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Advances in targeted proteomics and applications to biomedical research

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Abstract

Targeted proteomics technique has emerged as a powerful protein quantification tool in systems biology, biomedical research, and increasing for clinical applications. The most widely used targeted proteomics approach, selected reaction monitoring (SRM), also known as multiple reaction monitoring (MRM), can be used for quantification of cellular signaling networks and preclinical verification of candidate protein biomarkers. As an extension to our previous review on advances in SRM sensitivity herein we review recent advances in the method and technology for further enhancing SRM sensitivity (from 2012 to present), and highlighting its broad biomedical applications in human bodily fluids, tissue and cell lines. Furthermore, we also review two recently introduced targeted proteomics approaches, parallel reaction monitoring (PRM) and data-independent acquisition (DIA) with targeted data extraction on fast scanning high-resolution accurate-mass (HR/AM) instruments. Such HR/AM targeted quantification with monitoring all target product ions addresses SRM limitations effectively in specificity and multiplexing; whereas when compared to SRM, PRM and DIA are still in the infancy with a limited number of applications. Thus, for HR/AM targeted quantification we focus our discussion on method development, data processing and analysis, and its advantages and limitations in targeted proteomics. Finally, general perspectives on the potential of achieving both high sensitivity and high sample throughput for large-scale quantification of hundreds of target proteins are discussed.

Keywords

Biomarker; DIA; PRISM; PRM; Signaling pathway; SRM; Technology

1 Introduction

Mass spectrometry (MS)-based proteomics is a promising technology in characterization of human proteome at a genome scale [1–3]. For example, MS-based global proteomics allows achieving genome-scale proteome coverage and quantitative changes of thousands of proteins and their posttranslational modifications (PTMs) in response to perturbation when

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combined with labeling strategies [4, 5]. It has been mature and routinely used in systems biology for measuring proteome changes during signal transduction [6, 7] and biomedical research for discovering novel candidate protein biomarkers [8, 9]. However, such global measurements generally lack quantification precision and have inherently poor reproducibility for low-abundance proteins due to the stochastic selection of precursor ions for MS/MS fragmentation by the data-dependent acquisition (DDA) mode [10, 11]. Thus, DDA-based measurements are not suitable for reliable validation of candidate protein biomarkers because most informative biomarkers are typically low abundance proteins and the human proteome has a wide dynamic range in concentrations (e.g., >10 orders of magnitude in human plasma or serum, where serum is similar to plasma in composition but exclude clotting factors of blood [12]).

With significant advances in MS instrumentation thousands of candidate protein biomarkers have been generated from proteomics studies [13, 14], but sadly none of them has been successfully translated into an FDA-approved clinical test [15, 16]. One important reason is the lack of robust protein quantification tools for enabling precise measurements of such numerous candidate protein biomarkers simultaneously and effectively in large sample sets (e.g., ~1000 patient samples). Traditional antibody-based ELISA has a limited multiplexing power in biomarker validation (in general measuring one or several protein biomarker at a time) and the antibodies for new biomarker proteins are often not available, especially for variant proteins and PTMs [17, 18]. Targeted proteomics has been emerging as a powerful protein quantification technology in terms of reproducibility, multiplexing capability and quantification accuracy [17,19,20].

We can readily group three types of targeted proteomics approaches: selected reaction monitoring (SRM), also known as multiple reaction monitoring (MRM) [17,21]; parallel reaction monitoring (PRM) [22,23]; and data-independent acquisition (DIA) combined with targeted data extraction of the MS/MS spectra (e.g., sequential windowed acquisition of all theoretical product ion mass spectra, termed SWATH [11]). SRM is a classic targeted proteomics approach performed on a triple quadrupole mass spectrometer (QqQ MS) [17]. It exploits the unique features of QqQ with two levels of mass selection (i.e., Q1 and Q3 isolation of precursor ions and their product ions, respectively) and a relatively long dwell time (in general 10 ms per pair of precursor/product ions) over a narrow *m/z* window (± 0.02 *m/z*) (Fig. 1A), which result in significantly improved selectivity and sensitivity, at least one to two orders of magnitude higher than full scan global proteomics analysis [17,21]. Thus, conceptually SRM is similar to Western blotting [24]. More recently, hybrid mass spectrometers with the substitution of the third quadrupole with a high-resolution and accurate-mass (HR/AM) mass analyzer, such as the quadrupole-Orbitrap (i.e., Q Exactive) [22,23] and quadrupole-time of flight (e.g., the ‘Triple-TOF’) MS [25] instruments, were developed and operated in the PRM mode for targeted MS quantification (Fig. 1A). In PRM, the isolation and fragmentation of peptide precursors are very similar to those in SRM. The only difference for PRM is that full MS/MS spectra are acquired for each precursor (i.e., the parallel detection of all product ions) in the HR/AM mass analyzer; while in SRM only predefined product ions (i.e., transitions) are monitored by the low-resolution quadrupole mass analyzer. Thus, in complex biological samples PRM can provide higher selectivity than SRM for a better discrimination of the product ion signal of target peptides from the co-

eluting interferences [26]. With the use of internal standards both SRM and PRM enable reproducible accurate quantification of target proteins across many samples (Fig. 1B). However, both of them suffer from the limitations in the scale of quantification (i.e., multiplexing capability), typically ~500 peptides/125 proteins per a single SRM [21] or PRM analysis [27] for reliable quantification (assuming that each protein has two surrogate peptides with light and heavy versions per peptide). Increasing the number of target peptides requires the tight control of peptide LC elution time for accommodating more transitions or peptides in a narrow time window. Furthermore, with the scale of quantification increasing, the sensitivity could drop significantly, especially for PRM whose sensitivity is inversely proportional to the degree of multiplexing [28].

To alleviate the limitations in multiplexing, DIA-based targeted quantification (e.g., SWATH [11]) was recently introduced for proteome-wide quantification of target proteins of interest. This method consists of high specificity DIA for generating product ion maps of all detectable precursors and targeted data analysis with the use of the SRM concept for data extraction [11]. In a DIA analysis, a set of wide precursor acquisition windows are predefined to cover the whole *m/z* range of proteolytic peptides. All peptides within a defined mass-to-charge (*m/z*) window are fragmented and MS records a complete high accuracy product ion spectrum for each detectable peptide (Fig. 1A). Thus, DIA provides highly multiplexed product ion spectra for all the detectable peptides over the LC elution time, and requires more elaborate data processing and interpretation. A few software tools have been developed (e.g., OpenSWATH [29]) but DIA data analysis is still challenging. DIA-based targeted quantification is performed using a targeted data extraction strategy based on the utilization of *a priori* information (e.g., peptide retention time and product ion intensity) from spectral libraries for confident identification of target peptides of interest in the DIA product ion map, as well as using the most intense product ions for peptide quantification [29,30]. Because of its unbiased, broad range of precursor ion selection and fragmentation, DIA-based targeted quantification could potentially lead to a paradigm shift in targeted proteomics from small-scale to proteome-wide quantification in complex samples with good reproducibility and high accuracy (Fig. 1B). But when compared to SRM or PRM with only focusing on a small number of predefined target peptides, DIA-based targeted quantification has somewhat lower sensitivity, specificity, and reproducibility because of the much shorter dwell time for each individual peptide, a wider precursor isolation window, as well as the lack of using internal standards to correct MS run-to-run variability [11]. For example, SRM was demonstrated to offer at least 10-fold higher sensitivity than DIA-based targeted quantification [11].

In this review we provide an overview of recent advances in targeted proteomics and its broad applications in human bodily fluids, tissue and cell lines, including (i) recent advances in SRM sensitivity and its applications to biomedical research and systems biology (from 2012 to present), which expand our previous review article in advancing SRM sensitivity (covering the time period of 2002 to late 2011) [17], (ii) new development in targeted MS/MS quantification on fast hybrid MS operated in the PRM mode, (iii) DIA-based targeted proteomics for proteome-wide quantification of target proteins, and (iv) future perspectives in targeted proteomics for reliable validation of candidate protein biomarkers in a high throughput manner.

2 Recent advances in SRM sensitivity and its application

In principle, MS sensitivity is governed by the ability to provide sufficient target analyte ions for the MS detection and the overall resolving power of MS mass analyzers for a specific measurement of analyte signal in the presence of background/interference. Thus, enhancing the MS-based targeted proteomics sensitivity can be built upon the following three aspects: frond-end (sample preparation, fractionation, and LC separations), interface (ion sampling and ion transmission) and back-end (the resolving power of the mass analyzer for removal of co-eluting interferences) [17]. Compared to other targeted proteomics platforms SRM has been well documented with demonstrated performance and robustness both within and across laboratories and instrument platforms [31–34]. Therefore, the implementation of the front-end methods to improve the targeted proteomics sensitivity has been primarily evaluated on the SRM platform. However, in general all the front-end methods are equally applicable with other targeted proteomics platforms.

2.1 Affinity enrichment

2.1.1 Antibody—Antibody-based immunoaffinity enrichment coupled to SRM (i.e., immuno-SRM) has emerged as a promising technology for sensitive precise quantification of target proteins in complex matrices [17]. For example, it was applied for quantification of mutant RAS (G12D) at LOD of 12 amol (i.e., 0.25 pg) or 240 amol/mg of total protein in patient tumors by anti-RAS protein antibody at the protein-level enrichment [35] and FGF15 at LOD of 0.1 ng/mL in mouse plasma by anti-peptide antibody at the peptide-level enrichment (i.e., SISCAPA, stable isotope standard and capture by the anti-peptide antibody) [36]. Recently, the development of immuno-SRM has been primarily focused on multiplexing [37], assay sensitivity [35, 38], and rapid cost-effective generation of antibodies [39, 40]. For example, the sequential enrichment of target peptides was used for increasing the multiplexing capability. After enrichment with one set of anti-peptide antibodies the next set of anti-peptide antibodies was immediately added to the same incubation plate and repeated in an identical manner for the sequential capture of the target peptides. Immuno-SRM assays allow concurrently measuring up to 50 peptides at a single step enrichment with the similar data quality as a 10-plex configuration with five groups of antibodies in a sequential fashion (e.g., peptide recovery, assay reproducibility and accuracy) but with slightly lower sensitivity [37]. Further increasing the levels of multiplexing has been recognized as challenging because nonspecifically bound matrix components, likely on the surface of the stationary media will be increased [37]. This can lead to decreasing the S/N ratio of target peptides and lowering SRM sensitivity. For rapid generation of anti-peptide antibodies at low cost, Whiteaker et al. have recently evaluated the enrichment efficiency of replacing traditional monoclonal or polyclonal antibodies with recombinant antibody fragments (Fabs) by spiking exogenous target peptides into human plasma [39]. They concluded that at the peptide level high-affinity Fab-based SRM provided similar sensitivity (LODs of 0.3-2.9 ng/mL in human plasma) as monoclonal antibody-based SRM with similar reproducibility (~10% of average coefficient of variation (CV) at the LOD for both methods) by evaluation of three proteins (i.e., osteopontin, E-selection, and ADAM17). This suggests that high-affinity recombinant Fabs can be applied in peptide enrichment immuno-SRM assays. In another study they have shown the feasibility of using multiplex

immuno-SRM assays that are originally developed for unmodified peptides to quantify the pharmacodynamics of their corresponding phosphorylated peptides because those anti-peptide antibodies can concurrently enrich those phosphorylated peptides and SRM can easily distinguish the phosphorylated and unmodified forms of a given peptide [40]. A 69-plex peptide-level immuno-SRM assay targeting the DNA damage response network can provide sensitivity at a median LOQ of 2.0 fmol/mg and reproducibility with a median interassay variability of 10% CV for measuring endogenous phosphopeptides. This shows the potential of using immuno-SRM for rapid and precise quantification of cell signaling networks.

To further increase immuno-SRM sensitivity, tandem affinity enrichment approaches have recently been employed for highly specific enrichment of target peptides to quantify extremely low-abundance proteins in human blood (Table 1). With the use of the two-step sequential enrichment method (e.g., isolation of human β-nerve growth factor (β-NGF) protein using anti-NGF antibody from patient serum, followed by peptide immunoaffinity capture of the predefined surrogate peptide from the digested β-NGF protein) to generate a highly enriched peptide sample [41], LC-SRM can reliably measure the total β-NGF at concentration levels of 7.03–450 pg/mL in human serum with <15% interassay CV. Such sensitivity is at least 10-fold higher than that provided by regular immuno-SRM with single antibody-based enrichment. The other tandem affinity enrichment approach was to combine immunoaffinity depletion (e.g., plasma albumin depletion to remove the most abundant albumin protein that accounts for ~50% of the total plasma protein mass [42] or IgY14 depletion [43] to remove the 14 high abundance proteins that account for ~95% of the total plasma protein mass) for reducing the dynamic range of protein concentration and anti-peptide antibody enrichment for effectively enriching target peptides. The combination of plasma albumin depletion and affinity enrichment of the unique epitope peptide from TnI by anti-TnI mAb-coated mircoparticles [42] was demonstrated to significantly improve SRM sensitivity for measuring TnI, a low abundant protein in human plasma. This method was demonstrated to have low ng/mL (i.e., ~4 ng/mL) sensitivity and high reproducibility within 6% CV. Another example is to quantify low-abundance serum transferrin receptor (sTfR) [43]. Coupling the IgY14 depletion to SRM can only reliably quantify sTfR at a concentration of ≥1000 ng/mL. However, with anti-peptide affinity enrichment, ~10-fold enhancement in SRM sensitivity can be achieved (i.e., the LOQ of 100 ng/mL for IgY14-SISCAPA-SRM).

Besides the affinity enrichment at the peptide level, mass spectrometric immuno assay (MSIA)-SRM [38], in which immunoenrichment is performed at the protein level on a monolithic microcolumn activated with an anti-protein antibody rather than magnetic beads and fixed in a pipette tip, was developed for sensitive multiplexed quantification of target proteins ranging in concentration from pg/mL to mg/mL (Table 1). When compared to other immunoenrichment methods, MSIA has less non-specific binding and is flexible with a wide range of sample volumes, thus MSIA allows rapid and quantitative enrichment of very low abundance (pg/mL) plasma proteins from a large volume (e.g., 1 mL) of human plasma. MSIA-SRM assays also demonstrated high sensitivity (within published clinical ranges for target proteins or peptides). Very recently MSIA-SRM assay was used for sensitive multiplexed quantification of several forms of circulating PCSK9 in human plasma [44]. The

average LOQ of MSIA-SRM is ~20.8 ng/mL for PCSK9 in human plasma, which is below the clinical range. This sensitive PCSK9 MSIA-SRM assay revealed novel relationships between PCSK9 and metabolic phenotypes.

2.1.2 Aptamer—Single-stranded oligonucleotide (ssDNA) termed "aptamer" can be used for molecular detection in many screening platforms (e.g., the SOMAscan assay [45]). Similar to antibodies, high-quality aptamers have a strong binding affinity to target proteins with a dissociation constant (K_d) of ~10^{-9}-10^{-10} and can form stable aptamer-protein complexes. With the aptamers serving as both folded protein-binding entities with defined shapes and unique nucleotide sequences recognizable by specific hybridization probes, the target protein concentrations can be reliably determined by measuring their corresponding aptamer concentrations using a quantitative DNA microarray. Thus, DNA aptamers have been widely used as affinity reagents for identifying and validating protein biomarkers in complex fluids [45–52]. However, the use of the aptamers to enrich the target proteins for SRM analysis has never been reported until recently. An aptamer enrichment technique coupled to SRM was developed for absolute quantification of activated ReIA in cytokine-stimulated eukaryotic cells [53]. The isolated aptamer P028F4 was demonstrated to have a strong binding affinity (K_d: 6.4×10^{-10}) with the activated form of ReIA. Compared to direct SRM quantification of ReIA and associated proteins from crude cell extracts, aptamer-based enrichment dramatically reduced the sample complexity and the co-eluting interference, and thus significantly improved the S/N ratio of endogenous ReIA (~36-fold enhancement), quantification accuracy and reproducibility [53]. ReIA aptamer-SRM assay provided quantitative estimates of the number of activated ReIA molecules within a cell, which was in good agreement with previous Western blot experiments but with higher accuracy. ReIA aptamer-SRM is the first example of coupling of aptamer enrichment of target proteins to SRM-based targeted quantification. Aptamer-SRM is a promising alternative to immuno-SRM for reliable quantification of low-abundance target proteins in complex protein mixtures because of the demonstrated higher multiplexing capability of aptamer assays.

2.1.3 Other enrichment strategies—Other enrichment strategies (e.g., subcellular fractionation [54] and protein precipitation [55]) have been incorporated into SRM workflow for reducing sample complexity to enhance SRM sensitivity. Following the sequential protein extraction, in which the nuclear proteins were extracted from the whole cell lysate, the resulting protein mixture was then separated by SDS-PAGE, and the transcription factor (TF) protein bands were excised from the gel, low-abundance TFs were significantly enriched. Regular LC-SRM enabled sensitive reproducible quantification of the enriched TFs at ~250 copies per nucleus in mammalian cells [54]. This sensitive SRM method has been used for absolute quantification of the dynamic change of TFs during cellular differentiation. A single gel electrophoresis (Ge)-based enrichment method has recently been used for enriching KRAS at a low molecular weight protein fraction prior to SRM analysis. GeLC-SRM was demonstrated for reproducible quantification of KRAS mutant variants (14% CV for processing replicates) in a panel of cancer cell lines [56]. Next it was applied for robust sensitive quantification of mutant KRAS in pancreatic benign and cancer subjects at concentrations of 0.08–1.1 fmol/μg protein (Table 1). Recently, protein

precipitation with acetonitrile was used to extract low mass proteins and to remove medium and high mass proteins from human blood. This step also precipitated the high mass and high abundance plasma albumin and immunoglobulins, and thus significantly reducing the sample complexity. The enriched low mass target proteins were then measured by SRM [55]. This simple, low-cost SRM method was demonstrated to reliably quantify five low mass biomarker proteins at a medium to low abundance (56.1–20 547.0 ng/mL) in 40 individual blood samples. Protein precipitation was also integrated into multistep sample purifications for highly efficient enrichment of target peptides. With the application of the combined plasma albumin depletion to remove the highest abundance albumin, acetonitrile protein precipitation to remove plasma proteins, and C18-SCX multi-StageTip to remove unnecessary peptides from pretreated plasma by using appropriate buffer solvents [57], low-abundance APL1β peptides, a surrogate marker for Alzheimer's disease, in human plasma were significantly enriched and can be sensitively quantified by SRM without immunoaffinity enrichment. The quantifiable absolute concentration of APL1β peptides was identified as several hundred amol/mL, which is probably the lowest detection level of endogenous plasma peptides as claimed by the authors.

2.2 High-resolution liquid chromatography separations

2.2.1 PRISM—Similar to antibody-based immunoassays, affinity reagent-based SRM has significant potential for high-throughput studies, but it shares similar limitations in terms of availability of affinity reagents for new proteins or PTMs and multiplexing capability [17]. To address this issue, we recently developed an antibody-free chromatography-based technique termed PRISM (short for high-pressure, high-resolution separations with intelligent selection and multiplexing) that effectively enriches target peptides for sensitive LC-SRM analysis [18]. In PRISM, high pH reversed-phase capillary LC (RP cLC) separation is used to fractionate a peptide mixture into either 96- or 384-fractions, thus enriching target peptides by isolating them into specific fractions (Fig. 2). Selection of target fractions of interest is accomplished by online SRM monitoring of heavy isotope-labeled internal standards during sample fractionation. The accurate elution profiles of the internal standards allow precise determination of the locations of target peptides in the 96- or 384-well plate because the light and heavy forms of individual peptides are chemically identical so they co-elute during PRISM fractionation. This enables selection of the most informative target fractions for downstream LC-SRM analysis. Furthermore, a limited number of target fractions eluted at different times during the first-dimension separation can be multiplexed to further increase the overall analytical throughput because the high-and low-pH RP cLC are partially orthogonal. Therefore, only a small number of target fractions need to be analyzed, which effectively addresses the concern on the analytical throughput.

Combined with IgY14 immunoaffinity depletion (i.e., the removal of the 14 most abundant proteins), PRISM-SRM enables accurate and reproducible quantification of plasma proteins at the 50–100 pg/mL range [18], and without depletion, in the sub-ng/mL to low ng/mL range [58]. Compared to regular LC-SRM analysis, PRISM provides ≥ 200-fold enhancement in SRM sensitivity. Since its development, PRISM-SRM has been serving as a key enabling technology for rapid preclinical verification of low-abundance protein biomarkers in human bodily fluids and tissues. For example, PRISM-SRM was applied to

reliably measure candidate biomarker AGR2 at low pg/mL levels in prostate cancer patient urine and ~100 pg/mL levels in human serum when combined with the IgY14 depletion [59]. We also applied PRISM-SRM to measure TMPRSS2-ERG fusion protein products in prostate cancer cell lines and tumors [60]. PRISM-SRM enabled to confidently detect six unique ERG peptides in both TMPRSS2-ERG positive cell lines and tissues with the LOQ ranged from 2 to 50 amol/μg of total protein. Systematic comparison of three analytical platforms, ELISA, Western blotting, and PRISM assays, has shown that PRISM-SRM having a 20 pg detection limit is the most sensitive among the three platforms [61]. Very recently, we also applied PRISM-SRM for direct, site-specific quantification of the dynamics of PTMs (e.g., ERK phosphorylation dynamics and stoichiometry) [62]. PRISM-SRM was demonstrated to provide a level of sensitivity of ~1000 molecules per cell in quantifying ERK phosphorylation from as little as 25 μg tryptic peptides. Besides our broad applications PRISM-SRM was also applied by others to precisely quantify the total Aβ level in human plasma for avoiding the underestimation of Aβ by antibody-based assays [63]. This is the first study for measuring the plasma Aβ level by LC-SRM without antibody-based affinity enrichment.

Besides its application in PRISM fractionation, high pH reversed phase separation was also used to generate a limited number of fractions (e.g., 13 fractions) with pooling neighboring LC fractions that contained the same target peptides [64]. The neighboring fraction pooling enhances overall SRM signal but with the compromise of concurrently increasing the potential co-eluting interferences because the high-resolution reversed phase separation was not fully utilized. The partial orthogonality between the high pH and low pH RP separations only originates from the utilization of the difference in the ionic nature of peptides. Thus, 2D RPLC-SRM is very similar to SCX-RPLC-SRM because in both cases a limited number of continuous fractions were collected for LC-SRM analysis and the overall orthogonality between 2D RPLC and SCX-RP is similar [65–67]. 2D RPLC-SRM was demonstrated for enabling quantification of 31 proteins below 10 ng/mL and 41 proteins above 10 ng/mL [64] (Table 1).

Another example of using two dimensional LC to enhance SRM sensitivity is interfacing RP and hydrophilic interaction LC (HILIC) separations through an anionic cartridge [68]. RPHILIC-SRM was demonstrated for enabling quantification of PSA at 1 ng/mL level without any front-end immunoaffinity depletion. However, this 2D-LC-SRM method has two limitations, lacking multiplexing power (i.e., one target peptide at a time) and effectively trapping the selected target peptide on the anion exchange cartridge often requiring optimization of the buffer condition.

2.2.2 Long gradient (LG) separations—As an alternative to multidimensional sample fractionation strategies we recently evaluated the performance of the long-gradient separations coupled with SRM (LG-SRM) for targeted protein quantification [69]. Direct comparison of LG-SRM (5 h gradient) and regular LC-SRM (45 min gradient) showed that the long-gradient separations significantly reduced background interference levels and provided an 8- to 100-fold improvement in LOQ for target proteins in human female serum. LG-SRM enables reliable quantification of plasma proteins at ~10 ng/mL levels in non-depleted human plasma, which is comparable to most low-resolution fractionation-based

SRM methods in the overall sensitivity [17, 65]. Later a similar study for systematic evaluation of LG-SRM performance has also been conducted by Carr's group [70]. They concluded that LG-SRM could provide low ng/mL LOQs for proteins in the IgY14 depleted plasma when the optimal LC conditions were used. Such sensitivity is very consistent with our results of ~10 ng/mL LOQs in non-depleted human plasma because the IgY14 depletion resulted in removing the 14 most abundant proteins in human plasma [69] that constitute 90–95% of the total protein mass (i.e., 10-fold sample enrichment) [71]. Besides the improved sensitivity, LG-SRM potentially offers much higher multiplexing capacity than conventional LC-SRM due to an increase in average peak widths (~3-fold) for a 300-min gradient compared to a 45-min gradient [69]. This allows a QqQ to use ~3 times longer duty cycle for monitoring more transitions (i.e., ~3 times more proteins) in a given elution window without affecting the quality of quantification (e.g., the sufficient data points across the LC peak). Furthermore, when compared to LC fractionation-based SRM, LG-SRM has several advantages in terms of sample throughput (one analysis per sample without multiple fraction runs), minute amounts of starting materials (e.g., ~4 μg), simple implementation, and easy operation [69,70].

2.3 Broad applications in biomedical research and systems biology

With the recent advances in SRM technology, front-end sample preparation, and bioinformatics tools (e.g., Skyline [30], QuaSAR [72], and Qualis-SIS [73]), SRM-based targeted proteomics technique is nearly mature for routinely reliable quantification of 10–100 s of target proteins in complex biological samples [17, 19]. In recent years studies for systematic evaluation of SRM assays and their performance have been conducted, such as developing a system suitability protocol for quantitative assessment of intra- and inter-laboratory instrument performance in LC-SRM analysis [34, 74], large-scale development of standardized SRM assays [33, 75], inter-laboratory evaluation of SISCAPA-SRM [32], and defining three tiers of SRM assays using a “fit-for-purpose” approach [76]. In 2012 targeted proteomics was selected as Method of the Year in Nature Methods [77]. SRM-based targeted proteomics merits have been well documented in multiple reviews [17, 19, 20, 78] and commentary reports [24, 79, 80]. SRM has been broadly applied in systems biology and biomedical research fields for reliable protein quantification. Compared to antibody-based assays SRM assays have several advantages in terms of multiplexing, detection specificity, and the assay development time and success rate for new target proteins [17, 80]. Thus, it may be time to “turn the tables” in new target protein quantification with the more frequent use of SRM assays [24] because it is considerably easier to develop reliable SRM assay with high specificity [17, 81, 82] and SRM assay has been proved to have an excellent correlations with analytically validated ELISA [18,59,83–85].

2.3.1 Biomedical research—As SRM is mature with distinct advantages over antibody-based assay, in recent years a number of papers have been published on verification of protein biomarkers with SRM in different types of diseases (e.g., prostate cancer [59–61, [86]], lung cancer [87–92], colorectal cancer [93–95], pancreatic cancer [96,97], cardiovascular disease [84], Parkinson disease [98–101], Down syndrome [102–104], type 1 diabetes [8], and other rare diseases [105–109]) (Table 2). The candidate protein biomarkers are generated either from comparative proteomics (or genomics) discovery studies or from

previous literature reports. These candidate protein biomarkers are then measured in a medium or large matched set of clinical cohorts. For example, to identify lung cancer biomarkers in blood that are shed or secreted from lung tumor cells, comparative proteomics studies of protein expression levels between lung cancer tumor cells and normal lung cells have been conducted [91]. In combination with literature-resource biomarkers 388 protein candidates were selected for validation and performance assessment. SRM assays were applied in a three-site discovery study ($n = 143$) on plasma samples from patients with benign and stage IA lung cancer, producing a 13-protein classifier. The classifier was validated on an independent set of plasma samples ($n = 104$), exhibiting a negative predictive value of 90%. It provides a new metric, independent of current diagnostic risk factors, for assessing the molecular status of a lung nodule. Furthermore, to expedite SRM-based protein biomarker verification, SRM assays for most cancer-associated proteins were recently developed, and their sensitivity, reproducibility, quantitation dynamic range, and detectability were systematically evaluated in human bodily fluids (e.g., human plasma/serum [110–112], urine [110], CSF [113]). For example, SRM assays of more than 1000 cancer-associated proteins were generated and then used to determine the detectability of those proteins in human plasma and urine [110]. In depleted human plasma, 182 proteins were detected, spanning five orders of magnitude in abundance and reaching below a concentration of 10 ng/mL, whereas in human urine 408 proteins were confidently detected due to the narrower concentration range of proteins in urine.

Due to the lack of specificity to recognize individual protein isoforms, antibody-based assays can only be used to differentially measure certain protein isoforms [17]. SRM assays have been demonstrated to be well suited for site-specific quantification protein isoforms or PTMs. Isoform-specific SRM assays were recently developed for reliably quantifying protein isoforms in many studies (e.g., 14 UGT1As and UGT2Bs in liver matrices [114], three polyphenol oxidase isoforms in loquat fruits [115], 3 Apolipoprotein E (ApoE) isoforms that differ in only one or two amino acids in cerebrospinal fluid and plasma samples [116], and TMPRSS2-ERG fusion protein products in prostate cancer [60]). Two SAA isoforms, SAA1 and SAA2 that have high similarity (92%) amino acid sequences can be accurately measured by SRM assays for the first time [89]. Verification of SAA1 and SAA2 in clinical crude serum samples indicated that SAA2 could be a good biomarker for the detection of lung cancer. Another two elegant studies are to design SRM assays for identification of a novel PSA isoform coded by SNP-L132I (rs2003783) in clinical prostate samples [117], and to use de novo sequencing for unambiguous characterization of two VTG isoforms, Dc-VTG1 and Dc-VTG2, followed by SRM quantification of the two isoforms [118]. Plasma levels of Dc-VTG1 and Dc-VTG2 were found to be decreased as the nesting season proceeded, and were closely related to the increased levels of reproductive effort. In addition, several studies were reported for using PTM SRM assays for site-specific quantification of PTMs, such as selective measurement of free monoubiquitin (a potential neurodegenerative disease biomarker) in cerebrospinal fluid [101], and evaluation of a large set of candidate serum biomarker glycopeptides for malignant pleural mesothelioma (MPM), resulting in a seven glycopeptide signature with diagnostic potential for MPM [108].

2.3.2 Systems biology—Besides application in biomarker studies, SRM has also been used for accurate quantification of the dynamics of signaling pathways or networks in response to a given perturbation to improve our understanding of molecular mechanisms of signal transduction [7, 119–129]. Using the combination of affinity purification (AP) and quantitative SRM temporal regulation of EGF signaling networks by the scaffold protein Shc1 was investigated in detail, resulting in a significant finding that the Shc1 directs the temporal flow of signaling information after EGF stimulation [7]. AP-SRM has been widely used for measuring the concentrations of protein components including PTMs in target protein complexes. For example, it was used to quantify the dynamics of protein interaction networks in pre-60S particles after nuclear export [130]. From the dynamic data assembly factors were found to travel with pre-60S particles to the cytoplasm and a novel shuttling factor that facilitates nuclear export of pre-60S particles was identified. Other types of affinity enrichment combined with SRM were employed for comprehensively monitoring signal transduction pathway dynamics. For example, IMAC-SRM was used to measure phosphorylation dynamics of the PI3K-mTOR/MAPK signaling networks in oncogene-induced senescence [120].

Besides using SRM to measure the concentrations of a set of enriched proteins SRM is more broadly used for accurate quantification of the dynamics of specifically selected proteins (e.g., 76 proteins in the energy metabolic pathways and more than 80 cancer signaling proteins [119]) involved in the specific signal transduction pathways. 144 proteins involved in the insulin-signaling pathway and central metabolism in mouse liver homogenates were selected and measured by SRM across 36 samples (12 conditions and three replicates for each condition) [119]. The nearly complete quantitative pathway data revealed strain-specific changes in the mouse insulin and central metabolic pathways after a sustained high-fat diet. Very recently, SRM was combined with RNA-seq and rule-based pathway modeling to quantitatively explore the chemotaxis signaling pathway mediated by sphingosine-1-phosphate [126]. Using absolute protein concentrations measured by SRM as input parameters of a mathematic model, the resulting *in silico* pathway behavior matched experimental measurements. This finding demonstrates the feasibility and value of combining SRM with pathway modeling for advancing biological insight.

In addition, SRM was also applied in other systems biology studies, such as systematic measurement of transcription factor-DNA interactions [131], global kinetic analysis of proteolysis [132], and a sentinel protein assay for simultaneously quantifying cellular processes [133]. SRM assays of sentinel proteins (i.e., biological markers) were demonstrated for enabling simultaneously probed 188 biological processes in *Saccharomyces cerevisiae* under a set of environmental perturbations. Another elegant study is global analysis of protein structural changes in complex proteomes by an approach of coupling limited proteolysis with SRM [134]. This approach potentially enables probing both subtle and pronounced structural changes of proteins on a large scale.

3 New developments in other types of targeted MS/MS quantification and their applications

3.1 PRM

With recent introduction of fast data acquisition HR/AM MS (e.g., Q Exactive quadrupole Orbitrap (QqOrbi) [22, 23], and TripleTOF quadrupole TOF (QqTOF) [11, 25]), the third quadrupole is replaced by a HR/AM Orbitrap or TOF mass analyzer (Fig. 1A). Targeted MS/MS, when operated in PRM mode [11, 22, 23, 25], can be used for reliable quantification of target proteins in complex matrices. PRM was demonstrated to have similar analytical performance as SRM in sensitivity, reproducibility, accuracy, and dynamic range [22, 23, 135–137]. In the urine samples with 35 peptides spiked-in, PRM has shown to provide lower LOQs for 61% of 175 transitions than SRM and 20% of the transitions have similar LOQs between PRM and SRM [135]. For synthetic peptides spiked in yeast cell lysates PRM yielded quantitative data over a wider dynamic range than SRM [22]. Compared to SRM the analytical workflow of PRM is simplified without preselecting/optimizing target peptide transitions because it allows for detection of all product ions simultaneously [22,23]. Another advantage of PRM over SRM is the improved selectivity (or specificity) from the HR/AM quantification by discriminating target peptides more effectively from background interferences, which directly translates into the increased assay and data quality [22, 23]. With the use of trapping device in QqOrbi MS or longer accumulation time in QqTOF MS, PRM allows improving the S/N ratio (i.e., sensitivity) and thus lowering the LOQ values for detecting low-abundance target peptides [23, 27] but at the expense of duty cycle (i.e., multiplexing). For example, Majovsky et al. developed PRM assays on lower scan speed LTQ-Orbitrap Velos MS for quantification of protein degradation in plant signaling and concluded that the instrument duty cycle was a critical parameter limiting sensitivity and thus only a small number of proteins can be reliably quantified [138]. In addition, comparisons of PRM in QqTOF with SRM using six model peptides indicated that increasing the accumulation time in PRM was required to match the performance of SRM [139].

3.1.1 Multiplexing—For measuring limited peptide sets (typically 50 peptides or less) in complex matrices PRM was demonstrated to have high assay performance in terms of sensitivity and reproducibility [23,27]. However, similar to SRM it is still challenging for applying PRM to measure a large number of peptides without sacrificing the sensitivity and reproducibility [23,27]. The different PRM acquisition methods in QqOrbi MS (simplex, broadband, and multiplex) [23, 28] have been discussed in details (Fig. 3). In principle, the number of peptides that can be analyzed in one PRM run is determined by the cycle time, the transient time (the MS resolution), and the degree of multiplexing [23,28,140]. The cycle time is determined by the peak width of peptide LC elution (e.g., 8–10 data points across peptide peak for achieving precise quantification). The transient time (i.e., the resolving power) is directly correlated to the number of peptides to be monitored. The defined parameters determine the number of peptides measured in one cycle (i.e., the number of peptides = [cycle time × multiplexing degree]/transient time) [23, 28]. The PRM sensitivity is affected by the length of fill time [23,28,140]. The maximum fill time is generally adjusted according to the transient time and the multiplexing degree to ensure isolation and

fragmentation of a given peptide precursor ion concomitantly in the Orbitrap analysis (i.e., maximum fill time = [transient time / multiplexing degree]) [23, 28]. Thus, when the same fill time is used for both simplex and multiplex PRM the multiplex PRM could have similar sensitivity to the simplex PRM. By using multiplexing degree of 4, a resolving power of 35 000 at *m/z* 200 operated on the Q Exactive or Q Exactive Plus platform (i.e., 128 ms of transient time), and 2 s of cycle time, up to 64 peptides can be analyzed in one cycle but with compromising the fill time of 30 ms for each peptide precursor ion [28]. Thus, 4-plex PRM could have the similar multiplexing power as SRM, in which ~66 peptides can be simultaneously monitored in one 2 s cycle (i.e., the number of peptides ≈ [2000 ms / 30 ms]) when three transitions per peptide and the standard dwell time (10 ms per transition) are used. However, 4-plex PRM provides full MS/MS spectra with all the fragment ions while SRM only monitors the selected three fragment ions for the precursor ion. With the implementation of the new generation Q Exactive HF platform, which has nearly two times faster in scan speed [141], up to 128 peptides can be measured in one cycle under the same condition.

For a given number of peptides different combinations of parameters could lead to a trade-off between specificity (i.e., the transient time and quadrupole isolation window) and sensitivity (i.e., the fill time) [28]. Thus, large-scale quantification with PRM requires the adjustment of MS acquisition parameters, which dramatically affects the assay and data quality (e.g., sensitivity, accuracy, and reproducibility). Very recently, a new data acquisition method, internal standard triggered-PRM (i.e., IS-PRM) [27] was designed to maximize the efficient use of the instrument time for sensitive quantification of endogenous peptides. The data acquisition scheme switches between two PRM modes: a fast low-resolution “watch mode” for scanning heavy internal standards (e.g., the maximum fill time of 60 ms) and a slow high-resolution “quantitative mode” for scanning endogenous peptides (e.g., the maximum fill time of 360 ms). IS-PRM was demonstrated for achieving both the number of endogenous peptides and the quantification sensitivity. Application of IS-PRM to the analysis of large peptide sets (up to 600) in complex samples has shown an unprecedented combination of scale and analytical performance, with the LOQs at the low amol range [27]. However, this method involves the front truncation of the PRM signal and sophisticated MS software controls to trigger endogenous peptide measurement, which could affect the quantification accuracy and reliability. Thus, at present the broad utility of IS-PRM is not clear in the targeted proteomics field.

3.1.2 Application—Unlike SRM, the application of PRM in targeted quantification is still in its infancy, though PRM method development has been well documented in several reviews [26, 28, 140, 142, 143] and the data analysis of PRM is very similar to that of SRM [30, 72, 73]. In the past three years there are only a handful of reports of applying PRM for target protein quantification. Multiplexed PRM assays were recently developed for isotype-specific SAA1 (1α, 1β, 1γ) and SAA2 (2α, 2β) variants in human plasma and successfully applied to analyze plasma samples from lung cancer patients [88]. The high data quality from PRM assays allows interference-free quantification of all variants in large clinical cohorts of lung cancer patient plasma. The combination of immunoaffinity purification and PRM (i.e., immuno-PRM) allows fast screening driver mutations in tissue and tumor

markers in human plasma [144]. Very recently, Thomas et al. developed multiplexed PRM assays for quantification of 43 N-linked glycosite-containing peptides in prostate patient sera [86]. A total 41 N-linked glycosite-containing peptides (corresponding to 37 proteins) were reproducibly quantified with four proteins showing differential significance between nonaggressive and aggressive prostate cancer patient sera. Kim et al. developed and implemented multiplexed PRM assays for quantitative profiling 83 protein tyrosine kinases in human cancer cell lines [145]. The PRM assays enabled to detect 308 proteotypic peptides from 54 receptor tyrosine kinases and 29 nonreceptor tyrosine kinases in a single run. In addition, PRM was also used for sensitive quantification of protein modifications (e.g., histone lysine acetylation and methylation [146, 147], and polyubiquitin chains [148]).

3.2 DIA for proteome-wide quantification

Currently the top choice for protein targeted quantification is still SRM primarily because it has matured for the past two decades [21]. However, SRM quantification involves front-end assay development (e.g., peptide selection, transition and collision energy optimization, and interference-free transition selection, etc.) [19, 21]. The recently introduced PRM has partially alleviated this issue with more simplified analytical workflow. However, the scale of SRM/PRM quantification is limited, typically to several hundred of peptides per a single run for reliable quantification [21, 23]. Increasing the number of target peptides results in a significant trade-off in quantification sensitivity [27]. Often times, additional experiments are needed for achieving both the scale of quantification and sensitivity.

3.2.1 DIA method development—Data-independent acquisition (DIA) is an emerging MS technique to combine advantages of shotgun (i.e., large-scale based on DDA) and targeted proteomics (i.e., high reproducibility and accuracy) [11, 149, 150]. The data creation of DIA is more flexible and simpler compared to DDA or SRM/PRM experiments. DIA collects all MS/MS scans irrespective of precursor ion selections from a survey scan or full MS scan, in which DDA necessitates. The predefinition of target lists, which SRM/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 inclusive proteome-wide quantification using targeted data extraction strategy. However, when compared to SRM/PRM DIA-based targeted method in general provides lower sensitivity, specificity, and reproducibility as well as smaller dynamic range in protein quantification [11].

The term DIA was introduced by Venable et al. using LTQ-linear ion trap (LIT) mass spectrometer [151]. They used wide precursor isolation window (10 *m/z*) to perform sequential isolation and fragmentation of a predefined *m/z* range. Compared to the MS level quantification, the S/N ratio was greatly improved (higher than 350%) with a good linear dynamic range. They also demonstrated the applicability of ion extractions in MS/MS level of DIA quantification. However, such low resolution DIA MS/MS with the wide precursor isolation window decreased the mass accuracy and the confidence in peptide identification, which potentially resulted in increasing the false positive discovery rate. Since then, other modified DIA have been introduced. In 2005, MS^E was introduced by Silva et al. and operated on QqTOF instrument [152]. Precursor acquisition independent from ion count

(PAcIFIC) [153,154] was a modified DIA version, and initially performed on LIT MS [153] and later on a faster LTQ Velos MS [153]. The use of smaller isolation windows (2.5u) led to the improvement of protein identification though the overall data acquisition covering the whole target mass range required multiple injections (67 injections for 5 days). A much faster scanning ion trap MS (e.g., LTQ Orbitrap Velos MS) reduced the whole data acquisition time to ~2 days. In 2010, with an introduction of a bench top Exactive MS, Geiger et al. demonstrated the application of all-ion fragmentation (AIF) [155], in which peptides were injected to HCD collision cell for fragmentation without precursor selection and the fragments were returned back to C-trap and analyzed through Orbitrap mass analyzer. The assignment of fragment ions to co-eluting precursor ions was facilitated by high resolution (100 000 at *m/z* 200) and high mass accuracy. This concept significantly decreases the duty cycle time, but introduces more interferences from AIF at a certain retention time. In the same year, another DIA approach was introduced by Carvalho *et al.*, namely extended data-independent acquisition (XDIA) [156], which was performed on a different type of Orbitrap MS with the capability of electron transfer dissociation (ETD). When compared to DDA-based ETD analysis, the DIA-based ETD approach significantly increased the number of identified spectra (~250%) and the number of unique peptides (~30%), which could potentially facilitate the low-abundance PTM study. However, it is worth noting that ETD was performed on a low-resolution ion trap, and thus there are some challenges in differentiating isobaric peptides. FT-all reaction monitoring (FT-ARM) developed by Weisbrod et al. is to fragment all ions within a broad *m/z* window (100 *m/z*) [157]. Different types of fragmentation were applied (e.g., CID or infrared multiphoton dissociation).

Significant improvement in DIA has been achieved along with development of fast scanning HR/AM instruments for the past 3 years. Gillet et al. introduced a variation of DIA using QqTOF MS, termed SWATH, to conceptually refer the utilization of a broad isolation window (typically 25 *m/z*) consisting of multiplexed spectra [11]. One key feature of using QqTOF MS is the fastest data acquisition rate. Another DIA strategy was introduced by Egertson et al. with the use of QqOrbi MS, in which a novel acquisition method, namely MSX [158], was incorporated to improve the instrumental speed, selectivity, and sensitivity. The isolation window was set to 4 *m/z* covering a mass range from 500 to 900 *m/z*. Among those 100 windows (400 *m/z* divided by 4 *m/z*), five random windows were selected, stored in C-trap, and transferred to Orbitrap analyzer. The resultant spectra are multiplexed MS/MS of the selected windows [158]. However, more intelligent data processing was required to de-multiplex spectra for reliable identification and quantification of peptides. Two more recent improvements in DIA data acquisition and processing were aided by newer version of Orbitrap MS instruments. The first one termed *p*SMART, which was implemented on Q Exactive MS, utilized asymmetric isolation windows over the mass range: 5 Da window covering 400~800 *m/z*, 10 Da window covering 800–1000 *m/z*, and 20 Da window covering 1000–1200 *m/z* [159]. In another DIA method termed wide selected-ion monitoring DIA (wiSIM-DIA) [160], which was implemented on the new hybrid Q-HCD-Orbitrap-LIT MS (i.e., Orbitrap Fusion and Lumos), three HR/AM SIM scans with 200 Da isolation windows were used to cover all precursor ions of 400–1000 *m/z*. In parallel with each SIM scan, 17 sequential ion-trap MS/MS with 12-Da isolation windows were acquired to cover the

associated 200-Da SIM mass range. Different from the standard wide-window MS/MS-only DIA methods, for *p*SMART and wiSIM-DIA the MS1 data (i.e., HR/AM precursor data with a longer fill time and enough precursor ion data points across the LC elution profile) is used for sensitive detection and quantification, while the MS/MS data from the fast ion trap MS/MS scan is used only for peptide identification/confirmation. Compared to the standard DIA methods with a complete recording of all fragment ions of the detectable peptide precursors but with sophisticated data analysis, *p*SMART and wiSIM-DIA can only provide a smaller number of MS/MS spectra for the detectable precursors with relatively easier data analysis because the majority of the duty cycle time is used for generating the high-quality MS1 data [159, 160]. Thus, their sensitivity and precision are higher than those provided by the standard DIA methods, while their quantification accuracy (i.e., specificity or selectivity) could be somewhat lower than that from the standard DIA methods due to the increased chances for having co-eluting interference from MS1 than MS/MS. Very recently, Bruderer et al. introduced a novel DIA method termed hyper reaction monitoring (HRM) [161]. This consists of comprehensive DIA acquisition on a Q Exactive MS platform and target data analysis with retention-time-normalized (iRT) spectral libraries. HRM was demonstrated to outperform shotgun proteomics both in the number of consistently identified peptides and in reliable quantification of different abundant proteins across multiple measurements.

3.2.2 Bioinformatics tools for DIA data analysis—DIA spectra are highly multiplexed and thus more elaborate data interpretation algorithm is needed compared to DDA or SRM/PRM (reviewed in [162]). Currently, there are three approaches that are used to decipher DIA spectra. The first one is to construct pseudo-DDA spectra from DIA spectra, such as Demux [163], MaxQuant [155], XDIA processor [156, 164, 165], and Complementary Finder [166]. Those reconstructed pseudo-DDA spectra are then processed through conventional search engine tools such as MSGF+ [167], MaxQuant [168], MASCOT [169], or other spectra libraries [170, 171]. Some of the schemes implemented the use of chromatographic elution profiles to improve identification of peptides [155, 165, 172]. The recent publication by Tsou et al. described the development of a computational approach, termed DIA-Umpire [172]. DIA-Umpire starts with two dimensional (*m/z* and retention time) feature-detection algorithm to discover all possible precursor and fragment ion signals in MS and MS/MS data. Fragment ions are grouped with a precursor ion that has a correlation of LC elution peak and retention time at a peak apex. Generated pseudo-MS/MS spectra for each precursor-fragment group are then processed with conventional database search engine including the abovementioned tools. The other approach is to match multiplexed MS/MS to theoretical spectra of peptides (e.g., ProbIDtree [173], Ion Accounting [174], M-SPLIT [175], MixDB [176], and FT-ARM [157]). The scoring algorithms are directly based on how many theoretical fragment ions of a peptide from sequence databases or spectral libraries are found on multiplexed spectra with a high mass accuracy. The first two approaches have greatly tackled identification of peptides from DIA spectra prior to further quantification. Freely-available automated or semi-automated tools such as Skyline [30,177], mProphet [178], OpenSWATH [29], and DI-ANA [179] and two commercial software, PeakView® from AB/Sciex and Spectronaut™ from Biognosys, have been employed to process quantitative targeted DIA data using the targeted data extraction

strategy [11, 29, 158, 177]. However, more powerful computational tools are still needed for more effective analysis of DIA data.

3.2.3 Recent applications of DIA-based targeted quantification—DIA-based targeted proteomics provides SRM-like reproducibility, linearity, and accuracy while the sensitivity is still less than SRM [133,149,180]. Gillet et al. showed an excellent linear correlation of fold changes between SRM and SWATH with $R^2 \geq 0.95$, and a linear dynamic range of 4 orders of magnitude for SWATH [11]. Compared to DDA the quantification sensitivity was increased by 2–8 fold but still ~10 fold lower than SRM/PRM. Egertson et al. applied MSX strategy to increase the selectivity of precursors, showing LOD of 0.41 fmol with $R^2 \geq 0.95$ [158]. Compared to SRM/PRM the major advantage of DIA-based targeted quantification is the proteome-scale of quantification and thousands of proteins can be quantified simultaneously [149, 150]. Another advantage is that one-time DIA data procurement can serve as both protein identification and quantitation, which should accelerate the biomarker discovery process [149,150].

Recently targeted DIA approaches were applied for large-scale protein quantification. When combined with pressure cycling technology SWATH-MS allows detection and quantification of more than 2000 proteins with a high degree of reproducibility across 18 small biopsy samples from 9 patients [181]. Selevsek et al. demonstrated the reproducibility and consistency for quantification of more than 15 000 peptides and 2500 proteins of *Saccharomyces cerevisiae* using SWATH-MS [182]. They completed measuring proteins for 18 osmotic shock time course samples in less than 3 days. With the use of affinity purification for enriching protein complexes, targeted DIA has been applied for efficient quantification of large-scale proteome dynamic changes (e.g., interactomes of the 14-3-3 system [183] and the HSP90 inhibitor NVP-AUY922 or melanoma-associated mutations in the human kinase CDK4 [184]). In combination with solid phase extraction of glycopeptides (SPEG) Liu et al. applied SWATH MS for glycoproteomic analysis of prostate cancer tissues [185]. This approach was validated in human plasma with the LOQ of 5 ng/mL and a dynamic range of 4 orders of magnitude [180]. Quantitative glycoproteomics analysis showed that 220 glycoproteins appeared to be significant associated with prostate cancer aggressiveness and metastasis. Another example is the application of targeted DIA to investigate the effects of the histone deacetylase inhibitor SAHA (suberoylanilide hydroxamic acid) on the global histone PTM state of MCF7 cells [186]. A total of 62 unique histone PTMs were quantified accounting for less than 0.01% of a total peptides, which was also supported by another histone PTM study with SWATH MS (i.e., remarkably low abundance of hi-stone PTMs (H3K9me2S10ph, relative abundance <0.02%) in mouse trophoblast stem cells [187]).

4 Future perspectives

Targeted proteomics has become an indispensable tool for cost-effective and reliable measurement of hundreds of candidate protein biomarkers or signaling pathway proteins across many samples in term of specificity, reproducibility, multiplexing capability, and being independent of affinity reagents [17,19,149,188]. However, the major constraint of current target proteomics approaches is the lack of sufficient sensitivity for measuring low-

abundance proteins in complex biological matrices. Enhancing the sensitivity is often accomplished by the front-end sample enrichment or fractionation followed by subsequent LC separation (a typical 60 min run time) to increase analyte concentration and reduce sample complexity but with significantly sacrificing sample throughput (see [17]). Thus, it is a formidable challenge in time and cost for validation of multiple low-abundance protein biomarkers in large clinical cohorts (e.g., only two publications were found to use SRM to measure more than 1000 patient samples [189,190]) with the current targeted proteomics platforms.

Recent advances in coupling SISCAPA enrichment to RapidFire SPE-SRM [191] or MALDI-TOF MS [192, 193] significantly improve sample throughput with an average ~7 s per sample for RapidFire SPE-SRM and ~30 s per sample for MALDI-TOF MS. It could be practical for measuring ~1000 clinical samples per day if all components including sample processing (e.g., trypsin digestion and SISCAPA enrichment), MS measurement, and data analysis are effectively co-ordinated. However, quantification accuracy and assay reproducibility from the two SISCAPA-based methods primarily rely on peptide antibody quality (i.e., the purity of enriched target peptides) because there are no additional separation steps that are used to further remove co-eluting interferences. This one-step SISCAPA enrichment without additional separation also limits the multiplexing power (e.g., less than ten target peptides with monitoring only one transition per peptide [191]) and lowers the detection sensitivity (e.g., 100-fold less sensitive for SISCAPA-MALDI-TOF MS when compared to SISCAPA-LC-SRM [191]). Furthermore, SISCAPA-based targeted quantification shares the similar shortcomings as the antibody-based immunoassays with the difficulty of generating high-quality antibodies and a high failure rate.

To achieve high-throughput sensitive measurement of hundreds of target proteins we believe that advancing MS instrumentation is probably the most effective way to improve both sample throughput and MS sensitivity because any additional front-end sample handling will enhance MS sensitivity but at the expense of overall sample throughput [17]. This can be achieved by implementing advanced MS interface technologies (e.g., gas-phase ion mobility separations in long-path structures for lossless ion manipulation separation, termed SLIM [194–196] and the use of cutting-edge ion sources [197]), as well as increasing the resolving power of mass analyzer [23, 137]. For example, in the PRM mode HR/AM mass analyzer was demonstrated to provide high specificity and sensitivity in targeted quantification [23, 137]. A combination of high-resolution LC and gas-phase SLIM IMS separations could lead to achieve extremely high peak capacity because the two separation methods are fully orthogonal and each separation method should have at least 100 s of peak capacity in a short separation time (e.g., ~150 of peak capacities in less than 10 min LC separation [198]). Thus, LC×SLIM IMS may have great potential to achieve full-baseline separation of target peptides from complex biological matrices in a high-throughput manner without the development of specific antibodies for target proteins or peptides. It is very optimistic that in the near future ~1000 clinical samples with 100 s of target proteins can be analyzed by advanced targeted MS within a week. We expect that the new generation MS will revolutionize current targeted MS quantification and be more broadly and readily adopted in many biomedical and clinical laboratories.

5 Concluding remarks

Targeted proteomics has emerged as a powerful protein quantification tool for reliably measuring target protein concentrations across many biological or clinical samples in a consistent, reproducible, and accurate manner. SRM is a classic targeted proteomics approach and has matured over the past two decades. It has been widely applied for accurate quantification of cellular signaling networks or pathways and pre-clinical verification of candidate protein biomarkers. With recent advances in enhancing SRM sensitivity (e.g., PRISM-SRM [18, 58]), SRM enables quantification of target proteins in human plasma/serum at 50–100 pg/mL levels [18] and in human cells at ≤100 copies per cell [199]. Such sensitivity is comparable to or even better than that provided by many analytically validated ELISA. This could bring immediate impact on systems biology and biomedical research because the new informative protein biomarkers or feedback regulatory proteins in signaling pathways are generally less abundant and antibodies are available only for several protein biomarkers or regulatory proteins. However, SRM still has limitations in selectivity and requires more assay development/validation for achieving high quality quantification assays because it is operated on a low-resolution QqQ MS.

The recent introduction of fast HR/AM MS (e.g., QqOrbi MS and QqTOF MS) resulted in two new types of targeted MS quantification, PRM and DIA. They offer new avenues for HR/AM quantification of target proteins and overcome some SRM limitations. Compared to SRM the analytical workflow of PRM is simplified without the need for transition selection and optimization. For a smaller number of target peptides PRM was demonstrated to have similar performance as SRM in terms of sensitivity, multiplexing, reproducibility, and linear dynamic range and better performance than SRM in specificity (selectivity). Similar to SRM the daunting challenge for PRM is the scale of quantification because increasing the degree of multiplexing leads to significantly lowering sensitivity. Thus, further method development of PRM is required for enabling effective combination of scale and sensitivity for large-scale protein quantification. In contrast to SRM or PRM, DIA requires minimal assay development and offers proteome-scale targeted quantification using the targeted data extraction strategy. However, DIA has lower sensitivity and specificity, poorer reproducibility, smaller dynamic range, and much less matured informatics tools than SRM or PRM. Thus, at present DIA is suited for initial screening of a large number of high- to moderate-abundance proteins, and SRM or PRM is then used for sensitive accurate quantification of the remaining set of low-abundance proteins. It has been recognized by the proteomics community that a paradigm has already progressively shifted from un-targeted global proteomics to targeted proteomics as well as from small-scale to proteome-wide targeted quantification. With further advances in instrumentation and bioinformatics tools, we anticipate that targeted proteomics quantification will become a routinely accessible protein measurement tool for enabling cost-effective, rapid, robust quantification of target proteins including low-abundance proteins, isoforms and PTMs at a proteome scale in a high throughput manner. This could lead to significantly improving our understanding of aberrant signal transduction networks underlying human diseases for identification of rational therapeutic targets as well as accelerating biomarker verification for timely translating promising biomarkers into clinical use.

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Abbreviations

AP	affinity purification
DDA	data-dependent acquisition
DIA	data-independent acquisition
HILIC	hydrophilic interaction liquid chromatography
HR/AM	high-resolution accurate-mass
LG	long gradient
MRM	multiple reaction monitoring
MSIA	mass spectrometric immunoassay
PRISM	high-pressure, high-resolution separations with intelligent selection and multiplexing
PRM	parallel reaction monitoring
QqOrbi	quadrupole-Orbitrap
QqQ	triple quadrupole mass spectrometer
QqTOF	quadrupole-TOF
RP	reversed-phase
SIM	selectedion monitoring
SISCAPA	stable isotope standard and capture by the anti-peptide antibody
SRM	selected reaction monitoring
SWATH	sequential windowed acquisition of all theoretical fragment ion mass spectra

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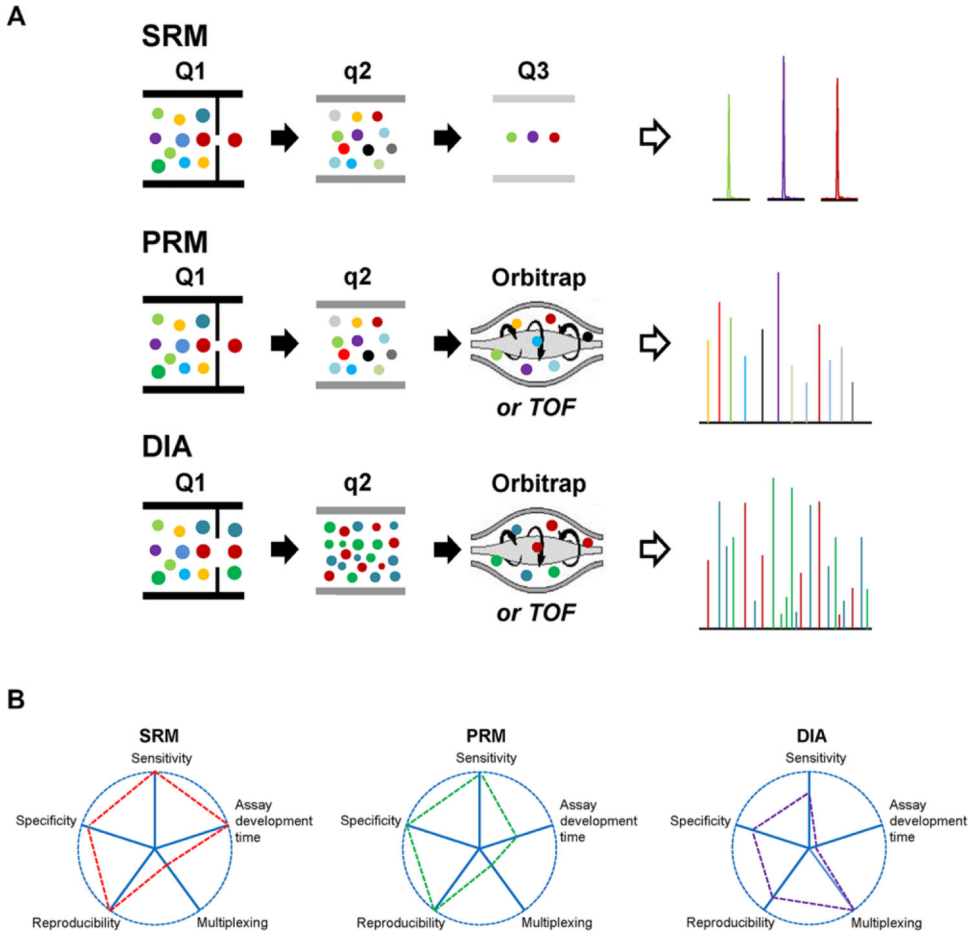

Figure 1.

(A) Schematic diagrams of typical SRM, PRM, and DIA. In SRM, the precursor ion of a specific peptide is selected in Q1, and then transmitted into q2 for fragmentation; specific product ions from the target peptide (typically three product ions) are selected in Q3 for detection. In PRM, the first two steps are the same as SRM; whereas in the last step all fragment ions from the target peptide are monitored by HR/AM mass analyzer. In DIA, all peptide ions within a defined mass-to-charge (*m/z*) window are isolated in Q1, then fragmented together in q2, and the highly complex mixture of all product ions are monitored by HR/AM mass analyzer; the analysis is repeated as MS progresses the full *m/z* range in a stepwise fashion. (B) Targeted quantification performance comparisons of SRM, PRM, and DIA. In the charts each analytical variable is represented by a spoke (the radial handle projecting from the center). The length of a spoke indicates the magnitude of the variables. PRM has similar multiplexing capability as SRM but with higher specificity due to the use of HR/AM mass analyzer to monitor the product ions. Note that the sensitivity comparison between SRM and PRM is based on quantification of the relatively small number of target peptides (e.g., ~50 peptides) in a single analysis.

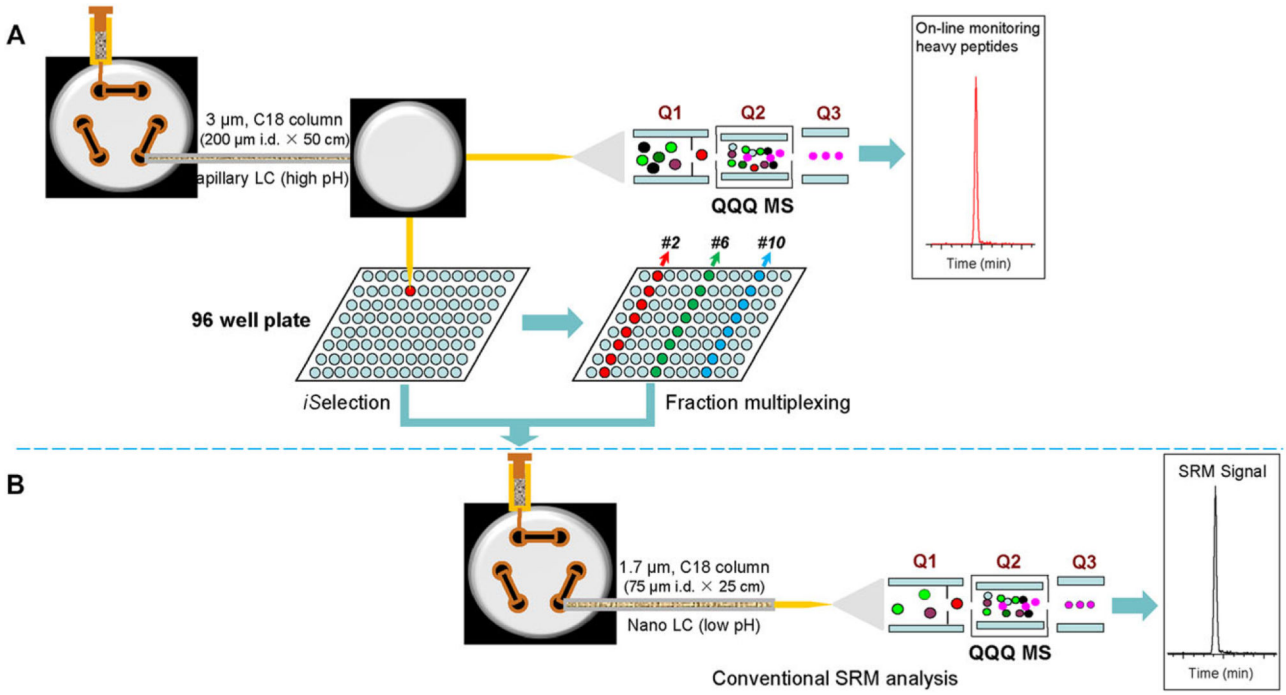

Figure 2.

Schematic diagram of the PRISM-SRM workflow reproduced from Shi et al. [18] with permission. (A) PRISM workflow. Peptide sample (typically 25 μg) spiked with internal standard (IS) heavy peptides was injected and separated by a high-resolution reversed-phase capillary LC (cLC) system using high-pH mobile phases. The eluent from the cLC column at a flow rate of 2.2 μL/min was split into two flowing streams via a Tee union (the split ratio of flow rates is 1:10): a small fraction (9%) of the column eluent went to a triple quadrupole mass spectrometer for on-line SRM monitoring IS peptides; a large fraction (91%) of the column eluent was automatically collected every minute into a 96-well plate during a ~100-min LC run. The specific target peptide fractions were either selected based on the same elution times of IS being monitored by the on-line SRM (*i*Selection) or multiplexed. For example, 96 fractions collected along with the first-dimension LC separation can be multiplexed into 12 fractions by combining 8 fractions from the first-dimension LC separation into 1 fraction for downstream LC-SRM analyses, such as pooling fractions 2, 14, 26, 38, 50, 62, 74, and 86 (marked in red color) into one sample (#2) for the second-dimension LC-SRM. (B) Conventional LC-SRM workflow. Following *i*Selection, a target peptide fraction was either directly subjected to nanoLC-SRM with 4 μL of sample per injection (~50 ng of peptides on the nanoLC column) or multiplexed with other target fractions with a final volume of 20 μL before nanoLC-SRM analysis.

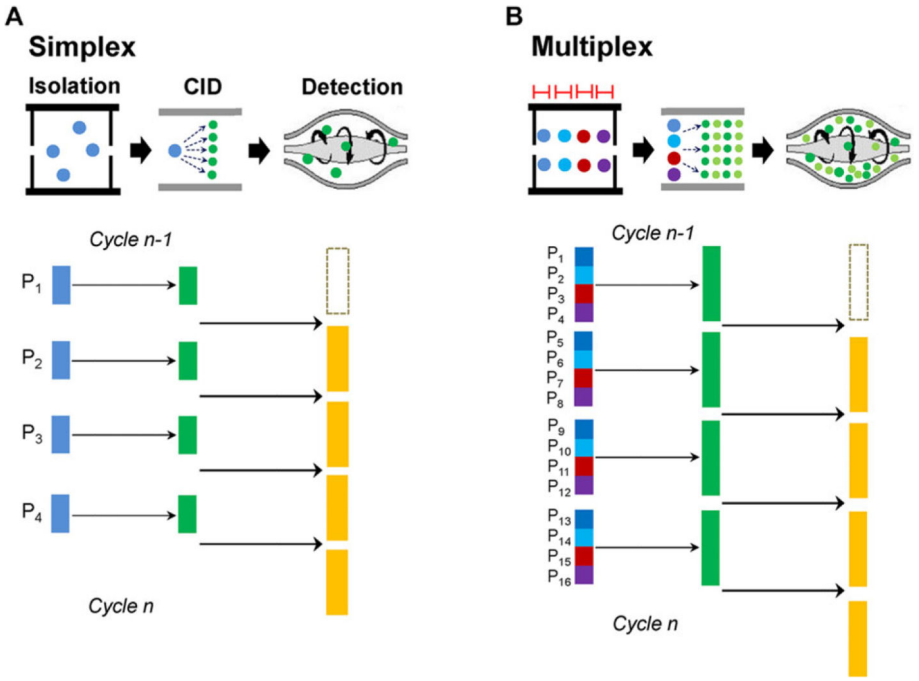

Figure 3.

Data acquisition methods in PRM modified from Gallien et al. [23, 28] with permission. (A) In the *simplex* mode, the co-eluting peptides are sequentially analyzed (i.e., isolating one precursor of interest at a time for MS/MS and then measuring its product ions). (B) In the *multiplex* mode the precursor ions of subsets of co-eluting peptides are isolated sequentially, and then sequentially fragmented in the HCD cell, where all products are trapped and accumulated in the C-trap. When the last precursor ion is fragmented, the mixture of product ions is transferred into the Orbitrap mass analyzer for detection, where a single combined MS/MS spectrum is acquired.

Table 1

A survey of recent advances in front-end strategies (2012-present) for enhancing SRM sensitivity of detection and quantification of target proteins in blood plasma or serum (with starting volume for sample analysis) as well as in human cell or tissue (with the description written in italics)

Strategy	Volume[a)]	Proteins[b)]	LOD	LOQ	CV	Ref.
SISCAPA	30 μL	1[c)]	0.1 ng/mL			[36]
	10 μL	3	0.3-3 ng/mL		4–15%	[39]
		69[d)]	*1–46 fmol/mg*[e)]	*1–400 fmol/mg*[e)]	*2–39%*	[40]
Protein enrichment		*1*	*240 amol/mg*[e)]	*280 amol/mg*[e)]	*4-20%*	[35]
Protein enrichment, SISCAPA	600 μL	1[f)]		~10-450 pg/mL	<15%	[41]
Depletion, SISCAPA	35 μL	1		~4 ng/mL[g)]	1-6%	[42]
		1		100 ng/mL[h)]	≤15%	[43]
MSIA (protein enrichment)	1-1000 μL[i)]	14		20 pg/mL-300 μg/mL	≤20%	[38]
	75 μL	1		~20 ng/mL	<25%	[44]
Gel extraction		4[j)]		*80–1100 fmol/mg*[e)]	*~14%*	[56]
NPE-Gel extraction[k)]		*10*		*~250 copies/nucleus*[e)]	*<15%*	[54]
Protein precipitation	7 μL	5		50–1250 ng/mL	<15%	[55]
Depletion, C18-SCX StageTip	800 μL	3[j)]	75-130 amol/mL[g)]	150-320 amol/mL[g)]	<20%	[57]
PRISM	~2 μL	3	<0.1-1 ng/mL	0.5-5 ng/mL	~10%	[58]
	~2 μL	1[j)]		~0.6 ng/mL	<20%	[63]
		7[j)]	*0.5-5 fmol/mg*[e)]	*2–50 fmol/mg*[e)]	*<15%*	[60]
Depletion, PRISM	10 μL	4	≤0.05 ng/mL[h)]	0.05-0.1 ng/mL[h)]	~10%	[18]
	10 μL	1		~130 pg/mL[h)]	<10%	[59]
μRPLC[l)]	30 μL[m)]	2[n)]		19 ng/mL-36 μg/mL		[64]
2D RPLC	30 μL[m)]	31[n)]		0.5-10 ng/mL	~10%	[64]
		41[n)]		>10 ng/mL	~10%	[64]
RP-HILIC	100 μL	1		1 ng/mL	6-8%	[68]
LG	~2 μL	2	~5 ng/mL	~10 ng/mL	~7%	[69]
		16	~0.5-13 ng/mL[o)]	~1-39 ng/mL[o)]	<10%	[70]

[a)] The starting volume of human plasma/serum for SRM quantification.

[b)] The number of target proteins in plasma or serum.

[c)] Mouse plasma.

[d)] Peptides including phosphopeptides.

[e)] Cells or tissues.

f) β-NGF neuropeptide.

g) Plasma albumin depletion.

h) IgY14 depleted human serum.

i) Plasma volume depending on the abundance of target proteins.

j) Peptides.

k) Nuclear protein extraction followed by gel extraction.

l) One dimensional microflow RPLC separation followed by SRM measurement.

m) 30 μL of human plasma used for both the 1D and 2D parallel experiments.

n) 2 undetected proteins by 2D RPLC-SRM but with detection by 1D RPLC-SRM; 31 and 41 detected proteins by 2D RPLC-SRM but without detection by 1D RPLC-SRM.

o) MARS14 depleted human plasma.

Table 2

Preclinical verification of candidate protein biomarkers by targeted proteomics assays in 2012–2015

Candidate protein biomarker	Disease type	Specimen	Assay type	Ref.
AGR2	Prostate	Urine	PRISM-SRM	[59]
TMPRSS2-ERG fusion protein products	Prostate	Tissue	PRISM-SRM	[60,61]
CD44 antigen, immunoglobulin γ-2 heavy chain, ITIH2, cadherin-13	Prostate	Serum	SPEG-LC-PRM[a)]	[86]
zyxin	Non-small-cell Lung	Plasma	LC-SRM	[87]
SAA1, SAA2	Lung	Plasma	LC-PRM	[88]
SAA1, SAA2	Lung	Serum	LC-SRM	[89]
TFPI, MDK, OPN, MMP2, TIMP1, CEA, CYFRA 21–1, SCC	Lung	Tissue, serum	LC-SRM	[90]
LRP1, BGH3, COIA1, TETN, TSP1, ALDOA, GRP78, ISLR, FRIL, LG3BP, PRDX1, FIBA, GSLG1	Lung	Plasma	IgY14-LC-SRM	[91]
ALCAM, CDH1, MUC1, SPINT1, THBS4, SVEP1	Lung	Plasma	MARS6-LC-SRM	[92]
ITGA5, GPRC5A, PDGFRB, TFRCC, 8orf55	Colorectal	Tissue	LC-SRM	[93]
CP, TIMP1, LRG1, PON1, SERPINA3	Colorectal	Plasma	Glyco-LC-SRM[b)]	[94]
HLA-A, CFH, CD44, PTPRJ, HP, CDH5	Colorectal	Plasma	Glyco-LC-SRM[b)]	[95]
LDH-B, CKMB, myoglobin, troponin I	Cardiovascular	Serum	MARS6-LC-SRM	[84]
PRNP, HSPG2, MEGF8, NCAM1	Parkinson	Plasma	LC-SRM	[98]
SPP1, LPR1, CSF1R. EPHA4, TIMP1	Parkinson	CSF	LC-SRM	[99]
GSN, MSN, LSP1, SEPT6, TALDO1, TWF2, VIM	Parkinson	T-lymphocyte	LC-SRM	[100]
Ubiquitin	Neurodegenerative	CSF	LC-SRM	[101]
CEL, CPA1, MUC13, CLCA1, MUC5AC, HAPLN1	Down syndrome	Amniotic fluid	LC-SRM	[102]
CEL, MUC13, CPA1, DPP4, MMP2	Down syndrome	Amniotic fluid	LC-SRM	[103]
SAP, C1-inhibitor	Down syndrome	Plasma	LC-SRM	[104]
244 NLF proteins	Upper airway	NLF[c)]	LC-SRM	[200]
C3, CD3E, DPT, MCM4, PMEL, S100A8, S100A13, S100B, TAGLN2	Malignant melanoma	Tissue	LC-SRM	[201]
Proline-hydroxylated α-fibrinogen	Pancreatic	Plasma	LC-SRM	[96]
SFN, GSN, LUM, TIMP1	Pancreatic	Plasma	IgG-LC-SRM	[97]
A1BG, GFH, IGFALS, PROC, RBP4	Hepatic fibrosis	Serum	IgY14-LC-SRM	[105]
ORM1, PFN1, H4, H2AFX, MPO, M2BP, C4BP, CRP, S100A9, MMP3, DEFA1, CD5L	Psoriatic arthritis	Synovial fluid	LC-SRM	[106]
A4, APOE, KLK6, MY15B, PEDF, 1433F, 1433G, AACT, CAD13, TAU, NFH, NFL, NFM, NRCAM, OSTP, SAMP	Multiple sclerosis	CSF	LC-SRM	[107]
ICAM1, HSPG2, ANTXR1, PON1, HYOU1, THBS1, MSLN	Malignant pleural mesothelioma	Serum	SPEG-LC-SRM[a)]	[108]
PPBP, SERPING1	Type 1 diabetes	Serum	LC-SRM	[8]
A1AG1, AACT, A1AT, CERU	Liver	Plasma	Lectin-LC-SRM	[109]

[a)] SPEG: solid-phase extraction of *N*-linked glycopeptides.

[b)] Glycoprotein enrichment.

[c)] NLF: nasal lavage fluid.