRM studies greatly reduces per sample assay cost and increases the number of samples that can be interrogated with a limited supply of polyclonal antibody reagent. This allows more characterization for promising and desirable targets prior to committing funds and efforts to conversion to a renewable monoclonal antibody.

Graphical Abstract

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Author Contributions

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Notes

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targeted proteomics; quantitative proteomics; peptide assays; immunoaffinity enrichment; antibody; mass spectrometry

INTRODUCTION

Targeted proteomics has the potential for widespread impact in clinical, biopharmaceutical, and fundamental biological studies. Targeted mass spectrometry approaches, like multiple reaction monitoring (MRM), have found broad applicability in targeted proteomic quantification with several advantages over traditional immunoassays, including the ability to multiplex, the relative ease of development, and inter-laboratory transferability.^{1,2} Peptide immunoaffinity enrichment can be coupled with MRM to improve sensitivity for low abundance and/or modified peptides, reduce upstream sample handling requirements, and improve throughput.^{3–5} The resulting immuno-MRM assays have shown utility for precise, specific, reproducible, and sensitive measurements in a number of sample types and matrices.^{6–13}

Most work to date has used affinity-purified polyclonal antibodies, although there are several examples of monoclonal antibodies^{8,9,14–18} and recombinant antibody fragments.¹⁹ Polyclonal antibodies are relatively inexpensive and can be generated in a few months; however, the yield of such reagents can vary²⁰, and they are limited in supply. Once exhausted, re-immunization of new animals is required to obtain more antibodies, and the immune response of different animals can be highly variable. Thus, the ‘one-time’ nature of polyclonals limits their use to preliminary studies of limited capacity. Converting reagents to renewable monoclonal antibodies has been successful^{14–17}, but requires a considerable investment in time and money, and thus only the most well characterized and desirable assays are chosen for monoclonal development. Extending the use of the polyclonal antibody resource would enable larger studies to fully evaluate assay targets and also reduce the long-term cost of using the assays prior to investing in generating a monoclonal reagent.

Based on the experience that antibodies can be denatured and re-natured without loss of activity^{21,22}, affinity reagents are regenerated and reused in many applications, including commercially available columns and preparations (e.g. protein A/G columns, affinity

depletion columns, etc.) and peptide enrichment using an in line bead trap device.²³ Thus, we predicted that antibodies used in solution phase immuno-MRM assays (run in a 96-well format) could be reused, lowering per sample assay cost and increasing the number of samples that can be analyzed.

In this study, we evaluate the removal of bound peptides and the performance of regenerated antibodies/beads in capturing a multiplexed panel of phosphorylated and non-modified peptides and demonstrate that analytical performance of the multiplexed panel is consistent for at least ten times of washing and re-use. The findings are significant because per sample costs are reduced, and the number of samples that can be analyzed is greatly expanded using the recycling approach.

EXPERIMENTAL

Reagents

Dimethyl pimelimidate dihydrochloride (DMP #80490) was purchased from Sigma-Aldrich (St. Louis, MO). Trypsin (#V511X) was obtained from Promega (Madison, MI). Synthetic peptide (light) and stable isotope-labeled peptide standards (SIS) were synthesized by New England Peptide (Gardner, MA). For stable isotope-labeled peptides, the C-terminal arginine or lysine was labeled with [¹³C] and [¹⁵N] labeled atoms. Peptide stock concentration was determined by amino acid analysis performed at New England Peptide. Affinity purified polyclonal antibodies were generated by Epitomics, an AbCam Company (Burlingame, CA). Dynabeads® protein G beads (MyOne™ #109150) were purchased from Invitrogen (Grand Island, NY).

Cell culture

The human mammary epithelial cell line MCF10A was obtained from the ATCC (Manassas, VA) and grown at 37°C and 5% CO₂ in DMEM/F12 1:1 (Invitrogen #11320) supplemented with 5% horse serum (Invitrogen), 10 µg/mL of insulin (Sigma #I6634), 20 ng/mL of EGF (PeproTech #AF-100-15), 0.5 mg/mL of hydrocortisone (Sigma #H-0888), 100 ng/mL of cholera toxin (Sigma #C-8052), 100 units/mL of penicillin, and 100 µg/mL streptomycin. Cells were grown to 80% confluency in 100 mm plates. After incubation, growth medium was removed, and cells were rinsed in 0.25% trypsin/EDTA solution (Gibco #25200-056) and lifted off of the plates by incubation in a fresh aliquot of 0.25% trypsin/EDTA solution at 37°C, 5% CO₂. When cells had lifted from the plate, the trypsin was quenched by the addition of 3 volumes of DMEM/F12 with 5% horse serum.

Cell lysis, digestion, and desalting

Cells were harvested in pre-chilled tubes, aliquots were removed for counting, and cells were spun down and washed 2 times in an equal volume of ice cold PBS. Cell count was determined with a Beckman Coulter Z1 Particle Counter. Cells were lysed at 5x10⁷ cells / mL in freshly prepared ice cold Urea Lysis Buffer (6M Urea, 25 mM Tris (pH8.0), 1 mM EDTA, 1 mM EGTA containing protease and phosphatase inhibitors (Sigma, #P0044, #P5726, and #P8340)). Lysates were sonicated 2 x 12 sec. and then cleared by centrifugation at 20,000 x g, 10 minutes at 4°C. Supernatants were transferred to cryo-vials, stored in

liquid nitrogen and thawed on ice. Protein lysate concentration was measured in triplicate using Micro BCA Protein Assay Kit (Thermo # 23235). The cell lysate was denatured by addition of 9 M Urea (final concentration at 6M) and 6% of 500 mM *tris*(2-carboxyethyl)phosphine (TCEP) and incubated at 37°C for 30 minutes on shaker (700rpm). After denaturation, 500 mM iodoacetamide was added to the mixture (final concentration at 50 mM) and cell lysate was incubated in dark under ambient condition for 30 minutes. Before addition of trypsin, 200 mM Tris buffer, pH 8.0 was added to cell lysate to reduced the urea concentration to approximately 0.6 M, and then sequencing grade trypsin was added to the mixture at a ratio of 1:50 (enzyme: protein). After two hours, trypsin was added again at 1:100 (enzyme: protein). The cell lysate was then incubated at 37°C for 16 hours, following which trypsin activity was quenched with addition of concentrated formic acid (final concentration 1%). The digested cell lysate was desalted on Oasis HLB cartridge (Waters). The cartridge was conditioned with 4x400 uL of 0.1% formic acid in 80% acetonitrile (ACN) and equilibrated with 4x400 uL of 0.1% formic acid. The digest was applied on cartridge, the column was washed with 4x400 uL of 0.1% formic acid, and peptides were eluted with 3x400 uL 0.1% formic in 50% acetonitrile. The cell digest was lyophilized, stored in -80°C, and resuspended in PBS prior to affinity capture.

Preparation of magnetic beads and immunoaffinity enrichment

Antibodies were individually cross-linked as previously described²¹ with the following modifications. 750 µL of the MyOne™ beads were added to 1.6 mL Eppendorf tubes and washed once with 500 µL of 1X PBS, followed by addition of 500 µg of antibody and 1X PBS-0.03% CHAPS for a ratio of 1 µg antibody-to-1.5 µL protein G beads. The mixture was tumbled overnight at 4 °C. The next day the supernatant was discarded and 500 µL fresh 20 mM DMP in 200 mM triethanolamine was added and incubated for 30 minutes at RT in a ventilated hood. The beads were washed once with 150 mM monoethanolamine for 30 minutes at RT, twice with 5% acetic acid / 0.3% CHAPS, then washed with PBS, and resuspended in PBS, 0.01% azide. Immunoaffinity enrichment was performed by mixing 500 µg of digested cell lysate, SIS peptides, light peptides, and 1.5 uL magnetic protein G beads coupled with 1 µg antibodies / peptide target into a final volume of 200 uL in PBS, 0.03% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate). Five microliters of 1M Tris (pH 8.0) was added to the resuspended mixture and the mixture was incubated over night at 4°C. The beads were washed and peptide elution was performed on a Kingfisher automated magnetic particle processor as previously described²⁴. Elution solution was 5% acetic acid with 3% acetonitrile in water (pH 2.0). The eluate was stored at -80°C until analysis by mass spectrometry.

Liquid chromatography - mass spectrometry

An Eksigent Ultra 2DLC system (Eksigent Technologies, Dublin CA) equipped with a nanoautosampler was used for liquid chromatography. Solvents were water, 0.1% formic acid (mobile phase A) and 90% acetonitrile, 0.1% formic acid (mobile phase B). The sample was loaded on to a trap column (0.3 X 5mm, LC Packings PepMap Acclaim C18) for 1.5 min at 10uL/min with 3% mobile phase B. The trap was connected to a 0.075 x 100 mm column (IntegraFrit, New Objective, Woburn, MA) packed with 3 um Reprosil C18-AQ particles (Dr. Maisch, Germany). The LC gradient was delivered at 300 nL/min with a linear

gradient of mobile phase B from 3 to 40% B in 10 minutes, then ramped to 90% B for 5 minutes. The columns were re-equilibrated at 3% B at the end of the run. The nano LC system was connected to a hybrid triple quadrupole/ion trap mass spectrometer (6500 QTRAP, ABSCIEX). The typical instrument settings included spray voltage of 2.2 kV, an ion source temperature of 150°C, a GS1 (nebulizer gas) of 6, and a curtain gas of 20. Values for collision energy were derived from linear regression of optimized values in the Skyline²⁵ software.

Data analysis

MRM data acquired on the 6500 QTRAP were analyzed by Skyline.²⁵ Peak integrations were reviewed manually and transitions from analyte peptides were confirmed by the precise alignment of retention times of the light synthetic peptides and heavy stable isotope-labeled peptides. Data were exported from Skyline for analysis and plotting. Correlation plots were performed in R using linear regression and Pearson's correlation.

RESULTS AND DISCUSSION

Our goals were to extend the productive life of polyclonal antibodies, a limited resource, and reduce the per sample cost of immuno-MRM assays. To achieve these goals, we tested the feasibility of re-using antibody-protein G magnetic beads in the affinity enrichment step. Concerns in re-using the antibody-coupled beads include the possibility of antigen carryover and degradation in performance.^{26,27} Therefore, we specifically asked two questions: i) how many elution washes were needed to strip all antibody bound peptides, and ii) were regenerated antibodies capable of binding peptides without a loss in performance following acid elution? A panel of 44 polyclonal antibodies (covering 60 peptides) targeting phosphorylated and non-modified peptides was used in multiplexed immuno-MRM experiments to address the above questions (see Supplemental Table 1 for a list of peptides).

Removing bound peptides

A summary of the experiments used to evaluate the removal of bound peptide analytes and the effects of stripping the antibodies on antibody life span is shown in Figure 1. We first examined the removal of peptides by repeated acid elution (Figure 1A). The multiplexed assay panel was used to enrich a mixture of 750 fmol of heavy peptides and 150 fmol of light peptides spiked into a background of MCF10A cell lysate digest. Optimized elution conditions (5% acetic acid / 3% acetonitrile) were determined from Whiteaker et. al.²⁸ in addition to a test of high pH elution (0.5 M NaOH), which showed 17% recovery compared to acid elution. Following initial elution with 5% acetic acid in 3% acetonitrile, the beads were resuspended in PBS, 0.01% azide, and then washed twice with fresh 5% acetic acid/3% acetonitrile. The process was repeated a total of 5 times. Samples from each acid elution of the antibody/beads were analyzed using LC-MRM-MS targeting the 60 peptide analytes in the eluates.

Figure 2 shows an example of the reduction in peptide signal following consecutive acid elution. The chromatogram in Figure 2A shows the signal of peptides from the capture of spiked peptides (750 fmol heavy plus 150 fmol light) plus endogenous analyte in the

MCF10A lysate background. The peptides span a large retention time window of the chromatogram and vary in relative intensity with overall high intensities and good signal-to-noise. Figure 2B shows the chromatogram following two acid wash cycles. The signal of most peptides was significantly lower following the wash steps. One peptide, NLSIDIDQSFNK, is predominant in the chromatogram. This is the most resistant peptide to removal with acid. Overall, inspection of the chromatograms indicated that we could effectively remove the vast majority of antibody-bound peptides by washing with acid.

To quantify the reduction of peptide signal following acid washing, we evaluated the signal measured in each elution relative to the amount of signal in the first elution of captured peptides (reported as carryover) and relative to the amount of background signal in a blank measurement (i.e. relative to the noise level). To determine the background (i.e. noise) level, a blank sample consisting of captured peptides from 500 µg of digested MCF10A cell line lysate with no heavy peptide added was analyzed in triplicate. The average signal in the heavy channel was used as a measure of the background (due to presence of endogenous peptide in the light channel). Next, identical aliquots of a sample containing 500 µg of the cell lysate digest with additions of 150 fmol light and 750 fmol heavy SIS peptides was analyzed using the multiplexed immuno-MRM assay. The antibody-beads were then washed up to 5 times with acid and the elution buffer analyzed by LC-MRM. Peak areas for peptides eluted from beads were compared to the signals in the original elution (reported as carryover) and compared to the background levels determined from the blank sample. Figure 3 shows the response for heavy SIS peptides (Figure 3A) and light peptides (Figure 3B) (The peptide NLSIDIDQSFNK was removed from the figure to better display the results for the other peptides). Values for each peptide are reported in Supplemental Table 2 (heavy peptides) and Supplemental Table 3 (light peptides). As shown in Figure 3, acid washing was effective in removing the peptides, resulting in low carryover and peak areas lower than the background, even for the challenging spike level of 750 fmol peptides. Carryover for heavy peptides is predominantly below 1%. The peptide NLSIDIDQSFNK and its phosphorylated counterpart, NLpSIDIDQSFNK, had the highest carryover (Supplemental Table 2). The peak areas of the SIS peptides were nearly equal to or lower than the background after two acid wash stripping cycles. Area intensities for all SIS peptides were continually reduced with subsequent acid wash cycles (Figure 3A). Signals from light peptide show carryover for the majority of assays is below 10% (Figure 3B). Differences in carryover between light and heavy peptides are reflective of lower amounts of light peptide in the initial enrichment compared to heavy. Light peptides also generally showed lower intensity (relative to background) in the acid wash samples (Figure 3B). The area intensity for all light peptides were less than background after two acid wash cycles, and continued washing resulted in the peptides' signals being measured at less than 0.5 relative to background after 5 acid wash cycles. In summary, acid washing was sufficient to remove the peptide bound on antibodies to an undetectable level.

Investigation of the most resistant peptide sequence, NLSIDIDQSFNK, shows that persistent washing (e.g. 4–5 cycles) is effective at reducing the carryover to below 1%, consistent with other peptides. However, because initial levels were very high, the signal intensity remains elevated relative to the blank background measurement. The initial signal intensity of the phosphorylated peptide, NLpSIDIDQSFNK, was relatively much lower. Given the amount of

carryover relative to the original signal, residual signal would have a small effect on endogenous measurement in a subsequent experiment. However, this result suggests an evaluation of each assay target should be conducted to determine the extent of any carryover.

Antibody activity on protein G beads after multiple exposures to acid

We next evaluated the performance of antibodies when repeatedly capturing peptides following successive cycles of acid washing (Figure 1B). The antibody-coupled beads were used for immunoaffinity enrichment of three levels of heavy peptide (low: 7.5 fmol, medium: 75 fmol, high: 750 fmol) and a constant amount of light peptide (150 fmol) spiked into 500 μ g MCF10A cell digest. Following elution of the captured peptides, the antibody-coupled beads were washed twice with acid and resuspended in PBS for a subsequent capture using aliquots of the same spiked samples. The process was repeated for up to 10 regeneration cycles. The eluates obtained from each cycle were analyzed by LC-MRM-MS, and the measured peak area ratios and absolute peak areas were compared for each cycle to the signal from the first elution.

If the antibody activity were preserved, we would expect to see good correlation and agreement between peak area ratios and peak areas across consecutive cycles of antibody regeneration. Examination of the measured peak area ratios for each regeneration cycle compared to cycle 0 (the first elution) showed that the correlation and agreement were high (see Figure 4A). Correlation coefficients ranged from 0.81 to 0.97, and the slopes ranged from 0.92 to 0.98 (correlation data for individual peptides can be found in Supplemental Table 4). It is possible that the measured peak area ratios could be consistent following re-using beads, but that the overall signal could decline due to loss of antibody activity. To examine this, we plotted the correlation of raw peak areas for each measured peptide for each regeneration cycle compared to cycle 0 (Figure 4B). Like the peak area ratios, a good correlation of peak areas at all levels of peptide concentrations was observed. The correlation coefficients ranged from 0.83 to 0.99. Agreement was also good for each concentration, with slopes from the linear regression ranging from 0.96 to 1.03.

In the peak area ratio and peak area comparisons, the highest spread in the correlation plots was in the low concentration sample (7.5 fmol). This was not surprising, since the highest intrinsic variability was expected in the low concentration samples, and the correlation and agreement were good. The results here indicated that antibodies cross-linked on protein G beads were able to bind to peptides equally well after 10 recycling procedures compared to the non-treated ('new') beads/antibodies.

The repeatability (i.e. precision) of the multiplexed assay was also examined using recycled beads. Figure 5 shows distributions of the coefficient of variation (%CV) from the measured area ratios (SIS/light) of all peptides for each sample. The median %CVs were 15%, 8%, and 9% for the low, medium, and high samples, respectively. As is evident in the figure, there was no change in measured precision as a result of reusing the antibody-coupled beads, showing that the overall performance and repeatability were maintained.

The ability to reuse antibody-coupled magnetic beads has significant implications for extending the supply of affinity-purified polyclonal antibodies. In our experience, median

antibody yields are typically 4.5 mg,²⁰ allowing for analysis of 4500 sample enrichments, using 1 µg of antibody per sample. Extending the use of these reagents by an order of magnitude through recycling creates a much larger and more flexible potential resource for performing experiments. In addition, antibody yields can be highly variable, with some purifications yielding less than 1 mg of antibody. For these assays, the ability to extend the number of samples that can be analyzed by a factor of ten is a significant benefit. In our experience, the antibodies are very stable when coupled to magnetic beads, allowing for reproducible enrichments over months (Supplementary Figure 1), but periodic testing is essential for assuring stable performance over time. This allows a wider application of the assay, permitting more and more cost-effective characterization of the target and evaluation of its usefulness in the intended biological or clinical applications. For promising or essential targets, the polyclonal antibody can be converted to a renewable monoclonal antibody.

Another benefit to reusing beads is cost savings. Antibodies and beads equally contribute to the per sample cost (estimated at \$40–50 per sample for the multiplexed assay) of assay implementation. As multiplexing levels increase, the costs of antibodies and beads increase while other costs associated with sample processing remain the same. Thus, recycling antibody beads contributes a large savings in the percentage of total cost (we estimate a savings of 75% for the multiplexed assay used in this study). This significant reduction in cost makes larger studies more feasible and attractive.

CONCLUSION

For the assays tested, use of acid washing to remove detectable peptide from antibody-coupled magnetic beads is effective for reusing the reagents in immuno-MRM assays. The performance of the antibodies was not affected, allowing for consistent reuse of the antibody beads for up to 10 times. This is significant for cost savings and obtaining a larger return on investment when generating affinity reagents for immuno-MRM assays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

MRM	multiple reaction monitoring
SIS	stable isotope-labeled standards

References

1. Picotti P, Bodenmiller B, Aebersold R. Proteomics meets the scientific method. *Nat Methods*. 2013; 10(1):24–27. [PubMed: 23269373]
2. Gillette MA, Carr SA. Quantitative analysis of peptides and proteins in biomedicine by targeted mass spectrometry. *Nat Methods*. 2013; 10(1):28–34. [PubMed: 23269374]
3. Whiteaker JR, Paulovich AG. Peptide immunoaffinity enrichment coupled with mass spectrometry for peptide and protein quantification. *Clin Lab Med*. 2011; 31(3):385–396. [PubMed: 21907104]
4. Madian AG, Rochelle NS, Regnier FE. Mass-linked immuno-selective assays in targeted proteomics. *Anal Chem*. 2013; 85(2):737–748. [PubMed: 22950521]
5. Ackermann BL, Berna MJ. Coupling immunoaffinity techniques with MS for quantitative analysis of low-abundance protein biomarkers. *Expert Rev Proteomics*. 2007; 4(2):175–186. [PubMed: 17425454]
6. Whiteaker JR, Lin C, Kennedy J, Hou L, Trute M, Sokal I, Yan P, Schoenherr RM, Zhao L, Voytovich UJ, et al. A targeted proteomics-based pipeline for verification of biomarkers in plasma. *Nat Biotechnol*. 2011; 29(7):625–634. [PubMed: 21685906]
7. Neubert H, Gale J, Muirhead D. Online high-flow peptide immunoaffinity enrichment and nanoflow LC-MS/MS: assay development for total salivary pepsin/pepsinogen. *Clin Chem*. 2010; 56(9):1413–1423. [PubMed: 20472821]
8. van den Broek I, Nouta J, Razavi M, Yip R, Bladergroen MR, Romijn FPHTM, Smit NPM, Drews O, Paape R, Suckau D, et al. B-100 in clinical samples using an automated SISCAPA-MALDI-TOF-MS workflow. *Methods*. 2015; 81:74–85. [PubMed: 25766926]
9. Schoenherr RM, Whiteaker JR, Zhao L, Ivey RG, Trute M, Kennedy J, Voytovich UJ, Yan P, Lin C, Paulovich AG. Multiplexed quantification of estrogen receptor and HER2/Neu in tissue and cell lysates by peptide immunoaffinity enrichment mass spectrometry. *Proteomics*. 2012; 12(8):1253–1260. [PubMed: 22577026]
10. Kuhn E, Addona T, Keshishian H, Burgess M, Mani DR, Lee RT, Sabatine MS, Gerszten RE, Carr SA. Developing Multiplexed Assays for Troponin I and Interleukin-33 in Plasma by Peptide Immunoaffinity Enrichment and Targeted Mass Spectrometry. *Clin Chem*. 2009; 55(6):1108–1117. [PubMed: 19372185]
11. Hoofnagle AN, Becker JO, Wener MH, Heinecke JW. Quantification of thyroglobulin, a low-abundance serum protein, by immunoaffinity peptide enrichment and tandem mass spectrometry. *Clin Chem*. 2008; 54(11):1796–1804. [PubMed: 18801935]
12. Ahn YH, Lee JY, Lee JY, Kim YS, Ko JH, Yoo JS. Quantitative analysis of an aberrant glycoform of TIMP1 from colon cancer serum by L-PHA-enrichment and SISCAPA with MRM mass spectrometry. *J Proteome Res*. 2009; 8(9):4216–4224. [PubMed: 19645485]
13. Berna M, Ott L, Engle S, Watson D, Solter P, Ackermann B. Quantification of NTproBNP in rat serum using immunoprecipitation and LC/MS/MS: a biomarker of drug-induced cardiac hypertrophy. *Anal Chem*. 2008; 80(3):561–566. [PubMed: 18179251]
14. Schoenherr RM, Saul RG, Whiteaker JR, Yan P, Whiteley GR, Paulovich AG. Anti-peptide monoclonal antibodies generated for immuno-MRM assays have a high probability of supporting Western blot and ELISA. *Mol Cell Proteomics*. 2014
15. Schoenherr RM, Zhao L, Whiteaker JR, Feng LC, Li L, Liu L, Liu X, Paulovich AG. Automated screening of monoclonal antibodies for SISCAPA assays using a magnetic bead processor and liquid chromatography-selected reaction monitoring-mass spectrometry. *J Immunol Methods*. 2010; 353(1–2):49–61. [PubMed: 19961853]
16. Pope ME, Soste MV, Eyford BA, Anderson NL, Pearson TW. Anti-peptide antibody screening: selection of high affinity monoclonal reagents by a refined surface plasmon resonance technique. *J Immunol Methods*. 2009; 341(1–2):86–96. [PubMed: 19041872]
17. Razavi M, Pope ME, Soste MV, Eyford BA, Jackson AM, Anderson NL, Pearson TW. MALDI immunoscreening (MiSCREEN): a method for selection of anti-peptide monoclonal antibodies for use in immunoproteomics. *J Immunol Methods*. 2011; 364(1–2):50–64. [PubMed: 21078325]
18. Razavi M, Johnson LD, Lum JJ, Kruppa G, Anderson NL, Pearson TW. Quantification of a proteotypic peptide from protein C inhibitor by liquid chromatography-free SISCAPA-MALDI

- mass spectrometry: application to identification of recurrence of prostate cancer. *Clin Chem.* 2013; 59(10):1514–1522. [PubMed: 23857672]
19. Whiteaker JR, Zhao L, Frisch C, Ylera F, Harth S, Knappik A, Paulovich AG. High-affinity recombinant antibody fragments (Fabs) can be applied in peptide enrichment immuno-MRM assays. *J Proteome Res.* 2014; 13(4):2187–2196. [PubMed: 24568200]
 20. Whiteaker JR, Zhao L, Abbatiello SE, Burgess M, Kuhn E, Lin C, Pope ME, Razavi M, Anderson NL, Pearson TW, et al. Evaluation of large scale quantitative proteomic assay development using peptide affinity-based mass spectrometry. *Mol Cell Proteomics.* 2011; 10(4):M110.005645. [PubMed: 21245105]
 21. Harlow, E.; Lane, D. *Antibodies: A Laboratory Manual.* Cold Spring Harbor Laboratory Press; Cold Spring Harbor, New York: 1988.
 22. Fang X, Zhang WW. Affinity separation and enrichment methods in proteomic analysis. *J Proteomics.* 2008; 71(3):284–303. [PubMed: 18619565]
 23. Anderson NL, Jackson A, Smith D, Hardie D, Borchers C, Pearson TW. SISCAPA peptide enrichment on magnetic beads using an in-line bead trap device. *Mol Cell Proteomics.* 2009; 8(5): 995–1005. [PubMed: 19196707]
 24. Whiteaker JR, Zhao L, Anderson L, Paulovich AG. An automated and multiplexed method for high throughput peptide immunoaffinity enrichment and multiple reaction monitoring mass spectrometry-based quantification of protein biomarkers. *Mol Cell Proteomics.* 2010; 9(1):184–196. [PubMed: 19843560]
 25. MacLean B, Tomazela DM, Shulman N, Chambers M, Finney GL, Frewen B, Kern R, Tabb DL, Liebler DC, MacCoss MJ. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinforma Oxf Engl.* 2010; 26(7):966–968.
 26. Hage, DS. *Handbook of Affinity Chromatography.* Taylor and Francis Group; Boca Raton, FL: 2006. Application and Elution in Affinity Chromatography; p. 944
 27. Moser AC, Hage DS. Immunoaffinity chromatography: an introduction to applications and recent developments. *Bioanalysis.* 2010; 2(4):769–790. [PubMed: 20640220]
 28. Whiteaker JR, Zhao L, Zhang HY, Feng LC, Piening BD, Anderson L, Paulovich AG. Antibody-based enrichment of peptides on magnetic beads for mass-spectrometry-based quantification of serum biomarkers. *Anal Biochem.* 2007; 362(1):44–54. [PubMed: 17241609]

A Evaluation of bound peptide removal **B Determination of regenerated bead performance** **Figure 1.**

Overview of experimental approach. A. Peptide removal from antibody-coupled beads was evaluated by measuring the signal for a multiplexed panel of peptide assays enriched from a background of cell lysate digest. Following enrichment, the captured peptides were washed twice with 5% acetic acid in 3% acetonitrile. The process was repeated for five total cycles. The eluate from each cycle was analyzed by LC-MRM-MS. B. Performance of reused beads was evaluated by measuring three levels of peptides spiked into a cell lysate digest and comparing the results to the initial elution. In between analysis of the samples, the beads were washed with two cycles of acid washing. The samples were collected from each cycle and the peak areas and peak area ratios measured by LC-MRM-MS for each set of peptides.

Figure 2.

Acid washing removes detectable peptide. A. Chromatogram of peptides (light and heavy) from initial elution of 750 fmol heavy peptide and 150 fmol light peptide spiked into 500 µg of cell lysate digest. B. Chromatogram showing total light and heavy peptide signal remaining after 2 acid wash cycles of the antibody beads. The inset zooms in on the y-axis scale.

Figure 3.

Intensity of detected peptides is indistinguishable from noise. A. The carryover (fraction of peptide compared to initial enrichment) and intensity relative to background signal of detected heavy peptides in acid wash cycles 2 through 5. Heavy peptides were originally spiked into 500 μg cell lysate digest at 750 fmol. B. The carryover (fraction of peptide compared to initial enrichment) and intensity relative to background signal of detected light peptides in acid wash cycles 2 through 5 determined for the peptide assays in 500 μg cell lysate. Light peptides were originally spiked into the cell lysate digest at 150 fmol. Box plots show median (bar), inner quartiles (box), 5–95 percentiles (line), and outliers (points).

A Comparison of Peak Area Ratio **B Comparison of Peak Area** **Figure 4.**

Performance of the antibody beads is maintained after reuse. A. Peak area ratios for spiked peptides at three concentrations (low: 7.5 fmol, medium: 75 fmol, and high: 750 fmol) are plotted for reused antibody beads (Cycles 1–10, y-axis) compared to values measured in the initial elution (Cycle 0, x-axis). Slopes and correlation coefficient squared (R^2) are reported from linear regression of the scatter plots for each cycle. B. Same analysis as above using raw peak areas of the measured peptides. Measurements for all 60 peptides were included in the analysis.

Figure 5.

Distribution of CVs shows consistency of reusing beads. The CVs of triplicate measurement of peak area ratio (SIS/light) measured at three levels (low, medium, and high) plotted for antibody beads reused between 1 and 10 times. Cycle 0 refers to the initial eluate. Box plots show the median (bar), inner quartiles (box), and 5–95th percentiles (whiskers), with individual outliers as points.