Published in final edited form as:

Methods. 2013 June 15; 61(3): 304–312. doi:10.1016/j.ymeth.2013.03.006.

Constrained Selected Reaction Monitoring: Quantification of selected post-translational modifications and protein isoforms

Xiaoqian Liu^{1,*}, Zhicheng Jin^{1,*}, Richard O'Brien², Joan Bathon³, Harry C. Dietz⁴, Eric Grote^{1,*}, and Jennifer E. Van Eyk^{1,5}

¹Department of Medicine, Johns Hopkins University, New York, NY

²Department of Neurology, Johns Hopkins Bayview Medical Center, New York, NY

³Department of Medicine, Columbia Medical School, New York, NY

⁴Department of Pediatrics, Johns Hopkins University, Baltimore MD

⁵Department of Biological Chemistry and Biomedical Engineering, Johns Hopkins University, Baltimore MD

Abstract

Selected reaction monitoring (SRM) is a mass spectrometry method that can target signature peptides to provide for the detection and quantitation of specific proteins in complex biological samples. When quantifying a protein, peptides are generated using a specific protease such as trypsin, allowing the choice of signature peptides with robust signals. In contrast, signature peptide selection can be constrained when the goal is to monitor a specific post-translational modification (PTM) or protein isoform as the signature peptide must include the amino acid residue(s) of PTM attachment or sequence variation. This can force the selection of a signature peptide with a weak SRM response or one that is confounded by high background. In this article, additional steps that can be optimized to maximize peptide selection and assay performance of constrained SRM assays are discussed including tuning instrument parameters, fragmenting product ions, using a different protease, and enriching the sample. Examples are provided for selection of phosphorylated or citrullinated peptides and protein isoforms.

Keywords

Quantification; Selected reaction monitoring; Post-translational modification; Protein isoform

© 2013 Elsevier Inc. All rights reserved.

Corresponding author: Eric Grote, PhD, The Hopkins Bayview Proteomics Center, 5200 Eastern Ave, Mason F Lord Building, Center Tower, Room 607, Baltimore, MD 21224, Tel: 410-550-8507 Fax: 410-550-8512, egrote@jhsp.edu, Jennifer Van Eyk, The Hopkins Bayview Proteomics Center, 5200 Eastern Ave, Mason F Lord Building, Center Tower, Room 604, Baltimore, MD 21224, jvaneyk1@jhmi.edu.

*equal contribution

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1 Introduction

Selected reaction monitoring (SRM), a targeted mass spectrometry-based method also known as multiple reaction monitoring (MRM), has been implemented for peptide and protein quantification [1]. SRM is a standard quantitative method for metabolites and other small molecules that has been adapted for the analysis of larger molecules, like peptides. For protein quantification, target peptide(s) that uniquely represent the protein are isolated and quantified within the mass spectrometer. Specificity is achieved in part by monitoring multiple transitions from each peptide, with each transition representing the mass-to-charge ratio (m/z) of a pair of precursor and product ions arising from collision-induced fragmentation of the targeted peptide. Now that SRM assays targeting peptides representing whole proteins are becoming more routine, the next challenge is to apply SRM to the quantification of post-translational modifications (PTMs) or isoforms of a target protein. In these cases, the representative peptides targeted in the SRM assay must encompass the specific amino acid(s) that can be modified or used to differentiate between protein isoforms. This restriction imposes constraints on SRM assay development that do not apply when protein quantitation alone is the objective, the most important of which is an increased difficulty in targeting the fragment of interest due to its physiochemical properties. In this article we will discuss the process of developing an SRM assay with particular emphasis on additional steps that may be required to quantify peptides encompassing PTMs or amino acids that differ between isoforms.

In an SRM assay, a mixture of peptides is first resolved by high performance liquid chromatography (HPLC). As the peptides elute from the column, they are ionized and directed into a mass spectrometer which is programmed to detect selected peptide ions with predefined m/z for precursor ions and fragment ions. Triple quadrupole mass spectrometers are preferred for SRM due to their high sensitivity and scan speed. Indeed, the sensitivity and precision of SRM assays on a triple quadrupole can meet the stringent criteria for clinical use [2]. SRM-like assays can be performed on other instruments including ion trap and quadrupole time-of-flight mass spectrometers.

The first step in the development of a SRM assay is to select target peptides and transitions to represent the protein, isoform or PTM of interest. Trypsin is generally the preferred protease for MS due to its cleavage specificity and ruggedness. Trypsin cleaves after Lys or Arg residues, which insures that the resulting peptides have a positive charge at both ends and therefore produce informative b and y ions upon collision-induced dissociation. The b series of ions contain the N-terminal amino acid residue of the peptide whereas the y series contains the C-terminal amino acid residue. Having fragments from both ends of the peptide increases the confidence of spectra interpretation [3]. Lys and Arg residues are relatively common, so tryptic peptides are generally with the size range detectable by triple quadrupole instruments. Peptide selection is already somewhat constrained when the goal is to simply quantify the total amount of a protein because the signature peptides must be unique to the target protein and should not have natural sequence variations. Peptides suitable for SRM assays consist of 6 to 20 amino acid residues [4]. In addition, peptides with the potential for *in vitro* artifacts such as incomplete protease cleavage, methionine

oxidation, cysteine modification, and asparagines or glutamine deamination should be avoided when possible.

If a purified peptide is available, it can be used to identify transitions with a robust SRM signal [5]. Alternatively, potential transitions can be identified from discovery MS data or predicted based upon the amino acid sequence. The sensitivity of an SRM assay depends upon the response of each transition, so it is crucial to select peptides with good ionization efficiency and their most abundant fragment ions. Multiple transitions should be targeted for each peptide to increase confidence in the quantitative results, because even the most responsive transition may ultimately turn out to be uninformative if it co-elutes with another peptide or a matrix component with the same nominal m/z of precursor and product ions.

Quantitation is facilitated by the use of heavy isotope-labeled internal standards otherwise equivalent to the endogenous target peptides [6, 7]. For absolute quantitation, a calibration curve is generated by spiking varying amounts of target proteins and fixed amounts of isotope-labeled peptides into a matrix similar to that of the biological samples (but lacking endogenous analytes). Heavy peptides are then spiked into the biological samples, and the ratio of peak areas for the heavy and light peptides is used to calculate the endogenous peptide concentration based on the standard curve. Internal standards make it possible to correct for instrument drift and signal suppression or enhancement by the matrix, and can also be used to account for any differences in sample preparation between samples. Measurements of the signal-to-noise ratio (S/N) on the standard curve are used to determine lower limit of detection (LLOD) and lower limit of quantitation (LLOQ), which are defined as the concentrations which yield peaks with S/N of 3 and 10 respectively. For accurate quantitation, the signal from a sample must be above the LLOQ.

Multiple peptides can be quantified simultaneously by SMR in a single LC/MS/MS run. If the HPLC elution profile of targeted peptides is known and reproducible (± 2 min), throughput can be increased even further by scheduling acquisition times. When monitoring metabolites and other small molecules, more than 250 analytes can be quantified in a 15-minute HPLC run [8]. For peptide quantification, longer dwell times are typically required to ensure appropriate precision and reproducibility, which reduces the number of peptides that can be quantified in a single LC/MS/MS analysis [1].

PTMs and protein isoforms present an additional challenge in the development of SRM methods because the assay must be targeted to specific sites in a protein's amino acid sequence that may not necessarily be amenable to LC/MS/MS analysis. Specific transitions must be identified for every isoform of the targeted protein, and if PTMs can be linked to more than one site within a given peptide, a separate assay must be developed for every combination of modified and unmodified peptides. In some cases, these peptides will be difficult to detect in the mass analyzer due to low ionization efficiency or signal intensity. Large peptides may fall outside of the available m/z range of the mass analyzer, and short hydrophilic peptides may not bind to the HPLC column. If the tryptic peptide encompassing the PTM or sequence variant is not suitable for SRM, other enzyme such as Lys-C or Glu-C may be considered.

A generic workflow for developing a constrained SRM assay is illustrated in Figure 1. In the following section, we present examples of constrained SRM assays for phosphopeptides from extracellular-signal-regulated kinase 1 (Erk1), for a citrullinated peptide from neurogranin (NRGR), and for the three isoforms of transforming growth factor beta (TGF- β). In order to be able to quantify these analytes, it was necessary to overcome challenges resulting from constrained peptide selection by tuning instrument parameters, including additional fragmentation (MS³), using alternative proteases, and/or enriching target proteins.

2 Methods

2.1 Peptide selection and instrument optimization

Constraining peptide selection to the site of a PTM or nonhomologous amino acid residue(s) can limit the choice of SRM transitions, resulting in low signals. In this situation, it is desirable to take advantage of all reasonable approaches to increase signal strength and thereby increase the S/N ratio. To illustrate, we present the development of an SRM assay for Erk1 activation. Erk1 is a protein kinase that regulates various cellular processes including proliferation, differentiation, stress, and cell cycle progression. It is activated by dual phosphorylation of a TEY sequence conserved among all members of the mitogen-activated protein (MAP) kinase family [9].

As a first step to identify possible SRM transitions, purified and phosphorylated Erk1 was digested with trypsin and run on an LTQ Orbitrap mass spectrometer (Thermo). The resulting spectra were analyzed by Mascot (a search engine which finds matches between acquired mass spectra and theoretical spectra generated by fragmenting the peptides expected to be generated by digesting all proteins in a genomic/proteomic database, <http://www.matrixscience.com/>) and found to cover 82% of the expected amino acid sequence of Erk1, including a 19 amino acid peptide encompassing the TEY phosphorylation site. Three forms of the TEY peptide were detected with 0, 1, and 2 phosphorylations. For single-phosphorylated TEY, the site was mapped to tyrosine, not threonine, consistent with the reported order of phosphate addition by MEK kinase [10].

Preliminary transitions to measure both activated and total Erk1 were designed based on mass spectra obtained from the LTQ Orbitrap. Complete cleavage of Erk1 by trypsin would yield 44 peptides (with no miscleavages), but only 5 of these peptides (including TEY) remained after eliminating 19 small peptides with less than 6 amino acids, 5 other peptides that were not identified by the Mascot search engine, 3 peptides with a missed cleavage site, and 15 peptides containing methionine or cysteine (Figure 2A). From the Orbitrap *m/z* spectra, 12 transitions were selected for the dual phosphorylated target (TpEYp) representing activated Erk1, and four to seven transitions were selected for each of the other peptides. The trypsin-digest of Erk1 was then run on a triple quadrupole instrument. Considering only the top transitions for each target peptide, the signal for the best peptide, ALD, was 400-fold higher than the signal for TpEYp (Figure 2B).

Tuning each transition by infusing purified target peptide and systematically adjusting instrument parameters can dramatically increase the SRM signal and S/N. On triple quadrupole instruments from AB Sciex, optimization of the entrance potential (EP),

depolarization potential (DP), collision energy (CE), and collision cell exit potential (CXP) can be performed using automated methods. Adjustments to the CE and CXP typically have the greatest effect. Optimal settings do not always translate from one MS instrument to another, or even between two instruments of the same make and model.

The optimal CE can be predicted based upon the m/z and charge of precursor ions, but these predictions are not necessarily optimal for all product ions. In general, longer peptides and peptides with less charge require higher collision energies. MacClean and colleagues empirically determined the relationship between CE and m/z for +2 and +3 precursor ions on 9 different platforms. They concluded that tuning the CE for individual peptides could only increase the signal by an average of 7.8% compared to using the calculated CE values, but this conclusion only applies for y ions [11]. In a systematic study of 80 peptides, Holstein found that b ions tend to require lower collision energies than y ions [12]. Although there have been no systematic studies regarding the optimal CE for phosphopeptides, neutral loss of a phosphate is the lowest energy fragmentation pathway for most peptides phosphorylated on serine or threonine (but not tyrosine) [13], so the collision energy for detecting the corresponding peptides is typically less than would be predicted using a linear relationship based solely on m/z and over all charge.

The advantages of tuning are illustrated for a synthetic peptide representing the dual phosphorylated Erk1 peptide containing the TEY site, which was difficult to detect using calculated SRM parameters (see Figure 2B). A total of 20 transitions were observed as the CE was varied from 0 – 100 V, and each transition had a unique profile (Figure 2C). The fragment generated by a neutral loss of phosphate yielded its strongest signal at 8–10 V less than the calculated CE, resulting in a substantial increase in signal. Other changes in instrument parameters that contributed to the ability to quantify activated Erk1 include tuning CXP and DP, as well as a significant increase in dwell time (Supplementary tables 1–2).

The optimized SRM assay for Erk1 was tested by quantifying Erk1 activation in EGF-treated vascular smooth muscle cells. The lysate for each time point was separated into two aliquots: one for trypsin digestion followed by SRM mass spectrometry with internal heavy isotope-labeled standards, and the other for a traditional western blot using a phospho-specific antibody. Exemplary extracted ion chromatograms show that EGF induced a significant increase in dual phosphorylated Erk1, with a corresponding decrease in unphosphorylated Erk1 (Figure 3A). Low levels of single phosphorylated Erk1 were also detected (not shown). Quantitation of the SRM and western blotting data yielded substantially identical results (Figure 3B). Thus, after optimization, the SRM assay for Erk1 activation provides an accurate result.

2.2 Enzyme selection

Although trypsin is usually the preferred enzyme for MS, circumstances often arise when trypsin cannot be used to detect and quantify a specific PTM or sequence alteration. Sometimes, the targeted amino acid residue(s) are located on a tryptic peptide that is either too long or too short to be detected on a triple quadrupole instrument. Others are located near a suboptimal tryptic cleavage site, which distributes the PTM onto more than one

peptide fragment. And some PTMs can interfere with ionization or fragmentation depending on the context of the peptide on which they are presented. If a PTM of interest cannot be detected in a tryptic digest, other proteases and cleavage reagents including Lys-C, Glu-C, chymotrypsin, and cyanogen bromide should be investigated, as these produce a complementary set of peptides. Multiple cleavage agents can also be used in combination to produce a peptide within the size-range useful for SRM.

PTMs on arginine residues (Arg) can inhibit trypsin cleavage. Arg is subject to modification by methylation, dimethylation and citrullination, a stable modification converting the guanidinium group of Arg to a ureido group [14]. Modified Arg can be more readily detected and quantified if the protein is digested with a protease other than trypsin. Online tools such as Peptide Cutter (http://web.expasy.org/peptide_cutter/) can be used to identify proteases that can produce a reasonably sized peptide encompassing the PTM. For example, the citrullinated Arg residues in glial fibrillary acidic protein (GFAP) and myosin basic protein (MBP) were monitored to determine the efficiency of cleavage using Glu-C or Lys-C. Different peptides were generated and only a subgroup was appropriate for constrained SRM development. When a single protease is insufficient, two or more proteases can be used sequentially to generate a peptide that can be used for a constrained assay. However, this adds to the complexity by requiring optimized digestion conditions for every enzyme to ensure complete proteolysis and generation of the required target peptide.

Neurogranin (NRGN), which can be citrullinated near its C-terminus, presents a complex protease selection problem (Figure 4). Citrullination blocks a trypsin cleavage site, so trypsin cleavage of citrullinated NRGN yields a single long C-terminal peptide encompassing the modification site, whereas unmodified NRGN yields two shorter peptides. The spectrum from the citrullinated tryptic peptide is dominated by a neutral loss of isocyanic acid (HNCO) peak (Figure 4B) [15]. Cleavage with Lys-C yields two peptides of the same length, since Lys-C does not cleave after either Arg or citrulline. The Lys-C peptides are one amino acid shorter than the citrullinated tryptic peptide because trypsin failed to cleave after a lysine residue at the N-terminal end (Figure 4A). This had the unexpected consequence of producing a spectrum for the citrullinated Lys-C peptide that was not dominated by a neutral loss peak, thereby offering a greater selection of transitions for quantitative SRM. Cleavage with Glu-C also yielded citrullinated and unmodified peptides of the same length, but the spectrum of the citrullinated peptide was dominated by a neutral loss ion, as seen with trypsin cleavage. These results highlight the point that although sequence analysis can help identify potential signature peptides, there is no substitute for experimentation to determine which peptides are best for quantitative SRM assays.

2.3 MS³ improves selectivity

Co-eluting non-target peptides can interfere with an SRM assay. This problem is especially acute in complex biological samples if a specific peptide with weak ionization must be targeted because it contains a PTM or sequence change of interest. One approach to reduce background noise and thereby increase the specificity and S/N for a more robust SRM assay is to move to MS³ [16], which is only available on some Q-trap instruments.

In MS³ mode, precursor ions are isolated in quadrupole mass filter 1 (Q1) and fragmented in the second quadrupole (Q2). The primary fragment ions are then isolated and fragmented further in Q3 operated in ion trap mode. The full scan spectrum of the secondary fragment ions is then collected. The signals from multiple secondary fragment ions can be combined to increase signal intensity [17]. MS³ transitions can be monitored in the same SRM run as conventional MS/MS transitions, but they require longer dwell times. Since peptides elute in relatively sharp peaks and accurate quantitation requires multiple measurements across the peak for each transition, the long dwell time for MS³ transitions can reduce the amount of time available to monitor other transitions. MS³ technology was recently implemented on a hybrid quadrupole-linear ion trap mass spectrometer for the quantification of drug molecules in biological fluids. Detection limits were improved significantly in the presence of interference [18]. Similarly, peptide specificity is improved by this additional fragmentation event, and background noise can be significantly reduced.

We incorporated MS³ into a method to quantify cystatin C in serum samples as a means to monitor kidney function, (Figure 5). Cystatin C is small protein of 146 amino acids with a limited number of tryptic peptides [19]. Among these, ALDFAVGEYNK was selected as a signature peptide. Even after depleting 14 abundant proteins from the serum sample with an IgY depletion column, the chromatographic peak of the 613.5/1042.5 transition in MS/MS mode has high background noise and interference ions which affect peptide quantification (Figure 5A, C). In MS³ mode, an adjacent interference peak was removed and another major interference peak was reduced significantly (Figure 5B, D). The increased specificity in MS³ mode makes it possible to increase throughput by reducing the LC run time.

2.4 Protein enrichment and isoform quantitation

SRM assays for proteins present at low concentration in complex biological samples are challenging because peptides generated from more highly abundant proteins can mask those obtained from less abundant proteins. This makes it more difficult to detect and quantify low abundance signature peptides. The challenge is even greater when targeting PTMs with low site occupancy. The solution to this problem generally involves some form of prefractionation or enrichment. For example, human cardiac troponin I has at least 20 phosphorylation sites [20]. The total concentration of troponin I in human myocardium is approximately 14 ng per mg of heart tissue; however, the specific phosphorylation sites are only occupied in 0.2–15% of the total troponin I protein. Therefore, in order to measure site occupancy for any specific PTM, troponin I must be enriched by isolating myofilaments followed by 1D-gel electrophoresis [20], or by immunoprecipitation [21].

In addition to directly enriching a specific protein of interest, the detection of less abundant proteins can also be facilitated by depleting the more abundant proteins that would otherwise mask the signals arising from less abundant proteins. IgY immunodepletion columns are available to deplete the top 14 or 20 most abundant proteins from serum or plasma, thereby enriching less abundant proteins by approximately 20-fold [22]. Another option is to enrich specific PTMs. For example, phosphorylated peptides can be enriched on immobilized metal affinity or TiO₂ columns, and glycopeptides can be enriched by chemically modifying them with a biotin tagged reagent and then capturing them on a streptavidin column [23–25]. One

limitation of these PTM enrichment methods is that it is no-longer possible to measure site occupancy when the unmodified protein is not enriched.

Protein isoforms can also be quantified. For example, transforming growth factor beta (TGF- β) has three isoforms, each of which are present at or below 1 ng/ml in human and mouse plasma [26]. Mature TGF- β is comprised of only 112 amino acids, which limits the number of peptides available for SRM assays. The amino acid sequence identity among all three isoforms is 68%, and there is 71–79% identity between any two isoforms. Furthermore, all three isoforms are rich in cysteine residues, which are suboptimal for SRM due to the possibility of multiple forms if alkylation is incomplete. Therefore, the selection of peptides which can differentiate between these three isoforms is extremely limited (Figure 6A).

The three TGF- β isoforms were enriched by immunoprecipitation with a monoclonal antibody that binds to all three isoforms with similar affinity (Figure 6B and online supplemental material). After enriching TGF- β by immunoprecipitation, interference from non-specific sample matrix components was removed, and the assay sensitivity was dramatically increased. In a single IP, we detected signature peptides from all three isoforms, with three SRM transitions for each peptide. IP followed by SRM assays were carried out on mouse plasma and on human saliva obtained from three individuals with genetic vascular disease. Extracted ion chromatograms for the TGF- β 1, 2, and 3 signature peptides showed a clean signal with little background (Figure 6C), and the same LC retention times were recorded for peptides from the IP and peptides from recombinant TGF- β 1, 2, and 3 proteins. The TGF- β 1, 2, and 3 concentrations from three saliva samples are presented in Figure 6D. For comparison, TGF- β 2 and β 3 could not be detected in saliva by ELISA because their concentration is below the limit of detection.

3 Conclusions

SRM assays provide an avenue to quantify PTMs and protein isoforms. However, the constraints inherent in selecting peptides that encompass a specific site within a protein often result in the need to target a signature peptide with a suboptimal mass spectrometer response. This limitation can be overcome in a variety of ways including depleting abundant proteins, concentrating the protein of interest to remove interfering peptides, selecting different protease(s) that produce better peptides, and optimizing detection in the mass spectrometer by tuning or additional fragmentation (MS³). Using these methods to develop quantitative SRM assays for PTMs and protein isoforms will make it possible to address a variety of interesting and important biological and clinical questions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank members of our lab for assistance including Weihua Ji for managing the triple quadrupole facility, Qin Fu for running proteins on the LTQ Orbitrap and advice regarding the TGF- β SRM assays, John Tra for predicting CEs with Skyline, Justyna Fert-Bober for ELISA assays, and Sarah Parker for running western blots.

Mouse sera and human saliva were kindly provided by Jennifer Habashi (Johns Hopkins University). This work was supported by grants: Johns Hopkins ICTR/CTSA grant 1U54RR023561-01A1 (JVE); the NHLBI Proteomics contract NHLBI-HV-10-05 (2) (JVE); 1R01OD011635-01 (HD, JVE); American College of Rheumatology Within Our Reach Campaign (to JMB).

Abbreviations

PTM	post-translational modification
MS	mass spectrometry
Erk 1	extracellular-signal-regulated kinase 1
NRGR	neurogranin
TGF-β	transforming growth factor beta
S/N	signal-to-noise ratio
IP	immunoprecipitation

References

- Addona TA, Abbatiello SE, Schilling B, Skates SJ, Mani DR, Bunk DM, Spiegelman CH, Zimmerman LJ, Ham AJL, Keshishian H, Hall SC, Allen S, Blackman RK, Borchers CH, Buck C, Cardasis HL, Cusack MP, Dodder NG, Gibson BW, Held JM, Hiltke T, Jackson A, Johansen EB, Kinsinger CR, Li J, Mesri M, Neubert TA, Niles RK, Pulsipher TC, Ransohoff D, Rodriguez H, Rudnick PA, Smith D, Tabb DL, Tegeler TJ, Variyath AM, Vega-Montoto LJ, Wahlander A, Waldemarson S, Wang M, Whiteaker JR, Zhao L, Anderson NL, Fisher SJ, Liebler DC, Paulovich AG, Regnier FE, Tempst P, Carr SA. Multi-site assessment of the precision and reproducibility of multiple reaction monitoring-based measurements of proteins in plasma. *Nat. Biotechnol.* 2009; 27:633–U85.
- Fortin T, Salvador A, Charrier JP, Lenz C, Lacoux X, Morla A, Choquet-Kastylevsky G, Lemoine J. Clinical Quantitation of Prostate-specific Antigen Biomarker in the Low Nanogram/Milliliter Range by Conventional Bore Liquid Chromatography-Tandem Mass Spectrometry (Multiple Reaction Monitoring) Coupling and Correlation with ELISA Tests. *Mol. Cell. Proteomics.* 2009; 8:1006–1015. [PubMed: 19068476]
- Steen H, Mann M. The ABC's (and XYZ's) of peptide sequencing. *Nat. Rev. Mol. Cell Bio.* 2004; 5:699–711. [PubMed: 15340378]
- Han B, Higgs RE. Proteomics: from hypothesis to quantitative assay on a single platform. Guidelines for developing MRM assays using ion trap mass spectrometers. *Briefings in Functional Genomics and Proteomics.* 2008; 7:340–354. [PubMed: 18579614]
- Grote E, Fu Q, Ji W, Lui X, Van Eyk JE. *Methods Mol Biol.* 2012 *In press.*
- Barr JR, Maggio VL, Patterson DG, Cooper GR, Henderson LO, Turner WE, Smith SJ, Hannon WH, Needham LL, Sampson EJ. Isotope dilution mass spectrometric quantification of specific proteins: Model application with apolipoprotein A-I. *Clin. Chem.* 1996; 42:1676–1682. [PubMed: 8855153]
- Kirkpatrick DS, Gerber SA, Gygi SP. The absolute quantification strategy: a general procedure for the quantification of proteins and post-translational modifications. *Methods.* 2005; 35:265–273. [PubMed: 15722223]
- Yuan M, Breitkopf SB, Yang X, Asara JM. A positive/negative ion-switching, targeted mass spectrometry-based metabolomics platform for bodily fluids, cells, and fresh and fixed tissue. *Nat. Protoc.* 2012; 7:872–881. [PubMed: 22498707]
- Roskoski R. ERK1/2 MAP kinases: Structure, function, and regulation. *Pharmacol. Res.* 2012; 66:105–143. [PubMed: 22569528]
- Ferrell JE, Bhatt RR. Mechanistic studies of the dual phosphorylation of mitogen-activated protein kinase. *J. Biol. Chem.* 1997; 272:19008–19016. [PubMed: 9228083]

11. MacLean B, Tomazela DM, Abbatiello SE, Zhang SC, Whiteaker JR, Paulovich AG, Carr SA, MacCoss MJ. Effect of Collision Energy Optimization on the Measurement of Peptides by Selected Reaction Monitoring (SRM) Mass Spectrometry. *Anal. Chem.* 2010; 82:10116–10124. [PubMed: 21090646]
12. Holstein CA, Gaffken PR, Martin DB. Collision Energy Optimization of b- and y-Ions for Multiple Reaction Monitoring Mass Spectrometry. *J. Proteome Res.* 2011; 10:231–240. [PubMed: 20968307]
13. Rozman M. Modelling of the gas-phase phosphate group loss and rearrangement in phosphorylated peptides. *J. Mass Spectrom.* 2011; 46:949–955. [PubMed: 21915960]
14. Harauz G, Musse AA. A tale of two citrullines - Structural and functional aspects of myelin basic protein deimination in health and disease. *Neurochem. Res.* 2007; 32:137–158. [PubMed: 16900293]
15. Hao G, Wang DC, Gu J, Shen QY, Gross SS, Wang YM. Neutral Loss of Isocyanic Acid in Peptide CID Spectra: A Novel Diagnostic Marker for Mass Spectrometric Identification of Protein Citrullination. *J. Am. Soc. Mass Spectr.* 2009; 20:723–727.
16. Hopfgartner G, Varesio E, Tschappat V, Grivet C, Bourgogne E, Leuthold LA. Triple quadrupole linear ion trap mass spectrometer for the analysis of small molecules and macromolecules. *J. Mass Spectrom.* 2004; 39:845–855. [PubMed: 15329837]
17. Shi XW, Liu M, Sun MM, Wang Q, Liu WN, Cao DY. Development of a LC-ESI-MS3 method for determination of nitrendipine in human plasma. *J. Pharmaceut. Biomed.* 2011; 56:1101–1105.
18. Cesari N, Fontana S, Montanari D, Braggio S. Development and validation of a high-throughput method for the quantitative analysis of D-amphetamine in rat blood using liquid chromatography/MS3 on a hybrid triple quadrupole-linear ion trap mass spectrometer and its application to a pharmacokinetic study. *J. Chromatogr. B.* 2010; 878:21–28.
19. Sanchez JC, Guillaume E, Lescuyer P, Allard L, Carrette O, Scherl A, Burgess J, Corthals GL, Burkhard PR, Hochstrasser DF. Cystatin C as a potential cerebrospinal fluid marker for the diagnosis of Creutzfeldt-Jakob disease. *Proteomics.* 2004; 4:2229–2233. [PubMed: 15274116]
20. Zhang P, Kirk JA, Ji W, Dos Remedios CG, Kass DA, Van Eyk JE, Murphy AM. Multiple Reaction Monitoring to Identify Site-Specific Troponin I Phosphorylated Residues in the Failing Human Heart. *Circulation.* 2012; 126:1828–1837. [PubMed: 22972900]
21. Zhang J, Guy MJ, Norman HS, Chen YC, Xu QG, Dong XT, Guner H, Wang SJ, Kohmoto T, Young KH, Moss RL, Ge Y. Top-Down Quantitative Proteomics Identified Phosphorylation of Cardiac Troponin I as a Candidate Biomarker for Chronic Heart Failure. *J. Proteome Res.* 2011; 10:4054–4065. [PubMed: 21751783]
22. Schiess R, Wollscheid B, Aebersold R. Targeted proteomic strategy for clinical biomarker discovery. *Mol. Oncol.* 2009; 3:33–44. [PubMed: 19383365]
23. Zhang H, Li XJ, Martin DB, Aebersold R. Identification and quantification of N-linked glycoproteins using hydrazide chemistry, stable isotope labeling and mass spectrometry. *Nat. Biotechnol.* 2003; 21:660–666. [PubMed: 12754519]
24. Schmidt A, Csaszar E, Ammerer G, Mechtler K. Enhanced detection and identification of multiply phosphorylated peptides using TiO₂ enrichment in combination with MALDI TOF/TOF MS. *Proteomics.* 2008; 8:4577–4592. [PubMed: 18972529]
25. Villen J, Gygi SP. The SCX/IMAC enrichment approach for global phosphorylation analysis by mass spectrometry. *Nat. Protoc.* 2008; 3:1630–1638. [PubMed: 18833199]
26. Matt P, Schoenhoff F, Habashi J, Holm T, Van Erp C, Loch D, Carlson OD, Griswold BF, Fu Q, De Backer J, Loeys B, Huso DL, McDonnell NB, Van Eyk JE, Dietz HC. Circulating Transforming Growth Factor-beta in Marfan Syndrome. *Circulation.* 2009; 120:526–532. [PubMed: 19635970]

Highlights

- SRM assays can be used to monitor PTMs and specific isoforms as well as whole proteins.
- Extra effort is often required to optimize MS signals when the choice of signature peptide is constrained.
- MS signals can be enhanced by tuning instrument parameters, fragmenting product ions, using a different protease, and enriching the sample.

Constrained SRM Workflow

Figure 1. Schematic workflow of a constrained SRM assay

Step1: 1. Biological samples include body fluids (such as blood, saliva), cell lysate, and tissue. 2. PTM proteins and protein isoforms are extracted from cell lysate and tissue. 3. Protein enrichment methods are applied for low abundant proteins. 4. PTMs and protein isoforms are digested by enzymes, including trypsin, Lys-C, etc. 5. After digestion, modified, unmodified peptides, and peptides representing the whole protein are selected.

Step2: 1. Isotopic labeled peptides are synthesized, including modified, unmodified peptides, and peptides representing the whole protein. 2. Mass spectrometer parameters are

optimized by synthesized peptides. 3. PTMs and isoforms are quantified by SRM or MS³ assay. Isotopic labeled peptides are internal standards and spiked in the samples.

Figure 2. Developing aSRM assay for a tryptic digest of active recombinant Erk1
 (A) Peptide selection. The amino acid sequence of Erk1 (with its TEY phosphorylation site in red) was separated into 41 predicted tryptic peptides. Boxes represent peptides detected on a LTQ Orbitrap mass spectrometer. Short peptides (S, <6 a.a.), peptides with missed cleavages (M/C), and peptides containing methionine (Met) or cysteine (Cys) were excluded. The remaining 5 peptides (red boxes) were evaluated for the SRM assay. (B) A preliminary SRM assay was performed using transitions detected on the LTQ Orbitrap, except for the TEY peptide with pT, which was not detected on the instrument. Peak

intensities are presented for the top 3 transitions from each peptide. (C) Tuning the collision energy (CE) for transitions from the doubly phosphorylated TEY peptide.

Figure 3. Quantitative assays for ppErk1
(A) Erk1 phosphorylation was measured by a constrained SRM assay in trypsin-digested extracts from EGF-stimulated vascular smooth muscle cells. Extracted ion chromatograms are presented with the area under the curve displayed in the top right corner. Heavy isotope-labeled internal standards were utilized for peak identification and normalization. (B) SRM and western blot assays show a similar time-course of Erk1 phosphorylation in response to EGF.

A

Peptide	Enzyme
(E) <u>R</u> GRKGPGGPGGGAGGAR <u>R</u> GGAGGGPSGD(-)	Glu-C
(R)KGPGGGPGGGAGGAR <u>R</u> GGAGGGPSGD(-)	Trypsin
(K)GPGGPGGGAGGAR <u>R</u> GGAGGGPSGD(-)	Lys-C

B

Figure 4. Enzyme selection to monitor citrullination of bovine NRGN
(A) Peptides from the C-terminus of NRGN. Citrullinated Arg residues are underlined. (B) High-resolution spectra of the C-terminal NRGN peptides. The charge state of the precursor ions was +2.

MS/MS **MS³**

Figure 5. MS³ vs. SRM for the quantification of Cystatin C in IgY14 depleted serum
 (A) MS/MS spectrum of the isotope labeled signature peptide from cystatin C. (B) One MS³ spectrum of the isotope labeled peptide. The selected 1st fragment ion is y9 ion at m/z 1050.5. (C) SRM peak (retention time, 11.2 min) of the endogenous peptide of cystatin C in IgY14 depleted normal serum. (D) MS³ peak of the endogenous peptide in IgY14 depleted normal serum.

Figure 6. A constrained-SRM assay for TGF- β isoforms
(A) TGF- β isoform amino acid sequence homology. One specific peptide was selected from TGF- β isoform 1, 2, and 3. (B) Schematic workflow for immunoprecipitation of TGF- β isoforms from mouse plasma and human saliva samples. (C) Representative extracted ion chromatograms of peptides for TGF- β 1 from mouse plasma, TGF- β 2, and 3 from human saliva samples. The signature peptides and SRM transitions are listed. (D). TGF- β isoform concentrations were measured from three individual human saliva samples with generic vascular disease. For quantitation, peak areas were compared with calibration curves prepared by running SRM assays on digests of recombinant forms of the three TGF- β isoforms.