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The clinical impact of recent advances in LC-MS for cancer biomarker discovery and verification

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Abstract

Mass spectrometry (MS) -based proteomics has become an indispensable tool with broad applications in systems biology and biomedical research. With recent advances in liquid chromatography (LC) and MS instrumentation, LC–MS is making increasingly significant contributions to clinical applications, especially in the area of cancer biomarker discovery and verification. To overcome challenges associated with analyses of clinical samples (for example, a wide dynamic range of protein concentrations in bodily fluids and the need to perform high throughput and accurate quantification of candidate biomarker proteins), significant efforts have been devoted to improve the overall performance of LC–MS-based clinical proteomics platforms. Reviewed here are the recent advances in LC–MS and its applications in cancer biomarker discovery and quantification, along with the potentials, limitations and future perspectives.

Keywords

LC–MS; cancer biomarker; proteomics; targeted quantification; selected reaction monitoring; multiple reaction monitoring; isobaric labelling; label-free; PRISM

The term biomarker, also referred to as a molecular marker or a signature molecule, connotes a measurable biological molecule found in cells, tissues or bodily fluids that can serve either as an indicator of a normal or abnormal process, or as a sign of a condition or disease state. The presence or absence of a biomarker may be correlated with early diagnosis, prognosis and prediction of various diseases, including cancer.[1–4] With recent advances in genomics and proteomics technologies, the number of potential candidate DNA, RNA and protein biomarkers has significantly increased. Compared to nucleic acid biomarkers, protein biomarkers provide more functional information and reflect a more precise physiological cellular state, which simply cannot be revealed by genome-level information. Therefore, candidate protein biomarkers are considered to be highly promising, specific biomarkers for both cancer diagnosis and prognosis in the clinical setting.[5– 8]

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However, due to the wide dynamic range of protein abundances, the potential presence of an array of post-translational modifications (PTMs) and the absence of protein amplification methods, candidate protein biomarkers pose tremendous challenges for the reliable and robust measurement of low-abundance protein biomarkers.[9,10] Therefore, new developments in protein quantification technologies that provide higher sensitivity and specificity are expected to greatly accelerate protein biomarker discovery and verification.

The coupled analytical tool, LC-MS, has become a powerful platform for protein identification and quantification. The combination of high-resolution LC separations with fast and sensitive MS detection methods, LC-MS has enabled genome-scale proteome coverage and quantitative measurements of tens of thousands of proteins and their PTMs, even in complex clinical specimens, such as human blood plasma/serum, urine and tissue. [11–15] More importantly, LC-MS presents unique advantages in protein biomarker discovery and verification compared to other biological techniques. For example, for the sensitive detection and quantification of cancer-specific missense mutant protein biomarkers, it is typically challenging to distinguish the abnormal protein from the wild-type form with antibodies. In contrast, LC-MS readily addresses this issue by precisely measuring the isoform-specific fragmentation patterns.[16] LC-MS can also be used for accurately monitoring hundreds of proteins simultaneously in the targeted fashion.

Two general types of LC-MS-based proteomics approaches are widely used for biomarker-related applications: global quantitative proteomics for biomarker discovery and targeted quantitative proteomics for candidate biomarker verification.[11,12,17] Global proteomics analysis primarily relies on either label-free or stable isotope labeling approaches to incorporate different mass tags into the peptides and is mainly used for unbiased biomarker discovery (Figure 1). The relative protein abundances can be determined by comparing signal intensity or peak area of corresponding peptides, or reporter ions in the case of isobaric labeling approaches such as “isobaric tags for relative and absolute quantitation” (iTRAQ) and “tandem mass tags” (TMT).[18–22] The global proteomics method is best suited for initial discovery of potential biomarkers or large-scale screening of protein biomarker candidates. However, such global measurements have inherent poor reproducibility (i.e., “missing values”) due to the stochastic sampling nature of the data-dependent acquisition (DDA) mode.[23] In contrast, targeted proteomics methods, such as selected reaction monitoring (SRM, also referred to as multiple reaction monitoring (MRM)) or parallel reaction monitoring (PRM),[24,25] are well suited for reproducible and accurate quantification of target proteins across many samples and also have higher sensitivity than global proteomics. [23,26–28] With known concentrations of stable isotope-labeled heavy peptides (i.e., internal standards) spiked into clinical samples, targeted MS can be used to accurately quantify many peptide targets in a single analysis by comparing the peak intensities or peak areas of light endogenous peptides with heavy internal standards. These unique features make targeted proteomics a perfect quantification tool for verification of candidate protein biomarkers without the need for affinity reagents, for example, antibodies.

The application of LC-MS techniques in protein biomarker studies has been discussed previously.[9,29–31] This review describes advances in LC-MS-based proteomics technologies in recent five years (2010–2015) and their applications for cancer biomarker

quantification. While the unbiased “global” discovery techniques are briefly covered, this review focuses more on the targeted proteomics techniques, given its critical role in preclinical verification of candidate biomarkers, the current bottleneck in biomarker development. More detailed discussions on the advances and application of LC-MS techniques for unbiased global proteome characterization can be found in other excellent reviews published previously.[17,32,33]

Brief overview of global proteomics approaches for cancer biomarker discovery

LC-MS/MS-based global proteomics approaches can routinely detect tens of thousands of proteins and PTMs while providing relative quantification information about protein abundance levels. Thus, it has become a powerful analytical tool for uncovering complex proteomes and discovering candidate protein biomarkers.[11,12,30–35] There are two types of quantification methods for global proteomics: label-free and stable isotope labeling.

Label-free quantification

Label-free quantification is a simple and straightforward method for relative quantification of proteins, which is typically achieved by comparing ion intensities [36] or peak areas [37] at the MS level, or spectral counts [38] at the MS/MS level for the same peptide. Performing label-free quantification offers broad proteome coverage with quantitative information, which is suitable for large-scale proteome-wide comparisons. This approach has been applied for various quantitative clinical proteomics studies including biomarker discovery. [39–41] However, it has a major limitation in terms of missing data, especially for low-abundance peptides due to the limited MS sampling duty cycle (i.e., “undersampling”) and the DDA operation mode. Sample fractionation may help to alleviate this issue to some extent, however, at the expense of lowering sample throughput. Besides that, the variation observed between different LC-MS analyses is another problem in label-free quantification, which results in decreased precision in quantitation. Therefore, to obtain reliable quantification by applying the label-free techniques, high level of reproducibility in the LC-MS analysis and additional normalization during data analysis is often required.[12]

Isotope labeling-based quantification

Stable isotope labeling-based quantification addresses the major issues in label-free quantification methods because its quantitation precision is independent of the LC-MS reproducibility. [42,43] In this type of approaches, different samples are labeled with heavy or light isotope-coded reagents with the same chemical structures, then mixed and analyzed by LC-MS/MS. Peptides with different labels (i.e., from different samples) are co-eluted and detected by MS in the same analysis, providing better precision and accuracy than that afforded by label-free approaches. Quantification is based on the comparison of the peak intensities of light and heavy peptides at the MS level or report ions at the MS/MS level.

Commonly used nonisobaric methods include labeling by stable isotope labeling by amino acid in cell culture (SILAC), [44,45] ^{18}O , [46–48] dimethylation, [49,50] isotope-coded protein label (ICPL) [51] or mTRAQ (MRM tags for relative and absolute quantitation).

[52,53] In the SILAC approach, the heavy isotopes are incorporated into cultured cells through the use of growth medium containing stable isotope-labeled amino acids, such as ^{13}C and/or ^{15}N labeled arginine or lysine. This type of metabolic labeling method provides the most precise quantitation possible because the isotope labeling is completed before any proteomic sample processing, for example, protein extraction and digestion. Human tumor proteomes can also be accurately quantified by combining a mixture of SILAC-labeled cell lines with the individual human carcinoma tissue samples (i.e., Super-SILAC [54]). Enzymatic ^{18}O labeling is typically achieved using post-digestion ^{16}O -to- ^{18}O exchange at the C-terminal carboxyl group of the peptide through which a 4 Da mass shift is introduced. ^{18}O labeling approach is simple and low cost, and can be applied for all types of samples. The earlier technical issues such as low label efficiency and back-exchange from ^{18}O to ^{16}O (introducing the ^{18}O during digestion step) [55,56] have also been effectively addressed by the later developed trypsin-assisted post-digestion exchange approach.[46] Dimethylation is a straightforward, rapid and inexpensive chemical labeling method. Peptide labeling is accomplished by reacting formaldehyde in deuterated water and peptide primary amines. Due to different isotopomers for formaldehyde and cyanoborohydride, 2, 4, 6 or 8 Da mass shift can be obtained between different samples. However, in some conditions, 2 Da mass difference between resulting peptides leads to difficulties in quantification because there might be overlap between labeled peptides, especially for the ones carrying multiple charges.[57] ICPL is a protein-level chemical labeling method that relies on the carbodiimide chemistry to introduce isotopic tag (d4 or d0) onto primary amine groups in lysine residues and N-termini on intact protein. Because ICPL-labeled lysine is protected against proteolytic digestion, endoproteases with cleavage sites at lysine (e.g., Lys-C) cannot be used in ICPL-labeling samples. For the commonly used trypsin digestion, only the C-terminal of arginine can be cleaved; therefore, post-enzymatic digestion is required to avoid long peptides that are not easily detected by MS. These disadvantages limit the application of the ICPL labeling method.[58] mTRAQ is another amine-specific nonisobaric labeling technology. Peptides labeled with mTRAQ reagents have identical retention time and ionization efficiency, but different masses (0, 4 or 8 Da for arginine-containing peptides, and 0, 8 or 16 for lysine-containing peptides for triplex labeling). This labeling technique is commonly used in targeted quantification [52,59] with few studies in global measurement.[60] The comparison of different nonisobaric labeling strategies mentioned above is summarized in Table 1. More details are available in other reviews.[42]

Isobaric labeling methods utilize several tags with identical masses for labeling different samples (e.g., cases versus controls, biological replicates and longitudinal samples); the labeled samples are then mixed for LC-MS/MS analysis. The relative quantification is facilitated by comparing the intensities of reporter ions generated from the different isobaric mass tags upon fragmentation in the mass spectrometer (i.e., MS/MS level). The most popular and commercially available isobaric labeling tags are iTRAQ [21,61–63] and TMT. [19,64] Compared to nonisobaric labeling methods, the isobaric labeling approaches provide much higher sample throughput (e.g., iTRAQ 8-plex and TMT 10-plex allow for simultaneous analysis of up to 8 and 10 samples, respectively) with good precision and a wider dynamic range (when coupled with sample fractionation), and thus have been more

broadly applied for proteome-scale protein quantification.[43] To further increase sample throughput, recently, Everley et al. developed a 54-plex TMT labeling strategy. In this method, two novel 6-plex isobaric tags were added to the original 6-plex to get a total of 18-plex in a single analysis, and then, three mass variants (light/medium/heavy) of the target peptide were labeled with 18-plex giving a 54-plex quantification in a single analysis. Thus, the analysis time and reagents needed were reduced significantly and the overall throughput was improved approximately 50-fold.[65] However, the demands associated with labeling when a large number of samples need to be analyzed require stringent controls on sample preparation reproducibility. Moreover, the scope of applying the isobaric labeling methods is certainly not limited by the inherent multiplexing capability for each type of reagent (e.g., 10 samples in TMT 10-plex labeling) as any of the isobaric labeling methods can be used for quantification of large sample cohorts by including a common reference sample in each multiplexed experiments (i.e., similar to the “universal reference” [66] or Super-SILAC [54] ideas). Similar to the label-free quantification approaches, isobaric labeling methods with DDA also have the missing value issue caused by inherent MS duty cycle limitation; however, in isobaric labeling, this is alleviated to some extent through sample fractionation, while maintaining reasonable sample throughput (with sample multiplexing). Other novel isobaric mass tags, such as *N,N*-dimethyl leucine and deuterium isobaric amine reactive tag, serve as cost-effective alternatives for iTRAQ and TMT with comparable labeling efficiency. [67,68] Novel reagents iTRAQH (iTRAQ hydrazide) and iodoTMT are especially designed for selective labeling and relative quantitation of carbonyl groups and cysteine residues, respectively.[69,70] More isobaric labeling-related discussion can be found in other review papers.[43,71]

Global proteomics in recent clinical applications

Global proteomics has been extensively applied to cancer biomarker discovery, including prostate cancer,[72] ovarian cancer, [73] renal cell carcinoma,[74] etc. By quantitative analysis of the patient and normal control, or the different disease stage levels, the proteins with significant differential expression were screened as the potential biomarker candidates. Among all the global proteomics technologies mentioned above, the most commonly used quantitative strategy is iTRAQ-labeling combined with strong cation exchange chromatography (SCX) fractionation followed by LC-MS/MS identification.[74–76] Other chromatographic separation technique, such as high pH reversed-phase LC, was also used for fractionation of isobarically labeled peptides.[77] For more complex serum or plasma sample, immunodepletion [73,78] or other protein-level enrichment approaches, such as SDS-PAGE,[78] combinatorial peptide ligand library protein enrichment,[79] are usually applied before iTRAQ labeling to remove the high abundance proteins. Another isobaric labeling, TMT, is also commonly used by combining with SCX fractionation.[80] SILAC labeling for cancer cell line quantitative studies were also reported.[81] Interestingly, Yeh et al. integrated two labeling methods, SILAC-based quantitative proteomics for hepatocellular carcinoma cell lines and iTRAQ labeling for hepatocellular carcinoma xenograft quantification. This dual labeling quantitative proteomics approach allowed for a broad, systematic examination of the changes in the proteome associated with disease.[82] In general, the sample cohort size used in the global discovery works has been relatively small (e.g., <20 patient/control specimens) with a few exceptions. For example, Yang et al. utilized

a large sample set with 54 cancer patients and 46 controls for label-free quantification of bladder cancer-specific urinary glycoprotein biomarkers. A total of 265 glycoproteins showed differential expression by spectral count observation.[83] With proper experimental design, cancer biomarker discovery with large sample size is expected to provide higher-quality biomarker candidates that are more likely to have better performance in the verification studies using targeted proteomics approaches. All the applications mentioned above are summarized in Table 2.

Targeted proteomics approaches for preclinical verification of candidate protein biomarkers

Global LC-MS/MS-based proteomics suffers inherent problems of quantification accuracy and reproducibility (i.e., missing data in samples with low-abundance proteins that fail to be identified) in large-scale studies due to the stochastic sampling nature of shotgun MS/MS. [12,84] Targeted MS-based proteomics is an emerging field of technologies designed to largely overcome these shortcomings.[85] Targeted proteomics approaches including SRM, PRM and data-independent acquisition (DIA) with targeted data extraction are designed to achieve precise and accurate quantification (i.e., actual protein concentration) of the proteins, when combined with heavy-labeled internal standards. By spiking in known amounts of stable isotope-labeled synthetic peptides as internal standards, quantification can be achieved by comparing the peak areas of “heavy” standard peptides versus “light” endogenous peptides in extracted ion chromatograms. [84,85]

Three types of targeted proteomics quantification

SRM is typically performed on a triple quadrupole (QQQ) MS instrument where Q1 and Q3 serve as precursor ion and fragment ion filters, respectively, and Q2 acts as collision cell (Figure 2). The predefined parent/fragment ion pairs of targeted peptides, referred to as transitions, are scanned during LC-SRM measurements. The two-stage mass filter with a narrow isolation window provides high specificity and low background for SRM. Compared to the conventional shotgun technique, the sensitivity of LC-SRM is enhanced by 1–2 orders of magnitude. Usually, LC-SRM provides a linear range of 4–5 orders of magnitude in response and low attomole levels for the limit of detection (LOD). The high sensitivity, wide dynamic range, in combination with the inherent properties of MS (e.g., good reproducibility and high accuracy), make SRM an ideal tool for targeted quantitative analysis of complex clinical samples. [29,86–88]

In addition to SRM, other alternative targeted quantitative proteomics approaches have been explored for their suitability for biomedical applications. One example is PRM that can be executed on high-resolution accurate-mass (HR/AM) MS (e.g., Q Exactive MS instrument from Thermo Scientific). Different from the QQQ, the third quadrupole is substituted with a HR/AM Orbitrap mass analyzer, allowing all precursor ions (instead of a few selected) of the target peptides to be monitored in parallel (Figure 2). Compared to SRM, PRM provides peptide identification and all transition information with high confidence and does not require extensive assay development for large-scale studies (e.g., optimization of transitions and collision energies, and selection of interference-free transitions). PRM also achieves

comparable dynamic range and linearity, but with less precision.[24] Other PRM-related studies, such as quality control investigation,[89] high-performance parameter optimization,[25] and precise quantification, and workflow of PRM data acquisition and processing,[90] have been reported recently. Combined with immunoaffinity depletion (ID), PRM has been successfully applied for rapid screening and validation of mutant proteins as predictive lung cancer biomarkers.[91] These results provide an indication for the potential of PRM to enhance large-scale clinical applications of biomarker discovery.

Although PRM can accelerate the targeted quantification to some extent, it is still limited by the scale of quantification, typically 50–100 proteins per a single run for reliable quantification. An alternative technique, targeted DIA (Figure 2) combines the advantages of global proteomics (i.e., large scale based on DDA) and targeted proteomics (i.e., high reproducibility and accuracy). Compared to SRM or PRM, the data creation of DIA is more flexible and simpler. DIA collects all MS/MS scans irrespective of precursor ion selections from a survey scan or full MS scan. The predefinition of target lists, which SRM or PRM requires, is unnecessary for DIA experiment. A broad range of precursors and corresponding transitions can be extracted after the data procurement. Thus, in targeted proteomics, DIA aims at proteome-wide quantification using a targeted data extraction strategy.[92] A novel DIA technique, sequential window acquisition of all theoretical mass spectra (SWATH) shows great potential for large-scale clinical applications. In the targeted SWATH-MS approach, the fragment ion spectra of target peptides of interest are extracted during the targeted quantification analysis. As a targeted DIA method, SWATH-MS provides an attractive alternative for quantitative proteomics with a wide dynamic range, high reproducibility and large-scale quantification.[93] The quantitative measurement of N-linked glycoproteins in human blood plasma demonstrated that, compared to SRM, SWATH-MS showed a similar level of reproducibility, a slightly worse sensitivity (limit of quantification (LOQ): 0.0456 fmol for SWATH-MS versus 0.0152 fmol for SRM), and good correlation with SRM results ($R^2 = 0.978$).[94] This technique has been applied for analysis of the N-linked glycoproteome of prostate cancer and resulted in the verification of two glycoproteins as novel potential biomarkers for prostate cancer aggressiveness.[95] Although SWATH is a promising technology with great potential, the extraction of targeted peptides from complex spectra still remains challenging. Moreover, large isolation width leads to the increased complexity of data and noise, but with significantly reduced selectivity. It can be expected that SWATH-MS would benefit from enhanced bioinformatics tools for increasing its effectiveness.

Challenges in targeted proteomics quantification

One of the biggest challenges in biomarker verification, with targeted proteomics quantification, is the lack of sufficient sensitivity for detecting extremely low-abundance proteins,[27] especially in bodily fluids. For example, blood plasma/serum has a wide dynamic range of 10–12 orders of magnitude in protein concentrations, and the top 20 most abundant proteins (e.g., albumin, immunoglobulins, transferrin, etc.) constitute approximately 99% of the total protein mass, while thousands of other proteins, including the potential protein biomarkers that are typically present at ng/ml or sub-ng/ml levels, account for the remaining 1%.[96–98] Therefore, method development for enriching low-

abundance proteins or specific targeted proteins, depleting high-abundance interference components, fractionating to reduce the sample complexity, will greatly improve the detection capabilities of LC-SRM.[99] Commonly used fractionation/enrichment methods in proteomics studies (e.g., multidimensional protein identification technologies (MudPIT), immuno affinity depletion techniques, immunoaffinity enrichment, etc.) can also be coupled to targeted quantification to reduce the sample complexity for better detection of low-abundance proteins.[99] In addition, to verify the large-scale number of clinical biomarker candidates generated from the discovery studies, it is necessary to establish high-throughput targeted analysis workflow with rapid assay development, high multiplexing and fast data processing capabilities.[26]

In the following sections, the authors review recent advances in various enrichment and fractionation techniques (Figure 2), as well as high sample throughput techniques for rapid quantification of targeted proteins across many samples. The relevant LODs and LOQs for each strategy to enhance targeted quantification sensitivity are listed in Table 3.

Protein-level enrichment

To increase the sensitivity for quantification of low-abundance proteins in bodily fluids, one of the most effective approaches is ID of high-abundance proteins. For example, a single-step, multicomponent ID removes the top 7–14 high-abundance proteins simultaneously from blood plasma/serum, leading to a 10- to 20-fold enrichment of the remaining lower-abundance proteins.[99,100] Alternatively, some low-abundance target proteins may also be specifically enriched using immobilized antibodies [115]. For example, Wang et al. utilized immuno-precipitation (IP) combined with SRM to quantify mutant Ras protein as a pancreatic cancer biomarker. The KRAS protein was selectively enriched by immobilized anti-Ras antibody on magnetic beads, eluted and then analyzed by LC-SRM. This strategy provided high specificity, and the LOD was as low as 10 fmol/mg of total protein.[16] The same strategy was repeated by Puppen-Canas et al. using a more sensitive mass spectrometer, resulting in two orders of magnitude improvement in sensitivity. The wild-type and mutant KRAS proteins in patient tumor and xenograft human tissue were quantified, and the LOD was as low as 0.24 fmol/mg of total protein. [101] The Reubsaet group reported their work using immunocapture-SRM.[102,116] Recently, they developed a multiplexing immunocapture technique, in which two kinds of antibody beads were utilized to co-extract different targeted markers simultaneously. The strategy was applied to identify the small cell lung cancer biomarkers progastrin releasing peptide (ProGRP), neuron-specific enolase (NSE) and their isoforms or isoenzymes. These two biomarkers are routinely determined by two different assays separately (immunofluorometric assay for ProGPR and immunoradiometric assay for NSE); however, they can be quantified in a single SRM experiment with the lower LOQ (LLOQ) of 24 and 15 pM for ProGRP and γ -NSE in human serum, respectively.[102] Although immunoaffinity-enrichment techniques can improve the sensitivity of targeted quantification, the requirements of high-quality antibodies and large sample amounts (usually milligram levels) still limit its broad application. Chen et al. combined IP and SRM-MS technologies and quantified prostate-specific antigen (PSA) and proPSA at low ng/ml level. The IP-SRM result is correlated with that of radioimmunoassay.

The strategy provides an attractive alternative to immunoassay for reliable measurement of proPSA.[103]

Besides immunocapture approaches, several other enrichment strategies have been performed at the protein level for quantitative targeted proteomics analysis. Liebler group utilized SDS-PAGE-based fractionation prior to SRM detection (GeLC-SRM). Compared to IP-SRM, the GeLC-SRM approach was more accessible, inexpensive and fast. More importantly, the required sample size for GeLC-SRM was significantly smaller, where only 5–50 µg was needed for a single analysis. Using GeLC-SRM, KRAS peptides were quantified at 1.1 fmol/µl protein from pancreatic cyst fluids.[104] In addition, the solubilizing and denaturing capacity of SDS allows for extraction of membrane proteins. GeLC-SRM was also successfully applied to human skin biopsies,[117] serum/plasma[118] and liver tissue samples.[119] Nevertheless, this approach may not be suitable for some post-translationally modified proteins with a broad molecular weight range; the enrichment efficiency may also vary significantly for different proteins.[104] Isoelectric focusing (IEF) has also been applied for protein enrichment prior to LC-SRM analysis. Zaenglein et al. analyzed the enzymatic catalase and Ho-1 by off-gel IEF followed by scheduled SRM-MS. This assay showed good correlation with western blot results, but with improved linearity, precision and sensitivity.[120]

Peptide-level enrichment

Stable isotope standards and capture by anti-peptide antibodies (SISCAPA) is a peptide-enrichment technique introduced by Anderson et al. in which antibodies against specific peptides are generated in rabbit and used for immunocapturing target peptides.[121] As high as 120-fold enrichment of the antigen peptide relative to other nonantigen peptides can be achieved using this method. SISCAPA was demonstrated for enabling to multiplex a number of targets during both antibody development and application stages. Combined with SRM, SISCAPA provides enhanced sensitivity and higher throughput for quantification of biomarker proteins.[122] Significant advances have been made in SISCAPA since its inception. In 2011 a multiplexed immunization strategy was reported and the overall success rate for making a sensitive immuno-SRM assay (i.e., SISCAPA coupled to SRM) for a target protein was >90%.[123] In addition, the multiplexed immuno-SRM assay showed high reproducibility across different laboratories, achieving an inter-laboratory coefficient of variance of <14%.[124] This workflow was further improved to allow multiplexing up to 50 peptides simultaneously in a single assay with sequential enrichment, with both high correlation ($r^2 = 0.98$) and good agreement (bias = 1%) compared to a 10-plex configuration.[125] An alternative strategy of using recombinant antibody fragments has been demonstrated recently. The antibody fragments demonstrate similar performance as conventional monoclonal antibodies, but with shorter generation times (e.g., 2 months as opposed to 6 months typically required for traditional monoclonal antibodies).[105] Moreover, it has been recently reported that the anti-peptide monoclonal antibodies generated for immuno-SRM have a high probability of supporting traditional immunoaffinity technologies, such as western blot and ELISA.[126] Despite the advantages and advances, SISCAPA has only been exploited by a few groups thus far, presumably due to the long lead

time, high reagent cost and expertise required for successful antibody and assay development.

In addition to immunoaffinity enrichment, chromatography-based approaches, such as SCX [127] and hydrophilic interaction liquid chromatography (HILIC), have also been considered for peptide-level pre-fractionation. Krisp et al. developed a MudPIT-SRM platform combining robust SCX fractionation to SRM analysis. Taking advantage of a 10-port valve and an independent SCX/RP hybrid column, all the analytical procedures including sample loading, SCX fractionation, eluting and desalting of fractions, as well as the final LC-SRM detection, were accomplished online. The novel system improved the peak area of abundant plasma proteins by average increase of almost 90% when compared to conventional SRM, which offers performance advantages to enhance sensitivity for biomarker studies.[106] Simon et al. proposed an RP-SPE-HILIC-SRM system that was based on the online transfer of analytes by an anion exchange SPE cartridge between RP and HILIC. This setup demonstrated good reproducibility and allowed for quantification of PSA in human plasma with an LOQ of 1 ng/ml without either upfront ID or intense offline fractionation.[107]

IEF has been used for pre-fractionation at the peptide level. Liebler's group fractionated peptides using the immobilized pH gradient strips followed by LC-SRM to verify potential single-gene mutations in colorectal cancer (CRC). From a subset of proteins differentially expressed between the adenomatous polyposis coli mutant and the restored CRC cell lines, as determined by LC-MS/MS proteomics, 22 proteotypic peptides were verified by LC-SRM.[128] Rafalko et al. developed a chip/chip/SRM platform for quantification of low-abundance protein biomarkers in human plasma. This platform was based on the IEF enrichment of peptides on a digital ProteomeChip, followed by the SRM quantification using a QQQ MS coupled to an LC-Chip. By combining the immunodepletion of albumin and IgG, this device can quantify PSA spiked in female plasma at 1–2.5 ng/ml.[108] Schafer et al. compared off-gel electrophoresis (OGE) and SCX for peptide fractionation prior to online LC-SRM. The result of 18 candidate proteins from mouse liver sample shows the median gain of sensitivity is 8.8-fold for SCX and 12.4-fold for OGE, indicating OGE is a more effective fractionation technique.[109]

Shi et al. have developed a high-pressure, high-resolution separation coupled with intelligent selection and multiplexing (PRISM)-SRM method for highly sensitive targeted protein quantification. PRISM-SRM uses a first dimension separation of high pH reversed phase LC (RPLC), through which the peptides of interest are separated and enriched with only their corresponding fractions selected intelligently via online monitoring, followed by further separation using the second-dimension low pH RPLC and detected by conventional SRM. Benefiting from the orthogonality in the separations, PRISM-SRM allows for specific enrichment of the target peptides while greatly reducing sample complexity and background/interferences prior to the final SRM analysis. Unlike the SCX fractionation method,[127] the resulting high pH RPLC fractions are compatible with the downstream SRM analysis, so that no additional cleanup is needed. PRISM-SRM uses a 200 μ m I.D. column for 1D separation (as opposed to the conventional set up such as 75 μ m I.D. column) that effectively increases the loading amount by 50-times while maintaining the high-resolution separation. Applying front-end IgY14 ID and PRISM-SRM, PSA spiked into female plasma was

quantified at 50–100 pg/ml level [110]; PRISM-SRM without ID still allows for high sensitivity detection of target proteins at low ng/ml levels.[111] Compared to conventional SRM, the PRISM-SRM assay provides more than 100-fold improvement in LOQ. Several initial applications demonstrated the effectiveness and robustness of this enabling targeted proteomics strategy in biological and biomedical applications. Anterior gradient 2 was quantified in urine using PRISM-SRM (LLOQ is 10 pg/100 µg protein mass in urine), and urinary AGR2/PSA concentration ratios showed significant difference between prostate cancer and noncancer subjects.[112] PRISM-SRM has enabled the high-sensitivity detection of protein products of fusions between transmembrane protease serine 2 (TMPRSS2) and ETS-related gene (ERG), one of the most specific biomarkers for prostate cancer, in cells, tissue and urine, and for the first time, detection of two distinctive ERG protein isoforms simultaneously expressed in the TMPRSS2:ERG-positive tissues.[113] The sensitivity of PRISM-SRM assay was compared to other technologies such as ELISA and qRT-PCR.[114] Additionally, the capability of PRISM-SRM platform for low-abundance PTMs has also been demonstrated in determining phosphorylation stoichiometry of eight ERK isoforms in human mammary epithelial cells without affinity enrichment. Compared with immobilized metal-ion affinity chromatography (IMAC) coupled to the SRM assay, PRISM-SRM improved the overall sensitivity by more than 10-times.[129] In summary, PRISM-SRM clearly demonstrated exceptional sensitivity compared to other proteomics techniques; however, further improvement in PRISM-SRM sample throughput is necessary to make it more practical for large-scale applications.[130]

Various protein PTMs have been proven relevant to cancer, indicating that PTMs may be potential cancer biomarkers.[131] Phosphorylation is one of the most important PTMs in eukaryotic cells and plays an important role in cell cycle regulation and signaling pathways. Alterations in phosphorylation are highly correlated to pathway activities, which lead to oncogenesis.[132] However, due to low abundance and stoichiometry, accurate quantification of phosphosites remains a challenge. Narumi et al. performed a large-scale phosphoproteome quantification and subsequent SRM-based verification for breast cancer biomarker research. The protein digests with internal standard spike-in was enriched for phosphopeptides with IMAC prior to SRM analysis. Among 19 phosphopeptide candidates with differential expression between the high- and low-risk groups, 15 were successfully quantified.[133] To determine phosphorylation stoichiometry, phosphatase dephosphorylation followed by indirect quantification of nonphosphopeptides by LC-MRM has been reported. Because the nonphosphorylated peptides usually have higher signal intensity than its phosphopeptide counterparts, this method showed high sensitivity.[134] Besides phosphorylation, glycosylation is another common and important PTM associated with cancer. However, detection and quantification of glycoproteins remain challenging due to the presence of multiple isoforms. Recently, Tao et al. presented an approach combining HILIC separation with LC-SRM, enabling the baseline separation and quantification of sialic acid (SA) linkage glycan isomers.[135]

Enhancing SRM throughput

The analytical efficiency of targeted proteomics quantification would reap further improvement in sample throughput by incorporating isotope labeling techniques into

targeted quantification workflow. Yin et al. proposed a hyperplex-SRM approach combining mTRAQ and iTRAQ labeling for absolute quantification of multiple samples simultaneously. In their strategy, four different tissue samples were labeled with 4-plex iTRAQ reagents, respectively (the mass shift is the same as mTRAQ (4)). In parallel, an equivalent amount of standard peptides were labeled with light (0) or heavy (8) mTRAQ reagents as double references. The total amount of iTRAQ-labeled peptide (4-like) can be calculated by the peak area with mTRAQ-labeled transitions from the SRM trace, while the relative ratio for each target peptide among four biological samples can be estimated by comparing the peak intensity of iTRAQ reporter ions in the MS/MS spectra. This approach was applied to the validation of human CRC biomarkers and displayed high accuracy, sensitivity and reproducibility.[136]

In addition to sample throughput issues, challenges associated with bioinformatics analyses represent another barrier in large-scale targeted proteomic applications.[137] Various novel algorithm and related software tools have been developed to accelerate and simplify assay development, data collection and data analysis.[138] In addition, enhancing automation is another promising aspect for targeted quantification of analytes with large sample sets.[139] The Aebersold group proposed a robust and automated SRM workflow integrating data processing (mProphet), statistical analysis (SRMstats) and dissemination (PASSEL) allowed for screening 35 biomarker candidates in 83 blood plasma samples with ovarian cancer within 1–2 weeks.[140] Other improvements related to assay development, for example, better prediction algorithms,[141] and approaches for improved implementation and utilization of existing discovery-based data [142] would also accelerate and enhance large-scale targeted quantification.

Recent use of targeted proteomics in clinical applications

Due to clinical and biological variability, the verification of candidate biomarkers needs a large sample set covering a broad section of patient cohorts. The dearth of robust analytical techniques with high sensitivity and reproducibility for the validation of biomarker candidates in large patient cohorts is one of the potential barriers in biomarker development. Targeted quantification technologies can alleviate this problem.[143] Here, the authors showed a couple of successful examples of analyzing a large set of samples. Hüttenhain et al. built a library of SRM assays for 5568 N-glycosites via SRMATlas for the multiplexed evaluation of clinically relevant N-glycoproteins as biomarker candidates. In total, 120 human plasma specimens from cancer patients and healthy controls were analyzed using this resource. N-glycoproteins with five orders of magnitude differences in abundance were able to be quantified, which demonstrated the feasibility of LC-SRM in large clinical sample cohorts.[143] Sjöström et al. combined shotgun MS and the targeted LC-SRM strategy for discovery and validation of breast cancer protein biomarkers. Tumor tissue samples from 80 patients with or without development of distant recurrence (DR±) were collected. N-glycosylated peptides were enriched and quantified by label-free LC-MS/MS. A breast cancer N-glycosylated proteome map containing 1515 glycopeptides from 778 proteins was created. By verification of targeted SRM assays, 10 proteins displayed the differential expression between DR+/DR– tumors. Five proteins were further validated at the gene expression level. LC-SRM data were also consistent with the clinically reported HRE2

status. All of these results demonstrated the potential of targeted MS for clinical biomarker verification.[144] Steiner et al. utilized SRM technique to accurately quantify HER2 in a cohort of 40 archival formalin-fixed paraffin-embedded tumor tissues from women with invasive breast carcinomas. The SRM assay showed good performance and high agreement with immunohistochemistry and FISH data.[145] The applications discussed above were selected to demonstrate the utility of targeted proteomic quantification as a powerful approach for biomarker verification with large sample sets.

For the large-scale targeted MS quantification, SWATH-MS that serves as a novel targeted data extraction technique shows the advances on the field of biomarker discovery and clinical research. Guo et al. combined pressure cycling technology for sample preparation and SWATH-MS to “draw a proteome map” for each clinical specimen. A set of 18 biopsy samples from nine patients with renal cell carcinoma were analyzed. More than 2000 proteins with high reproducibility were quantified and were able to clearly distinguish between tumorous kidney tissues and healthy specimens. The digital library obtained by DIA mode stores more protein information without bias and the library can be utilized for deep data mining in the future.[146] Krisp et al. developed an integrated online peptide fractionation and multiphasic microfluidic LC chip system with the SWATH-MS quantification. This approach provides more peptide identification, less sample consumption and lower limits of quantification.[147]

Expert commentary

With recent advances in sensitivity, quantification and throughput, LC-MS has emerged as a powerful tool for translational research such as biomarker discovery and verification. Global proteomics methods combined with either label-free or isobaric labeling allow in-depth, simultaneous, semi-quantitative profiling of thousands of proteins across biological conditions. Such comparative analysis of quantitative global data between normal and patient clinical specimens leads to the identification of useful sets of potential protein biomarkers including PTMs. However, the global discovery oftentimes leads to a relatively large set of candidate biomarkers and it is difficult to prioritize biomarkers for validation. It is also a formidable challenge for preclinical verification of hundreds of candidate protein biomarkers using traditional antibody-based assays because of the limited multiplexing capability and the unavailability of antibodies for new protein biomarkers and protein modifications.

Targeted proteomics offers a promising alternative for large-scale multiplexed verification of hundreds of candidate protein biomarkers in terms of specificity, reproducibility and multiplexing capability, without relying on affinity reagents. However, the major constraint of current SRM assays is the lack of sufficient sensitivity for measuring low-abundance protein biomarkers, especially in the case of bodily fluid samples where there is typically a tremendous “masking” effect present. To overcome this obstacle, sample pre-fractionation methods (e.g., affinity enrichment, high-resolution PRISM-SRM) are often required for enriching targets of interest and reducing sample complexity in order to significantly increase targeted MS sensitivity. The technology platform should be chosen based on the specific requirements of the application (e.g., sensitivity, specificity and throughput). The

sensitivity and throughput requirements still remain a dilemma for targeted proteomics and further advances are still needed in order to achieve high measurement sensitivity without compromising the throughput of targeted quantification.

Five-year view

When compared to traditional affinity reagent-based techniques, MS-based targeted proteomics strategies have been demonstrated as a powerful analytical tool for candidate protein biomarker verification.[148] Low-abundant protein biomarkers in complex clinical samples that cannot be detected by antibody-based assays can be successfully quantified by LC-MS analysis. For example, the LOQ for PRISM-SRM quantification of ARG2 in human urine is ~10 pg/100 µg total urinary protein. It is nearly impossible to detect by ELISA.[112] While multiple advancements have been noted for targeted proteomics methods utilizing MS, overall they remain lacking in sufficient sensitivity and throughput for the routine measurement of low-abundance target proteins in large cohorts of clinical samples. Hence, a compromise between sensitivity enhancement and sample throughput (e.g., as a result of sample pre-fractionation/enrichment) has to be made. During the next five years, with continuous advances in targeted MS sensitivity and throughput, these approaches will become more feasible for measuring patient protein concentrations in large numbers of clinical specimens. Enhancing sensitivity without significantly sacrificing throughput can be achieved by either simplifying or further increasing the efficiency of the front-end sample fractionation or by further advancements in MS instrumentation. Newer MS instrumentation incorporating advanced interfacing technologies (e.g., gas-phase separations and novel ion sources) may dramatically improve the overall analytical throughput. New, next-generation MS tools have the potential to revolutionize the field of clinical chemistry by providing the sensitivity, accuracy and throughput necessary for broad applications in clinical laboratories.

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Key issues

- Protein biomarker discovery and verification is of significant importance and in high demand for early detection, prognosis and treatment of cancer.
- LC-MS has become a robust analytical tool for clinical proteomics research, especially for protein biomarker studies.
- LC-MS-based global proteomic quantification can be used for large-scale protein biomarker discovery.
- Targeted quantitative proteomics provides the high sensitivity, accuracy and precision needed for verifying highly credentialed, candidate protein biomarkers in large numbers of biological samples.
- Protein-level or peptide-level enrichment techniques can further improve the measurement sensitivity.
- PRISM-SRM provides a sensitive, simple and robust two-dimensional SRM analytical system; however, sample throughput still needs improvement.
- To achieve high sensitivity and high throughput at the same time remains a challenge for targeted MS quantification, and further advances will be necessary.

Figure 1. Workflow of LC-MS-based proteomics for cancer biomarker quantification

Biomarker discovery relies on either label-free or stable isotope labeling global proteomics approaches. Further biomarker verification utilizes targeted proteomics techniques. Various enrichment techniques can be applied prior to LC-MS detection. SILAC: Stable isotope labeling by amino acid in cell culture; iTRAQ: Isobaric tags for relative and absolute quantitation; TMT: Tandem mass tags.

Figure 2. An overview of enrichment and fractionation techniques applied in combination with MS-based targeted approaches that are described in this review

Various enrichment and fractionation strategies such as SDS-PAGE, immunodepletion, isoelectric focusing (IEF) and immunoprecipitation at the protein level and stable isotope standards and capture by antipeptide antibodies (SISCAPA), IEF, strong cation exchange chromatography (SCX), hydrophilic interaction liquid chromatography (HILIC) and PRISM at the peptide level are applied prior to targeted proteomics measurements. Three targeted

MS strategies including selected reaction monitoring (SRM), parallel reaction monitoring (PRM) and targeted data-independent acquisition (DIA) are pictured.

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Table 1

Commonly used isotope labeling techniques for global proteomics quantification.

Method	MS	Labeling	Multiplex	mass	Reference
Stable isotope labeling by amino acid in cell culture	MSI	Metabolic	2	6, 8	[44,45]
¹⁸ O	MSI	Enzyme catalytic	2	4	[46–48,56]
Dimethylation	MSI	Chemical	2	2, 4, 6, 8	[49,50]
Isotope-coded protein label	MSI	Chemical	2	4	[51]
mTRAQ	MSI	Chemical	3	4, 8/8, 16	[52,53]
iTRAQ-8Plex	MS/MS	Chemical	8	0	[43]
TMT-10Plex	MS/MS	Chemical	10	0	[43]

Table 2

Global proteomics quantification in recent clinical application.

Labeling	Fractionation (protein)	Fractionation (peptide)	Cancer type	Sample type	Sample no.	Protein no. [†]	Reference
iTRAQ		SCX	Renal cell carcinoma	Tissue	20P, 20N	1591 (55)	[74]
iTRAQ		SCX	Esophageal squamous cell carcinoma	Tissue	10P, 10N	687 (238)	[75]
iTRAQ		SCX	Breast	Tissue	9P × 2	5122 (49)	[76]
iTRAQ		High pH RP	Human papillomavirus infection	Smear	23S	3200 (2300)	[77]
iTRAQ	Immunodepletion	SCX	Prostate	Serum	5P × 4	122 (23)	[72]
iTRAQ	Immunodepletion		Ovarian	Serum	6P, 6N	220 (14)	[73]
iTRAQ	Immunodepletion / SDS-PAGE	SCX	Oral squamous cell carcinoma	Serum	6P, 6N	319/218	[78]
iTRAQ	Combinatorial peptide ligand library protein enrichment	SCX	Breast	Plasma	12P, 12N	397 (23)	[79]
TMT		SCX	Epithelial ovarian cancer	Cell line	2C	946 (65)	[80]
SILAC	SDS-PAGE		Breast	Cell line	3C	1266 (1228)	[81]
SILAC iTRAQ		High pH RP	Hepatocellular carcinoma	Cell line xenograft	2C 6E × 2	2450 (156)	[82]
		Lectin	Bladder	Urinary	54P, 46N	421 (265)	[83]

[†] Identified protein number (quantifiable protein number).

×2; 2 groups; C: Cell line; E: Exnograph tumor; N: Normal control; P: Patient, S: Specimen; SCX: Strong cation exchange chromatography.

Table 3
Recent enrichment strategies used for enhancing the sensitivity of targeted proteomic quantification.

Method	Sample [†]	Protein	Cancer	Limit of detection	Limit of quantification	Reference
ID-SRM	Serum	PSA	Prostate	0.8 ng/ml	2.03 ng/ml	[100]
IP-SRM	Plasma/serum	KRAS	Pancreatic	25 fmol/mg		[16]
IP-SRM	Tissue	KRAS	Pancreatic	12 amol		[101]
IP-SRM	Serum	ProGRP/NSE	Small cell lung	7.2/4.5 pM	24/15 pM	[102]
IP-SRM	Serum	proPSA	Prostate	0.28 fmol	1.23 fmol	[103]
Ge-SRM	Urine	KRAS	Pancreatic	0.01 fmol/μg		[104]
SISCAPA-SRM	Plasma	3 proteins		0.3–2.9 ng/ml		[105]
MudPIT-SRM	Plasma	24 proteins	Liver	Low ng/ml		[106]
RP-HILIC-SRM	Plasma	PSA	Prostate		1 ng/ml	[107]
ChipIEF-SRM	Plasma	PSA	Prostate	0.06–0.12 ng/ml	1–2.5 ng/ml	[108]
IEF-SCX-SRM	Mouse liver	18 proteins		1 fmol/μg		[109]
PRISM-SRM	Plasma/serum	PSA	Prostate	0.1–1 ng/ml	0.5–5 ng/ml	[110,111]
PRISM-SRM	Urine	AGR2	Prostate		10 pg/100 μg	[112]
PRISM-SRM	Cell/tissue/urine	TMPRSS2-ERG	Prostate	0.5–5 fmol/mg	2–50 amol/μg	[113,114]
SWATH-MS	Plasma	N-glycoprotein	Prostate		0.0456 fmol	[94]

[†]Tissue, plasma, serum or other body fluid sample was from human except additional annotation.

ID: Immunodepletion; IP: Immunoprecipitation; Ge: Gel electrophoresis; SISCAPA: Stable isotope standards and capture by anti-peptide antibodies; MudPIT: Multidimensional protein identification technologies; RP: Reversed phase; HILIC: Hydrophilic interaction liquid chromatography; IEF: Isoelectric focusing; PRISM: High-pressure, high-resolution separation coupled with intelligent selection and multiplexing; SWATH: Sequential window acquisition of all theoretical mass spectra; PSA: Prostate specific antigen; proGRP: Progastrin releasing peptide; NSE: Neuron specific enolase; AGR2: Anterior gradient 2; TMPRSS2: Transmembrane protease serine 2; ERG: ETS related gene.