Chapter 9

Multiplexed Immunoaffinity Enrichment of Peptides with Anti-peptide Antibodies and Quantification by Stable Isotope Dilution Multiple Reaction Monitoring Mass Spectrometry

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Abstract

Immunoaffinity enrichment of peptides using anti-peptide antibodies and their subsequent analysis by targeted mass spectrometry using stable isotope-labeled peptide standards is a sensitive and relatively high-throughput assay technology for unmodified and modified peptides in cells, tissues, and biofluids. Suppliers of antibodies and peptides are increasingly aware of this technique and have started incorporating customized quality measures and production protocols to increase the success rate, performance, and supply of the necessary reagents. Over the past decade, analytical biochemists, clinical diagnosticians, antibody experts, and mass spectrometry specialists have shared ideas, instrumentation, reagents, and protocols, to demonstrate that immuno-MRM-MS is reproducible across laboratories. Assay performance is now suitable for verification of candidate biomarkers from large scale discovery "omics" studies, measuring diagnostic proteins in plasma in the clinical laboratory, and for developing a companion assay for preclinical drug studies. Here we illustrate the process for developing these assays with a step-by-step guide for a 20-plex immuno-MRM-MS assay. We emphasize the need for analytical validation of the assay to insure that antibodies, peptides, and mass spectrometer are working as intended, in a multiplexed manner, with suitable assay performance (median values for 20 peptides: CV = 12.4 % at 740 amol/μL, LOD = 310 amol/μL) for applications in quantitative biology and candidate biomarker verification. The assays described conform to Tier 2 (of 3) level of analytical assay validation (1), meaning that the assays are capable of repeatedly measuring sets of analytes of interest within and across samples/experiments and employ internal standards for each analyte for confident detection and precise quantification.

Key words Anti-peptide antibody, Protein assay, Peptide assay, Multiplexed, Quantification, Mass spectrometry, Immunoaffinity enrichment, Reverse curves, Plasma, Biomarkers, Multiple reaction monitoring, Selected reaction monitoring, Parallel reaction monitoring

1 Introduction

Sensitive and selective detection and quantification of peptides using targeted mass spectrometry has become an essential component of verification studies of candidate disease biomarkers and is being

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increasingly used in biology and clinical diagnostics (1–10 and elsewhere in this book). Historically these mass spectrometry-based peptide assays have been most widely developed and applied using triple quadrupole mass analyzers using a method known as multiple reaction monitoring (MRM) (also referred to as selected reaction monitoring, SRM) experiments [11–14], and assays multiplexed up to several hundred analytes are now achievable [15]. In these experiments a subset of sequence-defining fragment ions (usually 3–5) are selected from the precursor peptide and monitored to increase sensitivity and selectivity of analysis [16, 17]. With improvements in the sensitivity and data acquisition speed of mass spectrometers, these assays can now be robustly developed and applied using instruments that acquire full mass spectra at high resolution and mass accuracy, greatly increasing the selectivity and specificity of analysis, a method referred to as parallel reaction monitoring or PRM [18–20]. Adding stable isotope-labeled versions of the analyte peptides as internal standards [21–23], or labeled proteins when available [24, 25] is necessary to insure that the desired analyte is being measured and that the quantification is precise.

MRM assays can now be configured to quantitatively measure peptides and modified peptides from nearly any protein. However, sample complexity and the wide dynamic range of protein abundance in sample matrices like plasma and tissue require additional steps be taken besides assay development to insure detection of analytes that are present at low abundance in biological samples. Several approaches have become standard for plasma analysis, including the use of immunoaffinity depletion columns which remove the 6–60 most abundant proteins thereby facilitating detection of proteins present at lower abundances [26–30]. Fractionating peptide digests of depleted plasma by ion exchange [5, 8, 12] or high pH reversed-phase chromatography [7, 31–33] prior to targeted analysis by MS, a process referred to as fractionMRM (fMRM), reduces sample complexity and enhances sensitivity and specificity of analyte measurement. Combining immunoaffinity depletion with fMRM has resulted in robust, practical methods to quantitatively measure, in high multiplex, peptides from proteins that are present in the high picogram to low nanogram/mL concentration in plasma [5, 12, 15]. Even greater sensitivities can be achieved for small numbers of analytes by taking fMRM to extremes, isolating small numbers of peptides into very small volumes suitable for direct analysis using targeted MS [34].

Antibodies have been used by biologists and clinical laboratories for decades to enrich analytes of interest from biological samples by immunoprecipitation (IP) for detection and quantification [35, 36]. In 2004, Anderson et al. [37] described the use of antibodies raised against proteotypic tryptic peptides to immunoprecipitate analyte peptides from proteolytic digests of plasma. The enriched peptides were subsequently analyzed by LC-MRM-MS

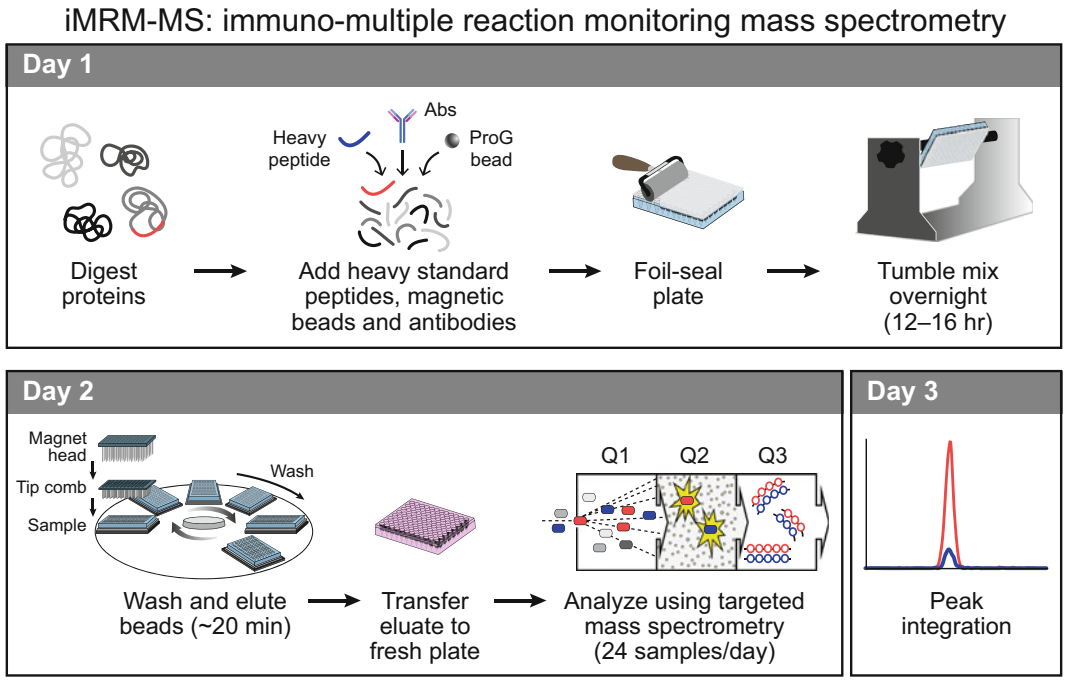


Fig. 1 Outline of the automated iMRM Assay Workflow. On day 1, peptide standards, protein G magnetic beads, and a mixture of antibodies were added to wells on a plate containing digested plasma proteins (digestion workflow not shown). After using a roller to firmly cover the plate with foil, samples were tumble-mixed 12–16 h overnight. On day 2, the Protein G beads, with antibodies and peptides bound, were washed and peptides eluted using a Kingfisher magnetic bead handler. Supernatants from the eluate plate were transferred to a fresh plate and analyzed by LC-MRM-MS. On day 3, data are analyzed. The heavy (*blue colored oval*) and light peptide (*red colored oval*) peak areas were integrated and the peak area ratios used to determine the molar concentration of the peptides in each sample

and quantified using stable isotope-labeled peptides added to the sample prior to IP and co-enriched with the analyte peptides (Fig. 1). This method was termed SISCAPA for Stable Isotope Standards and Capture by Anti-peptide Antibodies; more recently it has been referred to as immunoaffinity-MRM (iMRM). iMRM provides a one-step enrichment method capable of providing sufficient amounts of analyte peptides for MS analysis from even low abundance proteins. Prior removal of abundant proteins or fractionation at the protein or peptide level is not required [37–42]. Another advantage is that only a single capture Ab is required as the mass spectrometer substitutes for a secondary detection Ab, providing high sequence specificity and readily distinguishing the desired analyte from nonspecifically enriched peptides. The approach works equally well for modified peptides such as phosphopeptides [43], and it can adapted for and combined with capture at the protein level [44, 45]. Immunoaffinity enrichment of peptides requires generation of custom Abs for each peptide

target. This can be a relatively lengthy and expensive process especially if the goal is to generate monoclonal Abs that can be distributed to labs throughout the world. However, the success rate for obtaining anti-peptide Abs useful in iMRM assays is substantially higher than for generating IP-competent anti-protein Abs [7, 37, 41, 46]. Highly purified peptide antigens are synthesized with ease and a single rabbit can be immunized in batches of up to five peptide antigens to yield mg quantities of IgG that IP sufficiently well and function in iMRM assays [7, 37, 41, 46]. Throughput can be significantly improved by using Protein G coated magnetic beads and bead-handling robotics to automate peptide capture, wash, and elution steps [38, 46]. iMRM assays can be multiplexed to as high as 50 antibodies in a single sample [6, 7, 41, 47–49]. Interlaboratory studies have shown that iMRM assays are robust and reproducible across laboratories, with detection limits approximating ca. 1 ng of protein per mL of plasma and assay CVs of 15 % or less [49]. The interested reader is directed to the growing body of literature describing configuration and use of iMRM assays for biology, preclinical and clinical measurements [6, 7, 40–50].

There are several distinct steps to the generation, analytical validation, and application of an iMRM assay. First, peptides for use as internal standards and for assay development are selected. This step is informed by what peptides have been previously observed for the proteins of interest. In the absence of experimental data, in silico methods have been developed and can be used. Peptides are examined for uniqueness to the candidate protein as well as to any other protein sequence in the sample to be studied, and nonspecific peptides discarded. When multiple peptides for a protein meet these criteria, those exhibiting the highest MS response, as well as those predicted to have good retention behavior on reversed-phase, are favored. Second, MRM transitions are selected and optimized for the heavy synthetic peptide standards to configure the LC-MRM-MS portion of the assay. Third, anti-peptide antibodies are made and the resulting Abs are evaluated for their ability to capture target peptides in a simplified iMRM assay in a matrix background that suitably mimics the matrix planned for final analysis (e.g. digested plasma for plasma assays, digested tissue from the same source of tissue, similar cell lysate backgrounds, etc). This step identifies which of the 2–5 immunogen peptides developed for each protein is efficiently captured and detected by iMRM, and is therefore suitable for full assay development. In addition, some evidence of how well the endogenous analyte is detected can also be derived at this step [46]. The performance (i.e., linearity, precision, LOD/LOQ) of the antibodies and selected peptides are then systematically evaluated. This is typically done using response curves generated by a method of standard addition in which increasing amounts of light peptide are added to the matrix while keeping the concentration of heavy peptide internal

standard constant [51]. Alternatively, in cases where endogenous analyte was found or is expected to be present in the matrix, the heavy peptide may be added over a concentration range and a constant amount of light peptide (either added or endogenous) used as the internal standard. This approach is commonly referred to as "surrogate analyte" [52, 53]. Additional experiments may be used to further define the range and applicability of the iMRM assay, including repeatability, selectivity, stability, and reproducibility of endogenous detection [54]. In addition, experiments may be performed to optimize the amount of antibody per assay and determine the range of multiplexing (quantity of individual antibodies purified against separate peptide antigens used in a single capture) where performance is maintained [48].

Here we describe the generation of a 20-plex iMRM assay and the methods used to assess its performance in the context of a plasma matrix. The methods used are generalizable to smaller or larger multiplexes of Abs and are equally applicable to use in cell lines, tissues, or other biofluids like CSF.

2 Materials

1. Tryptic peptide standards (light versions): Amino acid sequences unique to a single protein (proteotypic) synthesized as free acids with unblocked termini (*see* **Note 1**), purified by RPLC, verified by MALDI and quantified by AAA. Light peptides are diluted, aliquoted, and formulated in 30 % acetonitrile/0.1 % formic acid at 100 pmol/μL. Refer to sequences and gene names in Table 1.
2. Tryptic peptide standards (heavy versions): Amino acid sequences that match the sequences of the light versions in 2.1 are synthesized with the C-terminal Arg or Lys residue labeled with heavy stable isotopes of carbon (^{13}C) or nitrogen (^{15}N) using $^{13}C_6$ l-Lysine, $^{13}C_6$ l- Arginine, or $^{13}C_6$, $^{15}N_4$ l-Arginine. The MRM-MS experiments rely on co-elution of the light and heavy versions of the peptides on RP-HPLC. Use of deuterium is not recommended for use in synthesis of heavy-labeled peptides as the isotope effect of deuterium (especially multiple deuterium atoms) can shift the retention time of the heavy versus the light version of the peptide on RP-HPLC. Heavy isotope-labeled peptides are diluted, aliquoted, and formulated identically to the light versions of the peptides as described, above. Refer to sequences and gene names in Table 1 (*see* **Note 2**).
3. Human plasma: plasma isolated from blood using potassium EDTA (purple tubes) from an individual or a pool of healthy individuals (Bioreclamation—K2EDTA), shipped in 1 mL aliquots and stored at −80 °C (*see* **Note 3**).

Table 1
Summary of peptides, proteins, and antibodies used in 20-plex iMRM assay evaluation

No.	mAb or pAb	Gene symbol	Uniprot protein name	Peptide sequence	Conc. (μg/μL)
1	pAb	TNNI	Troponin I	NITEIADLTQK	0.98
2	mAb	IL33	IL-33	TDPGVFIGVK	1
3	mAb	FTL	Ferritin light chain	LGGPEAGLGEYLFER	0.56
4	mAb	AFP	Alpha-fetoprotein	GYQELLEK	0.71
5	pAb	AFP	Alpha-fetoprotein	YIQESQALAK	0.12
6	mAb	ERBB2	Her-2	AVTSANIQEFAGCK	0.71
7	pAb	MUC1	Mucin-1	EGTINVHDVETQFNQYK	1.04
8	mAb	MUC16	Mucin-16	ELGPYTLDR	0.71
9	mAb	TG	Thyroglobulin	FSPDDSAGASALLR	0.8
10	pAb	TG	Thyroglobulin	VIFDANAPVAVR	1.39
11	pAb	ERBB2	Her-2	GLQSLPTHDPSPLQR	1.65
12	pAb	ERBB2	Her-2	VLGSGAFGTVYK	0.57
13	pAb	ANXA1	Annexin A1	GVDEATIIDILTK	0.68
14	pAb	CLIC1	Chloride intracellular channel 1	GFTIPEAFR	1.43
15	pAb	IL18	Interleukin 18	ISTLSCENK	2.22
16	pAb	NFKB2	Nuclear factor NF-kappa-B p100 subunit	IEVDLVTHSDPPR	0.82
17	pAb	FSCN1	Fascin	LSCFAQTVSPAEK	0.78
18	pAb	TAGLN	Transgelin	AAEDYGVIK	1.05
19	pAb	EZR	Ezrin	SQEQLAAELAEYTAK	0.94
20	pAb	PRDX4	Peroxiredoxin 4	QITLNDLPVGR	1.03

4. Polyclonal antibodies: polyclonal antibodies generated against target tryptic peptide sequences in rabbits (*see* **Note 4**), quantified by protein assay and formulated in 25 % glycerol/1× PBS/0.1 % sodium azide.
5. Monoclonal antibodies: monoclonal antibodies generated by clonal expansion of the rabbit immune cells isolated from the spleens harvested from the rabbits used in 4 (*see* **Note 5**), quantified by protein assay and formulated in 25 % glycerol/1× PBS pH 7.4/0.1 % sodium azide.
6. Peptide storage solvent: 30 % acetonitrile/0.1 % formic acid. 300 mL LC-MS grade acetonitrile, 700 mL HPLC grade water, 1 mL formic acid.

7. Antibody storage solution: 25 % glycerol/1× PBS pH 7.4/0.1 % sodium azide. 250 mL Glycerol, 1 packet PBS (Sigma), 1 g sodium azide dissolved into a final volume of 1 L HPLC grade water.
8. Sample diluent/Antibody Elution Solvent: 3 % Acetonitrile/5 % Acetic acid. 3 mL LC-MS grade acetonitrile, 5 mL acetic acid dissolved into a final volume of 100 mL HPLC grade water.
9. Trypsin, TPCK treated (Worthington).
10. TCEP solution: 0.5 M TCEP (BioRad).
11. Desalting Equilibration Solvent: 80 % Acetonitrile (ACN)/0.1 % trifluoroacetic acid (TFA). 800 mL of ACN, 1 mL TFA to a final volume of 1 L with HPLC grade water.
12. Desalting Load and Wash Solvent: 0.1 % TFA. Add 1 mL TFA to a final volume of 1 L with HPLC grade water.
13. Desalting Elution Solvent: 45 % ACN/0.1 % TFA. 450 mL of ACN, 1 mL TFA to a final volume of 1 L with HPLC grade water.
14. Antibody wash buffer 1: 1× PBS pH 7.4, 0.03 % CHAPS. 300 mg CHAPS, one packet of PBS (Sigma) dissolved in 1 L HPLC grade water.
15. Antibody wash buffer 2: 0.1× PBS pH 7.4, 0.03 % CHAPS. Add 100 mL of 1× PBS pH 7.4 and 300 mg CHAPS into 900 mL HPLC grade water.
16. Antibody storage buffer: 1× PBS/0.03 % CHAPS/0.1 % sodium azide. Dissolve 30 mg CHAPS and 1 g sodium azide in 1 L 1× PBS.
17. Antibody collection buffer: 1× PBS/100 mM Tris–HCl pH 8.1/0.03 % CHAPS/0.1 % sodium azide. Dissolve one packet of PBS (Sigma) and 28 g Tris–HCl pH 8.1 crystals (Sigma) 30 mg CHAPS and 1 g sodium azide in 1 L HPLC grade water.
18. Tris HCl solution: 200 mM Tris–HCl pH 8.1: Dissolve 14 g of Tris–HCl pH 8.1 crystals (Sigma) in 500 mL HPLC grade water.
19. 1 μm Protein G magnetic beads (Dynal) (NB: the 1 μm beads are no longer commercially available, but 2.8 μm beads are and can be used for the 20-plex level described here. An alternate source (Pierce/Life technologies/Thermo) of 1 μm Protein G magnetic beads may also be used).
20. KingFisher 96 magnetic particle processor (Thermo).
21. KingFisher 250 μL polypropylene 96-well plates (Thermo).
22. Barnstead Thermolyne Lab Quake Shaker (VWR).
23. Polypropylene 96-well hard-shell skirted PCR plates (BioRad).
24. Oasis HLB cartridges (Waters).

3 Methods

The key steps in developing and analytically validating iMRM assays are described below and illustrated in Fig. 1. Detailed descriptions of LC-MRM-MS data collection and analysis of MRM data can be found in references 3, 4, 12–17 and elsewhere in this volume.

3.1 Plasma Digestion and Desalt (Adapted from refs. 13, 49)

1. Remove 3 × 1 mL of plasma from the −80 °C freezer and thaw at ambient temperature (~30 min).
2. Turn on floor mixer incubator and set to 37 °C and rpm to 180. Configure with a 50 mL tube holder if necessary.
3. Add the following to one 50 mL Falcon tube, return any excess plasma to −80 °C freezer: 3 mL plasma, 2.73 g Urea, 1 mL 1 M Tris pH 8.0, 600 µL 0.5 M TCEP.
4. Mix briefly by gentle vortexing and place in incubator at 37 °C and 180 rpm. Once Urea has dissolved, incubate for additional 30 min.
5. Remove from incubator and cool to room temperature.
6. Weigh 462 mg of Iodoacetamide and dissolve in 5 mL 0.2 M Trizma pH 8.1 (500 mM IAA).
7. Add 2 mL of 500 mM IAA into 50 mL tube containing denatured plasma.
8. Mix briefly by gentle vortexing and let stand in the dark at room temperature for 30 min (*see* **Note 6**).
9. Add 40 mL 0.2 M Trizma pH 8.1. Total volume should be ~48 mL (*see* **Note 7**).
10. Verify pH ≥ 8.0 by pipetting 5 µL onto a 5–10 pH range pH test strip (EMD).
11. Carefully weigh 3 mg of TPCK-treated trypsin powder into a tared 15 mL Falcon tube (*see* **Note 8**).
12. Dissolve in 3 mL 0.2 M Trizma pH 8.1 and transfer to digestion mixture.
13. Incubate overnight (12–16 h) at 37 °C at 180 rpm.
14. Add 0.8 mL formic acid. Mix briefly by vortexing and verify pH < 3 by pipetting 5 µL of the digestion mixture onto a 2–5 pH range pH test strip.
15. Store at 4 °C until desalt step.

    If desalt is postponed until a later day, freeze digest mixture at −80 °C.
16. Prepare and label 3 × 1 g Oasis cartridges, one for each third of the total digest volume.
17. Install all three cartridges onto the vacuum manifold using pipet tip adaptors.

18. Condition each cartridge using 3×20 mL Desalting Equilibration Solvent (80 % ACN/0.1 % TFA).
19. Equilibrate each cartridge using 4×20 mL Desalting Wash and Loading Solvent (0.1 % TFA).
20. Add an additional 4 mL Desalting Wash and Loading Solvent (0.1 % TFA) to each cartridge but do not apply vacuum.
21. Divide the total digest volume into three equal volumes and add each third in 4 mL increments onto one of the three Oasis cartridges. Draw vacuum and load additional volume until the entire one-third of the total digest is loaded across the three cartridges.
22. Wash each cartridge using 3×20 mL Desalting Wash and Loading Solvent (0.1 % TFA).
23. Elute from each cartridge using into a fresh tube using 2×6 mL Desalting Elution Solvent (45 % ACN/0.1 % TFA).
24. Pool the eluates from all three Oasis cartridges into a single 50 mL Falcon tube.
25. Mix briefly by gentle vortexing and dispense an equivalent volume (e.g. 1.5 mL) into ten 2 mL polypropylene tubes (Sarstedt).

    Alternatively, the entire volume may be dried by lyophilization into a single tube.
26. Reduce volume in each tube to less than 0.5 mL/tube by rotary evaporation.
27. Add an additional equivalent volume (e.g. 1 mL) into each of the ten 2 mL tubes and dry to less than 0.5 mL by rotary evaporation. Continue until the remaining volume of the Oasis cartridge eluate is equally dispensed across all tubes.
28. Dry each tube completely by rotary evaporation.
29. Store at −80 °C until use.

3.2 Reverse Curve Preparation (Adapted from ref. 57)

1. Thaw peptide stock solutions on wet ice.
2. Combine 5 µL of each light peptide 100 pmol/µL solution into one tube (100 µL). Label as "Light Stock, 5 pmol/µL, 30 % ACN/0.1 % FA."
3. Combine 5 µL of each heavy peptide 100 pmol/µL solution into one tube (100 µL). Label as "Heavy Stock, 5 pmol/µL, 30 % ACN/0.1 % FA."
4. Resuspend 1×0.3 mL equivalent of digested lyophilized plasma into 270 µL 1× PBS, 0.03 % CHAPS and 30 µL 1 M Tris pH 8.0. Vortex and mix well for 30 min at RT.
5. Prepare 10 mL of peptide dilution buffer (1× PBS, 0.03 % CHAPS, 0.2 % digested plasma). Add 20 µL of resuspended digested plasma into 10 mL of 1× PBS, 0.03 % CHAPS.
6. Prepare 100 fmol/µL light peptide mix: add 10 µL of light peptide stock (5 pmol/µL) into 490 µL peptide dilution buffer (1× PBS, 0.03 % CHAPS, 0.2 % digested plasma).

7. Prepare Reverse Curve background plasma matrix: Pipet 5685 µL of 1× PBS, 0.03 % CHAPS pH 7.4 into a 15 mL Falcon tube. Add the resuspended 0.3 mL of digested plasma. Add 15 µL of 100 fmol/µL light peptide mix. Mix briefly by gentle vortexing.
8. Label 1.5 mL polypropylene centrifuge tubes No. 1–8.
9. Add 1045 µL of Reverse Curve Background Matrix to tube 8 and 700 µL into tubes 1–7. Keep tubes on wet ice.
10. Prepare 200 fmol/µL heavy peptide mix: add 20 µL of heavy peptide stock (5 pmol/µL) into 480 µL peptide dilution buffer (1× PBS, 0.03 % CHAPS, 0.2 % digested plasma).
11. Add 5 µL of 200 fmol/µL heavy peptide mix to tube 8. Mix briefly by gentle vortexing.
12. Transfer 350 µL of tube 8 into tube 7. Mix briefly by gentle vortexing.
13. Continue serial dilution repeating the process in similar manner transferring 350 µL from tube 7 to tube 6, tube 6 to tube 5 down to tube 2 remove 350 µL from tube 2 and discard (tube 1 is blank and contains light peptide only).
14. Freeze in −80 °C until next step (*see* **Note 9**).

3.3 Crosslinking Antibodies to Protein G Beads (Optional: See *Note 10. Skip to Subheading 3.4 for Procedure Without Crosslinking Antibodies to Beads)*

1. Prepare antibody crosslinking solutions:
   (a) Antibody equilibration solution: 200 mM triethanolamine (TEA) pH 8.5. Add 10 mL triethanolamine into 400 mL HPLC-grade water. Adjust pH to 8.5 using a target of 2 mL 5 N HCl. Add 1.8 mL of 5 N HCl, mix well and add the remaining 200 µL dropwise until pH is 8.5 (*see* **Note 11**).
   (b) Antibody crosslinking solution: 20 mM Dimethyl pimelimidate (DMP) in 200 mM TEA pH 8.5. Dissolve 1.03 g of DMP in 200 mL of Antibody Equilibration Solution.
   (c) Antibody quenching solution: 150 mM monoethanolamine (MEA) pH 9.0: Add 3.6 mL monoethanolamine in 400 mL HPLC-grade water. Adjust pH to 9.0 using a target of 7.5 mL 5 N HCl. Add 7.3 mL of 5 N HCl, mix well and add the remaining 200 µL dropwise until pH is 9.0.
   (d) Antibody wash solution: 5 % acetic acid/0.03 % CHAPS: Add 50 mL glacial acetic acid and 30 mg of CHAPS into a final volume of 1 L HPLC grade water.
   (e) Antibody Storage buffer: 1× PBS/0.03 % CHAPS/0.1 % sodium azide. Dissolve 30 mg CHAPS and 1 g sodium azide into a final volume of 1 L 1× PBS.
2. Add 1550 µL magnetic beads to volume containing the 775 µg required for this curve analysis in a 15 mL Falcon tube.
3. Tumble mix or rock mixture gently for 1–2 h at room temperature.

4. Place the magnet next to the tube and allow the beads to collect on the side of the tube, remove and discard supernatant.
5. Resuspend beads in 900 µL Antibody wash buffer 1 (1× PBS pH 7.4/0.03 % CHAPS). Mix briefly by gentle vortexing and store at 4 °C or on wet ice until use.
   The following crosslinking steps are performed at room temperature.
6. Place the magnet next to the tube and allow the beads to collect on the side of the tube.
7. Remove and discard supernatant. Add 1 mL Antibody equilibration solution and mix by gentle vortexing for 5 min.
8. Use magnet to collect beads on side of tube. Remove and discard supernatant and repeat equilibration with 1 mL Antibody equilibration solution.
9. Use magnet to collect beads on side of tube. Remove and discard supernatant. Add 1 mL Antibody crosslinking solution and mix by gentle vortexing. Continue tumble mixing for 30 min.
10. Use magnet to collect beads on side of tube. Remove and discard supernatant. Add 1 mL Antibody quenching solution and mix by gentle vortexing for 5 min. Continue tumble mixing for 60 min.
11. Use magnet to collect beads on side of tube. Remove and discard supernatant. Add 1 mL Antibody wash solution and mix by gentle vortexing for 5 min.
12. Use magnet to collect beads on side of tube. Remove and discard supernatant and repeat wash with 1 mL Antibody wash solution.
13. Use magnet to collect beads on side of tube. Remove and discard supernatant.
14. Add 1250 µL of Antibody storage buffer and mix by gentle vortexing. Store at 4 °C until use.

3.4 Antibody Affinity Enrichment (Adapted from ref. 49)

Day One

1. Thaw antibody stock solutions on wet ice.
2. Add 50 µg of each polyclonal antibody and 15 µg of each monoclonal antibody in a labeled 2 mL polypropylene centrifuge tube. Refer to Table 1 for antibody concentrations. Keep tubes on wet ice (*see* **Note 12**).
3. Bind antibodies to Protein G beads without crosslinking (optional—*see* **Note 10** and Subheading 3.3 for crosslinking antibodies to beads).
4. Add 1550 µL magnetic beads to volume containing the 775 µg required for this curve analysis in a 15 mL Falcon tube.
5. Tumble mix or rock mixture gently for 1–2 h at room temperature.

6. Place the magnet next to the tube and allow the beads to collect on the side of the tube.
7. Remove the supernatant and resuspend the beads in 1250 µL of 1× PBS, 0.03 % CHAPS pH 7.4. Mix briefly by gentle vortexing and store at 4 °C until use.
8. Thaw tubes containing the reverse curves prepared above.
9. Pipet 200 µL of tube 1 (blank sample, no heavy peptide added) into well A1. Pipet two more replicates of 200 µL of tube 1 into wells A2 and A3 of a Thermo 250 µL KF 96-well plate.
10. Repeat in series down the rows, pipetting three replicates of 200 µL of tube 2 into wells B1, B2, and B3 until three replicates of tube 8, which contains the highest concentration of heavy peptide (200 fmol total) are added into wells H1, H2, and H3. Refer to the plate maps in Table 2.
11. Add 50 µL of antibody bead mixture to each well, pipetting up and down 3–4 times to mix completely. Use a fresh pipet tip for each well.
12. Seal plate securely using a roller to press adhesive foil seal over all wells.
13. Place plate on Labquake mixer using rubber bands, Velcro strips, or ties and turn on to mix slowly inverting overnight (12–16 h) at 4 °C.

Day Two

14. Install the PCR magnet head on the Kingfisher bead handling platform.
15. Prepare and load the following plates on the Kingfisher:

    Plate 1: incubation plate (digested plasma, peptides, antibodies, and beads (~250 µL)).

    Plate 2: 250 µL Antibody wash buffer 1 (1× PBS/0.03 % CHAPS).

    Plate 3: 250 µL Antibody wash buffer 1 (1× PBS/0.03 % CHAPS).

    Plate 4: 250 µL Antibody wash buffer 2 (0.1× PBS/0.03 % CHAPS).

    Plate 5: 30 µL Antibody Elution Solvent: (3 % ACN/5 % acetic acid).

    Plate 6: 200 µL Antibody collection buffer: (1× PBS/100 mM Tris pH 8.0/0.03 % CHAPS/0.1 % sodium azide).

    Plate 7: tip comb.

    All solutions prepared for plates 1–4 and 6 are pipetted into KingFisher 250 µL wellplates. Solutions for elution (plate 5) are pipetted into a 96-well PCR plate. The tip comb is held in an empty 250 µL wellplate from plate 7. It is picked up at the beginning of the method to cover and protect the magnet and returned to plate 7 upon completion.

Table 2
Plate maps used for replicate samples of concentration points in the reverse curve by (A) concentration point and replicate and (B) by total heavy peptide amount

	1	2	3	4	5	6
(A) By concentration point and replicate						
A	pt1—1	pt1—2	pt1—3			
B	pt2—1	pt2—2	pt2—3			
C	pt3—1	pt3—2	pt3—3			
D	pt4—1	pt4—2	pt4—3			
E	pt5—1	pt5—2	pt5—3			
F	pt6—1	pt6—2	pt6—3			
G	pt7—1	pt7—2	pt7—3			
H	pt8—1	pt8—2	pt8—3			
(B) By total heavy peptide amount (fmol)						
A	0	0	0			
B	0.3	0.3	0.3			
C	0.8	0.8	0.8			
D	2.5	2.5	2.5			
E	7.4	7.4	7.4			
F	22.2	22.2	22.2			
G	66.7	66.7	66.7			
H	200	200	200			

16. Load the KingFisher program. Use the up ˄ and down ˅ arrows to scroll through methods until the one described in Table 3 is displayed (*see* **Note 11**).
17. Remove plate from Labquake and centrifuge at 1400 RPM (130–400 × *g* depending on the type of centrifuge and rotor) for 30–60 s to remove liquid that may be on the seal surface. Typically, a SpeedVac concentrator centrifuge equipped with a microplate rotor is used.
18. Remove the foil seal carefully (*see* **Note 13**).
19. Place the incubation plate in plate position 1 on the Kingfisher.
20. Press "start" to begin the method.
21. When the KingFisher method is finished (approximately 20 min (*see* **Note 14**))—seal the incubation plate (plate 1) and the collected antibody bead plate (plate 6) with adhesive foil and store at −80 °C and 4 °C, respectively.

Table 3
Description of the plate layouts and protocol steps in the KingFisher program

Instrument: KingFisher 96				
Protocol template version: 2.6.0				
Created: April 26, 2011				
Plate layout:				
No. and position	Type	Description	Contents	Volume (μL)
1	KingFisher 96–250 μL	Plate_1_beadAbPep	Plasma, ProG beads, Abs, peptides, 1× PBS pH 7.4, 0.03 % CHAPS	250
2	KingFisher 96–250 μL	Plate_2_wash_1	1× PBS pH 7.4, 0.03 % CHAPS	250
3	KingFisher 96–250 μL	Plate_3_wash_2	1× PBS pH 7.4, 0.03 % CHAPS	250
4	KingFisher 96–250 μL	Plate_4_wash_3	0.1× PBS pH 7.4, 0.03 % CHAPS	250
5	PCR—100 μL	Plate_5_elution	3 % Acetonitrile/5 % acetic acid	30
6	KingFisher 96–250 μL	Plate_6_collection	1× PBS pH 7.4, 0.03 % CHAPS, 0.1 % NaN_3	200
7	KingFisher 96–250 μL	Plate_7_tips	KF 96 tip comb	Empty

Protocol steps:					
Step no.	Plate no.	Description	Beginning of step	Wash/elution parameters	End of step
1	1	Ab and Pep Capture	Release = yes Time = 10 s Speed = slow	Time = 5 m Speed = bottom slow	Collect = yes Count = 5
2	2	Wash 1	Release = yes Time = 10 s Speed = slow	Time = 1.5 m Speed = slow	Collect = yes Count = 5
3	3	Wash 2	Release = yes Time = 10 s Speed = slow	Time = 1.5 m Speed = slow	Collect = yes Count = 5
4	4	Wash 3	Release = yes Time = 10 s Speed = slow	Time = 1.5 m Speed = slow	Collect = yes Count = 5
5	5	Elution	Release = yes Time = 10 s Speed = bottom slow	Time = 5 m Speed = bottom slow Heating = no	Remove beads = Yes, collect count = 10, disposal plate = Plate 6

22. Remove elution plate (Plate 5) from the KingFisher and place onto the autosampler magnet plate on wet ice.
23. Label a fresh PCR plate "iMRM reverse curves" and add 5 µL of 3 % ACN/5 % HOAc to the wells designated in the plate map in Table 2.
24. Using a multichannel pipet set to 20 µL, draw the eluate supernatant without touching the bottom of the well and transfer into corresponding wells of a fresh PCR well plate (*see* **Note 15**).
25. Cover the PCR plates with silicon plate mats and transfer onto autosampler for LC-MRM-MS analysis (*see* **Note 16**).

3.5 LC-MRM-MS Analysis (Refer to refs. 3, 4, 12–17)

1. Inject one-third of the sample onto a triple quadrupole MS instrument configured with nanoflow liquid chromatograph and autosampler configured with a trap and analytical column and perform MRM-MS experiments, unscheduled or scheduled using the transition masses in Table 4 (*see* **Note 17**).
2. Verify the LC-MRM-MS system is ready for analysis by injecting and analyzing an appropriate system suitability standard [58], typically a mixture of peptide standards analyzed by MRM.
3. Inject samples in order from lowest concentration point to highest, replicates one, two and three for each point (e.g. pt1—0 fmol rep1, rep2, rep3) followed by an injection of blank (3 % ACN/5 % HOAc). Continue in sequence for the rest of the samples up to point 8 (200 fmol) (*see* **Note 18**).
4. Prepare a Skyline [59] document (version 3.1 https://brendanx-uw1.gs.washington.edu/labkey/project/home/software/Skyline/begin.view) that contains the peptide sequences with the light and heavy peptide masses of the peptides analyzed by MRM-MS (*see* **Note 19**).
5. Under Peptide Settings, confirm that the heavy label matches that heavy amino acid used for heavy peptide and select the light peptide as standard in the checkbox.
6. Import the reverse curve MS raw data in Skyline from the File, Import, Results drop-down window, selecting the appropriate data files.
7. Open and view the Result Grid, choose and add the columns "SampleGroup," "Concentration," and "IS Spike." Enter the curve designation (e.g. "pt1") and the concentration of heavy peptide (e.g. "0") in "Concentration" and concentration of light peptide (e.g. "20") in "IS Spike" (*see* **Note 20**).
8. Select "Integrate All" from the "Setting" drop-down window (a check mark will appear when selected). This makes sure that the integration for one version of the peptide (light or heavy) is applied to the other peptide (light or heavy) automatically.

Table 4
Unscheduled MRM method for 20 light and 20 heavy peptides, three transitions each ($n = 120$ transitions total)

Q1	Q3	Dwell	ID	DP	CE
623.3379	1018.542	10	TNNI3.NITEIADLTQK.+2y9.light	76.6	29.9
623.3379	788.4512	10	TNNI3.NITEIADLTQK.+2y7.light	76.6	29.9
623.3379	675.3672	10	TNNI3.NITEIADLTQK.+2y6.light	76.6	29.9
626.348	1024.562	10	TNNI3.NITEIADLTQK.+2y9.heavy	76.6	29.9
626.348	794.4714	10	TNNI3.NITEIADLTQK.+2y7.heavy	76.6	29.9
626.348	681.3873	10	TNNI3.NITEIADLTQK.+2y6.heavy	76.6	29.9
516.7898	816.4978	10	IL33.TDPGVFIGVK.+2y8.light	68.8	25.7
516.7898	719.445	10	IL33.TDPGVFIGVK.+2y7.light	68.8	25.7
516.7898	662.4236	10	IL33.TDPGVFIGVK.+2y6.light	68.8	25.7
519.7999	822.5179	10	IL33.TDPGVFIGVK.+2y8.heavy	68.8	25.7
519.7999	725.4652	10	IL33.TDPGVFIGVK.+2y7.heavy	68.8	25.7
519.7999	668.4437	10	IL33.TDPGVFIGVK.+2y6.heavy	68.8	25.7
804.4068	1154.584	10	FTL.LGGPEAGLGEYLFER.+2y10.light	89.8	37.2
804.4068	1083.547	10	FTL.LGGPEAGLGEYLFER.+2y9.light	89.8	37.2
804.4068	913.4414	10	FTL.LGGPEAGLGEYLFER.+2y7.light	89.8	37.2
807.4169	1160.604	10	FTL.LGGPEAGLGEYLFER.+2y10.heavy	89.8	37.2
807.4169	1089.567	10	FTL.LGGPEAGLGEYLFER.+2y9.heavy	89.8	37.2
807.4169	919.4615	10	FTL.LGGPEAGLGEYLFER.+2y7.heavy	89.8	37.2
490.2584	759.4247	10	AFP.GYQELLEK.+2y6.light	66.9	24.6
490.2584	631.3661	10	AFP.GYQELLEK.+2y5.light	66.9	24.6
490.2584	502.3235	10	AFP.GYQELLEK.+2y4.light	66.9	24.6
493.2684	765.4448	10	AFP.GYQELLEK.+2y6.heavy	66.9	24.6
493.2684	637.3862	10	AFP.GYQELLEK.+2y5.heavy	66.9	24.6
493.2684	508.3437	10	AFP.GYQELLEK.+2y4.heavy	66.9	24.6
575.8088	987.5469	10	AFP.YIQESQALAK.+2y9.light	73.1	28
575.8088	874.4629	10	AFP.YIQESQALAK.+2y8.light	73.1	28
575.8088	746.4043	10	AFP.YIQESQALAK.+2y7.light	73.1	28
578.8188	993.5671	10	AFP.YIQESQALAK.+2y9.heavy	73.1	28
578.8188	880.483	10	AFP.YIQESQALAK.+2y8.heavy	73.1	28

(continued)

Table 4
(continued)

Q1	Q3	Dwell	ID	DP	CE
578.8188	752.4244	10	AFP.YIQESQALAK.+2y7.heavy	73.1	28
549.2934	949.485	10	ERBB2.GLQSLPTHDPSPLQR.+3y8.light	71.2	31.5
549.2934	812.4261	10	ERBB2.GLQSLPTHDPSPLQR.+3y7.light	71.2	31.5
549.2934	697.3991	10	ERBB2.GLQSLPTHDPSPLQR.+3y6.light	71.2	31.5
552.6295	959.4933	10	ERBB2.GLQSLPTHDPSPLQR.+3y8.heavy	71.2	31.5
552.6295	822.4344	10	ERBB2.GLQSLPTHDPSPLQR.+3y7.heavy	71.2	31.5
552.6295	707.4074	10	ERBB2.GLQSLPTHDPSPLQR.+3y6.heavy	71.2	31.5
599.827	986.4942	10	ERBB2.VLGSGAFGTVYK.+2y10.light	74.8	29
599.827	842.4407	10	ERBB2.VLGSGAFGTVYK.+2y8.light	74.8	29
599.827	714.3821	10	ERBB2.VLGSGAFGTVYK.+2y6.light	74.8	29
603.8341	994.5084	10	ERBB2.VLGSGAFGTVYK.+2y10.heavy	74.8	29
603.8341	850.4549	10	ERBB2.VLGSGAFGTVYK.+2y8.heavy	74.8	29
603.8341	722.3963	10	ERBB2.VLGSGAFGTVYK.+2y6.heavy	74.8	29
748.3641	1325.615	10	ERBB2.AVTSANIQEFAGC[CAM]K.+2y12.light	85.7	34.9
748.3641	1224.568	10	ERBB2.AVTSANIQEFAGC[CAM]K.+2y11.light	85.7	34.9
748.3641	1066.499	10	ERBB2.AVTSANIQEFAGC[CAM]K.+2y9.light	85.7	34.9
748.3641	839.3716	10	ERBB2.AVTSANIQEFAGC[CAM]K.+2y7.light	85.7	34.9
751.3742	1331.636	10	ERBB2.AVTSANIQEFAGC[CAM]K.+2y12.heavy	85.7	34.9
751.3742	1230.588	10	ERBB2.AVTSANIQEFAGC[CAM]K.+2y11.heavy	85.7	34.9
751.3742	1072.519	10	ERBB2.AVTSANIQEFAGC[CAM]K.+2y9.heavy	85.7	34.9
751.3742	845.3917	10	ERBB2.AVTSANIQEFAGC[CAM]K.+2y7.heavy	85.7	34.9
674.657	928.4523	10	MUC1.EGTINVHDVETQFNQYK.+3y7.light	80.3	37.7
674.657	827.4046	10	MUC1.EGTINVHDVETQFNQYK.+3y6.light	80.3	37.7
674.657	699.3461	10	MUC1.EGTINVHDVETQFNQYK.+3y5.light	80.3	37.7
677.3284	936.4665	10	MUC1.EGTINVHDVETQFNQYK.+3y7.heavy	80.3	37.7
677.3284	835.4188	10	MUC1.EGTINVHDVETQFNQYK.+3y6.heavy	80.3	37.7

(continued)

Table 4
(continued)

Q1	Q3	Dwell	ID	DP	CE
677.3284	707.3603	10	MUC1.EGTINVHDVETQFNQYK.+3y5.heavy	80.3	37.7
532.2746	821.4152	10	MUC16.ELGPYTLDR.+2y7.light	69.9	26.3
532.2746	764.3937	10	MUC16.ELGPYTLDR.+2y6.light	69.9	26.3
532.2746	667.341	10	MUC16.ELGPYTLDR.+2y5.light	69.9	26.3
532.2746	382.7005	10	MUC16.ELGPYTLDR.+2y6+2.light	69.9	26.3
535.2846	827.4353	10	MUC16.ELGPYTLDR.+2y7.heavy	69.9	26.3
535.2846	770.4139	10	MUC16.ELGPYTLDR.+2y6.heavy	69.9	26.3
535.2846	673.3611	10	MUC16.ELGPYTLDR.+2y5.heavy	69.9	26.3
535.2846	385.7106	10	MUC16.ELGPYTLDR.+2y6+2.heavy	69.9	26.3
703.8492	960.5109	10	TG.FSPDDSAGASALLR.+2y10.light	82.4	33.2
703.8492	845.4839	10	TG.FSPDDSAGASALLR.+2y9.light	82.4	33.2
703.8492	687.4148	10	TG.FSPDDSAGASALLR.+2y7.light	82.4	33.2
706.8592	966.531	10	TG.FSPDDSAGASALLR.+2y10.heavy	82.4	33.2
706.8592	851.5041	10	TG.FSPDDSAGASALLR.+2y9.heavy	82.4	33.2
706.8592	693.4349	10	TG.FSPDDSAGASALLR.+2y7.heavy	82.4	33.2
636.359	1059.558	10	TG.VIFDANAPVAVR.+2y10.light	77.5	30.5
636.359	912.4898	10	TG.VIFDANAPVAVR.+2y9.light	77.5	30.5
636.359	726.4257	10	TG.VIFDANAPVAVR.+2y7.light	77.5	30.5
639.369	1065.578	10	TG.VIFDANAPVAVR.+2y10.heavy	77.5	30.5
639.369	918.5099	10	TG.VIFDANAPVAVR.+2y9.heavy	77.5	30.5
639.369	732.4458	10	TG.VIFDANAPVAVR.+2y7.heavy	77.5	30.5
694.3876	916.5714	10	ANXA1.GVDEATIIDILTK.+2y8.light	81.7	32.8
694.3876	815.5237	10	ANXA1.GVDEATIIDILTK.+2y7.light	81.7	32.8
694.3876	702.4396	10	ANXA1.GVDEATIIDILTK.+2y6.light	81.7	32.8
698.3947	924.5856	10	ANXA1.GVDEATIIDILTK.+2y8.heavy	81.7	32.8
698.3947	823.5379	10	ANXA1.GVDEATIIDILTK.+2y7.heavy	81.7	32.8
698.3947	710.4538	10	ANXA1.GVDEATIIDILTK.+2y6.heavy	81.7	32.8
519.2744	833.4516	10	CLIC1.GFTIPEAFR.+2y7.light	69	25.8
519.2744	732.4039	10	CLIC1.GFTIPEAFR.+2y6.light	69	25.8
519.2744	619.3198	10	CLIC1.GFTIPEAFR.+2y5.light	69	25.8

(continued)

Table 4
(continued)

Q1	Q3	Dwell	ID	DP	CE
524.2785	843.4598	10	CLIC1.GFTIPEAFR.+2y7.heavy	69	25.8
524.2785	742.4122	10	CLIC1.GFTIPEAFR.+2y6.heavy	69	25.8
524.2785	629.3281	10	CLIC1.GFTIPEAFR.+2y5.heavy	69	25.8
526.2581	938.4248	10	IL18.ISTLSC[CAM]ENK.+2y8.light	69.5	26.1
526.2581	851.3927	10	IL18.ISTLSC[CAM]ENK.+2y7.light	69.5	26.1
526.2581	637.261	10	IL18.ISTLSC[CAM]ENK.+2y5.light	69.5	26.1
530.2652	946.439	10	IL18.ISTLSC[CAM]ENK.+2y8.heavy	69.5	26.1
530.2652	859.4069	10	IL18.ISTLSC[CAM]ENK.+2y7.heavy	69.5	26.1
530.2652	645.2752	10	IL18.ISTLSC[CAM]ENK.+2y5.heavy	69.5	26.1
493.2597	809.39	10	NFKB2.IEVDLVTHSDPPR.+3y7.light	67.1	28.7
493.2597	708.3424	10	NFKB2.IEVDLVTHSDPPR.+3y6.light	67.1	28.7
493.2597	618.3226	10	NFKB2.IEVDLVTHSDPPR.+3y11+2.light	67.1	28.7
496.5958	819.3983	10	NFKB2.IEVDLVTHSDPPR.+3y7.heavy	67.1	28.7
496.5958	718.3506	10	NFKB2.IEVDLVTHSDPPR.+3y6.heavy	67.1	28.7
496.5958	623.3267	10	NFKB2.IEVDLVTHSDPPR.+3y11+2.heavy	67.1	28.7
719.3558	930.4891	10	FSCN1.LSC[CAM]FAQTVSPAEK.+2y9.light	83.6	33.8
719.3558	859.452	10	FSCN1.LSC[CAM]FAQTVSPAEK.+2y8.light	83.6	33.8
719.3558	731.3934	10	FSCN1.LSC[CAM]FAQTVSPAEK.+2y7.light	83.6	33.8
723.3629	938.5033	10	FSCN1.LSC[CAM]FAQTVSPAEK.+2y9.heavy	83.6	33.8
723.3629	867.4662	10	FSCN1.LSC[CAM]FAQTVSPAEK.+2y8.heavy	83.6	33.8
723.3629	739.4076	10	FSCN1.LSC[CAM]FAQTVSPAEK.+2y7.heavy	83.6	33.8
483.2506	823.4196	10	TAGLN.AAEDYGVIK.+2y7.light	66.3	24.3
483.2506	694.377	10	TAGLN.AAEDYGVIK.+2y6.light	66.3	24.3
483.2506	579.3501	10	TAGLN.AAEDYGVIK.+2y5.light	66.3	24.3
487.2577	831.4338	10	TAGLN.AAEDYGVIK.+2y7.heavy	66.3	24.3
487.2577	702.3912	10	TAGLN.AAEDYGVIK.+2y6.heavy	66.3	24.3
487.2577	587.3643	10	TAGLN.AAEDYGVIK.+2y5.heavy	66.3	24.3
826.4123	1066.542	10	EZR.SQEQLAAELAEYTAK.+2y10.light	91.4	38.1
826.4123	995.5044	10	EZR.SQEQLAAELAEYTAK.+2y9.light	91.4	38.1
826.4123	924.4673	10	EZR.SQEQLAAELAEYTAK.+2y8.light	91.4	38.1

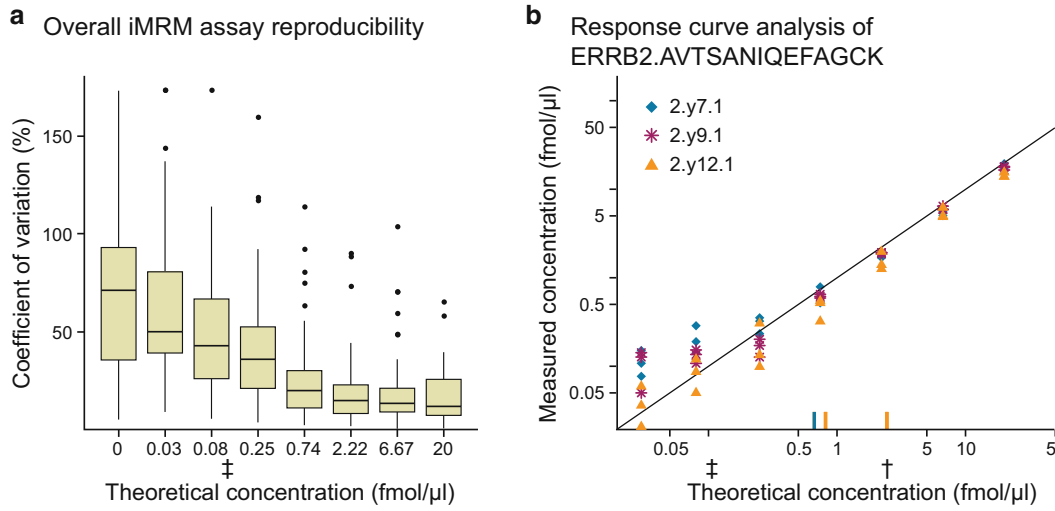
(continued)

Table 4
(continued)

Q1	Q3	Dwell	ID	DP	CE
830.4194	1074.556	10	EZR.SQEQLAAELAEYTAK.+2y10.heavy	91.4	38.1
830.4194	1003.519	10	EZR.SQEQLAAELAEYTAK.+2y9.heavy	91.4	38.1
830.4194	932.4815	10	EZR.SQEQLAAELAEYTAK.+2y8.heavy	91.4	38.1
613.3486	984.5473	10	PRDX4.QITLNDLPVGR.+2y9.light	75.8	29.5
613.3486	770.4155	10	PRDX4.QITLNDLPVGR.+2y7.light	75.8	29.5
613.3486	656.3726	10	PRDX4.QITLNDLPVGR.+2y6.light	75.8	29.5
618.3527	994.5555	10	PRDX4.QITLNDLPVGR.+2y9.heavy	75.8	29.5
618.3527	780.4238	10	PRDX4.QITLNDLPVGR.+2y7.heavy	75.8	29.5
618.3527	666.3809	10	PRDX4.QITLNDLPVGR.+2y6.heavy	75.8	29.5

9. Confirm peak integration. Select "Retention Times, Replicate Comparison" under the "View" drop-down window and use the Retention Time plot to identify potential chromatograms requiring manual re-integration.
10. Activate QuaSAR from the "Tools" drop-down window [60].
11. Perform statistical analysis (LOD, LOQ, CV [61–64]) to assess assay performance. Check "plot each peptide," "CV table" and "LOD/LOQ table" under the "Generate" tab. Check "Standard present" and set "Analyte" and "Standard" fields as heavy area and light area, respectively. Accept default settings for AuDIT [63] and plot scales (*see* **Note 21**).
12. Evaluate analysis of the data and report assay performance using a combination of plots of the concentration curves, CV and LOD box and whisker plots for all peptides in the multiplex iMRM assay as shown in Fig. 2.

Fig. 2 (continued) the analysis. *QuaSAR implements a comprehensive and easy-to-use pipeline for the analysis of MRM-MS data and provides succinct visual summaries of various results including reproducibility, interferences, and detection limits. QuaSAR can be accessed at (http://genepattern.broadinstitute.org/gp/pages/index.jsf?lsid=QuaSAR). This link prompts the user to login at GenePattern, it also provides free registration at the GenePattern website upon choosing "click to register"; then under modules browse to "Proteomics" then to "Quasar" or search for the "Quasar" module directly. Transitions with interferences or high variability are detected using AuDIT [63], enabling focused reevaluation of the raw data and/or exclusion of erroneous transitions. Erroneous transitions are also visually marked in the data visualization plots



c AuDIT report

Peptide	Sample	Transition ID	Final *p*-value	Status	CV	CV status
AVTSANIQEFAGCK	iMRM-pt6	2.y11.1	0.6278	Good	0.086	Good
AVTSANIQEFAGCK	iMRM-pt6	2.y12.1	0.2670	Good	0.244 †	Bad
AVTSANIQEFAGCK	iMRM-pt6	2.y7.1	0.5893	Good	0.074	Good

d Assay performance summary

Peptide	Sample	Transition ID	LOD (fmol/µl)	LOQ (fmol/µl)	Slope	*y*-intercept
AVTSANIQEFAGCK	ERBB2	2.y9.1	0.258	0.773	0.894	–0.054
AVTSANIQEFAGCK	ERBB2	2.y12.1	0.111 ‡	0.332	0.712	0.011
AVTSANIQEFAGCK	ERBB2	2.y7.1	0.294	0.882	0.781	0.126

Fig. 2 Statistical analysis of assay performance. Key assay characteristics including LOD, LOQ, and CV [61, 62] as well as flagging of interferences observed in the specific transitions monitored were assessed using the tools QuaSAR* [64] and AuDIT [63]. (**a**) CVs of the heavy-to-light peptide peak area ratios at each theoretical concentration were calculated for every transition and plotted in the box and whisker format. Interquartile ranges are shaded in beige and outliers are displayed as *black dots*. The median CV for all measurements is represented by a *black line* within the box. (**b**) Example plot of observed vs. theoretical concentration (log scale) for each transition and each replicate of peptide AVTSANIQEFAGCK from the protein ERBB2. QuaSAR generates two separate plots (linear scale and log scale) for each peptide in the multiplex to evaluate individual peptide assay performance. *Color-keyed tick marks* on *x*-axis indicate specific transitions and the corresponding concentration points that are either inconsistent or more variable, and require manual inspection and interpretation of the integrated peak areas. Theoretical (*black solid*) line drawn with a slope = 1 for assessing the accuracy of the measurements. (**c**) *AuDIT Summary Report*. AuDIT determines whether the relative ratios for each transition for light and heavy peptide are consistent and flags those that are statistically inconsistent (p-value > 0.05). CV of the ratio of heavy to light peptide peak area (H/L) is used to filter transitions with unacceptably large variation (>20 %) between the replicates. CVs exceeding the threshold (transition ID 2.y12.1 (†)) are flagged in the table and designated by *color-keyed tick marks* along the *x*-axis on the plot. (**d**) *Assay Performance Summary Table*. The performance (LOD/LOQ, slope, *y*-intercept) is listed for each transition in QuaSAR summary tables to rank assay performance for each transition of each peptide. A second "CV final" table (not shown) reports CV for only the best transition, which is defined as the one with the lowest LOD. LOD is reported for each transition. The transition with the lowest LOD (‡) is consistent with the region of the curve in (**b**) where the curve begins to level off and a noticeable increase in replicate and transition variability is observed (**a**, **b**). A second "LOD final" table lists the transition that provides the lowest LOD for each peptide in

4 Notes

1. Prior experience using peptides synthesized with blocked termini (e.g. acetyl group on N-terminus, amide group on C-terminus) gave lower affinity anti-peptide Abs. Therefore we suggest not to use them. Trypsin treatment of proteins yields peptides with free termini. Peptide standards and the immunogen peptide sequence used for antibody generation, which may be synthesized with additional amino acids (e.g. Cys) for easier conjugation to KLH and purification media or spacer molecules (GSGS, or $dPEG_2$, $dPEG_4$) to increase immunogenicity, should be prepared as chemically similar to that expected from the native protein upon digestion with trypsin using amino acids with natural isotope (e.g. 12C > 13C > 14C) distribution and correspond to a tryptic sequence (R or K C-terminal amino acid).

   Peptides selected as target analytes should, in general, be unique to the protein of interest as well as being unique in the proteome (i.e. proteotypic). Evaluating if the sequence of a target peptide is unique can be determined by BLAST analysis http://www.uniprot.org/blast/ of each target peptide sequence or in batch mode for a group of peptides using the Peptide String Match utility in Spectrum Mill (http://proteomics.broadinstitute.org/). Species specificity depends on the source of samples intended for analysis. Peptide Selector, another utility in Spectrum Mill, provides an initial in silico ranking of peptides to monitor based on mass, sequence, and theoretical retention time.

   The best predictor of whether a given peptide will be useful for assay development is the observation of that peptide in your own data or public database containing high-quality MS and MS/MS data acquired on high-performance instrumentation at high mass accuracy and high resolution. Peptides can be ranked for assay development and synthesis based on frequency of detection, score (e.g., number and/or percentage of fragment ions in an observed spectrum that correspond to the target sequence) and retention time. Signal response by electrospray MS is another important parameter to consider, especially when deciding which of several peptides from a given protein to select for assay development. The response of tryptic peptides derived from the same protein can vary by up to 50-fold based on inherent physiochemical parameters [55]. In general, for peptides that are well recovered from sample processing, the peptides with the higher ESI-MS response will yield higher assay sensitivity. When this information is not readily obtained from available data, prediction tools can be used to algorithmically predict the highest responding peptides

from a given protein using software tools such as ESP (http://www.broadinstitute.org/cancer/software/genepattern/esppredictor) or PeptideSieve [56] (http://www.systemsbiology.org/peptidesieve).

For iMRM assays designed to measure total protein, peptides containing amino acids susceptible to modification during biochemical processing (e.g. methionine oxidation, cyclization of N-terminal Glu or carboxamidomethyl Cys) should be avoided [12]. However, it is not always possible to do so, in which case multiple forms of the peptide may need to be synthesized and included in the final assay. Small biochemically introduced modifications such as phosphorylation or acetylation can be readily synthesized and assays constructed in the same manner as for unmodified peptides. Peptides predicted or known to contain large modifications such as N-linked carbohydrate should be avoided.

Synthesized peptides are purified by reversed-phase liquid chromatography (RPLC) and assessed for identity and purity by matrix-assisted laser desorption ionization (MALDI) and RPLC, respectively. Quantity and concentration of peptide are determined by Amino acid analysis (AAA).

2. Tryptic peptides will contain a C-terminal Lys or Arg unless derived from the C-terminus of the protein. These amino acids are preferred for stable isotopic incorporation for several reasons. The y-ion series of ions for tryptic peptides are usually among the most abundant ions in their MS/MS spectra. Each y-ion fragment will contain either the light version or the heavy version of Lys or Arg, depending on which version was selected for fragmentation in the MS. Several of these y-ions are monitored in the MRM-MS experiment and used for both identification and quantification. Use of common heavy amino acids at the C-termini of synthetic tryptic peptides also simplifies synthesis.
3. Plasma was used for this evaluation because it is the matrix in which the assays were designed to measure analytes. Plasma is readily available from healthy subjects collected under appropriate IRB collection protocols, and can be obtained commercially.
4. Immunogen peptides are conjugated to KLH, formulated with adjuvant and administered to New Zealand white rabbits following 77–120 day immunization schedules depending on vendor and protocol. Antibody titer is determined by peptide ELISA, coating free peptide on the plate. Rabbit sera with the highest titers are collected and purified by peptide affinity chromatography [46]. Glycerol and sodium azide are added to 25 % and 0.1 % respectively to aid antibody stability during freeze/thaw for antibodies stored at −20 °C and longer term storage at 4 °C [65, 66]

5. Lymphocytes isolated from the spleens harvested from the rabbits used to generate polyclonal antibodies were isolated and fused with partner cells to generate a mixture of hybridoma cell clones which are expanded. After expansion and growth, subclones are picked, expanded further and tested until a single cell population (monoclone) with a positive screen against the target peptide (by ELISA or by automated iMRM format [67, 68]) is isolated. IgG is purified from monoclonal cell cultures or is sequenced and expressed as recombinant protein that are purified by antibody affinity chromatography or protein G chromatography, respectively.
6. Dissolve IAA fresh for every digestion and do not reuse solutions. Continuous mixing is not necessary after the IAA is initially mixed. Since IAA breaks down when exposed to light, it is suggested to either use amber-tinted tubes that can be handled on the bench or to place the tubes in a light-tight enclosure during reaction.
7. Concentration of urea needs to be reduced from the initial concentration of greater than 6 M to less than 1 M for optimum trypsin efficiency.
8. This trypsin is purified from bovine pancreas, treated with l-(tosylamido 2-phenyl) ethyl chloromethyl ketone (TPCK) to inhibit chymotryptic activity [69] and lyophilized after dialysis with 1 mM HCl. Trypsin in powder form is very flaky and light and highly susceptible to static interactions. Use a static gun and weigh without wearing gloves to reduce static electricity. Be cautious when pipetting dissolution solvent (next step) to avoid aerating and forcing trypsin out of the tube. The optimum pH for trypsin is 8.3. Trypsin becomes active immediately upon dissolution of its powder form in 0.2 M Tris–HCl pH 8.1. To terminate digestion, trypsin enzymatic activity is reduced by the addition of acid to reduce the pH below 3.0.
9. Even if the samples are continuing directly to antibody affinity enrichment freeze these tubes at −80 °C. That way, freeze/thaw steps will be consistent between replicates, which is especially important when additional curve replicates are prepared for future studies.
10. Under acidic conditions, peptides as well as immunoglobulin protein will elute from the protein G beads. The large amount of desorbed protein will eventually overload the 75 μm ID × 10 cm analytical column (packed with 3 μm C18) that we estimate has a loading capacity of approximately 1 μg. Crosslinking antibodies to protein G beads via primary amines with dimethyl pimelimidate (DMP) allows the antibodies to be washed to reduce the amount of nonspecific background, the amount of bound passenger peptide (see Note 12) and retains the anti-

body on bead for the potential reuse. Antibodies cross-linked to beads should be tested by a capture efficiency test or mini-curve [46] prior to use.

11. Bead processing begins by selecting and starting the method using the dialog box of the KingFisher magnetic bead handler. The method outlined in Table 3 was programmed using KingFisher software version 2.6 and then sent to the instrument via a serial port connection. It is not necessary to have the computer on or running during the method once it is programmed and loaded onto the KingFisher. Method settings, such as duration and intensity of bead mixing or bead collection times, can be changed when the method is open on the computer, saved and then re-sent to the KingFisher. Outline of bead processing steps performed on the KingFisher:
    - Wash the beads twice with 250 µL PBS/0.03 % CHAPS (1.5 min per wash).
    - Wash the beads once with 250 µL 0.1× PBS/0.03 % CHAPS (1.5 min per wash).
    - Elute the peptides in 25 µL of 3 % acetonitrile/5 % acetic acid (5 min).
    - Collect used beads into a fresh collection plate (5 min).

    This method collects the magnetic beads after elution into a fresh collection plate (plate 6) containing Antibody collection buffer (1×PBS/100 mM Tris pH 8.0/0.03 % CHAPS/0.1 % sodium azide) and subsequently pooled, washed, and reused. When beads with crosslinked antibodies are used (*see* **Note 11**) these beads may be pooled, washed, and reused after re-equilibration in storage solution, 1×PBS pH 7.4/0.03 % CHAPS/0.1 % NaN3. They can be stored for longer terms (6 months or longer at 4 °C depending on the antibody), but should be retested prior to reuse.
12. Antibody mixtures may be made in advance of reverse curve preparations and stored at 4 °C for 3 months. Antibodies stored for longer than 3 month at 4 °C after thawing should be evaluated by a capture efficiency test or mini-curve [46] prior to use. Total mixture volume is dependent on the individual antibody concentrations. There are six monoclonal antibodies and 14 polyclonal antibodies in this 20-plex iMRM assay. Since 2 µg of each polyclonal antibody and 0.5 µg of each monoclonal antibody are used per enrichment, there will be 31 µg of total IgG added to each well (refer to Table 1). Protein G binding capacities depend on the type and size of bead and should be tested empirically for each batch and bead type. Protein G is a bacterial-derived protein that binds to the Fc portion of immunoglobulin heavy chain 66], and is the recommended

ligand for binding antibodies derived from rabbit. Protein G is commercially available conjugated on many bead types and sizes, from larger 20 μm porous beads to smaller 1 μm magnetic beads. Magnetic beads were chosen to make the process more amenable to automation. Here, we found 2 μL of 1 μm protein G magnetic beads sufficient to bind 1 μg of antibody. At this plex level, 62 μL of 1 μm beads are required per enrichment. If larger beads are used (e.g., 2.8 μm beads), more beads will be required leading to some increase in nonspecific binding. Binding capacities of alternate sources of protein G magnetic beads should be tested prior to use.

13. Removing the foil seal can be tricky, especially after being well applied and tumble mixed overnight. It may tear off in pieces, requiring multiple grip and tear motions to completely remove. The plate must be securely controlled in one hand to prevent it from tipping and mixing the contents of neighboring wells on the plate.
14. The duration and overall lapse time for the steps was optimized to keep the KingFisher method wash and elution time to less than 30 min. This upper time limit is based on the estimated Kd of these rabbit polyclonal antibodies calculated from the off-time measured under constant flow conditions in 1× PBS pH 7.4 [70].
15. Transferring 20 μL of eluate supernatant to a fresh PCR plate (or to fresh wells on the same plate) was found to increase the reproducibility of the subsequent LC-MRM-MS analysis by removing particulates and other precipitous solids may form during the wash and elution process. After placing the PCR plate on the autosampler magnet plate, wait 1–3 min for the magnet to draw and collect residual magnetic beads to the side before drawing up the eluate supernatant. However, it is not imperative to remove the entire volume from each well nor equivalent volumes from all wells since the heavy peptide standard have already been added to account for the variation that may occur during this process.
16. In cases where MS analysis may not occur right away, plates may be resealed with aluminum foil seals and stored at –80 °C until the instrument is ready. Prolonged freezer storage of digested plasma or enrichments from digested plasma may produce addition particulates and precipitate, which should be removed as described above using a magnet plate holder. Plates may be centrifuged briefly after thawing a frozen plate, for less than 2 min at 250 × g, to collect particulates in the bottom of the well. Although it is preferred to analyze a plate soon after processing, in some situations, laboratory workflows may be segregated between individuals or sites or labs, which may

make immediate analysis difficult. For these samples, processed iMRM samples may be sealed and frozen immediately after the automated wash and elution steps on the KingFisher and not transferred to a fresh plate until the day of MS analysis. Although a full stability study has not been conducted, to reduce nonspecific losses to plastic and evaporation, minimize the length of storage of antibody-enriched plates at −80 °C (preferably within a month of processing). This method was designed to prepare samples intended to be injected onto a LC configured for trap/elute [46, 49], i.e. samples are injected onto a trap column, washed to remove salts, then the valves are switched to put the trap in-line with an analytical column. Alternatively, samples may be desalted off-line using StageTips [71] or other SPE/C18 cartridges or columns and subsequently injected onto a MS equipped with a single analytical column format.

17. Instrument operating parameters should be optimized separately using mixtures of light and heavy peptides formulated in 3 % ACN/5 % HOAc containing 0.2 % digested plasma at a concentration suitable to inject 100 fmol in a background of ~100 ng plasma peptides to optimize instrument dependent conditions, such as source gas and collision energy. To prepare 0.2 % digested plasma, resuspend a 0.3 mL equivalent of digested plasma in 1× PBS/0.03 % CHAPS as described in Subheading 3.2, **step 4**. Pipet 10 μL into 10 mL of 3 % ACN/5 % HOAc and use this matrix to dilute the heavy and light peptide mixtures to 50 or 100 fmol/μL. Alternatively, 10 μL of the digested plasma resuspended as described in Subheading 3.2, **step 4** can be added to 10 mL of 3 % ACN/5 % HOAc prior to the preparation of the reverse curve background matrix described in Subheading 3.2, **step 7**.

    Scheduled methods constrain the MRM scans to a defined a retention time window. Typically a retention time window of between 2 and 10 min is used when the number of peptides in an experiment exceeds 40 peptides. In this iMRM assay for example, MRM-MS analysis of 40 peptides, 20 light and 20 heavy, 3 transitions/peptide, requires a total of 120 scans for one measurement of each transition. Using a dwell time of 10 ms, it would take 1200 ms to complete one cycle. If the average peptide chromatographic peak width is 15 s (this may vary depending on the peptide retention time, LC, and column conditions) approximately 12.5 cycles or over 12 scans per transition could be acquired without scheduling. By only monitoring for peptides at their retention time (±2–5 min), scheduled MRM can be used to maintain the number of scans per transitions as the number of peptides (thus transitions) increases [15]. The number of peptides in this iMRM assay (20 peptide pairs, 40 total peptides, 3 transitions/peptide,

120 transitions total) can be acquired with over ten scans per transition, but the differences in precision offered by scheduled MRM can also be assessed. In our experience, ten or more scans over the elution profile of each peptide is needed for good inter- and intra-lab reproducibility [14, 49] although there are instances where this cannot be achieved and a lower number must be used to accommodate the plex size and chromatography conditions used. It is important for the publication of results and for method comparisons to state the number of scans used per peak area determination to compare results to across instrument platforms. These methods can be prepared, both for collision energy optimization and final data collection by exporting instrument-specific conditions from a Skyline document containing these peptides.

18. To minimize carryover, wash methods should be added in between each set of concentration points. Typically we insert two rapid reversed-phase gradients that cover the same or even a broader range of acetonitrile concentrations than used in the analytical gradient [58].
19. A Skyline document containing the peptide sequences and selected masses and transitions of the light and heavy peptides analyzed by MRM-MS may have been done earlier as part of iMRM methods generation. Spectral libraries for each peptide may have been generated and imported into the Skyline document for earlier data-dependent MS analysis. Spectral libraries displayed within Skyline are not required for integrating and processing these data, however, they provide helpful information troubleshooting data that are affected by interferences either from changes in matrix or chromatographic conditions.
20. Units of concentration are fmol/μL plasma (e.g. enter "20" for pt 8 which contains a total (*see* plate map in Table 2) of 200 fmol per well. Even though the total volume is 200 μL per well and the peptide concentration at the time of immunoaffinity capture is 1 fmol/μL, the concentration entered into the Results Grid is based on the starting amount of 10 μL of plasma per well. Concentrations are entered into the results grids as numbers without units.
21. When Quasar starts it will open an Immediate Window in Skyline where progress and any errors can be monitored. LOD, LOQ, and CVs for each transition will be calculated and summarized in two tables, one for all transitions and a second table for the performance of the best transition selected as the transition with the lowest CV. Box and whisker plots of these results as well as calibration or response curves will be generated as linear and log plots. Summary tables for the regression line, including R2 and standard errors, slope and intercept will be generated and saved in the same Skyline directory.

22. Under acidic conditions, peptides as well as immunoglobulin protein will elute from the protein G beads. The large amount of desorbed protein will eventually overload the 75 μm ID × 10 cm analytical column (packed with 3 μm C18) that we estimate has a loading capacity of approximately 1 μg. Crosslinking antibodies to protein G beads via primary amines with dimethyl pimelimidate (DMP) allows the antibodies to be washed to reduce the amount of nonspecific background, the amount of bound passenger peptide (see Note 122) and retains the antibody on bead for the potential reuse. Antibodies cross-linked to beads should be tested by a capture efficiency test or mini-curve [46] prior to use.

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