REVIEW

Mass spectrometry-based targeted quantitative proteomics: Achieving sensitive and reproducible detection of proteins

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Traditional shotgun proteomics used to detect a mixture of hundreds to thousands of proteins through mass spectrometric analysis, has been the standard approach in research to profile protein content in a biological sample which could lead to the discovery of new (and all) protein candidates with diagnostic, prognostic, and therapeutic values. In practice, this approach requires significant resources and time, and does not necessarily represent the goal of the researcher who would rather study a subset of such discovered proteins (including their variations or posttranslational modifications) under different biological conditions. In this context, targeted proteomics is playing an increasingly important role in the accurate measurement of protein targets in biological samples in the hope of elucidating the molecular mechanism of cellular function via the understanding of intricate protein networks and pathways. One such (targeted) approach, selected reaction monitoring (or multiple reaction monitoring) mass spectrometry (MRM-MS), offers the capability of measuring multiple proteins with higher sensitivity and throughput than shotgun proteomics. Developing and validating MRM-MS-based assays, however, is an extensive and iterative process, requiring a coordinated and collaborative effort by the scientific community through the sharing of publicly accessible data and datasets, bioinformatic tools, standard operating procedures, and well characterized reagents.

Keywords:
Biomarker verification / Multiple reaction monitoring mass spectrometry / Posttranslational modifications / Protein quantitation / Systems biology / Technology

1 Introduction

The development of modern proteomic technologies has exploded exponentially to accommodate the growing demand for biological and clinical research over the last 15–20 years due to their ability to profile a large number of proteins in a relatively high-throughput fashion [1–8]. Such approaches, including mass spectrometry (MS), protein and affinity-based arrays, and bead-based multiplex flow cytometry, are powerful tools for identifying and quantifying proteins and their variations, as well as posttranslational modifications (PTMs) in biological materials in a nontargeted manner. With additional sample preparation, including the depletion of highly and moderately abundant proteins from complex biological matrices such as plasma or serum or

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Abbreviations: ABPP, activity-based protein profiling; **AQUA**, absolute quantification; **CRC**, colorectal cancer; **FDA**, Food and Drug Administration; **GBM**, glioblastoma; **iMALDI**, immuno-MALDI; **IT**, ion trap; **PSAQ**, protein standard absolute quantification; **SID**, stable isotope dilution; **SIS**, stable isotope-labelled internal standard; **SISCAPA**, stable isotope standards and capture by anti-peptide antibodies; **SOP**, standard operating procedure; **QC**, quality control; **QQQ-MS**, triple quadrupole mass spectrometer

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multi-dimensional chromatographic fractionation coupled with MS, these technologies have further enabled the detection of low-abundance proteins [9, 10]. The goal of these types of "survey"- and "inventory"-based studies is to detect as many proteins and their modifications as possible, providing a broad coverage of the protein content in a particular biological system. Indeed, these technological advances have been successfully used to discover unique proteins associated with diseases such as cancer [11–14].

Biomarker research is one of the most exciting areas to apply proteomics where distinct protein/peptide biosignatures in clinical specimens such as tissue, proximal fluids, and blood have been discovered as potential clinical tools for diagnosis, prognosis, and therapeutic prediction of the disease of interest. For example, there are approximately >1261 protein biomarker candidates for cancer alone in the scientific literature [15]. However, few of them have reached a high threshold of evidence to be qualified as clinical biomarkers, as demonstrated by the stagnant rate of approval of protein biomarkers for all diseases by the Food and Drug Administration (FDA) averaging 1.5 proteins per year over the last 15 years [16], while assays for at least 96 analytes have been developed and used as laboratory-developed tests (LDTs). Furthermore, discovering disease-relevant biomarkers in biospecimens such as serum and plasma remains extremely daunting. This is due to the fact that the concentration ranges of current clinically used biomarkers (e.g. for cancer) are at and below the eighth order of magnitude in this desirable sample source spanning 11 orders of magnitude in dynamic range, which still presents huge analytical challenges for the research community in spite of recent technical advances. Concomitantly, there is a lack of analytical validation of a platform(s) for the precise and accurate measurements of "discovered" analytes in a smaller set of clinical samples prior to conducting costly and time-consuming large-scale clinical trials. In this regard, targeted proteomic technologies such as multiplexed MS, protein arrays, and various derivations of enzyme-linked immunosorbent assays (ELISAs) can fill this "middle" space by (i) confirming the observed differences reflecting changes in protein abundances or PTMs from initial exploratory experiments in a larger set of samples with statistical rigor, and (ii) providing quantitative measurement of protein/peptide candidates in a biological matrix of interest with greater accuracy and higher throughput. Stable isotope dilution (SID) multiple reaction monitoring MS (SID-MRM-MS) has quickly emerged as one of the powerful targeted proteomic tools in the past few years. It has the advantage of accurately calculating protein concentrations in a multiplexed and high-throughput manner, while potentially overcoming many of the difficulties associated with antibody-based protein quantification [17].

Herein, this review intends to provide a thorough description of this targeted MS technology and the challenges faced in the technical aspects of quantitative MS especially on its application for biomarker verification and validation. In light of the recent advances in functional proteomics and systems biology, as well as proteomics becoming increasingly quantitative, pathway mapping and modeling requiring knowledge of flux through individual steps in the pathway becomes critically important in the understanding of cellular processes, including cellular constituents and their quantities, dynamics, and interactions. Hence, this manuscript will highlight the unique capability of this targeted technology for the accurate quantitation of PTMs and functional subproteomes as well as protein isoforms and variations in order to go beyond the assessment of protein abundances alone. When combined with changes in protein abundances, fluctuations in the functional components of the cell will provide additional insights into the underlying molecular mechanisms of diseases such as cancer.

2 Targeted proteomics: MRM-MS

2.1 Principles of MRM-MS on triple quadrupole mass spectrometer (QQQ-MS)

MRM-MS is a targeted quantitative technology commonly performed on QQQ-MS that generates unique fragment ions associated with their corresponding precursor ions that can be quantified in a very complex matrix. Specifically, the MRM technique relies on selecting precursor-product ion pairs, or transitions. The first quadrupole (Q1) of a QQQ-MS is set to allow only a particular precursor (parent) peptide ion into the second quadrupole (Q2), where collisionally induced dissociation (CID) yields fragment (daughter) ions (MS/MS spectrum). A signature fragment ion of particular mass-to-charge ratio (m/z) or several fragment ions called signature transitions (usually with high intensity in the MS/MS spectrum) is then allowed into the third quadrupole (Q3) and subsequently measured by the MS detector. Finally, the quantitation of peptides (and thus proteins when assembled) is achieved by measuring the intensity of the fragment ions as previously illustrated [18].

2.2 SID-MRM-MS analysis for proteins

Although the use of MRM-MS to quantify biomolecules (e.g. drugs and metabolites) [19] was widely adopted in research and clinical laboratories, as well as the pharmaceutical industry (hormones, drugs, metabolites) many years ago, it only has been recently applied, in combination with SID-MS, to quantify peptides and proteins. Protein-based SID-MRM-MS assays are based upon the measurement of signature proteotypic (i.e. tryptic) peptides as surrogates that uniquely and stoichiometrically represent the protein candidates of interest for quantitation [20–22]. MRM-based assay development usually starts with a selection of three to five peptides per protein [23] to improve the specificity of the quantitative measurement for targeted analytes. Additionally, synthetic stable isotope-labeled versions of each peptide (or heavy

peptides) of known quantities, usually at the carboxyl-terminal lysine or arginine residues, are used as internal standard peptides (i.e. stable isotope-labeled internal standards or SIS, such as absolute quantification (AQUA) peptides) [22]. These SISs, chemically identical to their endogenous analyte peptide counterparts with the exception of their masses (usually 6–10 Da more), will co-elute with their endogenous counterparts during chromatographic separation. For accurate quantitation, specific fragment ion signals (peak heights or areas) derived from the endogenous unlabeled species are compared to those from the spike-in exogenously isotope-labeled peptides. Specifically, the ratios of peak areas under the monitored fragment ions of native (light) to that of a known amount of SIS (heavy) peptides are measured to calculate the concentration of that protein [21–23]. As expected, the specificity of such quantitative measurements improves as the number of monitored proteotypic peptides corresponding to a protein and the number of transitions for each peptide selected for monitoring increase [24]. More advantageously, when MRM-MS is coupled with SIS, the presence of SIS can calculate more accurate ratios with high sensitivity (~attomole) and across a wide dynamic range. Additionally, ion suppression and matrix effects as often encountered in MS-based proteomics are less problematic than label-free methods because the chemically identical internal standards and the endogenous forms are expected to be suppressed to the same extent. If no endogenous (unlabeled) signal is detected in the sample while there is an SIS signal present, one can be assured that the concentration of a peptide in the sample is below the detection limit of the instrument, confirming its proper functioning. In the case of an experiment entailing multiple treatments or conditions, the use of SIS can ensure that all of the treatments to the sample can be adequately compared with each other and control. However, the amount of SIS added depends on the protein's individual relative abundances within a sample and thus should be tested in a preliminary study, during which the amount of spike-in should be optimized to obtain low coefficients of variations (CVs) (5–10%). Along with high sensitivity and precision, this technique is ideal for sensitive and specific quantitation in a multiplex fashion (i.e. allowing many precursor/product ion pairs to be monitored in a single liquid chromatography-MS/MS [LC-MS/MS] run and increasing throughput), making it attractive for translational and clinical research [25–27].

2.3 MRM-MS assay development and available resources

Although developing and validating MRM-MS assays is a laborious process, once generated for a particular protein, this assay can be deployed and adopted across laboratories for the accurate quantification of that protein. Typical steps involved in the workflow of an MRM-MS protein assay on a large-scale include: (i) selection of surrogate or signature peptides diagnostic for each protein; (ii) protein extraction from biological matrices such as tissue or blood; (iii) proteolytic digestion of proteins (usually with trypsin); (iv) iterative testing of synthetic peptides and transitions by LC-MRM-MS (endogenous unlabeled and heavy isotopically labeled SIS); (v) assay validation on biological samples (analytically); and (vi) testing standard operating procedures (SOPs) and method documentation important for the reproducibility of measurements.

Bioinformatic support for such endeavor is undoubtedly critical in the customized planning of assay configuration and data analysis on target analytes. Therefore, we have summarized some publicly available software/algorithms and peptide/protein databases useful for successfully configuring MRM-MS-based assays in Table 1 . While current approaches to assess consistent peptide detection for step (i) are based upon initial discovery experimental observations from individual laboratories compiled in proteomic data repositories, which include PeptideAtlas [28], Global Proteome Machine (GPM) Proteomics Database [29], and PRIDE [30], in situations where experimental data are unavailable, e.g. low-abundant proteins poorly represented in data repositories, computational approaches to predict the best "MS-performing peptides" are usually adopted. Examples of several tools that have been published include ESP predictor [31], PeptideSieve [32], and PepFly [33]. Since MRM-MS sensitivity on QQQ-MS/MS is critically dependent on ionization conditions and tuning of instrument parameters, such as collision energy [34, 35] and cone voltage, for the generation of maximal product ion signal, having reference spectral fragmentation libraries of proteotypic peptides (with information on instrument parameters used) would be extremely useful for the proteomics community in MRM-MS assay development. An additional dimension of information that can be added to the library is chromatographic retention time. This can enable the scheduling of MRM-MS scans on target analytes based on their distinct retention time, which could ensure more accurate quantification on the desired ions when background interferences are significant (e.g. matrix effects from complex biological samples such as blood and urine). For example, Spicer et al. have developed a sequence-specific model for predicting slopes in the fundamental equation of linear solvent strength theory for the reversed-phase HPLC separation of tryptic peptides detected in a typical bottom-up proteomics experiment [36]. Retention time information, however, would require precise system stability of LC pumps and columns, putting high demands on the quality control (QC) aspects of LC systems and factors which affect LC performance such as column heater set-up (data not shown). In this regard, chip-based nano LC systems with microfluidic design advantages [37].

Currently, MRMer [38] and Skyline [39] are the most commonly used open source software for developing MRM-MS-based assays by the proteomics community. MRMer, developed for managing highly complex MRM-MS experiments, including quantitative analyses using heavy/light isotopic peptide pairs, and has the capability of importing data in a platform-independent mzXML format, which allows

Table 1. A list of bioinformatic tools for designing MRM-MS assays

Bioinformatic tools	Sources	Utilities
Global Proteome Machine (GPM) [29]	http://www.thegpm.org	• Serving as a multi-organism, publicly accessible data repository. • Including posttranslational modification data from different organisms including *Caenorhabditis elegans*, etc.
PeptideAtlas [28]	http://www.peptideatlas.org	• Serving as a multi-organism, publicly accessible compilation of peptides identified in a large set of tandem MS-based proteomic experiments. • Collecting MS output files for human, mouse, yeast, and several other organisms, and searching using the latest search engines and protein sequences. • Processing all results of sequence and spectral library searching subsequently through the Trans-proteomic Pipeline (TPP) to derive a probability of correct identification for all results to ensure a high-quality database (FDRs at the whole atlas level). • Querying and browsing results at the PeptideAtlas website; downloading raw data, search results, and full builds for other uses.
Proteomics IDEntifications (PRIDE) [30]	http://www.ebi.ac.uk/pride	• Serving as a centralized, standards compliant, public data repository for proteomics data. • Capturing details of PTMs coordinated relative to the peptides in which they have been found. • Serving as a compendium of targeted proteomic assays to detect and quantify proteins in complex proteome digests by MS. • Resulting from high-quality measurements of natural and synthetic peptides conducted on a QQQ-MS. • Intended as a resource for SRM/MRM-based workflows
SRMAtlas [40]	http://www.srmatlas.org	• Querying transitions from yeast, human, and mouse by users (the yeast library is based on both natural samples and synthetic peptides run on a 4000 QTRAP instrument, supplemented with ion-trap observations and predictions, where QQQ spectra are unavailable).
ESP predictor [31]	http://www.broadinstitute.org/cancer/software/genepattern/modules/ESPPredictor.html	• Implementing the enhanced signature peptide (ESP) predictor method. • Providing a means of predicting, from sequence alone, which peptides for any given protein are likely to work for MRM-MS assay development.
PeptideSieve [32]	http://tools.proteomecenter.org/software.php	• Predicting proteotypic propensity of a peptide based on its physicochemical properties. • Improving protein identification scoring functions of database search software, providing a panel of reagents for protein quantification, as well as the annotation of genomes for coding sequences. • Guiding peptide selection in targeted proteomic experiments.
PepFly [33]	Mallick, *et al*.	• Predicting peptides potentially observable for a given set of experimental, instrumental, and analytical conditions for 2D-LC-MS/MS datasets.
MRMer [38]	http://proteomics.fhcrc.org/CPL/MRMer.html	• Reading mzXML files and inferring precursor ion/product-ion relationships. • Displaying elution curves of product ions and allowing users to select or deselect them as candidates for MRM-based assays; also elution curves of precursor ions when MS1 scans are present in the mzXML file. • MRMer's calculations output to a tab-delimited file.

Table 1. Continued

Bioinformatic tools	Sources	Utilities
Skyline [39]	http://https://brendanx-uw1.gs.washington.edu/labkey/project/home/software/Skyline/begin.view	• Building SRM/MRM methods and analyzing the resulting MS data. • Employing cutting-edge technologies for creating and iteratively refining SRM methods for large-scale proteomic studies.
Automated detection of inaccurate and imprecise transitions (AuDIT) [43]	http://www.broadinstitute.org/cancer/software/genepattern/modules/AuDIT.html	• Comparing the relative product ion intensities of the analyte peptide to those of the internal peptide standard (SIS) and using a *t*-test with a *p*-value threshold to determine if they are significantly different. • Calculating CVs from the ratio of analyte and SIS peak areas from sample replicates. • Flagging transitions with excessive variation as being unsuitable.
Automated and targeted analysis with quantitative SRM (ATAQS) [41]	http://tools.proteomecenter.org/ATAQS/ATAQS.html	• Providing a high-throughput tool for organizing, generating, and verifying transition lists, and for the postacquisition analysis and dissemination of the data generated from applying the transition lists to studies of biological samples. • Using information from publicly accessible databases for the optimization of the protein and peptide target lists and for the optimization of a transition set.

data extraction, visualization, and analysis. When combined with MRMer, high-throughput quantitative studies of many samples can be easily accomplished, allowing expanded biomarker target monitoring for a large number of clinical samples. Skyline, developed by MacCoss's group in a format integratable with all major instrument platforms, has been successfully used to design MRM-MS assays and support data analysis including SIS (freely downloadable from https://brendanx-uw1.gs.washington.edu/labkey/project/home/software/Skyline/begin.view) [39]. By supporting all major publicly available spectral libraries from the GPM, National Institute of Standards and Technology (NIST), the Institute for Systems Biology, and the MacCoss Lab (links to these repositories are available on the Skyline web site), a library file from one of these sources can first be downloaded and inspected with the Skyline Spectral Library Explorer, after which it can be used to help choose peptide precursor and product ions to monitor specific proteins of interest. The data generated from a single Skyline-directed MRM analysis, for example, allows researchers to maximize the sensitivity and duty cycle by identifying the two to three best transitions and the proper retention time window for scheduled monitoring of each peptide. Skyline also provides platform-dependent collision energy predictions for major instrument manufacturers of QQQ-MS, which recently showed similar performance to that derived empirically [34].

SRMAtlas, a pioneering database of targeted proteomic assays to detect and quantify proteins in complex proteomes by this targeted mass spectrometric approach (http://www.mrmatlas.org), serves as a rich resource for the proteomics community applying this technology in research. The information in this database results from MRM-MS measurements of natural and synthetic peptides performed on a QQQ-MS [40]. Currently, this database allows users to query transitions from peptides from yeast, human, and mouse species obtained from QQQ-MS instruments, supplemented with ion trap (IT) observations and predictions where QQQ spectra are unavailable. Furthermore, the optimal coordinates for building an SRM assay can be easily uploaded to the MRM methods of a QQQ-MS instrument, which can be used to quantify a set of target proteins in any biological sample of interest in a high-throughput manner. This database is currently being expanded to include QQQ observations of 7000 human glycopeptides and 100 000 peptides from 19 000 proteins (i.e. ~95% proteome coverage).

More recently, Brusniak et al. introduced an open source software pipeline called ATAQS (Automated and Targeted Analysis with Quantitative SRM). This software suite consists of several modules that collectively support MRM assay development workflow (http://tools.proteomecenter.org/ATAQS/ATAQS.html) [41]. ATAQS provides a flexible pipeline for researchers by allowing the workflow to start or end at any point of the pipeline, and for computational biologists, by enabling their own algorithm plug-in or connection. This integrated system is expected to significantly facilitate the application of targeted proteomic technologies and contribute to the generation of highly sensitive, reproducible, and complete datasets important for the discovery and verification of targets. In combination with mProphet, a software tool to compute accurate error rates for the identification of targeted peptides in SRM datasets in a fully automated fashion [42], these tools can maximize specificity and sensitivity by combining relevant features in the data into a statistical model.

In reality, neither experimental or in silico approach is sufficient to define an optimal peptide set for a target protein, supporting the practice of selecting multiple peptides across the full-length sequence of a protein as a multiplexed measurement, unless the purpose of the assay is to quantify specific modified peptides and/or variants of a protein. Additional factors such as inaccurate and imprecise transitions encountered in peptide quantification by MRM-MS could further complicate assay development. Consequently, an algorithm called Automated Detection of Inaccurate and Imprecise Transitions (AuDIT) has been developed that can automatically identify inaccurate transition data based on the observation of interfering signal or inconsistent recovery among replicates [43]. This algorithm is designed to objectively evaluate MRM-MS data by comparing the relative product ion intensities of the analyte peptide to those of the SIS peptides, followed by a *t*-test to determine any significant difference. Subsequently, a CV is calculated from the ratio of the analyte peak area to the SIS peak area from the replicates. The algorithm has already demonstrated the capability of identifying problematic transitions and achieving accuracies of 94–100% for the correct identification of errant transitions.

2.4 MRM-MS applications for biomarker verification

MRM-MS analysis as an attractive quantitative method of measurement with multiplexing capability has been increasingly applied for biomarker verification [44–48]. For instance, in order to improve the fecal occult blood test currently used as the first-line method for colorectal cancer (CRC) screening with the disadvantages of low sensitivity and specificity, an initial discovery in human fecal samples from CRC patients yielded a library of 108 human fecal proteins. Forty nonredundant target proteins were selected and tested using multiplexed MRM screening in the fecal samples from eight CRC patients and seven normal volunteers [44]. It was found that 24 target proteins were consistently detected in all samples and nine proteins were detected only in CRC patients, showing the potential of this approach for the analysis of potential CRC biomarkers [44]. Another study focused on measuring levels of six proteins of clinical relevance to cardiac injury in six patient plasma samples undergoing alcohol septal ablation for hypertrophic obstructive cardiomyopathy [45]. These studies have demonstrated the high-throughput and multiplexing capability of this approach for protein biomarker verification in a highly complex sample matrix with reproducibility and a dynamic range greater than 10^4.

Recently, the network investigators of the National Cancer Institute (NCI) Clinical Proteomic Technologies for Cancer initiative developed a more refined analytical workflow to meet the needs of filling a void in current biomarker development pipeline [18, 49]. Specifically, an intermediary step termed "verification" that relies on targeted quantitative proteomics such as MRM-MS has been incorporated between discovery and clinical validation steps in protein biomarker research [18]. While global analysis of proteomes in biospecimens from or close to the tumor site, likely enriched for the target proteins, is an important first step to define the protein content in a biological system as it can provide a clue on the differences between normal (control) and diseased states (e.g. cancer), "verification" streamlines the entire proteomics pipeline by winnowing large lists of "discovered" protein targets by measuring their presence in blood (or proximal fluids) using targeted quantitative approaches in a larger cohort set. Thus, verification serves as a triage filter, ensuring that only the most credible protein candidates can move forward to clinical validation studies. Using this pipeline, several early biomarkers of cardiac injury from the blood of patients undergoing a therapeutic, planned myocardial infarction (PMI) for treatment of hypertrophic cardiomyopathy have been analytically verified [49].

2.5 Methods for increasing the sensitivity of MRM-MS assays

Blood, as a rich source of potential biomarkers, has an extraordinary dynamic range of >10 orders of magnitude of protein concentration [50]. Although MRM-MS alone provides biomarker verification down to 100–1000 ng/mL range, many biomarkers of current clinical importance, such as prostate-specific antigen (PSA), carcinoembryonic antigen (CEA), and the troponins (Tns), are present in the mid pg/mL to low ng/mL range in plasma below the lower limit of detection of a QQQ-MS. To use MRM-MS assays in the clinic for the measurement of protein biomarkers, this technology has yet to improve its sensitivity for detection and quantitation. Several approaches have been developed in this direction, one of which has demonstrated that a combination of abundant protein depletion (such as antibody-based depletion columns) with minimal fractionation of tryptic peptides by strong cation exchange (SCX) prior to SID-MRM-MS provides limit of quantification (LOQ) signal-to-noise ratios of >10 in the 1–20 ng/mL range with CVs of 10–20% at the LOQs for proteins in plasma [23]. An example of this is the development of assays for a number of known markers of cardiovascular disease [51], providing additional proof of the power of MRM-MS approaches for measuring proteins for which antibodies are unavailable or of low quality. Hossain et al. adopted a different approach of improving MRM-MS sensitivity by increasing ion transmission efficiency using a nanospray ionization multicapillary inlet/dual electrodynamic ion funnel interface in front of a commercial QQQ-MS [52]. As a result, significant enhancements in overall signal intensities and improved LOD were observed with the new interface compared with the original interface for MRM measurements of tryptic peptides from proteins spiked into nondepleted mouse plasma over a range of concentrations (average MRM peak intensity increase by >70-fold). One would expect that abundant protein depletion

strategy combined with instrument modification could further improve the LOD/LOQ of MRM measurements.

Although current MRM-MS technology has the ability to detect and quantify proteins in the low μg/mL to high ng/mL range, a MRM^3 strategy has recently demonstrated the improvement of the LOQ [53]. This strategy takes advantage of the capability of a hybrid QQQ-MS/linear IT (LIT) mass spectrometer to further fragment the product ions monitored in Q3. In this case, precursor ions are still selected in the Q1 quadrupole, fragmented in the Q2 collision cell, and product ions are collected in the LIT. A suitable product ion is isolated and fragmented in a second step using resonance excitation, following which second-generation product ions are collected and scanned out of the LIT to the detector. The area under the peak is used for quantitation in the same way as MRM. This technique has been applied to a number of biological samples that demonstrated three to five times improved LOQ using MRM3 analysis over MRM through removal of interferences with a final LOD of approximately 10 ng/mL when quantifying proteins in plasma. The standard concentration curves were linear over the three orders of dynamic range tested [53].

Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA) is an alternative approach developed to significantly increase the sensitivity of detection and quantitation of proteins in plasma by target peptide enrichment [54] and has previously been deployed in a clinical setting [55]. In this approach, anti-peptide antibodies are generated against the selected signature "tryptic" peptides from target proteins. If stable isotope-labeled recombinant protein standard is available (labeled as red asterisk), it should be added to the plasma (or other biological matrices) in the beginning of the assay workflow to control for proteolytic efficiency (Fig. 1, top). Following the digestion of the biospecimens and addition of known amounts of SIS (labeled as green asterisk), both exogenously (spike-in) and endogenous peptides are specifically enriched and their relative amounts are quantitated by MRM-MS. In this case, instead of a secondary antibody for visualization and quantitation, an MS detector provides quantitation through peak areas for targeted *m/z* values. Recent studies suggested that more than a 1000-fold enrichment can be achieved for plasma-digested peptides using this approach [56] and that SISCAPA assays can achieve low ng/mL LOQs in plasma with CVs <20% [52]. Once generated, these affinity reagents can provide enough material for hundreds to thousands of SISCAPA assays. Additionally, the coupling of SISCAPA to magnetic bead-handling robotics during assay workflow can significantly improve throughput and reproducibility [57, 58]. In summary, QQQ-MS coupled with upstream immunoaffinity enrichment of target peptides provide greater sensitivity, wider dynamic range quantitation, and detailed sequence-based characterization of multiple peptides digested from several proteins (unlike the lower specificity optical or electrochemical signals generated as surrogates for the analytes in conventional immunoassay) [59], which allows for simultaneous measurement and characterization of many targeted analytes. This approach improves protein assay development by: (i) allowing multiplexed measurement of peptides without significant interference; (ii) including analyte-identical internal standards of same structure (with different *m/z*) to control all aspects of the assay workflow except for digestion, which could otherwise be rectified by additional protein internal standards; (iii) resulting in reduced sample handling, high sensitivity, and wide dynamic range; and (iv) allowing site-specific quantitation of posttranslationally modified peptides (e.g. phosphopeptides [60], glycopeptides [61]) as important biomarker candidates. These advantages constitute a step forward in assay QC, potentially shifting some of the performance and reliability burden from technical standardization of reagents and instruments to real-time observation, and evaluation of the analytes themselves. Alternatively, intact protein targets from biospecimens, along with their recombinant heavy isotope-labeled internal protein standards, such as Protein Standard Absolute Quantification (PSAQ) approach [62, 63], if available (labeled as red asterisk), can both be immunoprecipitated with antibodies prior to proteolysis and SID-MRM-MS (Fig. 1, bottom). As shown in this schematic, several heavy-labeled protein and/or peptide standards are included at each step of the assay workflow to assess the efficiency of immunoprecipitation at the protein level (red label) and proteolysis (red/green double labels) to obtain the most accurate absolute quantitation of targeted proteins, with a spike-in known amount of target peptide (labeled as green asterisk), prior to MRM-MS measurement. The advantages and caveats of MRM-MS-based assays have been summarized in Table 2.

While SISCAPA is coupled with LC/MRM-MS, MALDI/MRM-MS detection is also possible. This alternative approach termed immuno-MALDI (iMALDI), where beads are directly placed on a MALDI target with the affinity-bound peptides still attached, utilizes MALDI matrix solvents to elute the bound peptides from the beads [64, 65]. The peak height or peak area of the peptide from an MS spectrum is used for quantitation, while peptide identities are confirmed with the MS/MS spectrum. In principle, iMALDI can be performed with only a MALDI-MS instrument, but it can also be used in the "MRM mode on a MALDI-MS/MS instrument" termed $iMALDI^+$ [66].

3 Standardization and QC for targeted MS

For targeted MS proteomics technology to be suitable for use in studies where large numbers of candidate protein biomarkers and patient samples (i.e. hundreds) must be rapidly screened – verified, it must be demonstrated that protein quantification can be achieved reproducibly within and across laboratories on different instrument platforms. Numerous efforts to standardize proteomic workflows in order to obtain reproducible results within and between laboratories

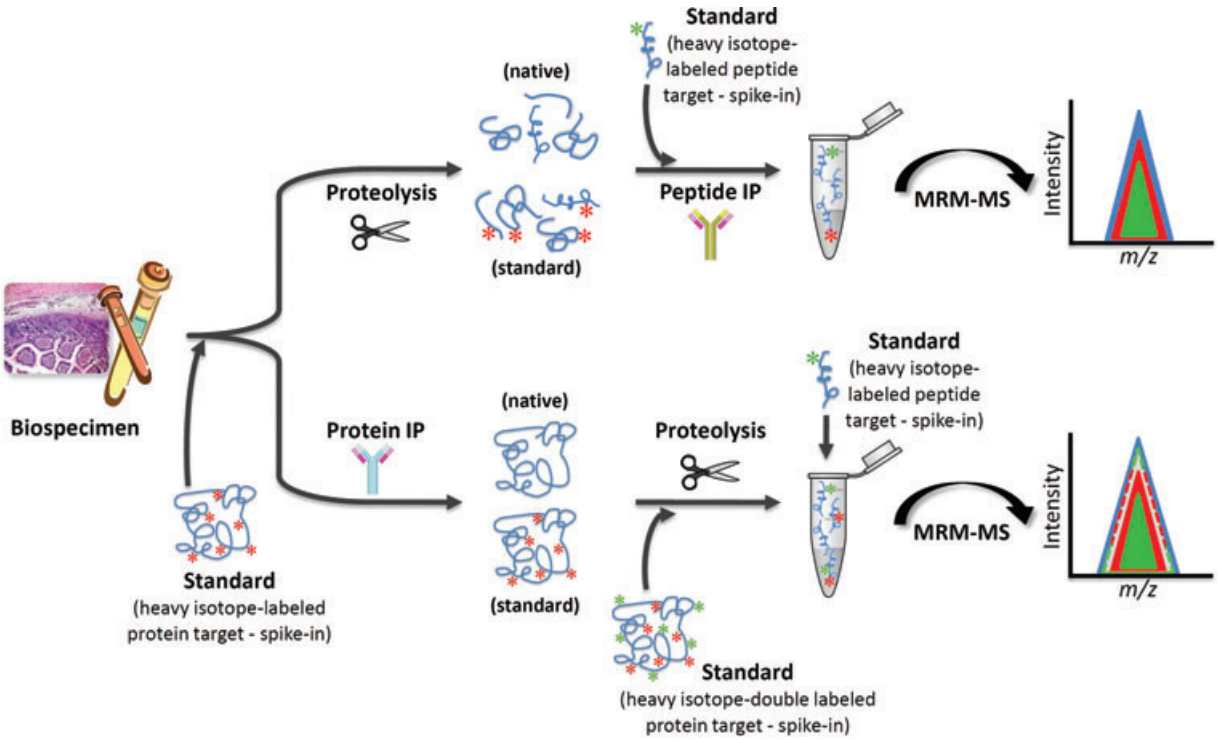

Figure 1. Commonly used MRM-MS-based assay workflows (± immunoaffinity enrichment of proteins or peptides) and its quality control (QC) procedures. SISCAPA workflow using proteolytic peptides as surrogates for their respective proteins, as illustrated in the top panel of the schematic, is a sensitive approach to measure protein concentrations using immunoaffinity enrichment of surrogate peptides prior to MRM-MS. Alternatively, an assay can start with immunoaffinity enrichment of intact target proteins from biospecimens using an internal heavy isotope-labeled protein standard (red asterisk, such as PSAQ approach) and an antibody, as illustrated in the bottom panel, followed by proteolysis with an internal standard of the same protein (double-labeled as shown by red/green double asterisks) and final quantitation of the target protein by peak areas with a spike-in internal peptide standard (green asterisk). An internal standard (either at protein or peptide level) is included at every step of the assay in order to obtain the "absolute" concentration of the target protein. In both cases, while the inclusion of an internal heavy isotope-labeled protein standard is desirable, its wide use is dependent upon the availability of recombinant proteins, and thus is not always feasible.

have been put forth in the past. Although non-MRM-centric, HUPO performed a test sample study in 2009 to identify errors leading to irreproducibility [67]. This study involved the use of an equimolar test sample comprising 20 recombinant human proteins, which was distributed to 27 laboratories. Each protein contained one or more unique tryptic peptides of 1250 Da to test for ion selection and sampling in the mass spectrometer. Of the 27 participating laboratories, only seven laboratories initially reported all 20 proteins correctly, while only one laboratory reported all tryptic peptides of 1250 Da. It was demonstrated that centralized raw data analyses improved the results by identifying missed identifications (false negatives), environmental contamination, database matching, and curation of protein identifications as sources of problems. Another study from the Association of Biomolecular Resource Facilities (ABRF) in 2009 centered on the theme of "Relative Protein Quantification in a Clinical Matrix-A targeted relative protein quantification study relevant for a biomarker validation project" [68]. This study was designed to explore the use of different approaches for determining quantitative differences for several target proteins in six samples of human plasma that were centrally prepared. While a wide range of techniques were used including ICAT, iTRAQ, SRM/MRM with and without SID, lessons learned from this study include the requirement of careful planning and expertise for success especially for complex quantitative proteomic experiments. ABRFs effort to assess individual laboratory's platforms, methods, and results was one of the first attempts to address variability in sample preparation and processing on different proteomic platforms.

In 2009, the NCI Clinical Proteomic Technologies for Cancer initiative network spearheaded a "round robin" study composed of three sub-studies designed to increase the level of difficulty in sample preparation (i.e. more sources of variability) at eight individual sites [69]. Study III represents the most complicated workflow that simulates a real biomarker discovery and verification study in a laboratory where variables can be introduced at every step of the experiment. Intralaboratory variability and reproducibility in all three sub-studies were evaluated by comparing the measured concentrations of seven target proteins including human C-reactive protein and PSA to the actual concentrations across the range of spiked-in

Table 2. A list of MRM-MS-based quantitative assays

Assays	Sample acquisition	Data analysis	Advantages, challenges, and caveats
Absolute quantification (AQUA) [22]	• Selection of proteotypic tryptic peptides to act as quantitation standards. • Synthesis of peptides with incorporated stable isotopes (^{13}C, ^{15}N, etc.) as internal standards to mimic native peptides (AQUA) formed by proteolysis. • Spike-in known amounts of AQUA peptides into samples. • Proteolysis of samples. • LC-MRM-MS analysis.	• Peptide identities by confirmation with MS/MS. • Method validation by spiking in varying amounts of a light (unlabeled) target protein and constant amounts of heavy proteotypic peptides for linearity. • Protein quantitation by calculating peak areas of product ions from selected peptides in MS/MS spectra (SRM) in reference to those of SISs (known amounts).	• Allowing peptide surrogate marker quantitation for proteins • Multiplexing capability • Compatibility with a wide array of proteins or modifications of interest. • Reduced limit of detection and limit of quantitation in comparison to its immunoaffinity enrichment version (see SISCAPA and iMALDI below). • Incomplete proteolysis affecting accurate quantitation: even with synthetic peptides, it is necessary to ensure that the equivalent analyte peptide is quantitatively released from the parent protein. • Higher concentration of endogenous proteins/peptides complicating the linearity of concentration curves. • Discrepancies between two or more peptides selected for the same protein (e.g. attributed to PTMs and/or protein miscleavages) complicating overall quantitation.
Stable isotope standards and capture by anti-peptide antibodies (SISCAPA) [54–58]	• Selection of proteotypic tryptic peptides to act as quantitation standards. • Anti-peptide antibodies produced and purified. • Known amounts of heavy SISs spike-in with samples. • Proteolysis of samples. • Immunoaffinity enrichment of proteotypic peptides from target proteins. • LC-MRM-MS analysis.	• Peptide identities by confirmation with MS/MS. • Establishment of concentration linearity with varying amounts of light (unlabeled) peptides and constant amounts of their respective heavy SISs, or vice versa. • Protein quantitation by peak areas of product ions of selected peptides from MS/MS spectra (SRM) in reference to those of SISs.	• Allowing peptide surrogate marker quantitation for proteins with higher sensitivity than AQUA. • Multiplexing capability. • Compatibility with a wide array of proteins or modifications of interest. • Enriching for target analytes while introducing additional steps for analyte loss. • Reduction of analytical variation from automated sample handling workstation (magnetic beads for immunoaffinity step, etc.). • Incomplete proteolysis affecting accurate quantitation: it is necessary to ensure that the equivalent analyte peptide is quantitatively released from the parent protein. • Endogenous proteins/peptides interfering with construction of linear concentration curves. • Discrepancies between two or more peptides selected for the same protein (e.g. attributed to PTMs and/or protein miscleavages) complicating overall quantitation.
iMALDI [64–66]	• Selection of proteotypic tryptic peptides to act as quantification standards. • Anti-peptide antibodies produced and immobilized on affinity beads. • Proteolysis of samples.	• Peptide identities by confirmation with MS/MS. • Construction of linear concentration curves with varying amounts of light (unlabeled) peptides and constant amounts of heavy SISs, or vice versa.	• Allowing peptide surrogate marker quantitation for proteins. • Multiplexing capability. • Enriching target analytes while introducing additional steps for analyte loss. • Reduction of analytical variation with robotics from start to finish on MALDI platforms. • MALDI platforms offering higher throughput than SISCAPA.
	• Known amounts of SISs spike-in with samples. • Immunoaffinity enrichment of proteotypic peptides from target proteins (both light and heavy) on beads directly placed on a MALDI target with the affinity-bound peptides attached in a microarray/spot format. • MALDI matrix solvent elution of bound peptides from the beads. • MALDI-MS or MS/MS analysis.	• Protein quantitation by peak heights of precursor ions of target peptides from MS spectra in MALDI-MS mode, or from MS/MS spectra in MALDI-MRM mode ($iMALDI^{+}$).	• Incomplete proteolysis affecting accurate quantitation. • Endogenous proteins/peptides complicating the linearity of the concentration curve. • Discrepancies between two or more peptides selected for the same protein (e.g. attributed to PTMs and/or protein miscleavages) complicating overall quantitation.
QconCAT [72]	• Selection of proteotypic tryptic peptides to act as quantification standards.	• Protein identification and confirmation (MS/MS and database search)	• Allowing the assessment of proteolytic efficiency and completeness by including an internal protein standard of a concatamer.

Table 2. Continued

Assays	Sample acquisition	Data analysis	Advantages, challenges, and caveats
	• QconCAT concatamer construct design, expression and purification as internal protein standards (both light and heavy). • Spike-in known amounts of recombinantly expressed, isotopically labeled QconCAT proteins into samples. • Proteolysis of samples. • LC-MRM-MS analysis.	• Linear concentration curve construction with varying amounts of light (unlabeled) peptides and constant amounts of heavy SISs, or vice versa • Protein quantitation (SRM): peak areas are used to compare the ratios of analytes to standards (following verification of tandem MS data from one or both of the heavy/light pairs)	• Labor-intensive design and production of a QconCAT concatamer including: ○ Selection of the appropriate proteotypic tryptic peptides to act as quantification standards based on the nomination of standard peptides from the expectation of efficient cleavage from the analyte protein and high-quality MS signals. ○ Incorporation of a restriction site midway through the construct and translation to a small linker peptide, and the separation of different peptides for each of the target proteins between the two halves, and the randomization of the order within each half. ○ Difficulties in expressing and purifying recombinant proteins in their native conformations. • Analyte recovery causing erroneous measures of quantities prior to proteomic analysis. • Confirmation of the complete release of standard peptides from the concatamer requiring prior knowledge of kinetics of release of the peptides from the proteins required. • Incomplete proteolysis affecting accurate quantitation: it is necessary to ensure that the equivalent analyte peptide is quantitatively released from the parent protein. • Endogenous proteins/peptides interfering with construction of linear concentration curves. • Discrepancies between two or more peptides for the same protein (e.g. attributed to PTMs and/or protein miscleavages) complicating overall protein quantitation.
Protein standard accurate quantification (PSAQ) [62–63]	• Production and purification of intact protein standards (both light and heavy). • Spike-in known amounts of recombinant, isotopically labeled proteins into samples. • Optional step: immunoaffinity enrichment using antibodies for intact proteins. • Proteolysis of samples with protein standards. • LC-MRM-MS analysis.	• Protein identification and confirmation (MS/MS and database search). • Establishing linear concentration curves with varying amounts of light (unlabeled) peptides and constant amounts of heavy SIS, or vice versa. • Protein quantitation (SRM): peak areas are used to compare the ratios of analytes to standards.	• Preventing differences in digestion yields between the standard and the analyte. • Addition of isotope-labeled protein standards to samples and peptide standards to trypsin digestion enabling the determination of recovery efficiency of proteins throughout the isolation procedure, including capture by the affinity reagents. • Compatible with any type of sample prefractionation provided that the "critical" biochemical properties involved in the partitioning process are shared by the recombinant, e.g. compatible with SDS-PAGE, protein hydrophobic capture, and immunocapture. • Offering the largest sequence coverage available for quantification (all detectable proteotypic peptides are considered): isoforms and variants may also be distinguishable, especially when immunoaffinity enrichment of intact proteins is included in sample preparation. • Constituting the relevant quantification standards for "top-down" quantification. • Labor-intensive and costly production of protein standards with potential difficulties expressing and purifying recombinant proteins in their native conformations. • Discrepancies between two or more peptides for the same protein (e.g. attributed to PTMs and/or protein miscleavages) complicating overall protein quantitation.

analytes (a total of nine concentration points with LOQ at 2.92 nM, i.e. 73.3 ng/mL for C-reactive protein), and by determining the CVs for these quantitative measurements. The results showed that the reproducibility and precision of these quantitative measurements for nine of ten peptides tested across eight laboratories ranged from 4–14%, 4–13%, and 10–23% interlaboratory CVs at or near the estimated LOQ of 2.92 nM for studies I, II, and III when SOPs were adopted. Intralaboratory CVs were usually <15% and <25% at the identical concentration for studies I, II, and III. The progressive increases in CVs from studies I–III clearly indicate that sample handling contributes more to assay variability than instrumental variability, further highlighting the high quality of MRM-MS data. Although the current MRM assay performance under real biomarker conditions (study III) is below that generally stated for clinical assays using ELISAs with CVs typically <5–10%, the performance achieved here is sufficient for the preclinical verification stage (middle) of candidate biomarkers present at more than ~2–6 μg/mL in plasma with a linear dynamic range spanning three orders of magnitude. Interlaboratory and intralaboratory CVs improved with increasing analyte concentration in all cases, whether by spiking in more analytes or by enrichment techniques. Furthermore, analytical variability is expected to improve with more streamlined sample preparation (such as with robotic automation), which, in conjunction with software development, should reduce labor-intensive workflow, variability between instrumentation platforms, and the need for high level of expertise currently required. Currently, the Clinical Proteomic Technologies for Cancer initiative network investigators along with others are undertaking a larger-scale interlaboratory study to assess the reproducibility of SRM assays across more laboratories and QQQ-MS instrument platforms than the initial study by spiking in 27 cancer-relevant biomarker candidates in depleted plasma (data not shown). SISCAPA interlaboratory studies within the NCI Clinical Proteomic Technologies for Cancer initiative network are currently underway, using metrics, SOPs, high-quality reagents, and reference materials against interesting cancer targets. The inclusion of internal standards (i.e. isotope-labeled proteins/peptides spiked into the biological matrices), whenever possible, is ideal throughout the rest of an assay workflow on biological samples to assess the purity, presence of correct peptide sequence and unexpected modifications, analyte retention time, and analytical recovery, etc. for the accurate measurement of proteins.

For SISCAPA, QC of the peptides and antibodies must be an integral part of the assay development process. Peptide QC can be implemented on an LC-UV-MS/MS system, together with amino acid analysis (AAA), to determine peptide quantity and purity, to characterize any modifications, and to obtain full MS/MS spectra [58]. This data can be fully examined for the presence of correct peptide sequences, the identification of any impurities, and analyte retention times, but also be assembled in a spectral library using Skyline, for example, for MRM method development. Furthermore, the removal of "passenger peptides" (i.e. free or modified peptides bound to the polyclonal antibody during antibody affinity purification) prior to MRM-MS measurement can ensure more accurate quantitation of peptides/proteins. This can be accomplished by "antibody scrubbing" or covalently linking peptides to resins followed by antibody binding to the resin and extensive washing [58]. For anti-peptide antibodies, monoclonal antibodies are an attractive alternative since they provide exquisite specificity, a renewable resource, and the potential for isolation of clones with very high affinities. Schoenherr et al. described an automated high-throughput SISCAPA method making screening of large numbers of hybridomas feasible while conserving time and resources [70].

Another important factor to consider for SISCAPA-type strategies is the percentage of analytical recovery of peptides in biological matrices such as human serum or plasma. To compensate for this loss, a calibration curve made in an appropriate matrix with internal controls and included with each batch of samples would permit the signal of each peptide in each sample to reflect an actual concentration in a multiplexed fashion. Furthermore, incomplete digestion due to the high concentration of matrix proteins likely plays a major role in the reduced yield of peptides. Proteolytic digestion variability in different samples can further complicate the measurements, while PTMs, single nucleotide polymorphisms (SNPs), other protein modifications, and interferences from other proteins [71] can also potentially affect quantitation. In both cases, spiking in an exogenous protein (e.g. PSAQ method), preferably the properly folded, stable isotope-labeled version of the native protein of interest, to each sample becomes essential for gauging and normalizing digestion efficiencies. However, the high cost and extensive efforts associated with the development of high-quality reagents, including heavy isotope versions of target proteins and peptides, as well as anti-protein/anti-peptide antibodies, could limit the broad use of these internal standards for QC purposes. Even if the production of these reagents is no longer the rate-limiting step, recombinant protein standards are still imperfect as PTMs affecting trypsin digestion in the biospecimens under analysis may not be present in the recombinant protein standards. Alternatively, artificial QconCAT proteins or concatamers of tryptic peptides for target proteins has been developed to aid in absolute quantification of proteins by assessing digestion variations [72]. QConCAT approach involves the design, expression, labeling, purification, characterization of QConCAT concatamer proteins, and their application steps in protein quantitation. The estimated total time required to complete the assay process (from the receipt of the QconCAT expression plasmids to the absolute quantitation of the set of proteins, together with the QconCAT proteins in an analyte sample) is approximately 1 month [72].

To expand the efforts of regulatory science in clearing multiplex protein-based in vitro Diagnostics (IVDs) based on these newly emerging proteomic platforms, the NCI Clinical Proteomic Technologies for Cancer initiative network investigators, in collaboration with the FDA and industry,

jointly published a first-of-its-kind publicly accessible mock 510(k) review document (i.e. PepCa10 test) based on the entire SISCAPA workflow and QC procedures [73]. The goal of this review document (not intended as a guidance document published by the FDA) was to provide the research community information on what the regulatory agency looks for in a medical device/assay system analytically and clinically if one wishes to submit such an assay for FDA clearance/approval [73]. Previously, such 510(k) documents were only available to corporations and entities that have formally filed with the FDA. This work provides a framework for the future creation of similar open-access regulatory materials that address a critical need in clinical proteomics.

4 Systems biology using targeted proteomics

With recent advances in protein-based technologies, it is expected that clinical proteomics, in the near future, will focus on developing highly multiplexed and automated technologies for more accurate quantification of proteins and their isoforms, as well as differences in PTMs between normal and diseased states in a statistically robust patient cohort size (i.e. biospecimens), that incorporates known genomic information whenever possible, in order to better understand the disease at the molecular level.

The completion of the human genome project has presented a more challenging task for scientists: the characterization of the complex and dynamic human proteome. Defining a comprehensive Human Proteome Project (HPP) poses more challenges due to several factors: (i) potentially a very large number of proteins with their PTMs involved in cellular function and signaling; (ii) the diversity of proteomic technology platforms involved (MS, arrays, ELISA, etc.); (iii) the variety of overlapping biological "units" into which the proteome might be divided for organized conquest; and (iv) the sensitivity limitation in detecting proteins present in low abundances especially in complex biofluids such as plasma and serum.

To better understand the linkage between proteotype and phenotype and the underlying molecular mechanisms associated with disease phenotype, researchers have to first be able to measure the target proteins/peptides in a biological system, for which multiplex MRM-MS assays can accomplish this task in a timely and cost-effective way. As the sensitivity, specificity, and reproducibility of this technology have been established in measuring small molecules and peptides [74–76], human Proteome Detection and Quantitation project (hPDQ) based on this approach proposes to build a complete suite of assays (e.g. two peptides from the protein product of each of the approximately 20 500 human genes), which would enable rapid and systematic verification of candidate protein biomarkers and lay a quantitative foundation for subsequent efforts to define the larger protein space of splice variants, PTMs, protein–protein interactions, and tissue localization [77]. This will enable measurement of the human proteome that could yield immediately useful results such as answering basic biological questions about the relationship between protein abundance (or concentration) and gene expression, phenotype, disease, and treatment response; while the strategy for a comprehensive HPP is being pursued. As a result, it is focused on the study of biological variation affecting protein expression rather than study of structure and mechanism, and does not initially directly address splice variants or most PTMs. It will take time to build a database of SRM assays for whole proteomes, but we are likely to see rapid growth in this area which will facilitate high-throughput and highly sensitive protein detection for a wide variety of experiments. Therefore, public sharing of information on already-built MRM assays for peptides would be extremely valuable to the research community to avoid duplicative efforts for cost-saving purposes.

Currently, large-scale multi-disciplinary cancer-based genomics initiatives, such as The Cancer Genome Atlas (TCGA) from the NCI and National Human Genome Research Institute (NHGRI), and The International Cancer Genome Consortium (ICGC), are advancing the comprehensive characterization of cancer genomes in order to understand cancer at a DNA and RNA level. As a result, genomic alterations associated with cancer including copy number aberration, mutation, microdeletion, and epigenetic dysregulation have been generated by multi-dimensional datasets and high-level integrative analysis. As an example, an integrative analysis of DNA copy number, gene expression, and DNA methylation aberrations in 206 glioblastomas (GBM), the most common form of adult brain cancer, revealed the roles of ERBB2, NF1, and TP53 and frequent mutations of the phosphatidylinositol-3-OH kinase regulatory subunit gene PIK3R1, as well as providing a network view of the altered pathways in the development of glioblastoma [78]. Another large-scale GBM study illustrated a robust gene expression based molecular classification of GBM into Proneural, Neural, Classical, and Mesenchymal subtypes, which differs in response to aggressive therapy, with the greatest benefit in the Classical subtype and no benefit in the Proneural subtype [79]. This finding potentially provides a glimpse into the molecular framework for GBM stratification, and if corroborated and expanded at the protein level, it would vastly advance our knowledge of cancer and ultimately improve the practice of medicine for patients. In fact, Wang et al. recently demonstrated the capability of targeted MRM-MS approach when coupled with immunoprecipitation of intact RAS protein isoforms to able to detect a single point mutation at the peptide level in K-RAS oncogenes from a cell line, tumor sample, and pancreatic cyst fluid at sensitivity of <25 fmol/mL, corroborating genomic data at the protein level [80]. This proof-of-concept proteomics study has demonstrated the capability of targeted MRM-MS approaches in accurately detecting and quantitating aberrant gene products, and will, with further studies, be able to decipher the effect of genomic variations on protein networks in diseases.

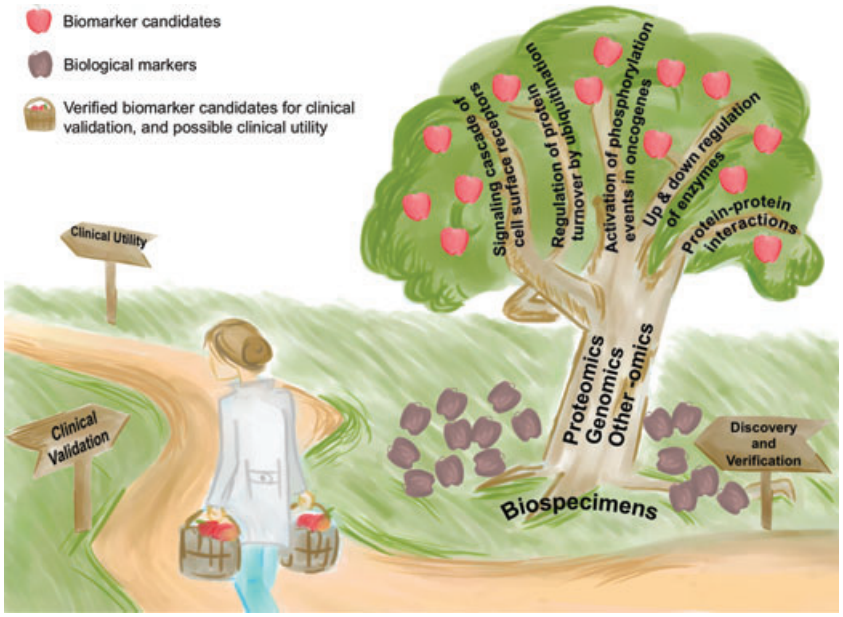

Figure 2. The systems biology approach for understanding the mechanisms of diseases at the molecular level by applying and integrating "-omics" technologies to gain knowledge for biology and hopefully generate "biomarkers" for clinical practice. This figure conveys the message that while protein/peptide biomarker candidates (the fruit of labor from extensive discovery efforts) are not always validated for their intended clinical use in a large unbiased cohort, they are still valuable in providing new insights into biology, provided that biologically relevant biomarkers can be analytically validated in a statistically appropriate number of biospecimens. Albeit not necessarily translational, the accurate quantitation of protein variants and PTMs, along with genomic information in a systems biology manner, is anticipated to generate multi-dimensional data for the better understanding of disease biology and pathology, including signaling pathways, protein network regulation and functional proteomes, etc.

Powered with these rich, multi-dimensional high-quality genomic data along with other "-omics" information, the next logical step is to apply a systems biology approach to study the effect of genomic aberration (as a result of cancer in this case) on the protein framework in the cell, including the up- or downregulation of proteins, protein–protein interaction, and dynamic signaling networks modulated in part by PTMs such as phosphorylation (Fig. 2). Albeit biology does not alway leads to clinical utility, accurate quantitation of protein variants and PTMs, along with genomic information is expected to generate multi-dimensional data for the better understanding of disease biology, including signaling pathways, network regulation, functional proteomes (e.g. phosphoproteomes), etc. This integrative knowledge will also aid in the development of biomarkers and assays for clinical use. In fact, an example is the well-known epidermal growth factor receptor (EGFR) that plays an important role in cancer by activating downstream signals important in growth and survival. Thus, EGFR inhibitors are frequently selected as viable treatment for cancer including lung cancer in the clinic. A recent comprehensive study on EGFR phosphorylation events related to somatic activating mutations and EGFR inhibitor (erlotinib) sensitivity has been performed using targeted MRM-MS approach [81]. A total of 30 phosphorylation sites were identified following EGFR immunoprecipitation and LC-MS. Specifically, several phosphorylation sites related to activating mutations in EGFR and to the sensitivity to erlotinib were identified in 31 lung cancer cell lines, three of which (pY1092, pY1110, pY1172) correlated with activating mutations, and three (pY1110, pY1172, pY1197) correlated with erlotinib sensitivity. In addition, Erlotinib-sensitive phosphosites was confirmed using LC-MRM-MS and quantitative Western blotting. This strategy can thus quantitatively assess site-specific protein phosphorylation events related to cancer signaling pathways and identify relationships between somatic mutations and/or drug sensitivity and these phosphorylation events.

Looking ahead, linking the progress made from the past 5 years of the NCI Clinical Proteomic Technologies for Cancer initiative at the analytical level to ensure standardization of clinical proteomic platforms to the ever growing amount of genomic data (e.g. from TCGA and ICGC) has the potential to provide a systematic approach for analytically verified biomarker candidates at the protein level. To this end, NCI recently launched the Clinical Proteomic Tumor Analysis Consortium (CPTAC) based on network-driven, multidisciplinary science approach (http://proteomics.cancer.gov). Since high-throughput proteomics can robustly analyze a statistically appropriate number of biospecimens associated with cancer (tissues, proximal fluids, and blood), the integration of proteomic with genomic information will provide unique insights on the effect of genomic variations on proteins and their interaction and networks for understanding cancer biology, as well as developing useful tools for clinical investigation and implementation. Multiplex quantitative assays can be configured for these protein targets for verification in clinically relevant and unbiased cohorts. The purpose of this integrative approach is to provide the broad scientific community with knowledge that links genotype to proteotype and ultimately phenotype. Importantly, data sets, analytically validated assays, as well as high-quality reagents generated by CPTAC will be made publicly accessible, which could further be applied by researchers anywhere around the world in larger scale clinical validation studies [82].

5 Combining functional proteomics with targeted approaches

With the rapid increase in the implementation of this targeted approach of understanding biology and facilitating clinical research (e.g. cancer signaling pathways), it is anticipated that similar databases to SRMAtlas may emerge, providing relevant information on protein abundances and PTMs that modulate cellular function. The Quantitative Assay Database (QuAD) as described by Remily-Wood et al., shares methods and reagents for measuring protein expression and modifications based on LC-MRM-MS assays developed using SDS-PAGE-fractionated lysates from cancer cell lines (http://proteome.moffitt.org/QUAD/) [83]. More interestingly, the pathway maps provide the biological relationships between proteins and demonstrate the concepts for multiplexed analysis. To date, LC-MRM screening has been used to detect 876 peptides from 218 cancer-related proteins in model systems including colon, lung, melanoma, leukemias, and myeloma, leading to the development of 95 quantitative assays including SISs. For example, protein expression measurements for heat shock proteins, with a comparison with ELISA and monitoring response to the HSP90 inhibitor, 17-DMAG, illustrated the components of the QuAD and its potential utility. This publicly available resource allows quantitative assessment of protein components of signaling pathways and biological processes, as well as systematic investigation of therapeutic monitoring for cancer.

The emerging role of targeting specific classes/families such as kinases in understanding the functional activities associated with diseases can further be coupled to MRM-MS for quantitative analysis. This development is, in part, due to the lack of selectivity of immunoaffinity and immobilized metal affinity chromatography (IMAC) approaches to capture all phosphopeptides including many highly abundant species that comprise the "phosphopeptide background," which poses a major limitation of current methods for phosphoproteomics. This high background can limit the sensitivity of targeted assays directed at key phosphopeptide intermediates in signaling networks [84]. In this regard, Cravatt's laboratory pioneered a chemical technology referred to as activity-based protein profiling (ABPP), which uses active site-directed probes to measure the functional state of many enzymes directly in whole proteomes [85]. Activity-based probes consist of at least two key elements: a reactive group for binding and covalently labeling the active sites of many members of a given enzyme class (or classes); and a reporter tag for the detection, enrichment, and identification of probe-labeled enzymes in proteomes. One unique feature of ABPP is that these active-site probes selectively label active enzymes, but not their inactive forms, thereby facilitating the characterization of changes in enzyme activity without corresponding alterations in protein expression. Since ABPP probes label enzymes based on shared catalytic properties rather than mere expression level, they provide access to low-abundance proteins in the proteomes that otherwise would not be detected, which can be further analyzed by gel electrophoresis and multi-dimensional protein identification technology (MudPIT). With this integrated approach, more than 30 primary human breast tumors and normal breast specimens probed have demonstrated that several enzyme activities are elevated in specific breast tumor classes [86]. More recently, a tag-free method for ABPP that utilizes the copper(I) catalyzed azide–alkyne cycloaddition reaction (i.e. "click chemistry") to analyze the functional state of enzymes in living cells and organisms has been introduced [87]. Thus, ABPP strategy is sufficiently versatile to be applied to virtually any cell or tissue (when the genome of the parental organism has been sequenced); and can be combined with a range of analytical methods for data acquisition. The drawback of this approach is that the specificity of ABPP probes is not absolute, and these probes can be toxic and thus disrupt biochemical pathways when applied to living systems. Despite this, they remain valuable for characterizing deregulated enzymatic activities in various cancer models and specimens. This approach has allowed the capture and characterization of several enzyme classes including many that have central roles in cancer, such as hydrolases and proteases [88–90], kinases and phosphatases [91–93], histone deacetylases [94], as well as deubiquitylases [95], which can subsequently be quantified with MRM-MS technology. This more focused approach will enable the elucidation of underlying mechanisms of disease biology/pathology and potential therapeutic intervention, as the quantitative information obtained from these analyses will reveal the intricate interplay of signaling transduction pathways responsible for cellular function such as differentiation and apoptosis.

6 Conclusions

Targeted MS-based technology has demonstrated an increasingly important role in biology and medicine, as it provides a sensitive and specific way to measure protein and peptide molecules simultaneously and accurately in a biological system [96]. From a practical perspective for clinical applications, it serves as an effective tool to confirm and triage lists of hundreds of protein (biomarker) candidates backed by statistical rigor, biological and/or clinical significance, and analytical validity prior to lengthy and costly large-scale clinical trials. With improvements in assay development (e.g. robotics, automation, and affinity enrichment), protein-based targeted MS may eventually become a routine practice in clinical laboratories as FDA-approved assay systems, especially when antibody-based tests such as ELISA encounter problems in a subset of patients due to the presence of autoantibody, hook effect, and cross-reactivity issues. In addition, the multiplexing capability of targeted proteomics can enable the measurement of proteins involved in cellular signaling pathways and enzymatic catalysis. When guided by large amount of genomic information from systematic interrogation of biospecimens, this approach will greatly advance the understanding

of molecular mechanisms of systems biology and disease pathology.

7 Disclaimer

Certain commercial equipment, instruments, or materials are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the NCI, NIH, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose. In this manuscript, SRM-MS and MRM-MS are used interchangeably. Although the FDA "approves" premarket application submissions and "clears" 510(k) submissions, the words "approved" and "cleared" have the same meaning in this report, but are not related to any proposed or real classification decision for any device.

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8 References

[1] Wang, P., Whiteaker, J. R., Paulovich, A. G., The evolving role of mass spectrometry in cancer biomarker discovery. *Cancer Biol. Ther.* 2009, *8*, 1083–1094.

[2] Lee, J., Soper, S. A., Murray, K. K., Microfluidic chips for mass spectrometry-based proteomics. *J. Mass Spectrom.* 2009, *44*, 579–593.

[3] Pierobon, M., Calvert, V., Belluco, C., Garaci, E. et al., Multiplexed cell signaling analysis of metastatic and non-metastatic colorectal cancer reveals COX2-EGFR signaling activation as a potential prognostic pathway biomarker. *Clin. Colorectal Cancer* 2009, *8*, 110–117.

[4] Liotta, L. A., Espina, V., Mehta, A. I., Calvert, V. et al., Protein microarrays: meeting analytical challenges for clinical applications. *Cancer Cell* 2003, *3*, 317–325.

[5] Krishhan, V. V., Khan, I. H., Luciw, P. A., Multiplexed microbead immunoassays by flow cytometry for molecular profiling: basic concepts and proteomics applications. *Crit. Rev. Biotechnol.* 2009, *29*, 29–43.

[6] Gonzalez-Malerva, L., Park, J., Zou, L., Hu, Y. et al., High-throughput ectopic expression screen for tamoxifen resistance identifies an atypical kinase that blocks autophagy. *Proc. Natl. Acad. Sci. U.S.A.* 2011, *108*, 2058–2063.

[7] Szutowicz, E., Dziadziuszko, R., Quantitative immunohistochemistry in lung cancer: clinical perspective. *Folia Histochem. Cytobiol.* 2010, *48*, 7–11.

[8] Fu, Q., Zhu, J., Van Eyk, J. E., Comparison of multiplex immunoassay platforms. *Clin. Chem.* 2010, *56*, 314–318.

[9] Wang, H., Wong, C. H., Chin, A., Kennedy, J. et al., Quantitative serum proteomics using dual stable isotope coding and nano LC-MS/MSMS. *J. Proteome Res.* 2009, *8*, 5412–5422.

[10] Qian, W. J., Kaleta, D. T., Petritis, B. O., 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.

[11] Rego, R. L., Foster, N. R., Smyrk, T. C., Le, M. et al., Prognostic effect of activated EGFR expression in human colon carcinomas: comparison with EGFR status. *Br. J. Cancer* 2010, *102*, 165–172.

[12] García-Foncillas, J., Bandrés, E., Zárate, R., Remírez, N., Proteomic analysis in cancer research: potential application in clinical use. *Clin. Transl. Oncol.* 2006, *8*, 250–261.

[13] Beirne, P., Pantelidis, P., Charles, P., Wells, A. U. et al., Multiplex immune serum biomarker profiling in sarcoidosis and systemic sclerosis. *Eur. Respir. J.* 2009, *34*, 1376–1382.

[14] Rigopoulos, D. N., Tsiambas, E., Lazaris, A. C., Kavantzas, N. et al., Deregulation of EGFR/VEGF/HIF-1a signaling pathway in colon adenocarcinoma based on tissue microarrays analysis. *J. B.U.ON.* 2010, *15*, 107–115.

[15] Polanski, M., Anderson, N. L., A list of candidate cancer biomarkers for targeted proteomics. *Biomark. Insights* 2007, *1*, 1–48.

[16] Anderson, N. L., The clinical plasma proteome: a survey of clinical assays for proteins in plasma and serum. *Clin. Chem.* 2010, *56*, 177–185.

[17] Ohtsuki, S., Uchida, Y., Kubo, Y., Terasaki, T., Quantitative targeted absolute proteomics-based ADME research as a new path to drug discovery and development: methodology, advantages, strategy, and prospects. *J. Pharm. Sci.* 2011, *100*, 3547–3559.

[18] Rodriguez, H., Rivers, R., Kinsinger, C., Mesri, M. et al., Reconstructing the pipeline by introducing multiplexed multiple reaction monitoring mass spectrometry for cancer biomarker verification: an NCI-CPTC initiative perspective. *Proteomics Clin. Appl.* 2010, *4*, 904–914. doi: 10.1002/prca.201000057.

[19] Schupke, H., Hempel, R., Eckardt, R., Kronback, T., Identification of talinolol metabolites in urine of man, dog, rat and mouse after oral administration by high-performance liquid chromatography-thermospray tandem mass spectrometry. *J. Mass Spectrom.* 1996, *31*, 1371–1381.

[20] Kuzyk, M. A., Smith, D., Yang, J., Cross, T. J. et al., Multiple reaction monitoring-based, multiplexed, absolute quantitation of 45 proteins in human plasma. *Mol. Cell. Proteomics* 2009, *8*, 1860–1877.

[21] Meng, Z., Veenstra, T. D., Targeted mass spectrometry approaches for protein biomarker verification. *J. Proteomics.* 2011, *74*, 1–10.

[22] Kettenbach, A. N., Rush, J., Gerber, S. A., Absolute quantification of protein and post-translational modification abundance with stable isotope-labeled synthetic peptides. *Nat. Protoc.* 2011, *6*, 175–186.

[23] Keshishian, H., Addona, T., Burgess, M., Kuhn, E., Carr S. A., Quantitative, multiplexed assays for low abundance proteins in plasma by targeted mass spectrometry and stable isotope dilution. *Mol. Cell. Proteomics* 2007, *6*, 2212–2229.

[24] Gerszten, R. E., Carr, S. A., Sabatine, M., Integration of proteomic-based tools for improved biomarkers of myocardial injury. *Clin. Chem*. 2010, *56*, 194–201.

[25] Keshishian, H., Addona, T., Burgess, M., Mani, D. R. et al., Quantification of cardiovascular biomarkers in patient plasma by targeted mass spectrometry and stable isotope dilution. *Mol. Cell. Proteomics* 2009, *8*, 2339–2349.

[26] Hoofnagle, A. N., Becker, J. O., Wener, M. H., Heinecke, J. W., Quantification of thyroglobulin, a low-abundance serum protein, by immunoaffinity peptide enrichment and tandem mass spectrometry. *Clin. Chem*. 2008, *54*, 1796–1804.

[27] Végvári, A., Marko-Varga, G., Clinical protein science and bioanalytical mass spectrometry with an emphasis on lung cancer. *Chem. Rev*. 2010, *110*, 3278–3298.

[28] Deutsch, E. W., Lam, H., Aebersold, R., PeptideAtlas: a resource for target selection for emerging targeted proteomics workflows. *EMBO Rep*. 2008, *9*, 429–434.

[29] Craig, R., Cortens, J. P., Beavis, R. C., Open source system for analyzing, validating, and storing protein identification data. *J. Proteome Res.* 2004, *3*, 1234–1242.

[30] Jones, P., Cote, R. G., Cho, S. Y., Klie, S. et al., PRIDE: new developments and new datasets. *Nucleic Acids Res.* 2008, *36*, D878–D883.

[31] Fusaro, V. A., Mani, D. R., Mesirov, J. P., Carr, S. A., Prediction of high-responding peptides for targeted protein assays by mass spectrometry. *Nat. Biotechnol.* 2009, *27*, 190–198.

[32] Mallick, P., Schirle, M., Chen, S. S., Flory, M. R. et al., Computational prediction of proteotypic peptides for quantitative proteomics. *Nat. Biotechnol*. 2007, *25*, 125–131.

[33] Sanders, W. S., Bridges, S. M., McCarthy, F. M., Nanduri, B. et al., Prediction of peptides observable by mass spectrometry applied at the experimental set level. *BMC Bioinformatics* 2007, *8*, S23.

[34] Maclean, B., Tomazela, D. M., Abbatiello, S. E., Zhang, S. et al., Effect of collision energy optimization on the measurement of peptides by selected reaction monitoring (SRM) mass spectrometry. *Anal. Chem*. 2010, *82*, 10116–10124.

[35] Holstein Sherwood, C. A., Gafken, P. R., Martin, D. B., Collision energy optimization of b- and y-ions for multiple reaction monitoring mass spectrometry. *J. Proteome Res*. 2011, *10*, 231–240.

[36] Spicer, V., Grigoryan, M., Gotfrid, A., Standing, K. G. et al., Predicting retention time shifts associated with variation of the gradient slope in peptide RP-HPLC. *Anal. Chem*. 2010, *82*, 9678–9685.

[37] Young, B., Lin, E., Hebert, N., Increased long term retention time stability in scheduled MRM peptide assays using chip based chromatography. *J. Biomol. Tech*. 2010, *21*, S33.

[38] Martin, D. B., Holzman, T., May, D., Peterson, A. et al., MRMer, an interactive open source and cross-platform system for data extraction and visualization of multiple reaction monitoring experiments. *Mol. Cell. Proteomics* 2008, *7*, 2270–2278.

[39] MacLean, B., Tomazela, D. M., Shulman, N., Chambers, M. et al., Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics* 2010, *26*, 966–968.

[40] Picotti, P., Lam, H., Campbell, D., Deutsch, E. W. et al., A database of mass spectrometric assays for the yeast proteome. *Nat. Methods* 2008, *5*, 913–914.

[41] Brusniak, M. Y., Kwok, S. T., Christiansen, M., Campbell D. et al., ATAQS: a computational software tool for high throughput transition optimization and validation for selected reaction monitoring mass spectrometry. *BMC Bioinformatics* 2011, 12, 78.

[42] Reiter, L., Rinner, O., Picotti, P., Hüttenhain, R. et al., mProphet: automated data processing and statistical validation for large-scale SRM experiments. *Nat. Methods* 2011, *8*, 430–435.

[43] Abbatiello, S. E., Mani, D. R., Keshishian, H., Carr, S. A., Automated detection of inaccurate and imprecise transitions in peptide quantification by multiple reaction monitoring mass spectrometry. *Clin. Chem*. 2010, *56*, 291–305.

[44] Ang, C. S., Rothacker, J., Patsiouras, H., Gibbs, P. et al., Use of multiple reaction monitoring for multiplex analysis of colorectal cancer-associated proteins in human feces. *Electrophoresis* 2011 *32*, 1926–1938.

[45] Keshishian, H., Addona, T., Burgess, M., Mani, D. R. et al., Quantification of cardiovascular biomarkers in patient plasma by targeted mass spectrometry and stable isotope dilution. *Mol. Cell. Proteomics* 2009, *8*, 2339–2349.

[46] Fu, Q., Schoenhoff, F. S., Savage, W. J., Zhang, P. et al., Multiplex assays for biomarker research and clinical application: translational science coming of age. *Proteomics Clin. Appl*. 2010, *4*, 271–284.

[47] Kim, K., Kim, S. J., Yu, H. G., Yu, J. et al., Verification of biomarkers for diabetic retinopathy by multiple reaction monitoring. *J. Proteome Res*. 2010, *9*, 689–699.

[48] Kuzyk, M. A., Smith, D., Yang, J., Cross, T. J. et al., Multiple reaction monitoring-based, multiplexed, absolute quantitation of 45 proteins in human plasma. *Mol. Cell. Proteomics* 2009, *8*, 1860–1877.

[49] Addona, T. A., Shi, X., Keshishian, H., Mani, D. R. 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.

[50] Anderson, N. L., Anderson, N. G., The human plasma proteome: history, character, and diagnostic prospects. *Mol. Cell. Proteomics* 2002, *1*, 845–867.

[51] 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.

[52] Hossain, M., Kaleta, D. T., Robinson, E. W., Liu, T. et al., Enhanced sensitivity for selected reaction monitoring mass spectrometry-based targeted proteomics using a dual stage electrodynamic ion funnel interface. *Mol. Cell. Proteomics* 2011, *10*, M000062–MCP201.

[53] Fortin, T., Salvador, A., Charrier, J. P., 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.

[54] Anderson, N. L., Anderson, N. G., Haines, L. R., Hardie, D. B. 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.

[55] Agger, S. A., Marney, L. C., Hoofnagle, A. N., Simultaneous quantification of apolipoprotein A-I and apolipoprotein B by liquid-chromatography-multiple- reaction-monitoring mass spectrometry. *Clin. Chem.* 2010, *56*, 1804–1813.

[56] Whiteaker, J. R., Zhao, L., Zhang, H. Y., Feng, L. C. et al., Antibody-based enrichment of peptides on magnetic beads for mass-spectrometry-based quantification of serum biomarkers. *Anal. Biochem.* 2007, *362*, 44–54.

[57] Anderson, N. L., 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.

[58] Whiteaker, J. R., Zhao, L., Abbatiello, S. E., 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.

[59] Kelleher, M. T., Fruhwirth, G., Patel, G., Ofo, E. et al., The potential of optical proteomic technologies to individualize prognosis and guide rational treatment for cancer patients. *Target Oncol.* 2009, *4*, 235–252.

[60] Ciccimaro, E., Hanks, S. K., Yu, K. H., Blair, I. A., 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.

[61] Ahn, Y. H., Lee, J. Y., Lee, J. Y., Kim, Y. S. et al., 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.

[62] Brun, V., Masselon, C., Garin, J., Dupuis, A., Isotope dilution strategies for absolute quantitative proteomics. *J. Proteomics* 2009, *72*, 740–749.

[63] Lebert, D., Dupuis, A., Garin, J., Bruley, C. et al., Production and use of stable isotope-labeled proteins for absolute quantitative proteomics. *Methods Mol. Biol.* 2011, *753* 93–115.

[64] Reid, J. D., Holmes, D. T., Mason, D. R., Shah, B. et al., Towards the Development of an Immuno MALDI (iMALDI) mass spectrometry assay for the diagnosis of hypertension. *J. Am. Soc. Mass. Spectrom.* 2010, *21*, 1680–1686.

[65] Jiang, J., Parker, C. E., Hoadley, K. A., Perou, C. M. et al., Development of an immuno tandem mass spectrometry (iMALDI) assay for EGFR diagnosis. *Proteomics Clin. Appl.* 2007, *1*, 1651–1659.

[66] Elliott, M. H., Smith, D. S., Parker, C. E., Borchers, C., Current trends in quantitative proteomics. *J. Mass. Spectrom.* 2009, *44*, 1637–1660.

[67] Bell, A. W., Deutsch, E. W., Au, C. E., Kearney, R. E. et al., HUPO test sample working group. A HUPO test sample study reveals common problems in mass spectrometry-based proteomics. *Nat. Methods* 2009, *6*, 423–430.

[68] Friedman, D. B., Andacht, T. M., Bunger, M. K., Chien, A. S. et al., The ABRF proteomics research group studies: educational exercises for qualitative and quantitative proteomic analyses. *Proteomics* 2011, *11*, 1371–1381.

[69] Addona, T. A., Abbatiello, S. E., Schilling, B., Skates, S. J. 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.

[70] Schoenherr, R. M., Zhao, L., Whiteaker, J. R., Feng, L. C. et al., Automated screening of monoclonal antibodies for SISCAPA assays using a magnetic bead processor and liquid chromatography-selected reaction monitoring-mass spectrometry. *Immunol. Methods.* 2010, *353*, 49–61.

[71] Hoofnagle, A. N., Wener, M. H., The fundamental flaws of immunoassays and potential solutions using tandem mass spectrometry. *J. Immunol. Methods* 2009, *347*, 3–11.

[72] Pratt, J. M., Simpson, D. M., Doherty, M. K., Rivers J. et al., Multiplexed absolute quantification for proteomics using concatenated signature peptides encoded by QconCAT genes. *Nat. Protoc.* 2006, *1*, 1029–1043.

[73] Regnier, F. E., Skates, S. J., Mesri, M., Rodriguez, H. et al., Protein-based multiplex assays: mock presubmissions to the US Food and Drug Administration. *Clin. Chem.* 2010, *56*, 165–171.

[74] Picotti, P., Rinner, O., Stallmach, R., Dautel, F. et al., High-throughput generation of selected reaction-monitoring assays for proteins and proteomes. *Nat. Methods* 2010, *7*, 43–46.

[75] Ossola, R., Schiess, R., Picotti, P., Rinner, O. et al., Biomarker validation in blood specimens by selected reaction monitoring mass spectrometry of N-glycosites. *Methods Mol. Biol.* 2011, *728*, 179–194.

[76] Picotti, P., Rinner, O., Stallmach, R., Dautel, F. et al., High-throughput generation of selected reaction-monitoring assays for proteins and proteomes. *Nat. Methods* 2010, *7*, 43–46.

[77] Anderson, N. L., Anderson, N. G., Pearson, T. W., Borchers, C. H. et al., A human proteome detection and quantitation project. *Mol. Cell. Proteomics* 2009, *8*, 883–886.

[78] Cancer Genome Atlas Research Network. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature* 2008, *455*, 1061–1068.

[79] Verhaak, R. G., Hoadley, K. A., Purdom, E., Wang, V. et al., Cancer genome atlas research network. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell* 2010, *17*, 98–110.

[80] Wang, Q., Chaerkady, R., Wu, J., Hwang, H. J. et al., Mutant proteins as cancer-specific biomarkers. *Proc. Natl. Acad. Sci. U.S.A.* 2011, *108*, 2444–2449.

[81] Zhang, G., Fang, B., Liu, R. Z., Lin, H. et al., Mass spectrometry mapping of epidermal growth factor receptor

phosphorylation related to oncogenic mutations and tyrosine kinase inhibitor sensitivity. *J. Proteome Res*. 2011, *10*, 305–319.

[82] Boja, E., Hiltke, T., Rivers, R., Kinsinger, C. et al., Evolution of clinical proteomics and its role in medicine. *J. Proteome Res*. 2011, *10*, 66–84.

[83] Remily-Wood, E. R., Liu, R. Z., Xiang, Y., Chen, Y. et al., A database of reaction monitoring mass spectrometry assays for elucidating therapeutic response in cancer. *Proteomics Clin. Appl*. 2011, *5*, 383–396.

[84] Wolf-Yadlin, A., Hautaniemi, S., Lauffenburger, D. A., White, F. M., Multiple reaction monitoring for robust quantitative proteomic analysis of cellular signaling networks. *Proc. Natl. Acad. Sci. U.S.A*. 2007, *104*, 5860–5865.

[85] Nomura, D. K., Dix, M. M., Cravatt, B. F., Activity-based protein profiling for biochemical pathway discovery in cancer. *Nat. Rev. Cancer* 2010, *10*, 630–638.

[86] Jessani, N., Niessen, S., Wei, B. Q., Nicolau, M. et al., A streamlined platform for high-content functional proteomics of primary human specimens. *Nat. Methods* 2005, *2*, 691–697.

[87] Speers, A. E., Cravatt, B. F., Profiling enzyme activities in vivo using click chemistry methods. *Chem. Biol.* 2004, *11*, 535–546.

[88] Liu, Y., Patricelli, M. P., Cravatt, B. F., Activity-based protein profiling: the serine hydrolases. *Proc. Natl. Acad. Sci. U.S.A*. 1999, *96*, 14694–14699.

[89] Sieber, S. A., Niessen, S., Hoover, H. S., Cravatt, B. F., Proteomic profiling of metalloprotease activities with cocktails of active-site probes. *Nat. Chem. Biol.* 2006, *2*, 274–281.

[90] Kato, D., Boatright, K. M., Berger, A. B., Nazif, T. et al., Activity-based probes that target diverse cysteine protease families. *Nat. Chem. Biol.* 2005, *1*, 33–38.

[91] Cohen, M. S., Hadjivassiliou, H., Taunton, J., A clickable inhibitor reveals context-dependent autoactivation of p90 RSK. *Nat. Chem. Biol.* 2007, *3*, 156–160.

[92] Patricelli, M. P., Szardenings, A. K., Liyanage, M., Nomanbhoy, T. K. et al., Functional interrogation of the kinome using nucleotide acyl phosphates. *Biochemistry* 2007, *46*, 350–358.

[93] Kumar, S., Zhou, B., Liang, F., Wang, W. Q. et al., Activity-based probes for protein tyrosine phosphatases. *Proc. Natl. Acad. Sci. U.S.A*. 2004, *101*, 7943–7948.

[94] Salisbury, C. M., Cravatt, B. F., Activity-based probes for proteomic profiling of histone deacetylase complexes. *Proc. Natl. Acad. Sci. U.S.A*. 2007, *104*, 1171–1176.

[95] Rolén, U., Kobzeva, V., Gasparjan, N., Ovaa, H. et al., Activity profiling of deubiquitinating enzymes in cervical carcinoma biopsies and cell lines. *Mol. Carcinog*. 2006, *45*, 260–269.

[96] Gallien, S., Duriez, E., Domon, B., Selected reaction monitoring applied to proteomics. *J. Mass Spectrom*. 2011, *46*, 298–312.

