Published in final edited form as:

Proteomics. 2012 April ; 12(8): 1074–1092. doi:10.1002/pmic.201100436.

Advancing the sensitivity of selected reaction monitoring-based targeted quantitative proteomics

Tujin Shi, Dian Su, Tao Liu, Keqi Tang, David G. Camp II, Wei-Jun Qian*, and Richard D. Smith*

Biological Sciences Division and Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory, Richland, WA 99352

Abstract

Selected reaction monitoring (SRM)—also known as multiple reaction monitoring (MRM)—has emerged as a promising high-throughput targeted protein quantification technology for candidate biomarker verification and systems biology applications. A major bottleneck for current SRM technology, however, is insufficient sensitivity for e.g., detecting low-abundance biomarkers likely present at the low ng/mL to pg/mL range in human blood plasma or serum, or extremely low-abundance signaling proteins in cells or tissues. Herein we review recent advances in methods and technologies, including front-end immunoaffinity depletion, fractionation, selective enrichment of target proteins/peptides including posttranslational modifications (PTMs), as well as advances in MS instrumentation which have significantly enhanced the overall sensitivity of SRM assays and enabled the detection of low-abundance proteins at low to sub- ng/mL level in human blood plasma or serum. General perspectives on the potential of achieving sufficient sensitivity for detection of pg/mL level proteins in plasma are also discussed.

Keywords

SRM; sensitivity; fractionation; ion funnel; enrichment

1. Introduction

Mass spectrometry (MS)-based proteomics has become a powerful discovery tool for systems biology and biomarker discovery applications; however, the limitations of global discovery proteomics with regard to quantification accuracy and throughput are well recognized [1-5]. In addition, these global discovery studies often lead to large datasets with specific proteins of interest supported by limited statistical confidence. During the past decade there have been a flood of proteomics publications related to biomarker discovery and the number of candidate biomarkers reported for diseases such as cancer has increased exponentially in the scientific literature. In spite of all this data however, no new protein biomarkers suitable for clinical practice have to date been successfully validated by proteomics platforms [6-29]. One of the primary reasons for this disconnect is that current technologies have yet to provide the combination of sufficient throughput with robust accuracy for analyzing the thousands of biomarker candidates required for verification and validation [30, 31].

*Authors for correspondence: Dr. Richard D. Smith Biological Sciences Division, Pacific Northwest National Laboratory, Richland, WA 99352 rds@pnl.gov Dr. Wei-Jun Qian Biological Sciences Division, Pacific Northwest National Laboratory, Richland, WA 99352 weijun.qian@pnl.gov.

The authors declare no conflicts of interest.

Accurate measurements of protein concentrations in biological or clinical specimens traditionally rely on the use of sensitive, fairly specific, and high-throughput immunoassays, such as the enzyme-linked immunosorbent assay (ELISA). While well-developed immunoassays often provide high sensitivity for the detection of blood plasma proteins in the pg/mL to ng/mL concentration range, immunoassays for new candidate proteins are generally not available [32-34]. The development of novel immunoassays for reliable quantification can be expensive (e.g., \$50,000-\$100,000 per ELISA assay) and requires long development times (on the order of a year) [1, 4, 30]. These limitations, as well as the limited multiplexing capability of ELISA, give rise to the need for alternative technologies for high-throughput targeted protein quantification in order to meet the needs for biomarker verification or systems biology applications.

Selected reaction monitoring (SRM)—also referred to as multiple reaction monitoring (MRM)—has emerged as a powerful alternative to immunoassays for targeted quantification of proteins in biofluids, cells, or tissues [30, 32, 35-46]. SRM is well suited for high-throughput multiplexed quantification of different proteins due to the multiplexing capability of the SRM mode in which e.g., more than 100 proteins can be quantified in a single LC-MS run by scheduled SRM [43, 44, 47, 48]. The specificity, reproducibility (or precision), as well as sensitivity of SRM for protein assays have been well demonstrated [32, 41, 43, 44, 49-53]. For example, Picotti *et al.* demonstrated the potential of SRM assays to detect and quantify proteins expressed at concentrations above 50-100 copies per cell [43]. Furthermore, SRM holds great potential for quantifying protein isoforms [54] or protein posttranslational modifications [37, 55-57], for which effective antibody-based assays often do not exist.

Despite the promising aspects of SRM-based targeted quantification, this technology still does not provide sufficient sensitivity for e.g., detecting many potential low-abundance protein biomarkers in plasma, extremely low-abundance signaling proteins, and protein post-translational modifications in cells. Most biomarker discovery studies involve human plasma or serum samples because blood is easy to obtain from patients, and is attractive for the potential discovery of specific biomarkers relevant to various human diseases and for establishing new clinical tests [58, 59]. Ideally, SRM assays would provide sufficient sensitivity for direct measurements of protein concentrations without pre-processing or fractionation, thus achieving the maximum throughput as well as the optimum precision; however, the detection of low-abundance proteins in plasma is challenged by the extremely wide dynamic range of protein concentrations [60, 61]. Without immunoaffinity depletion and pre-fractionation, LC-SRM-based assays are generally only able to quantify moderate-abundance proteins in the low $\mu\text{g/mL}$ concentration range using conventional platforms [32, 40], and potentially able to detect proteins in the 10 to 100 ng/mL concentration range when applying the dual-ion funnel interface [62]; however, potential disease-specific biomarker proteins in human plasma are often in the ng/mL to pg/mL concentration range [5, 63]. Following the application of front-end sample preparation strategies, such as the antibody-based enrichment of target proteins/peptides, or immunoaffinity depletion of high-abundance proteins, combined with LC fractionation, quantification of plasma proteins in the low ng/mL concentration range by LC-SRM has been reported [29, 34, 41, 64-67]. In general, any sensitivity improvements achieved by introducing additional enrichment or fractionation steps can lead to a sacrifice in the overall analytical throughput. In addition to developments in sample processing, recent advances in MS instrumentation, particularly the use of multi-inlets and ion funnel [62, 68] interfaces, along with utilization of improved resolving power of a mass analyzer [69], have also led to enhanced SRM sensitivity. Advances in MS technologies hold the promise for achieving high-throughput and high sensitivity SRM measurements without introducing additional sample processing steps, and generally without any concomitant loss of throughput.

In this review, we provide an overview of recent advances in different methodological and technological aspects relevant to the improved sensitivity of SRM assays. We also include perspectives on further advancing SRM sensitivity for reliable quantification of candidate protein biomarkers at even lower levels, e.g., low pg/mL range in plasma where many candidate biomarkers or proteins of clinical interest, such as cytokines, are found.

2. Principles and factors governing SRM sensitivity

MS-based quantitative approaches have been widely applied for “bottom-up” protein quantification by measuring peptides generated by proteolysis using enzymes such as trypsin. The peptide sequence unique to its corresponding protein is then utilized as a quantitative surrogate for the concentration of a given protein in different samples. Targeted quantification using SRM coupled with stable isotope dilution (SID) has been considered as a true quantitative technique due to its relatively high selectivity, accuracy, and precision for target quantification [40] using triple quadrupole (QQQ) mass spectrometers. In the SRM mode, there are two stages of mass selection. The mass of the intact targeted analyte (precursor ion) is selected in the first quadrupole (Q1). After the fragmentation of the Q1 mass-selected precursor ion by collision-induced dissociation in the second quadrupole (Q2), a desired fragment (product ion) is selected in the third quadrupole (Q3), which is then transmitted to the detector (Fig. 1A). The specific pair of precursor and product ions with specific m/z values is termed a “transition” and quantitative measurements are based on the intensity profile of each transition recorded by the MS detector. The two stages of mass selection result in high selectivity against co-eluting interferences. Unlike conventional MS-based proteomics where mass spectra with a broad m/z range are recorded, SRM only scans each individual transition with narrow m/z windows, or integrates signal at specific m/z values. The effectively non-scanning nature of SRM mode results in increased sensitivity or dynamic range by nearly one to two orders of magnitude [40] compared with the $\sim 10^3$ accessible dynamic range of detection in human plasma for conventional LC-MS/MS in ‘full scan’ modes [5]; however, this is still not sufficient to effectively cover the dynamic range of protein concentrations ($>10^{10}$) in plasma [60, 70]. In principle, the current MS challenges for achieving high SRM sensitivity in complex biological samples are primarily two-fold: 1) insufficient analyte signal into the MS, where the minimum detectable analyte ion signal is determined by the detection limit of the MS detector, and 2) the overall resolving power of the platform, i.e., the ability to distinguish between ions differing in m/z by potentially very small increments, to provide a specific measurement of analyte signal in the presence of interferences. Both aspects are extremely important for highly sensitive, quantitative detection of low-abundance species with high specificity. As illustrated in Fig. 1B, the front-end sample processing strategies are essential for providing samples with an increased analyte-to-background ratio, thus allowing more targeted analyte to be injected onto the LC column while at the same time reducing sample complexity to eliminate potential interferences as well as minimizing ion suppression in electrospray ionization (ESI) due to the presence of other components. Indeed, various combinations of fractionation methods for front-end sample processing, such as depletion of high-abundance proteins [41, 70-72], isolation of target proteins [73] or peptides [33, 34, 74-76], and strong cation exchange (SCX) separations [41, 64], have been shown to dramatically reduce sample complexity, enrich target analytes, and enhance the limit of detection (LOD) of low-abundance proteins in human plasma.

The ESI to MS interface technology is a second important aspect for effectively delivering analyte ions to the MS, and advances made to the interface directly enhance the overall sensitivity. One example is the recently introduced multi-capillary inlet/dual-stage ion funnel interface, which dramatically enhances the ion transmission efficiency from ESI to MS, thus allowing more than a factor of 10 improvement in SRM sensitivity [77]. Finally,

the resolving power of MS plays an important role to achieve the best signal-to-background ratio, since background interference can often be limiting in allowing an analyte to be specifically detected and quantified. Using additional stages of ion fragmentation to remove co-eluting interferences is key to the MRM³ strategy [69, 78] and is an example of utilizing MS capabilities to achieve better specificity and a better limit of quantification (LOQ) for low-abundance proteins. Shown in Table 1 is a summary of the LOQs reported for SRM quantification of proteins in blood plasma and serum using different strategies or platforms. In the following sections, we provide detailed discussions of recent advances in front-end sample processing strategies, interfaces, and MS technologies, and their contributions to overall SRM sensitivity.

3. Front-end sample processing strategies

To date, most efforts on enhancing SRM sensitivity have been focused on front-end sample processing strategies. As illustrated in Fig. 2, the common strategies for achieving higher SRM sensitivity include immunoaffinity depletion of multiple abundant proteins from human biofluids, protein- or peptide-level fractionation, and selective enrichment of sub-proteomes or specific target peptides/proteins using chemical or immunoaffinity approaches. All these strategies essentially enrich the analytes of interest while reducing the background complexity, thus improving MS detection.

3.1 Immunoaffinity depletion and fractionation strategies

Given the extremely wide dynamic range of the plasma proteome, multi-component immunoaffinity depletion of high-abundance proteins [79] has become a popular method for enriching low-abundance plasma proteins in both proteomics discovery and SRM verification studies. A single-step immunoaffinity depletion can routinely remove either the top 7, 12, or 14 high abundant proteins from human plasma with good reproducibility depending on the depletion column used [71, 80], allowing a 10-20 fold enrichment of low-abundance proteins due to the depletion of 90-95% of the total protein mass. While the new generation of MS instrumentation, e.g., the Agilent 6490, will provide further enhanced performance, conventional LC-SRM platforms typically provide a dynamic range of quantification at 4 to 5 orders of magnitude in protein concentration [40, 49], but generally only allows (without on-line separations) the detection of proteins at concentrations higher than ~1 µg/mL in plasma or serum without depletion or fractionation [32, 40, 74, 81]. With the initial integration of depletion of high-abundance proteins using either a MARS hu7 or a IgY12 column in 2007, Keshishian *et al.* demonstrated that the LOD and LOQ were in the concentration range of 25-100 ng/mL in human plasma [41], which represented at least a 10-fold enhancement in SRM sensitivity compared to the data obtained without depletion, where the LOD and LOQ were in the concentration range of ~1 µg/mL [32, 34, 40]. In addition, depletion of 12 high-abundance proteins using IgY12 showed consistent improvements in S/N, LOQ, and CV for all spiked proteins compared to depletion of only 7 high-abundance proteins using MARS hu7 [41]. In 2008 a tandem IgY12 (now available as IgY14 from Sigma)-SuperMix immunoaffinity separation strategy (See Fig. 3) was reported for the effective removal of ~60 high- and moderate-abundance proteins from human plasma and mouse plasma (the SuperMix column contains a mixture of purified immobilized antibodies against the moderate-abundance proteins in plasma) [70, 82], allowing ~100-fold enrichment of low-abundance proteins. It has been demonstrated that the tandem IgY12-SuperMix depletion further enhanced the detection of low-abundance human plasma proteins [70]. For instance, the original concentrations of two proteins, macrophage colony-stimulating factor 1 and matrix metalloproteinase-8, which were identified by LC-MS/MS in the SuperMix-depleted sample, were validated by ELISA as 202 pg/mL and 12.4 ng/mL, respectively [70]. The results suggest that the application of the tandem IgY-SuperMix

depletion will further improve the sensitivity and enable SRM-based quantification of plasma proteins with LOQ values potentially at 1-10 ng/mL levels.

Besides immunoaffinity depletion, different fractionation methods have been commonly applied to enhance the detection of low-abundance proteins by concentrating different target analytes to specific fractions. One of the common fractionation approaches is strong cation exchange chromatography (SCX) known for its excellent orthogonality with reversed-phase LC separations. SCX fractionation has been coupled with LC-SRM in a number of studies for quantification of putative clinical biomarkers in cancer tissues or human biofluids [83-86]. Immunoaffinity depletion coupled with SCX fractionation has also been used for LC-SRM quantification. In the study by Keshishian *et al.* [41], the application of a limited SCX fractionation after depletion further improved the LOQ of all 6 spiked-in proteins to the 1-10 ng/mL range with CVs ranging from 2 to 15%. The same strategy was also applied for quantification of cardiovascular biomarkers to achieve low ng/mL range quantification of 9 marker proteins relevant to cardiac injury [64, 87]. In another 2009 study, Fortin *et al.* [88] demonstrated that SRM-based quantification of prostate-specific antigen (PSA) in the low ng/mL range was achievable using a conventional bore LC system (a 2.1 mm inner diameter reversed-phase column) in combination with albumin depletion and a simple mixed cation exchange (MCX) peptide fractionation. Moreover, quantification of PSA concentrations in 9 clinical samples from patients correlated well with ELISA data from the same samples [88].

In addition to SCX, a number of other chromatography or electrophoresis based approaches have been applied for either protein-level or peptide-level fractionation. These approaches include reversed-phase LC [89, 90], size exclusion chromatography (SEC) [39], free-flow electrophoresis [91, 92], isoelectric focusing [48, 66, 93, 94], and gel-based fractionation [29, 65, 95, 96]. Each of these techniques can be integrated with LC-SRM to achieve improved detection of low-abundance proteins. For example, in 2009 Picotti *et al.* [43] applied isoelectric focusing based fractionation using off-gel electrophoresis followed by LC-SRM quantification of low-copy number yeast proteins. Their data demonstrated that the pI-based fractionation can improve the sensitivity for detection of target peptides from yeast extracts by an average of 10-fold compared to that recorded for an unfractionated sample [43]. Using digital chip isoelectric focusing for selective enrichment of peptides, a Chip/Chip/SRM platform reported in 2011 demonstrated quantification of low-abundance protein biomarkers in human plasma [66]. The combination of immunodepletion of albumin and IgG with peptide fractionation on the digital ProteomeChip, followed by LC-Chip SRM analysis, resulted in a limit of quantification for PSA spiked into female plasma at levels of ~1-2.5 ng/mL with a CV of ~13%. In 2010 Ang and Nice reported the use of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) followed by in-gel digestion and LC-SRM quantification of 19 putative protein biomarkers across 5 colorectal cancer (CRC) patients and 5 healthy volunteers, revealing 5 proteins (hemoglobin, myeloperoxidase, S100A9, filamin A and I-plastin) that are present only in the feces of CRC patients [95]. In 2011 Tang *et al.* demonstrated that a label-free GeLC-SRM workflow allowed more rapid and sensitive initial screening of large numbers of candidate biomarkers as a prelude to large-scale SID-SRM assays, or immunoassays for the most promising candidate biomarkers [29, 65]. Through the integration of 2D PAGE and SRM assays, Maass *et al.* demonstrated effective measurement of the absolute amounts of metabolic enzymes [96]. While this approach is limited by its throughput, the combination of the sensitivity of SRM with the resolving power of 2D PAGE for protein separation provides improved access to low-abundance proteins in complex biological systems.

As described above, depletion and/or fractionation approaches offer significant potential for improving the overall sensitivity of SRM quantification of low-abundance proteins at

physiologically relevant concentrations; however, the depletion and fractionation strategies in general suffer from the limitations of low sample throughput and potentially poor sample recovery. Any extensive fractionation reduces the overall sample throughput as the subsequent fractions have to be measured individually. Protein recovery is also a concern, especially with immunoaffinity depletion strategies. Proteins of interest may be either non-specifically or specifically bound to the antibody columns or partially bound to high-abundance proteins (e.g., albumin), leading to a partial or total loss of the analytes and resulting in an underestimation of protein concentrations for both absolute and relative quantification depending upon the consistency of protein recovery [64, 87]. For instance, in an evaluation of the loss of PSA protein as a function of various depletion methods, in which only albumin was immunoaffinity depleted from female plasma, it was shown that PSA recovery ranges from 5% to 90%, depending upon the depletion products being applied [88]. Therefore, a detailed assessment of the depletion methods or products is necessary for developing reliable SRM assays since the strategy may not be applicable to all analytes and its utility depends on the interaction between analyte and other proteins and antibodies as well as non-specific binding characteristics.

3.2 Enrichment strategies of target analytes

The depletion and fractionation approaches described above are “negative” enrichment strategies where non-targeted proteins (i.e., abundant proteins) are removed or reduced by immunoaffinity separation or LC fractionation. Positive enrichment strategies have also been extensively explored in proteomics for better detection of low-abundance peptides or proteins. These strategies include affinity enrichment of peptides or proteins, and chemical enrichment of different subsets of the proteome including PTMs such as N-linked glycopeptides and phosphopeptides. Many of the enrichment strategies reported for general proteomics are also applicable for SRM applications.

3.2.1 Affinity protein enrichment—Immunoaffinity capture of target proteins is likely the most effective method for sensitive detection of low-abundance proteins in complex samples [1, 44]. The immunoaffinity enrichment method coupled with MS has allowed reliable quantification of target proteins in the low ng/mL range [73, 97-99]. In 2008 Nicol *et al.* demonstrated the immunoaffinity-SRM approach for the quantification of protein biomarkers from 100 μ L of serum samples from lung cancer patients [73]. Using antibodies to enrich proteins of interest, followed by digestion of captured proteins and subsequent SRM analysis, this approach enabled the quantification of multiple protein biomarkers in lung cancer and normal human sera in the low ng/mL range [73]. Importantly, this immunoaffinity-SRM approach provided a specific and accurate assay for those proteins for which ELISA assays are not available. Kulasingam *et al.* reported the enrichment of endogenous PSA protein from 5 μ L of serum with a monoclonal antibody followed by product ion monitoring using a linear ion-trap mass spectrometer where a specific m/z of the peptide precursor was isolated and all product ions of its fragmentation were monitored using the ion-trap [97]. This method enabled quantification of PSA down to less than 1 ng/mL level with acceptable CVs [97]; however, we should note that SRM with a triple quadrupole MS has considerable advantages compared to the product ion monitoring mode on an ion-trap instrument due to the limitations of scan time and trapping efficiency as well as trap capacity. In 2011 Chénau *et al.* demonstrated that immunocapture of intact *Bacillus anthracis* spores with an antibody greatly enhanced the SRM sensitivity, allowing limits of detection of 7×10^3 spores/mL of milk or 10 mg of soil [100]. To address the potential sample throughput limitation of the affinity MS strategies, a study in 2009 reported the use of immunoprecipitation in a 96-well ELISA format followed by microwave-assisted protein digestion [99].

While the affinity MS approaches can clearly enhance the sensitivity for quantification of low-abundance proteins, the major limitation of this strategy is that antibodies are typically not available for most new candidate biomarkers discovered. It should be noted that the basis of affinity MS methods is similar to that of ELISA assays in that antibodies immobilized on various platforms are used for capturing target proteins, and the mass spectrometer acts analogously to “secondary antibodies” for subsequent detection and quantification. The need for different antibodies for individual proteins inherently limits the multiplexing power and the throughput for quantifying a large number of target proteins when employing affinity MS approaches.

3.2.2 Affinity peptide enrichment—Since proteomics typically performs measurements at the peptide level, an alternative for analyte enrichment is to directly capture target peptides using anti-peptide antibodies, in which target peptides act as surrogates for protein quantification. This concept was introduced in 2004 by Anderson *et al.* [74] using immobilized anti-peptide polyclonal rabbit antibodies to capture and subsequently, elute the target peptides of four blood plasma proteins along with isotope-labeled peptide standards for MS quantification. This approach, termed Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA), provided an average 120-fold enrichment of the antigen peptides relative to the other non-antigen peptides after capture and elution as measured by SRM [74]. The potential of the SISCAPA-SRM assay for clinical applications was further illustrated by Hoofnagle *et al.*, who developed the first SISCAPA assay implemented in a clinical laboratory environment for quantification of low-abundance serum thyroglobulin [101], an established tumor marker whose existing immunoassays are plagued by frequent interferences that negatively affect clinical performance [102, 103].

The initial SISCAPA implementation utilized small POROS columns carrying covalently bound rabbit antibodies [74]. This LC-column-based SISCAPA enrichment suffered from low throughput and limited multiplexing capability. To address these limitations, the SISCAPA strategy was further optimized using a magnetic-bead-based platform [104], which can be performed in an automated fashion using 96-well plates. Using the optimized platform, in 2007 Whiteaker *et al.* demonstrated that antibody enrichment followed by SRM can achieve ion signal enhancements on the order of 10^3 with good reproducibility, sufficient for quantifying biomarkers in human plasma at the low ng/mL level [104]. In 2009 Kuhn *et al.* demonstrated that SISCAPA-SRM assays for established cardiac biomarkers troponin I (cTnI) and interleukin-33 (IL-33) in human plasma can be multiplexed while retaining the necessary precision, reproducibility, and sensitivity (e.g., 1-10 ng/mL in human plasma) [33]. In 2010 Whiteaker *et al.* described the automated magnetic-bead-based platform for high-throughput sample processing, which was implemented in a multiplexed SISCAPA assay (nine target peptides in one assay) [34]. Using known concentrations of peptides spiked into 10 μ L of human plasma, combined with the automated and multiplexed SISCAPA platform, the LOQ in the physiological relevant ng/mL range was achieved with sufficient precision (median CV 12.6%). More sensitive detection in the 50-100 pg/mL range of protein concentration was reported when the initial plasma volume was increased from 10 μ L to 1 mL for SISCAPA enrichment [34]. In a related development, an off-line magnetic bead capture protocol followed by an in-line magnetic bead trap that enabled direct washing and eluting in the LC-SRM system was reported [75], which minimized the loss of low-abundance peptides. While most SISCAPA studies have used polyclonal antibodies, the potential advantage of monoclonal antibodies (mAbs) has been recognized due to their potentially higher specificity [105, 106]. In 2010 Schoenherr *et al.* demonstrated that mAbs can be used to configure SISCAPA assays for enhancing both specificity and affinity [105] and reported a platform for automated screening of mAbs. The method makes screening large numbers of hybridoma supernatants feasible, thereby facilitating the development of high affinity mAbs for sensitive SISCAPA assays [105]. In 2011 another antibody screening

method, matrix-assisted laser desorption/ionization (MALDI) immunoscreening (MiSCREEN) [106], was developed, enabling rapid screening and selection of high affinity anti-peptide mAbs by measurement of monovalent interactions of peptides with single antigen binding sites on the antibodies. The method allows the identification of antibodies that are able to bind specific peptides in solution from complex mixtures and that have low dissociation constants (kd) suitable for ultimate use in immuno-SRM assays.

Although the SISCAPA strategy provides significant potential for achieving high sensitivity detection, large-scale assay development, and high-throughput sample analysis [34, 74, 76], there are still limitations in the SISCAPA strategy that are worth noting. First, the dependence on individual antibodies for each target peptide results in a relatively high reagent cost (up to \$5,000 per assay) and long lead time (~24 weeks for assay generation) for SRM assay development [67]. Second, not all selected peptides can be used as surrogates of respective proteins due to the issues related to the success rate for producing suitable antibodies as well as the potentially low peptide recovery rate following SISCAPA capture [34]. Those selected peptides with recovery less than 5% are generally excluded from the list of target peptides. Third, although the antibody has a high specificity toward the analyte, the level of non-specificity binding to the bead surfaces and the recovery of low-abundance captured peptides will eventually limit the sensitivity of SISCAPA assays. In this aspect, efforts to reduce non-specific binding to bead surfaces and increase the recovery of captured peptides will be useful for further improvement in assay sensitivity.

3.2.3 Enrichment of sub-proteomes—Besides the enrichment of proteins and peptides using antibody-based approaches, the development of varying strategies for chemical enrichment of widely different sub-proteomes, including different types of PTMs, specific amino acid residues such as cysteine, as well as proteins with specific functions (i.e., activity-based proteomics) [107, 108], represent a significant advance in proteomics. These enrichment strategies can be coupled with SRM for quantification of important PTMs or specific enzyme classes. Two widely studied PTMs by proteomics, phosphorylation and glycosylation, are reviewed here as examples of SRM applications.

Phosphorylation is the most widely-studied PTM. Interestingly, absolute quantification of phosphoproteins was explored long before SRM was recognized as a promising tool for targeted protein quantification. In 2003 Gerber *et al.* first reported the concept of absolute quantification with internal standard peptides coupled with immobilized metal-ion affinity chromatography (IMAC) enrichment of phosphopeptides and LC-SRM for quantifying the dynamic changes in phosphoproteins [37]. In 2006 Mayya *et al.* extended this approach and validated its applicability for quantification of multi-site phosphorylation by quantitative immunoblotting [109]. With SRM-based assays, in 2007 Wolf-Yadlin *et al.* quantified hundreds of phosphorylation sites within a signaling network and across multiple conditions simultaneously and achieved highly reproducible quantitative results[55]. Using *in vitro* isotope labeled phosphorylated FAK protein standards as controls to account for loss during immunoprecipitation of FAK, and stable isotope labeled peptide standards with four endogenous amino acid overhangs at the trypsin digestion sites of both the amino and carboxyl C-terminus for avoiding incomplete digest, Ciccimaro *et al.* (2009) demonstrated the absolute quantification of phosphorylated and non-phosphorylated forms on the activation loop domain of FAK protein in cells [110]. In 2010, Jin *et al.* further demonstrated the measurement of protein phosphorylation stoichiometry using SRM for the protein tyrosine kinase Lyn [111]. These studies demonstrated that SRM is an important tool for quantitative studies of cell signaling pathways.

Glycosylation is another important PTM that plays an essential role in diverse cellular processes [112]. Targeted proteomics, combined with glycopeptide/glycoprotein enrichment

strategies, has been used for site-specific quantification of diseased-related glycoproteins under different pathological or physiological conditions [56, 113-116]. Zhang *et al.* first developed an effective method for selective enrichment of N-linked glycopeptides using hydrazide chemistry [117]. In this approach, the glycans were first oxidized followed by the capture of glycoproteins onto hydrazide resins. After tryptic digestion and removal of non-specific binding peptides, the formerly N-linked glycopeptides are specifically released via peptide- N-glycosidase F (PNGase F). The N-glycopeptide enrichment significantly reduces overall proteome complexity, thus allowing for detection of more low-abundance proteins. By coupling N-glycopeptide enrichment with LC-SRM, Stahl-Zeng *et al.* (2007) demonstrated the detection of plasma proteins in the low ng/mL range with accurate quantification over 5 orders of magnitude [56]. In a different approach using L-phytohemagglutinin(PHA) lectin enrichment of glycoproteins followed by SISCAPA-SRM analyses, Ahn *et al.* (2009) quantitatively monitored the variation of aberrant glycoforms of a glycoprotein, tissue inhibitor of metalloproteinase 1 (TIMP1), with the terminal addition of β -1,6-GlcNAc in colorectal cancer cell lines [115]. With this approach, an aberrant TIMP1 isoform carrying the glycan terminating with β -1,6-GlcNAc was quantified at approximately the 0.8 ng/mL level using only 1.7 μ L of colon cancer serum from a CRC patient. Comparative quantification of aberrant glycoforms was recently achieved by coupling the lectin enrichment with a SRM assay, providing a useful tool for monitoring changes in the abundance of aberrant glycosylation in proteins of interest [118]. In 2010 Kurogochi *et al.* developed a novel strategy called reverse glycoblotting-assisted SRM assays for quantitative glycoproteomics. Feasibility of this strategy was demonstrated by quantitative comparison of 25 targeted glycopeptides containing sialic acids from 16 proteins between mice with homo- and hetero-types of a diabetes disease model [116].

These studies on protein phosphorylation and glycosylation illustrate the great potential of coupling specific enrichment of PTMs with SRM for the quantification of low-abundance protein modifications in systems biology and biomarker discovery applications. For example, in a recent study (2010), SRM measurements enabled the quantification of site-specific cysteine oxidation status of endogenous p53 with high sensitivity and accuracy [57]. SRM-based targeted quantification should serve as a general tool for providing accurate quantification of many other important PTMs including but not limited to S-nitrosylation [119], acetylation[120, 121], ubiquitinylation [122, 123], and proteolytic processing [124].

4. Advances in MS instrumentation

Despite significant advances in the front-end fractionation and enrichment approaches, the overall sensitivity of current SRM assays is still not sufficient for reliable detection and quantification of proteins below the low ng/mL level in plasma or serum. Advances in MS instrumentation are essential for ultimately providing sufficient sensitivity and specificity for accurate protein quantification at extremely low levels. There are two key factors limiting peptide MS or MS/MS signal intensities measured by a MS detector: (1) ESI ionization efficiency, and (2) the significant ion loss (including that due to e.g., reduced detection duty cycle) during transmission from the ESI source to the MS detector. In conventional LC-MS with a flow rate 1 μ L/min, a primary limitation is a highly inefficient ESI, along with the potential for significant bias and ion suppression effects in the analysis [125]. Recent advances in nanoESI have demonstrated substantial advantages for ESI-MS analyses at very low flow rates in terms of ionization efficiency [126, 127], thus leading to overall enhanced sensitivity (as well as some reduced concerns with bias and suppression effects). Besides ionization efficiency, ion loss in the ESI-MS interface is also a significant issue. In typical LC-ESI-MS interfaces, the MS inlet, where the heated capillary is followed by an aperture to separate the first and the second vacuum chambers, only provides a combined ion sampling and ion transmission efficiency on the order of ~1% of ions created

by the electrospray [128] due to very limited ion sampling from the ESI source into the inlet and inefficient transmission of ions through the interface. Therefore, MS interface technologies play a key role in advancing overall LC-SRM sensitivity. In addition, improvements in MS resolving power should allow more specific monitoring of analyte ions with reduced chemical background noise, thus providing enhanced sensitivity. In this section we summarize recent advances in MS instrumentation that provide improved ion transmission and ionization efficiency and better MS resolving power.

4.1 Ion funnel technology

The electrodynamic ion funnel, which was originally developed and implemented at the Smith laboratory as a replacement for the ion-transmission-limiting skimmer, has proven to be a broadly applicable ion guide for ion capturing, focusing, and transmission at elevated pressures [129-131]. The original ion funnel interfaces, which operated at a maximum of ~5 Torr, were able to enhance signal intensity for a variety of MS analyzers [129, 132] by replacing the inefficient skimmer interface. While achieving near-lossless ion transmission under high vacuum, losses at the atmospheric pressure interface went unmitigated. With the introduction of a high-pressure ion funnel interface capable of operating at a maximum pressure of ~30 Torr, more efficient ion sampling at atmospheric pressure was achieved through the application of a multi-capillary inlet [133]. Since in many cases the SRM detection of low-abundance species is limited by signal intensity, increased signal intensity using ion funnels will further improve detection sensitivity and quantification accuracy.

4.1.1 Single-stage ion funnel interface—In an initial implementation of the ion funnel, >10-fold signal gains were observed when a standard low-pressure ion funnel interface was coupled to a triple quadrupole MS [132]. Enhanced SRM sensitivity by introduction of the ion funnel interface was also demonstrated by a direct comparison of the Thermo Scientific TSQ Quantum with the new TSQ Vantage, in which the ‘S-lens’, very similar in design to an ion funnel, is used to replace the tube lens and skimmer found in the TSQ Quantum. The implementation of the newly designed S-lens on the newer TSQ Vantage provided better reproducibility and precision for quantitative analysis compared to the TSQ Quantum due to improved ion transmission. To further increase SRM sensitivity, Belov *et al.* recently introduced an ion funnel trap prior to the quadrupoles by converting the conventional continuous stream of ions from the ion source into a pulsed ion beam [134]. In comparison with the conventional ion funnel approach, the pulsed SRM signals showed a 5-fold increase in signal intensities and a ~2-fold reduction in background noise.

4.1.2 Dual-stage ion funnel interface—The ion transmission through the single ion-funnel interface is still limited by the inlet size, which is governed by the operating pressure for the ion funnel and practical pumping speeds. To further reduce ion losses in the inlet region of the interface, a dual stage ion funnel interface was initially introduced by our laboratory in 2006 [133] and later coupled to a TSQ for SRM applications [62]. In this design the first stage high-pressure ion-funnel is operated at ~30 Torr allowing the use of a multi-capillary inlet for higher efficiency. The second ion funnel immediately behind the higher pressure ion funnel, differentially pumped in a separate vacuum chamber, is implemented to maintain the low pressure in the downstream MS analyzer chamber while ensuring high ion transmission efficiency through the interface. An additional 5-fold sensitivity enhancement was observed for using an 18-port, heated, multi-capillary inlet compared to a single inlet [131]. The multi-capillary inlet/dual ion funnel interfaces also allowed using the electrospray emitter array as an ion source [135], which effectively reduced the flow rate through each emitter and further improved the overall ionization efficiency.

By coupling the dual stage ion funnel interface with multi-capillary inlets to a commercial triple quadrupole mass spectrometer (TSQ Quantum Ultra; Thermo Fisher Scientific) (Fig. 4), Hossain *et al.* demonstrated that the new dual ion funnel interface significantly improved SRM sensitivity as evaluated by the LOD and CV values of peptides spiked into non-depleted mouse plasma [38]. Average SRM peak intensities were increased by ~70-fold for all peptides compared to the conventional interface, resulting in a lower LOD and improved reproducibility for SRM quantification. The LOD was improved by ~10-fold for the selected peptides, with notably reduced CVs. Using the dual ion funnel platform, 40 to 80 ng/mL of spiked-in proteins in mouse plasma can be detected without the application of front-end immunoaffinity depletion and fractionation. The dual ion funnel technology has recently been adapted and implemented by Agilent Technologies Inc. (Santa Clara, CA) in their new Agilent 6490 triple quadrupole mass spectrometer to increase ion sampling and ion transmission efficiency by a combination of 'Jet Stream' – ESI with thermal gradient ion focusing confinement, a hexabore sampling capillary to enable sampling of a much larger fraction of the ions, and a dual-stage ion funnel for efficient removal of large gas volumes and ion transfer to Q1 optics. The triple quadrupole mass spectrometer showed significant improvements in both sensitivity and reproducibility compared to instruments with standard interfaces [77]. More recently, Domanski *et al.* (2011) demonstrated the use of high flow-rate LC instead of nanoscale capillary LC for LC-SRM applications using the 6490 system [136]. The use of higher flow rates also should allow faster assays, increasing the overall throughput, at the cost of increased sample sizes that compensate for any loss of sensitivity compared to nanoscale LC. The significant improvement in detection sensitivity and reproducibility with such interface developments are expected to greatly aid a broad range of SRM applications.

4.2 Field asymmetric waveform ion mobility spectrometry (FAIMS)

Besides the improvements in ionization and ion transmission, technologies that provide overall higher resolving power to the MS-based platform have also been explored to enhance SRM sensitivity. FAIMS, an ion mobility separation technique that separates gas-phase ions at atmospheric pressure and room temperature based on their mobility differences in high and low electric fields [137-139], is one of the technologies being investigated for SRM applications. FAIMS separation is orthogonal to both chromatographic and mass spectrometric separation, thereby providing an additional dimension of resolving power by separating ions in the gas-phase to reduce chemical noise and improve the MS spectrum S/N by selective ion transmission, enhancing both sensitivity and reproducibility for LC-SRM analysis [140, 141]. The FAIMS QQQ MS was first commercialized by Thermo Fisher Scientific, Inc. With the introduction of SelecION™ technology, a planar differential mobility spectrometry (DMS) device was recently implemented between the curtain plate and orifice plate of the AB SCIEX 5500® system. With the application of LC-DMS-SRM method, Corr *et al.* (2011) confirmed again that DMS can significantly reduce matrix background and interferences, permitting quantification of target analytes which were previously not achievable, and thereby improving detection limits [142].

One major drawback of either FAIMS or DMS for SRM applications is that the cycle time for each SRM transition can be significantly increased due to the relatively long residence time of the devices, 10 ms to 100 ms, depending upon the type of FAIMS or DMS being used [68]. The observed signal intensities were also lower for LC-FAIMS-SRM than LC-SRM potentially due to the additional ion losses in the FAIMS and at the FAIMS/MS interface [141]. Future work focuses on improving the transmission of ions through the FAIMS device into the mass spectrometer, increasing the overall resolution of the separation, as well as reducing the residence time which should enable the application of

FAIMS for more sensitive SRM detection and broader biological and biomedical applications.

4.3 Selected reaction monitoring cubed (SRM³)

One of the advantages of SRM technology is the high specificity in target measurement due to the two-stage mass selection of the precursor and product ions for a given SRM transition. Despite this high resolving power of the two-stage mass selection, the detection of low-abundance peptides in complex samples such as blood plasma is often challenged by the high level of background interference or poor signal-to-background ratio. In 2009 Fortin *et al.* reported the use of monitoring MS³ fragment ions instead of the traditional measurement of MS² fragment ions for SRM transitions [69, 143]. In this SRM³ approach the analyte precursor ion is selected in the Q1 quadrupole, fragmented in the Q2 collision cell, and the primary product ion is trapped and activated in the Q3 linear ion trap (LIT) using the hybrid features of the QTRAP® 5500 system (triple quadrupole linear ion trap). In SRM³ mode, the primary product ion is isolated and fragmented in the LIT through a rapid increase in collision gas pressure and for the resonance ion excitation and collision induced fragmentation. The resulting secondary product ions from the selected primary product ion (MS³ spectrum) are subsequently detected by the MS detector. The intensities of MS³ ions can be used to reconstruct the SRM ion chromatogram for quantification in a similar manner as regular SRM, but with dramatically increased specificity by largely eliminating background interference, resulting in high sensitivity and a low baseline. It was demonstrated that this technique could enable the detection of protein biomarkers at the low ng/mL level in non-depleted human serum [69, 143]. With a simple two-step sample preparation workflow, a trypsin digestion of whole serum (100 µL) followed by enrichment of targeted tryptic peptides on a solid phase extraction column using MCX resin, QTRAP SRM³ can extend the dynamic range and LOQ of protein biomarkers to the low ng/mL range [69]. When compared to a traditional SRM mode for protein quantification in human plasma, the QTRAP SRM³ has been shown to provide a 3-5 fold lower LOQ in cases where the limitation is due to interferences [69, 143].

A disadvantage of using the QTRAP SRM³ mode for accurate quantification of candidate biomarkers is the relatively long cycle time of one MRM³ experiment (up to 350 ms), resulting in a decreased multiplexing capability and reduced number of data points across the chromatographic elution profile for a given peptide (hence, potentially decreased accuracy and reproducibility in quantification). Therefore, the QTRAP SRM³ assay is not suitable for quantifying a large number of protein biomarkers in a highly multiplexed fashion, whereas more than 1000 transitions or even ~6000 transitions can be multiplexed using conventional scheduled SRM [44, 49] or intelligent SRM [48] in a single LC-SRM experiment.

4.4 MALDI-SRM

While ESI is the most commonly applied ionization technique in LC-SRM experiments, MALDI has also been used as an alternative ionization technique to be coupled with SRM for quantification of small molecules with low molecular weight because of the limited m/z range of the quadrupoles (typically 1,500) and the limited collision energies for the singly charged ions, which precludes fragmentation of larger peptides [144, 145]. This technique does not necessarily require liquid chromatographic separation of samples prior to mass spectrometric analysis, and thus offers ultra-fast analysis. MALDI-SRM has been used for quantification of small drug compounds, such as several spirolide toxins in phytoplankton [146] and human liver microsome half-lives of 53 pharmaceutical compounds [147]. Luider *et al.* has demonstrated that MALDI-SRM technology is a versatile tool in the determination of concentrations of small molecules such as antiretroviral drugs (protease inhibitors [148,

149] and tenofovir [150]), anticancer drugs [151], and other types of drugs [146, 152, 153]. Wagner *et al.* demonstrated the potential of MALDI-SRM for the high-throughput quantification of saquinavir (a HIV-protease inhibitor drug) in human plasma without any prior chromatographic separation [152]. In 2011 Stoeckli *et al.* used MALDI-SRM to image moxifloxacin distribution in tuberculosis-infected rabbit lungs and granulomatous lesions [154]. The method has been shown to be rapid and sensitive for analysis of the distribution of anti-tuberculosis compounds with high quality images. However, for quantification of peptides using MALDI-SRM, only a few studies have been reported [155, 156]. The successful application of this technique in targeted proteomics for quantification of large numbers of peptides is hampered by the limited m/z range and modest resolution of most triple quadrupole and relatively poor reproducibility of SRM signals during quantitative analysis.

5. Perspective

SRM-based targeted quantification approaches have the potential to make a significant impact on high-throughput quantitative biology and biomarker verification. Significant advances have been made in recent years by exploiting SRM capabilities of multiplexing, relatively high sensitivity, accuracy, and reproducibility for quantifying proteins expressed at a small number of copies per cell [43] and low-abundance proteins in human plasma [34, 87]. However, improving the overall SRM sensitivity and throughput for target analyses still constitute major challenges. While significant advances have been made in the methods of front-end pre-fractionation and enrichment for enhanced detection of low-abundance proteins, these approaches generally share disadvantages associated with the added sample processing and the reduced overall throughput of SRM assays. Further technology advances on both the MS interface and MS instrumentation hold the promise for achieving further enhancement on SRM sensitivity without sacrificing throughput.

Current front-end pre-fractionation techniques, such as immunoaffinity depletion, SCX, and off-gel isoelectric focusing, all provide significant enrichment of analytes in the target fractions; however, despite these relatively extensive processing steps for analyte enrichment, the overall SRM sensitivity is still limited to the detection of proteins at concentrations above the ng/mL range in human plasma. It is anticipated that high resolution fractionation techniques, such as high pH reversed-phase LC, should provide further improvement on SRM sensitivity based on the advantage of using concatenated high pH reversed-phase LC as a first dimension fractionation strategy that has been demonstrated for gaining better coverage in global proteome profiling [90]. Among enrichment techniques, when applicable, the SISCAPA strategy appears a promising approach for achieving high sensitivity SRM quantification due to the high specificity of the immunoaffinity purification using anti-peptide antibodies. The potential of SISCAPA-SRM has been demonstrated in the context of an automated magnetic bead processing workflow, multiplexing experiments, as well as the integrated pipeline for assay development [34, 76], and an integrated platform is now feasible (Bravo Automated Liquid Handling platform and Agilent 6490 QQQ MS) [157]. The sensitivity of SISCAPA-SRM assays may be further enhanced through the utilization of monoclonal antibodies [105, 106] instead of the more commonly used polyclonal antibodies. Further developments in strategies providing minimal non-specific binding, optimal capture efficiency, and good recovery for captured peptides may further advance sensitivity. Eventually, the overall sensitivity of SISCAPA-SRM assays will be dependent on the LC-SRM platform sensitivity due to the amount of target peptides available, even if enriched with 100% peptide recovery, may still be below the detection limit of the LC-SRM platform for extremely low-abundance proteins. In such cases, the absolute sensitivity of the LC-SRM platform becomes of paramount importance, and the use

of e.g., small inner diameter capillary columns with nanoflow ESI [158, 159] will be an advantage for achieving higher sensitivity detection of low-abundance proteins.

The advances in either MS instrumentation or interface technologies play a major role in realizing the full potential of SRM for high-throughput quantitative biology. Indeed, the newer generation of commercially available MS instruments (e.g., ABI SCIEX 5500, Thermo TSQ Vantage, Agilent 6490, Waters Xevo TQ-S systems) from all major MS instrument companies have the potential to significantly enhance SRM sensitivity through effective ion transmission and utilization since the first reported use of SRM assays for plasma proteins [40]. Several other interface technologies, such as a subambient pressure ionization source with nanoelectrospray (SPIN) source [160], multi-emitter ESI source [135, 161], FAIMS [141, 162, 163], and differential mobility analysis [164], can potentially provide further improvements in ionization and ion transmission efficiency as well as greater resolving power for the MS platform, representing opportunities for additional enhancements in sensitivity for the SRM platform. Additional enhancements in SRM sensitivity may be achieved by creating platforms capable of performing multi-stage mass isolation [69].

We anticipate that the advances in highly automated front-end sample processing and analyte enrichments coupled with developments in MS technologies should enable reliable quantification of plasma proteins at the pg/mL to ng/mL levels as a routine practice within the next few years with SRM measurements of hundreds of clinical plasma samples per week being routine as opposed to presenting a formidable task. These exciting developments in SRM technology may also contribute to a significant paradigm shift where new SRM platforms play a major role for broad quantitative measurements in biological applications.

6 Concluding remarks

The advances in “omics” studies have produced a long list of candidate proteins and/or genes of interest for various human diseases; however, there exists a critical gap between biomarker discovery and development due to the drawbacks of “verification” technologies that provide insufficient sensitivity and throughput for analyzing large-scale clinical samples to reach statistically-sound conclusions. SRM is a promising tool for higher throughput qualification of target proteins and has the potential to eventually replace immunoassays for quantitative verification. Major challenges remaining for SRM assays are its presently insufficient sensitivity for detection of low-abundance proteins and inadequate throughput for large-scale studies. Enhancing the sensitivity of the SRM assays can involve, e.g., (1) extensive front-end pre-fractionation or analyte enrichment to reduce sample complexity and ion suppression; (2) efficient ESI ionization and ion transmission; (3) gas-phase ion separation to maximize the number of analyte ions into the MS and at the same time reduce the background noise; and (4) improvement in the MS resolving power for analyte detection with high specificity. While most front-end sample processing strategies aimed at advancing the SRM sensitivity sacrifice the overall throughput, it is possible that more effective automated sample processing workflows will significantly alleviate such limitations. Further advances in MS interface and instrumentation are important for eventually achieving sufficient sensitivity without sacrificing analytical throughput. Recent advances in SRM have demonstrated the ability to detect low ng/mL proteins in human plasma using several different strategies [34, 41, 64, 69, 77]. These developments illustrate the great potential of SRM as a quantitative verification technology for bridging the gap in biomarker discovery and clinical use. With further advances in sensitivity, we anticipate that SRM should have much broader applications in biomarker discovery and systems biology studies for quantification of candidate biomarkers in biofluids, protein isoforms, and even posttranslational modifications in cells and tissues.

Acknowledgments

Portions of this work were supported by the NIH Director's New Innovator Award Program 1-DP2OD006668-01, and NIH Grants CA111244, DK083447, and RR018522. The experimental work described herein was performed in the Environmental Molecular Sciences Laboratory, a national scientific user facility sponsored by the DOE and located at Pacific Northwest National Laboratory, which is operated by Battelle Memorial Institute for the DOE under Contract DE-AC05-76RL0 1830.

Abbreviations

CV	coefficient of variation
CRC	colorectal cancer
DMS	differential mobility spectrometry
ESI	electrospray ionization
ELISA	enzyme-linked immunosorbent assay
FAIMS	high-field asymmetric waveform ion mobility spectrometry
FAK	focal adhesion kinase
IMAC	immobilized metal-ion affinity chromatography
LOD	limit of detection
LOQ	limit of quantification
LIT	linear ion trap
LC	liquid chromatography
MALDI	matrix-assisted laser desorption/ionization
MCX	mixed cation exchange
mAbs	monoclonal antibodies
MRM	multiple reaction monitoring
MS	mass spectrometry
PSA	prostate-specific antigen
S/N	signal-to-noise ratio
SCX	strong cation exchange
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
SID	stable isotope dilution
SRM	selected reaction monitoring
SRM³	selected reaction monitoring cubed
SISCAPA	stable isotope standards and capture by anti-peptide antibodies
SPIN	subambient pressure ionization source with nanoelectrospray
QQQ	triple quadrupole mass spectrometer
PTM	posttranslational modification
TIMP1	tissue inhibitor of metalloproteinase 1

References

1. Rifai N, Gillette MA, Carr SA. Protein biomarker discovery and validation: the long and uncertain path to clinical utility. *Nat Biotechnol.* 2006; 24:971–983. [PubMed: 16900146]
2. Kulasingam V, Diamandis EP. Strategies for discovering novel cancer biomarkers through utilization of emerging technologies. *Nat Clin Pract Oncol.* 2008; 5:588–599. [PubMed: 18695711]
3. Hanash SM, Pitteri SJ, Faca VM. Mining the plasma proteome for cancer biomarkers. *Nature.* 2008; 452:571–579. [PubMed: 18385731]
4. Wang P, Whiteaker JR, Paulovich AG. The evolving role of mass spectrometry in cancer biomarker discovery. *Cancer Biol Ther.* 2009; 8:1083–1094. [PubMed: 19502776]
5. Qian WJ, Jacobs JM, Liu T, Camp DG 2nd, Smith RD. Advances and challenges in liquid chromatography-mass spectrometry-based proteomics profiling for clinical applications. *Mol Cell Proteomics.* 2006; 5:1727–1744. [PubMed: 16887931]
6. Edwards AV, White MY, Cordwell SJ. The role of proteomics in clinical cardiovascular biomarker discovery. *Mol Cell Proteomics.* 2008; 7:1824–1837. [PubMed: 18667414]
7. Jacquet S, Yin X, Sicard P, Clark J, et al. Identification of cardiac myosin-binding protein C as a candidate biomarker of myocardial infarction by proteomics analysis. *Mol Cell Proteomics.* 2009; 8:2687–2699. [PubMed: 19721077]
8. Fu Q, Van Eyk JE. Proteomics and heart disease: identifying biomarkers of clinical utility. *Expert Rev Proteomics.* 2006; 3:237–249. [PubMed: 16608436]
9. Anderson NL. The clinical plasma proteome: a survey of clinical assays for proteins in plasma and serum. *Clin Chem.* 2010; 56:177–185. [PubMed: 19884488]
10. Carr SA, Anderson L. Protein quantitation through targeted mass spectrometry: the way out of biomarker purgatory? *Clin Chem.* 2008; 54:1749–1752. [PubMed: 18957555]
11. Williams DK, Muddiman DC. Absolute quantification of C-reactive protein in human plasma derived from patients with epithelial ovarian cancer utilizing protein cleavage isotope dilution mass spectrometry. *J Proteome Res.* 2009; 8:1085–1090. [PubMed: 19196186]
12. Neubert H, Gale J, Muirhead D. Online high-flow peptide immunoaffinity enrichment and nanoflow LC-MS/MS: assay development for total salivary pepsin/pepsinogen. *Clin Chem.* 2010; 56:1413–1423. [PubMed: 20472821]
13. Domanski D, Murphy LC, Borchers CH. Assay development for the determination of phosphorylation stoichiometry using multiple reaction monitoring methods with and without phosphatase treatment: application to breast cancer signaling pathways. *Anal Chem.* 2010; 82:5610–5620. [PubMed: 20524616]
14. Nishimura T, Nomura M, Tojo H, Hamasaki H, et al. Proteomic analysis of laser-microdissected paraffin-embedded tissues: (2) MRM assay for stage-related proteins upon non-metastatic lung adenocarcinoma. *J Proteomics.* 2010; 73:1100–1110. [PubMed: 19944198]
15. Agger SA, Marney LC, Hoofnagle AN. Simultaneous quantification of apolipoprotein A-I and apolipoprotein B by liquid-chromatography-multiple-reaction-monitoring mass spectrometry. *Clin Chem.* 2010; 56:1804–1813. [PubMed: 20923952]
16. Lopez MF, Kuppusamy R, Sarracino DA, Prakash A, et al. Mass spectrometric discovery and selective reaction monitoring (SRM) of putative protein biomarker candidates in first trimester Trisomy 21 maternal serum. *J Proteome Res.* 2011; 10:133–142. [PubMed: 20499897]
17. Chen Y, Gruidl M, Remily-Wood E, Liu RZ, et al. Quantification of beta-catenin signaling components in colon cancer cell lines, tissue sections, and microdissected tumor cells using reaction monitoring mass spectrometry. *J Proteome Res.* 2010; 9:4215–4227. [PubMed: 20590165]
18. Kim K, Kim SJ, Yu HG, Yu J, et al. Verification of biomarkers for diabetic retinopathy by multiple reaction monitoring. *J Proteome Res.* 2010; 9:689–699. [PubMed: 20020744]
19. Yocum AK, Khan AP, Zhao R, Chinnaiyan AM. Development of selected reaction monitoring-MS methodology to measure peptide biomarkers in prostate cancer. *Proteomics.* 2010; 10:3506–3514. [PubMed: 20821805]

20. Mesmin C, Dubois M, Becher F, Fenaille F, Ezan E. Liquid chromatography/tandem mass spectrometry assay for the absolute quantification of the expected circulating apelin peptides in human plasma. *Rapid Commun Mass Spectrom*. 2010; 24:2875–2884. [PubMed: 20857448]
21. Rezeli M, Vegvari A, Ottervald J, Olsson T, et al. MRM assay for quantitation of complement components in human blood plasma - a feasibility study on multiple sclerosis. *J Proteomics*. 2011; 75:211–220. [PubMed: 21703369]
22. Elschenbroich S, Ignatchenko V, Clarke B, Kalloger SE, et al. In-depth proteomics of ovarian cancer ascites: combining shotgun proteomics and selected reaction monitoring mass spectrometry. *J Proteome Res*. 2011; 10:2286–2299. [PubMed: 21491939]
23. Thingholm TE, Bak S, Beck-Nielsen H, Jensen ON, Gaster M. Characterization of human myotubes from type 2 diabetic and nondiabetic subjects using complementary quantitative mass spectrometric methods. *Mol Cell Proteomics*. 2011; 10:M110 006650. [PubMed: 21697546]
24. Ijsselstijn L, Dekker LJM, Koudstaal PJ, Hofman A, et al. Serum Clusterin Levels Are Not Increased in Presymptomatic Alzheimer's Disease. *Journal of Proteome Research*. 2011; 10:2006–2010. [PubMed: 21280673]
25. Cho CK, Drabovich AP, Batruch I, Diamandis EP. Verification of a biomarker discovery approach for detection of Down syndrome in amniotic fluid via multiplex selected reaction monitoring (SRM) assay. *J Proteomics*. 2011; 74:2052–2059. [PubMed: 21624510]
26. Zhang H, Liu Q, Zimmerman LJ, Ham AJ, et al. Methods for peptide and protein quantitation by liquid chromatography-multiple reaction monitoring mass spectrometry. *Mol Cell Proteomics*. 2011; 10:M110 006593.
27. Selevsek N, Matondo M, Sanchez Carbayo M, Aebersold R, Domon B. Systematic quantification of peptides/proteins in urine using selected reaction monitoring. *Proteomics*. 2011; 11:1135–1147. [PubMed: 21360671]
28. Drabovich AP, Jarvi K, Diamandis EP. Verification of male infertility biomarkers in seminal plasma by multiplex selected reaction monitoring assay. *Mol Cell Proteomics*. 2011
29. Tang HY, Beer LA, Chang-Wong T, Hammond R, et al. A Xenograft Mouse Model Coupled with In-depth Plasma Proteome Analysis Facilitates Identification of Novel Serum Biomarkers for Human Ovarian Cancer. *J Proteome Res*. 2011
30. Makawita S, Diamandis EP. The bottleneck in the cancer biomarker pipeline and protein quantification through mass spectrometry-based approaches: current strategies for candidate verification. *Clin Chem*. 2010; 56:212–222. [PubMed: 20007861]
31. Meng Z, Veenstra TD. Targeted mass spectrometry approaches for protein biomarker verification. *J Proteomics*. 2011; 74:2650–2659. [PubMed: 21540133]
32. Addona TA, Abbatiello SE, Schilling B, Skates SJ, et al. Multi-site assessment of the precision and reproducibility of multiple reaction monitoring-based measurements of proteins in plasma. *Nat Biotechnol*. 2009; 27:633–641. [PubMed: 19561596]
33. Kuhn E, Addona T, Keshishian H, Burgess M, et al. Developing multiplexed assays for troponin I and interleukin-33 in plasma by peptide immunoaffinity enrichment and targeted mass spectrometry. *Clin Chem*. 2009; 55:1108–1117. [PubMed: 19372185]
34. Whiteaker JR, Zhao L, Anderson L, Paulovich AG. An automated and multiplexed method for high throughput peptide immunoaffinity enrichment and multiple reaction monitoring mass spectrometry-based quantification of protein biomarkers. *Mol Cell Proteomics*. 2010; 9:184–196. [PubMed: 19843560]
35. Barr JR, Maggio VL, Patterson DG Jr. Cooper GR, et al. Isotope dilution--mass spectrometric quantification of specific proteins: model application with apolipoprotein A-I. *Clin Chem*. 1996; 42:1676–1682. [PubMed: 8855153]
36. Barnidge DR, Dratz EA, Martin T, Bonilla LE, et al. Absolute quantification of the G protein-coupled receptor rhodopsin by LC/MS/MS using proteolysis product peptides and synthetic peptide standards. *Anal Chem*. 2003; 75:445–451. [PubMed: 12585469]
37. Gerber SA, Rush J, Stemman O, Kirschner MW, Gygi SP. Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. *Proc Natl Acad Sci U S A*. 2003; 100:6940–6945. [PubMed: 12771378]

38. Barnidge DR, Goodmanson MK, Klee GG, Muddiman DC. Absolute quantification of the model biomarker prostate-specific antigen in serum by LC-MS/MS using protein cleavage and isotope dilution mass spectrometry. *J Proteome Res.* 2004; 3:644–652. [PubMed: 15253448]
39. Kuhn E, Wu J, Karl J, Liao H, et al. Quantification of C-reactive protein in the serum of patients with rheumatoid arthritis using multiple reaction monitoring mass spectrometry and ¹³C-labeled peptide standards. *Proteomics.* 2004; 4:1175–1186. [PubMed: 15048997]
40. Anderson L, Hunter CL. Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins. *Mol Cell Proteomics.* 2006; 5:573–588. [PubMed: 16332733]
41. Keshishian H, Addona T, Burgess M, Kuhn E, Carr SA. Quantitative, multiplexed assays for low abundance proteins in plasma by targeted mass spectrometry and stable isotope dilution. *Mol Cell Proteomics.* 2007; 6:2212–2229. [PubMed: 17939991]
42. Aguiar M, Masse R, Gibbs BF. Mass spectrometric quantitation of C-reactive protein using labeled tryptic peptides. *Anal Biochem.* 2006; 354:175–181. [PubMed: 16723111]
43. Picotti P, Bodenmiller B, Mueller LN, Domon B, Aebersold R. Full dynamic range proteome analysis of *S. cerevisiae* by targeted proteomics. *Cell.* 2009; 138:795–806. [PubMed: 19664813]
44. Bisson N, James DA, Ivosev G, Tate SA, et al. Selected reaction monitoring mass spectrometry reveals the dynamics of signaling through the GRB2 adaptor. *Nat Biotechnol.* 2011; 29:653–658. [PubMed: 21706016]
45. Lange V, Malmstrom JA, Didion J, King NL, et al. Targeted quantitative analysis of *Streptococcus pyogenes* virulence factors by multiple reaction monitoring. *Mol Cell Proteomics.* 2008; 7:1489–1500. [PubMed: 18408245]
46. Zhao Y, Widen SG, Jamaluddin M, Tian B, et al. Quantification of activated NF-kappaB/RelA complexes using ssDNA aptamer affinity-stable isotope dilution-selected reaction monitoring-mass spectrometry. *Mol Cell Proteomics.* 2011; 10:M111 008771.
47. Costenoble R, Picotti P, Reiter L, Stallmach R, et al. Comprehensive quantitative analysis of central carbon and amino-acid metabolism in *Saccharomyces cerevisiae* under multiple conditions by targeted proteomics. *Molecular systems biology.* 2011; 7:464. [PubMed: 21283140]
48. Kiyonami R, Schoen A, Prakash A, Peterman S, et al. Increased selectivity, analytical precision, and throughput in targeted proteomics. *Mol Cell Proteomics.* 2011; 10:M110 002931. [PubMed: 20664071]
49. Lange V, Picotti P, Domon B, Aebersold R. Selected reaction monitoring for quantitative proteomics: a tutorial. *Mol Syst Biol.* 2008; 4:222. [PubMed: 18854821]
50. Izrael-Tomasevic A, Phu L, Phung QT, Lill JR, Arnott D. Targeting interferon alpha subtypes in serum: a comparison of analytical approaches to the detection and quantitation of proteins in complex biological matrices. *J Proteome Res.* 2009; 8:3132–3140. [PubMed: 19351188]
51. Prakash A, Rezai T, Krastins B, Sarracino D, et al. Platform for establishing interlaboratory reproducibility of selected reaction monitoring-based mass spectrometry peptide assays. *J Proteome Res.* 2010; 9:6678–6688. [PubMed: 20945832]
52. Bluemlein K, Ralser M. Monitoring protein expression in whole-cell extracts by targeted label- and standard-free LC-MS/MS. *Nat Protoc.* 2011; 6:859–869. [PubMed: 21637204]
53. Unwin RD, Griffiths JR, Whetton AD. A sensitive mass spectrometric method for hypothesis-driven detection of peptide post-translational modifications: multiple reaction monitoring-initiated detection and sequencing (MIDAS). *Nat Protoc.* 2009; 4:870–877. [PubMed: 19444244]
54. Wang Q, Chaerkady R, Wu J, Hwang HJ, et al. Mutant proteins as cancer-specific biomarkers. *Proc Natl Acad Sci U S A.* 2011; 108:2444–2449. [PubMed: 21248225]
55. Wolf-Yadlin A, Hautaniemi S, Lauffenburger DA, White FM. Multiple reaction monitoring for robust quantitative proteomic analysis of cellular signaling networks. *Proc Natl Acad Sci U S A.* 2007; 104:5860–5865. [PubMed: 17389395]
56. Stahl-Zeng J, Lange V, Ossola R, Eckhardt K, et al. High sensitivity detection of plasma proteins by multiple reaction monitoring of N-glycosites. *Mol Cell Proteomics.* 2007; 6:1809–1817. [PubMed: 17644760]
57. Held JM, Danielson SR, Behring JB, Atsriku C, et al. Targeted quantitation of site-specific cysteine oxidation in endogenous proteins using a differential alkylation and multiple reaction

- monitoring mass spectrometry approach. *Mol Cell Proteomics*. 2010; 9:1400–1410. [PubMed: 20233844]
58. Lathrop JT, Anderson NL, Anderson NG, Hammond DJ. Therapeutic potential of the plasma proteome. *Curr Opin Mol Ther*. 2003; 5:250–257. [PubMed: 12870434]
59. Anderson NL, Polanski M, Pieper R, Gatlin T, et al. The human plasma proteome: a nonredundant list developed by combination of four separate sources. *Mol Cell Proteomics*. 2004; 3:311–326. [PubMed: 14718574]
60. Anderson NL, Anderson NG. The human plasma proteome: history, character, and diagnostic prospects. *Mol Cell Proteomics*. 2002; 1:845–867. [PubMed: 12488461]
61. Shi T, Zhou JY, Gritsenko MA, Hossain M, et al. IgY14 and SuperMix immunoaffinity separations coupled with liquid chromatography-mass spectrometry for human plasma proteomics biomarker discovery. *Methods*. 2011
62. Hossain M, Kaleta DT, Robinson EW, Liu T, et al. Enhanced Sensitivity for Selected Reaction Monitoring Mass Spectrometry-based Targeted Proteomics Using a Dual Stage Electrodynamical Ion Funnel Interface. *Mol Cell Proteomics*. 2011; 10:M000062MCP000201. [PubMed: 20410378]
63. Jacobs JM, Adkins JN, Qian WJ, Liu T, et al. Utilizing human blood plasma for proteomic biomarker discovery. *J Proteome Res*. 2005; 4:1073–1085. [PubMed: 16083256]
64. Keshishian H, Addona T, Burgess M, Mani DR, et al. Quantification of cardiovascular biomarkers in patient plasma by targeted mass spectrometry and stable isotope dilution. *Mol Cell Proteomics*. 2009; 8:2339–2349. [PubMed: 19596694]
65. Tang HY, Beer LA, Barnhart KT, Speicher DW. Rapid verification of candidate serological biomarkers using gel-based, label-free multiple reaction monitoring. *J Proteome Res*. 2011; 10:4005–4017. [PubMed: 21726088]
66. Rafalko A, Dai S, Hancock WS, Karger BL, Hincapié M. Development of a Chip/Chip/SRM platform using digital chip isoelectric focusing and LC-Chip mass spectrometry for enrichment and quantitation of low abundance protein biomarkers in human plasma. *J Proteome Res*. 2011
67. Whiteaker JR, Lin C, Kennedy J, Hou L, et al. A targeted proteomics-based pipeline for verification of biomarkers in plasma. *Nat Biotechnol*. 2011; 29:625–634. [PubMed: 21685906]
68. Xia YQ, Wu ST, Jemal M. LC-FAIMS-MS/MS for quantification of a peptide in plasma and evaluation of FAIMS global selectivity from plasma components. *Anal Chem*. 2008; 80:7137–7143. [PubMed: 18652493]
69. Fortin T, Salvador A, Charrier JP, Lenz C, et al. Multiple reaction monitoring cubed for protein quantification at the low nanogram/milliliter level in nondepleted human serum. *Anal Chem*. 2009; 81:9343–9352. [PubMed: 19839594]
70. Qian WJ, Kaleta DT, Petritis BO, Jiang H, et al. Enhanced detection of low abundance human plasma proteins using a tandem IgY12-SuperMix immunoaffinity separation strategy. *Mol Cell Proteomics*. 2008; 7:1963–1973. [PubMed: 18632595]
71. Liu T, Qian WJ, Mottaz HM, Gritsenko MA, et al. Evaluation of multiprotein immunoaffinity subtraction for plasma proteomics and candidate biomarker discovery using mass spectrometry. *Mol Cell Proteomics*. 2006; 5:2167–2174. [PubMed: 16854842]
72. Bandow JE. Comparison of protein enrichment strategies for proteome analysis of plasma. *Proteomics*. 2010; 10:1416–1425. [PubMed: 20127685]
73. Nicol GR, Han M, Kim J, Birse CE, et al. Use of an immunoaffinity-mass spectrometry-based approach for the quantification of protein biomarkers from serum samples of lung cancer patients. *Mol Cell Proteomics*. 2008; 7:1974–1982. [PubMed: 18388126]
74. Anderson NL, Anderson NG, Haines LR, Hardie DB, et al. Mass spectrometric quantitation of peptides and proteins using Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA). *J Proteome Res*. 2004; 3:235–244. [PubMed: 15113099]
75. Anderson NL, Jackson A, Smith D, Hardie D, et al. SISCAPA peptide enrichment on magnetic beads using an in-line bead trap device. *Mol Cell Proteomics*. 2009; 8:995–1005. [PubMed: 19196707]
76. Whiteaker JR, Zhao L, Abbatiello SE, Burgess M, et al. Evaluation of large scale quantitative proteomic assay development using peptide affinity-based mass spectrometry. *Mol Cell Proteomics*. 2011; 10:M110 005645. [PubMed: 21245105]

77. Miller, C.; Yang, Y.; Waddell, K. ABRF 2011. San Antonio, TX: 2011. Highest sensitivity protein quantitation using a triple quadrupole with a dual ion funnel..
78. Schwartz JC, Schey KL, Cooks RG. A Penta-Quadrupole Instrument for Reaction Intermediate Scans and Other Ms-Ms-Ms Experiments. *International Journal of Mass Spectrometry and Ion Processes*. 1990; 101:1–20.
79. Huang L, Harvie G, Feitelson JS, Gramatikoff K, et al. Immunoaffinity separation of plasma proteins by IgY microbeads: meeting the needs of proteomic sample preparation and analysis. *Proteomics*. 2005; 5:3314–3328. [PubMed: 16041669]
80. Tu C, Rudnick PA, Martinez MY, Cheek KL, et al. Depletion of abundant plasma proteins and limitations of plasma proteomics. *Journal of proteome research*. 2010; 9:4982–4991. [PubMed: 20677825]
81. Kuzyk MA, Smith D, Yang J, Cross TJ, et al. Multiple reaction monitoring-based, multiplexed, absolute quantitation of 45 proteins in human plasma. *Mol Cell Proteomics*. 2009; 8:1860–1877. [PubMed: 19411661]
82. Zhou JY, Petritis BO, Petritis K, Norbeck AD, et al. Mouse-specific tandem IgY7-SuperMix immunoaffinity separations for improved LC-MS/MS coverage of the plasma proteome. *Journal of proteome research*. 2009; 8:5387–5395. [PubMed: 19722698]
83. DeSouza LV, Taylor AM, Li W, Minkoff MS, et al. Multiple reaction monitoring of mTRAQ-labeled peptides enables absolute quantification of endogenous levels of a potential cancer marker in cancerous and normal endometrial tissues. *J Proteome Res*. 2008; 7:3525–3534. [PubMed: 18630974]
84. DeSouza LV, Romaschin AD, Colgan TJ, Siu KW. Absolute quantification of potential cancer markers in clinical tissue homogenates using multiple reaction monitoring on a hybrid triple quadrupole/linear ion trap tandem mass spectrometer. *Anal Chem*. 2009; 81:3462–3470. [PubMed: 19323455]
85. DeSouza LV, Krakovska O, Darfler MM, Krizman DB, et al. mTRAQ-based quantification of potential endometrial carcinoma biomarkers from archived formalin-fixed paraffin-embedded tissues. *Proteomics*. 2010; 10:3108–3116. [PubMed: 20661955]
86. Shah SJ, Yu KH, Sangar V, Parry SI, Blair IA. Identification and quantification of preterm birth biomarkers in human cervicovaginal fluid by liquid chromatography/tandem mass spectrometry. *J Proteome Res*. 2009; 8:2407–2417. [PubMed: 19271751]
87. Addona TA, Shi X, Keshishian H, Mani DR, et al. A pipeline that integrates the discovery and verification of plasma protein biomarkers reveals candidate markers for cardiovascular disease. *Nat Biotechnol*. 2011; 29:635–643. [PubMed: 21685905]
88. Fortin T, Salvador A, Charrier JP, Lenz C, et al. 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]
89. Martosella J, Zolotarjova N, Liu H, Nicol G, Boyes BE. Reversed-phase high-performance liquid chromatographic prefractionation of immunodepleted human serum proteins to enhance mass spectrometry identification of lower-abundant proteins. *Journal of proteome research*. 2005; 4:1522–1537. [PubMed: 16212403]
90. Wang Y, Yang F, Gritsenko MA, Clauss T, et al. Reversed-phase chromatography with multiple fraction concatenation strategy for proteome profiling of human MCF10A cells. *Proteomics*. 2011; 11:2019–2026. [PubMed: 21500348]
91. Cho SY, Lee EY, Lee JS, Kim HY, et al. Efficient prefractionation of low-abundance proteins in human plasma and construction of a two-dimensional map. *Proteomics*. 2005; 5:3386–3396. [PubMed: 16047310]
92. Nissum M, Foucher AL. Analysis of human plasma proteins: a focus on sample collection and separation using free-flow electrophoresis. *Expert review of proteomics*. 2008; 5:571–587. [PubMed: 18761468]
93. Heller M, Michel PE, Morier P, Crettaz D, et al. Two-stage Off-Gel isoelectric focusing: protein followed by peptide fractionation and application to proteome analysis of human plasma. *Electrophoresis*. 2005; 26:1174–1188. [PubMed: 15704250]

94. Tang HY, Ali-Khan N, Echan LA, Levenkova N, et al. A novel four-dimensional strategy combining protein and peptide separation methods enables detection of low-abundance proteins in human plasma and serum proteomes. *Proteomics*. 2005; 5:3329–3342. [PubMed: 16052622]
95. Ang CS, Nice EC. Targeted in-gel MRM: a hypothesis driven approach for colorectal cancer biomarker discovery in human feces. *J Proteome Res*. 2010; 9:4346–4355. [PubMed: 20684568]
96. Maass S, Sievers S, Zuhlke D, Kuzinski J, et al. Efficient, global-scale quantification of absolute protein amounts by integration of targeted mass spectrometry and two-dimensional gel-based proteomics. *Anal Chem*. 2011; 83:2677–2684. [PubMed: 21395229]
97. Kulasingam V, Smith CR, Batruch I, Buckler A, et al. “Product ion monitoring” assay for prostate-specific antigen in serum using a linear ion-trap. *J Proteome Res*. 2008; 7:640–647. [PubMed: 18186600]
98. Berna MJ, Zhen Y, Watson DE, Hale JE, Ackermann BL. Strategic use of immunoprecipitation and LC/MS/MS for trace-level protein quantification: myosin light chain 1, a biomarker of cardiac necrosis. *Anal Chem*. 2007; 79:4199–4205. [PubMed: 17447729]
99. Berna M, Ackermann B. Increased throughput for low-abundance protein biomarker verification by liquid chromatography/tandem mass spectrometry. *Anal Chem*. 2009; 81:3950–3956. [PubMed: 19388669]
100. Chénau J, Fenaille F, Ezan E, Morel N, et al. Sensitive Detection of *Bacillus anthracis* Spores by Immunocapture and Liquid Chromatography-Tandem Mass Spectrometry. *Anal Chem*. 2011; 83:8675–8682. [PubMed: 21961787]
101. Hoofnagle AN, Becker JO, Wener MH, Heinecke JW. Quantification of thyroglobulin, a low-abundance serum protein, by immunoaffinity peptide enrichment and tandem mass spectrometry. *Clin Chem*. 2008; 54:1796–1804. [PubMed: 18801935]
102. Spencer CA, Takeuchi M, Kazarosyan M, Wang CC, et al. Serum thyroglobulin autoantibodies: prevalence, influence on serum thyroglobulin measurement, and prognostic significance in patients with differentiated thyroid carcinoma. *J Clin Endocrinol Metab*. 1998; 83:1121–1127. [PubMed: 9543128]
103. Hoofnagle AN, Wener MH. Serum thyroglobulin: a model of immunoassay imperfection. *Clin Lab Int*. 2006; 8:12–14.
104. Whiteaker JR, Zhao L, Zhang HY, Feng LC, et al. Antibody-based enrichment of peptides on magnetic beads for mass-spectrometry-based quantification of serum biomarkers. *Anal Biochem*. 2007; 362:44–54. [PubMed: 17241609]
105. Schoenherr RM, Zhao L, Whiteaker JR, Feng LC, et al. Automated screening of monoclonal antibodies for SISCAPA assays using a magnetic bead processor and liquid chromatography-selected reaction monitoring-mass spectrometry. *J Immunol Methods*. 2010; 353:49–61. [PubMed: 19961853]
106. Razavi M, Pope ME, Soste MV, Eyford BA, et al. MALDI immunoscreening (MiSCREEN): a method for selection of anti-peptide monoclonal antibodies for use in immunoproteomics. *J Immunol Methods*. 2011; 364:50–64. [PubMed: 21078325]
107. Leitner A, Lindner W. Chemistry meets proteomics: the use of chemical tagging reactions for MS-based proteomics. *Proteomics*. 2006; 6:5418–5434. [PubMed: 16972287]
108. Cravatt BF, Wright AT, Kozarich JW. Activity-based protein profiling: from enzyme chemistry to proteomic chemistry. *Annu Rev Biochem*. 2008; 77:383–414. [PubMed: 18366325]
109. Mayya V, Rezual K, Wu L, Fong MB, Han DK. Absolute quantification of multisite phosphorylation by selective reaction monitoring mass spectrometry: determination of inhibitory phosphorylation status of cyclin-dependent kinases. *Mol Cell Proteomics*. 2006; 5:1146–1157. [PubMed: 16546994]
110. Ciccimaro E, Hanks SK, Yu KH, Blair IA. Absolute quantification of phosphorylation on the kinase activation loop of cellular focal adhesion kinase by stable isotope dilution liquid chromatography/mass spectrometry. *Anal Chem*. 2009; 81:3304–3313. [PubMed: 19354260]
111. Jin LL, Tong J, Prakash A, Peterman SM, et al. Measurement of protein phosphorylation stoichiometry by selected reaction monitoring mass spectrometry. *Journal of proteome research*. 2010; 9:2752–2761. [PubMed: 20205385]

112. Spiro RG. Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds. *Glycobiology*. 2002; 12:43R–56R.
113. Hulsmeier AJ, Paesold-Burda P, Hennet T. N-glycosylation site occupancy in serum glycoproteins using multiple reaction monitoring liquid chromatography-mass spectrometry. *Mol Cell Proteomics*. 2007; 6:2132–2138. [PubMed: 17823199]
114. Zhao Y, Jia W, Wang J, Ying W, et al. Fragmentation and site-specific quantification of core fucosylated glycoprotein by multiple reaction monitoring-mass spectrometry. *Anal Chem*. 2011; 83:8802–8809. [PubMed: 21970473]
115. Ahn YH, Lee JY, Kim YS, Ko JH, Yoo JS. Quantitative analysis of an aberrant glycoform of TIMP1 from colon cancer serum by L-PHA-enrichment and SISCAPA with MRM mass spectrometry. *J Proteome Res*. 2009; 8:4216–4224. [PubMed: 19645485]
116. Kuroguchi M, Matsushita T, Amano M, Furukawa J, et al. Sialic acid-focused quantitative mouse serum glycoproteomics by multiple reaction monitoring assay. *Mol Cell Proteomics*. 2010; 9:2354–2368. [PubMed: 20571061]
117. Zhang H, Li XJ, Martin DB, Aebersold R. Identification and quantification of N-linked glycoproteins using hydrazide chemistry, stable isotope labeling and mass spectrometry. *Nature biotechnology*. 2003; 21:660–666.
118. Ahn YH, Kim YS, Ji ES, Lee JY, et al. Comparative quantitation of aberrant glycoforms by lectin-based glycoprotein enrichment coupled with multiple-reaction monitoring mass spectrometry. *Anal Chem*. 2010; 82:4441–4447. [PubMed: 20462175]
119. Jaffrey SR, Erdjument-Bromage H, Ferris CD, Tempst P, Snyder SH. Protein S-nitrosylation: a physiological signal for neuronal nitric oxide. *Nat Cell Biol*. 2001; 3:193–197. [PubMed: 11175752]
120. Wang Q, Zhang Y, Yang C, Xiong H, et al. Acetylation of metabolic enzymes coordinates carbon source utilization and metabolic flux. *Science*. 2010; 327:1004–1007. [PubMed: 20167787]
121. Zhao S, Xu W, Jiang W, Yu W, et al. Regulation of cellular metabolism by protein lysine acetylation. *Science*. 2010; 327:1000–1004. [PubMed: 20167786]
122. Peng J, Schwartz D, Elias JE, Thoreen CC, et al. A proteomics approach to understanding protein ubiquitination. *Nature biotechnology*. 2003; 21:921–926.
123. Phu L, Izrael-Tomasevic A, Matsumoto ML, Bustos D, et al. Improved quantitative mass spectrometry methods for characterizing complex ubiquitin signals. *Mol Cell Proteomics*. 2011; 10:M110 003756. [PubMed: 21048196]
124. Doucet A, Butler GS, Rodriguez D, Prudova A, Overall CM. Metadegradomics: toward in vivo quantitative degradomics of proteolytic post-translational modifications of the cancer proteome. *Molecular & cellular proteomics : MCP*. 2008; 7:1925–1951.
125. Tang K, Page JS, Smith RD. Charge competition and the linear dynamic range of detection in electrospray ionization mass spectrometry. *J Am Soc Mass Spectrom*. 2004; 15:1416–1423. [PubMed: 15465354]
126. Wilm MS, Mann M. Electrospray and Taylor-Cone theory, Dole's beam of macromolecules at last? *International Journal of Mass Spectrometry and Ion Processes*. 1994; 136:167–180.
127. Smith RD, Shen Y, Tang K. Ultrasensitive and Quantitative Analyses from Combined Separations-Mass Spectrometry for the Characterization of Proteomes. *Acc Chem Res*. 2004; 37:269–278. [PubMed: 15096064]
128. Page JS, Kelly RT, Tang K, Smith RD. Ionization and transmission efficiency in an electrospray ionization-mass spectrometry interface. *J Am Soc Mass Spectrom*. 2007; 18:1582–1590. [PubMed: 17627841]
129. Shaffer SA, Tolmachev A, Prior DC, Anderson GA, et al. Characterization of an improved electrodynamic ion funnel interface for electrospray ionization mass spectrometry. *Anal Chem*. 1999; 71:2957–2964. [PubMed: 10450147]
130. Page JS, Bogdanov B, Vilkov AN, Prior DC, et al. Automatic gain control in mass spectrometry using a jet disrupter electrode in an electrodynamic ion funnel. *J Am Soc Mass Spectrom*. 2005; 16:244–253. [PubMed: 15694774]
131. Kelly RT, Tolmachev AV, Page JS, Tang K, Smith RD. The ion funnel: theory, implementations, and applications. *Mass Spectrom Rev*. 2010; 29:294–312. [PubMed: 19391099]

132. Shaffer SA, Tang K, Anderson GA, Prior DC, et al. A Novel ion funnel for focusing ions at elevated pressure using electrospray ionization mass spectrometry. *Rapid Communication in Mass Spectrometry*. 1997; 11:1813–1817.
133. Ibrahim Y, Tang K, Tolmachev AV, Shvartsburg AA, Smith RD. Improving mass spectrometer sensitivity using a high-pressure electrodynamic ion funnel interface. *J Am Soc Mass Spectrom*. 2006; 17:1299–1305. [PubMed: 16839773]
134. Belov ME, Prasad S, Prior DC, Danielson WF 3rd, et al. Pulsed multiple reaction monitoring approach to enhancing sensitivity of a tandem quadrupole mass spectrometer. *Anal Chem*. 2011; 83:2162–2171. [PubMed: 21344863]
135. Kelly RT, Page JS, Marginean I, Tang K, Smith RD. Nanoelectrospray emitter arrays providing interemitter electric field uniformity. *Anal Chem*. 2008; 80:5660–5665. [PubMed: 18553942]
136. Domanski D, Smith DS, Miller CA, Yang Y, et al. High-flow multiplexed MRM-based analysis of proteins in human plasma without depletion or enrichment. *Clin Lab Med*. 2011; 31:371–384. [PubMed: 21907103]
137. Buryakov IA, Krylov EV, Nazarov EG, Rasulev UK. A new method of separation of multi-atomic ions by mobility at atmospheric pressure using a high-frequency amplitude-asymmetric strong electric field. *Int J Mass Spectrom Ion Process*. 1993; 128:143–148.
138. Purves RW, Guevremont R, Day S, Pipich CW, Matyjaszczyk MS. Mass spectrometric characterization of a high-field asymmetric waveform ion mobility spectrometer. *Rev Sci Instrum*. 1998; 69:4094–4105.
139. Purves RW, Guevremont R. Electrospray ionization high-field asymmetric waveform ion mobility spectrometry-mass spectrometry. *Anal Chem*. 1999; 71:2346–2357. [PubMed: 21662783]
140. Barnett DA, Ding L, Ells B, Purves RW, Guevremont R. Tandem mass spectra of tryptic peptides at signal-to-background ratios approaching unity using electrospray ionization high-field asymmetric waveform ion mobility spectrometry/hybrid quadrupole time-of-flight mass spectrometry. *Rapid Commun Mass Spectrom*. 2002; 16:676–680. [PubMed: 11921246]
141. Canterbury JD, Yi X, Hoopmann MR, MacCoss MJ. Assessing the dynamic range and peak capacity of nanoflow LC-FAIMS-MS on an ion trap mass spectrometer for proteomics. *Anal Chem*. 2008; 80:6888–6897. [PubMed: 18693747]
142. Corr, JJ.; Keshishian, H.; Abbatiello, SE.; Covey, TR. Quantitative analysis of proteins in plasma using differential mobility spectrometry and multiple reaction monitoring with nanoLC and capillaryLC.. 59th ASMS Conference on Mass Spectrometry and Allied Topics; Denver, CO. 2011;
143. Hunter C. MRM3 quantitation for highest selectivity of proteins in complex matrices. *J Biomol Tech*. 2010; 21:S34–S35.
144. van Kampen JJ, Burgers PC, de Groot R, Gruters RA, Luider TM. Biomedical application of MALDI mass spectrometry for small-molecule analysis. *Mass Spectrom Rev*. 2011; 30:101–120. [PubMed: 20169623]
145. Parker CE, Pearson TW, Anderson NL, Borchers CH. Mass-spectrometry-based clinical proteomics--a review and prospective. *Analyst*. 2010; 135:1830–1838. [PubMed: 20520858]
146. Sleno L, Volmer DA. Toxin screening in phytoplankton: Detection and quantitation using MALDI triple quadrupole mass spectrometry. *Analytical Chemistry*. 2005; 77:1509–1517. [PubMed: 15732938]
147. Gobey J, Cole M, Janiszewski J, Covey T, et al. Characterization and performance of MALDI on a triple quadrupole mass spectrometer for analysis and quantification of small molecules. *Anal Chem*. 2005; 77:5643–5654. [PubMed: 16131077]
148. Meesters RJ, van Kampen JJ, Reedijk ML, Scheuer RD, et al. Ultrafast and high-throughput mass spectrometric assay for therapeutic drug monitoring of antiretroviral drugs in pediatric HIV-1 infection applying dried blood spots. *Anal Bioanal Chem*. 2010; 398:319–328. [PubMed: 20632164]
149. van Kampen JJ, Reedijk ML, Burgers PC, Dekker LJ, et al. Ultra-fast analysis of plasma and intracellular levels of HIV protease inhibitors in children: a clinical application of MALDI mass spectrometry. *PLoS One*. 2010; 5:e11409. [PubMed: 20625386]

150. Meesters RJ, van Kampen JJ, Scheuer RD, van der Ende ME, et al. Determination of the antiretroviral drug tenofovir in plasma from HIV-infected adults by ultrafast isotope dilution MALDI-triple quadrupole tandem mass spectrometry. *J Mass Spectrom.* 2011; 46:282–289. [PubMed: 21394844]
151. Meesters RJ, Cornelissen R, van Klaveren RJ, de Jonge R, et al. A new ultrafast and high-throughput mass spectrometric approach for the therapeutic drug monitoring of the multi-targeted anti-folate pemetrexed in plasma from lung cancer patients. *Anal Bioanal Chem.* 2010; 398:2943–2948. [PubMed: 20865407]
152. Wagner M, Varesio E, Hopfgartner G. Ultra-fast quantitation of saquinavir in human plasma by matrix-assisted laser desorption/ionization and selected reaction monitoring mode detection. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2008; 872:68–76.
153. Hatsis P, Brombacher S, Corr J, Kovarik P, Volmer DA. Quantitative analysis of small pharmaceutical drugs using a high repetition rate laser matrix-assisted laser/desorption ionization source. *Rapid Commun Mass Spectrom.* 2003; 17:2303–2309. [PubMed: 14558130]
154. Prideaux B, Dartois V, Staab D, Weiner DM, et al. High-sensitivity MALDI-MRM-MS imaging of moxifloxacin distribution in tuberculosis-infected rabbit lungs and granulomatous lesions. *Anal Chem.* 2011; 83:2112–2118. [PubMed: 21332183]
155. Melanson JE, Chisholm KA, Pinto DM. Targeted comparative proteomics by liquid chromatography/matrix-assisted laser desorption/ionization triple-quadrupole mass spectrometry. *Rapid Commun Mass Spectrom.* 2006; 20:904–910. [PubMed: 16470697]
156. Locke SJ, Leslie AD, Melanson JE, Pinto DM. Deviation from the mobile proton model in amino-modified peptides: implications for multiple reaction monitoring analysis of peptides. *Rapid Commun Mass Spectrom.* 2006; 20:1525–1530. [PubMed: 16642461]
157. Anderson, L.; Pope, M.; Razavi, M.; Pearson, T., et al. Automation of a SISCAPA magnetic bead workflow for protein biomarker quantitation by mass spectrometry.. 59th ASMS Conference on Mass Spectrometry and Allied Topics; Denver, CO. 2011;
158. Shen Y, Tolic N, Masselon C, Pasa-Tolic L, et al. Ultrasensitive proteomics using high-efficiency on-line micro-SPE-nanoLC-nanoESI MS and MS/MS. *Analytical chemistry.* 2004; 76:144–154. [PubMed: 14697044]
159. Shen Y, Zhao R, Berger SJ, Anderson GA, et al. High-efficiency nanoscale liquid chromatography coupled on-line with mass spectrometry using nanoelectrospray ionization for proteomics. *Analytical chemistry.* 2002; 74:4235–4249. [PubMed: 12199598]
160. Page JS, Tang K, Kelly RT, Smith RD. Subambient pressure ionization with nanoelectrospray source and interface for improved sensitivity in mass spectrometry. *Anal Chem.* 2008; 80:1800–1805. [PubMed: 18237189]
161. Kelly RT, Page JS, Zhao R, Qian WJ, et al. Capillary-based multi nanoelectrospray emitters: improvements in ion transmission efficiency and implementation with capillary reversed-phase LC-ESI-MS. *Anal Chem.* 2008; 80:143–149. [PubMed: 18044958]
162. Barnett DA, Ells B, Guevremont R, Purves RW. Application of ESI-FAIMS-MS to the analysis of tryptic peptides. *J Am Soc Mass Spectrom.* 2002; 13:1282–1291. [PubMed: 12443018]
163. Shvartsburg AA, Smith RD. Optimum waveforms for differential ion mobility spectrometry (FAIMS). *J Am Soc Mass Spectrom.* 2008; 19:1286–1295. [PubMed: 18585054]
164. Hogan CJ Jr, Ruotolo BT, Robinson CV, Fernandez de la Mora J. Tandem differential mobility analysis-mass spectrometry reveals partial gas-phase collapse of the GroEL complex. *J Phys Chem B.* 2011; 115:3614–3621. [PubMed: 21395304]
165. Kuhn E, Whiteaker JR, Mani DR, Jackson AM, et al. Inter-laboratory evaluation of automated, multiplexed peptide immunoaffinity enrichment coupled to multiple reaction monitoring mass spectrometry for quantifying proteins in plasma. *Molecular & Cellular Proteomics : MCP.* Dec 22.2011 [Epub ahead of print].

Figure 1.

(A) Schematic diagram of a triple quadrupole mass spectrometer (QQQ MS). Typical SRM analyses of target proteins involve three stages: front-end, sample preparation, fractionation, and enrichment; interface, ion sampling, gas-phase separation, and ion transmission; back-end, the resolving power of the mass analyzer for removal of co-eluting interferences. **(B)** Techniques for enhancing SRM sensitivity.

Figure 2. Fractionation strategies for enhancing SRM sensitivity by reducing sample complexity.

Figure 3. Schematic illustration of the principles of tandem IgY12/SuperMix immunoaffinity depletion.

Figure 4.
Schematic diagram of a Thermo QQQ Quantum Ultra MS equipped with a dual electrodynamic ion funnel interface and a multi-capillary inlet.

A survey of recent strategies for enhancing SRM sensitivity in detection and quantification of target proteins in blood plasma or serum (with the exception of the yeast proteome in Ref. [48]).

Table 1

Instrumentation platforms	Enrichment methods	Proteins ^a	LOD	LOQ	Ref.
NanoLC-SRM with standard interface	None	47		~1 µg/mL	[40]
		45		15-168,014 amol ^b	[81]
		11		~2 ug/mL	[32]
		9	0.2-23.8 µg/mL	0.8-79.3 µg/mL	[34]
		~350 ^c		10 amol ^b	[48]
NanoLC-SRM with dual-funnel interface		5	40-80 ng/mL		[50]
High-flow LC-SRM with dual-funnel interface		92	not reported		[136]
High-flow LC-SRM with SRM ³	MCX	1	1.5 ng/mL	4.5 ng/mL	[69]
NanoLC-SRM with standard interface	Depletion	6	2.5-50 ng/mL ^d	25-100 ng/mL ^d	[41]
		6	5.0-100 ng/mL ^e	25-250 ng/mL ^e	[41]
	Depletion, SCX	6		2.5 ng/mL ^d	[41]
		9	1-49 ng/mL ^d	2-110 ng/mL ^d	[64]
	Depletion, SEC	1		60 ng/mL	[39]
		4	< 2 ng/mL	2-430 ng/mL	[73]
	Anti-protein immunoaffinity enrichment	2		0.1-0.95 ng/mL	[99]
	SISCAPA	1	2.6 ng/mL	3.8 ng/mL	[101]
		3	1.2-3.4 ng/mL	2.8-5.2 ng/mL	[33]
		9 ^f	0.9-101 ng/mL	2.4-269 ng/mL	[34]
		9 ^g	3.6-1026 pg/mL	7.2-1973 pg/mL	[34]
		8	0.2-2.8 ng/mL	0.5-8.3 ng/mL	[165]
		31	1.8-498.6 ng/mL	2.9-1280.3 ng/mL	[76]
	Lectin, SISCAPA	1		~ 0.8 ng/mL	[115]
	N-glycopeptide	5	low ng/mL	5 ng/mL	[56]

^aThe number of target proteins in plasma or serum.

*b*₁ The number of attomoles of peptide injected onto the column per analysis.

*c*_{Yeast} Proteins with a total of 757 target peptides.

*d*_{IgY12} depleted plasma.

*e*_{MARS hu7} depleted plasma.

*f*₁₀ μ L of plasma.

*g*₁ mL of plasma.