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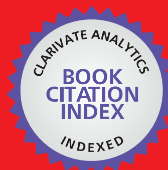

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Chapter 5

Targeted Proteomics in Translational and Clinical Studies

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Additional information is available at the end of the chapter

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Abstract

This chapter provides a concise overview on the methods and applications of targeted proteomics in the context of translational and clinical studies. Mass spectrometry-based targeted proteomics has emerged as a promising technique for protein and peptide quantification, presenting a great potential for clinical applications. While significant amount of discovery works have been carried out in both genomics and proteomics for an assortment of diseases, it has been challenging in further characterizing individual protein targets for their biological significance and clinical value due to the lack of effective and "universal" techniques. The development of targeted proteomics approach opened a unique avenue to bridge the discovery-based genomics and proteomics with candidate-based protein analysis, which is clinically highly relevant. Targeted proteomics analysis has been implemented on a variety of instrument platforms, and applied for a wide range of studies, from blood biomarker detection to pathway-driven mechanistic investigations, with the triple quadrupole-based selected reaction monitoring (SRM) technique being the most widely used method. With a right combination of calibration approach, internal standards, and sample preparation strategies, mass spectrometry-based targeted analysis has proven to be of inter-laboratory reproducibility and sensitivity in analyzing many clinical specimens. More recently, the advent of mass spectrometry with high frequencies and resolutions yielded the data independent acquisition (DIA) techniques, such as sequential window acquisition of all theoretical fragment ion spectra (SWATH). The unbiased nature of DIA methods would enable a wider analytical scope and a greater robustness in targeted analysis, representing a paradigm shift in targeted proteomics.

Keywords: Proteomics, Targeted proteomics, Mass spectrometry, Data independent acquisition

1. Introduction

The introduction of soft ionization techniques in mass spectrometry has ushered in a fascinating era in the analysis of large biomolecules, including metabolites and proteolytic peptides

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and proteins from complex biological matrixes [1,2]. Mass spectrometry-based proteomics is nowadays utilized in a wide arena of translational and clinical applications for global profiling of biological matrixes to explore disease mechanisms and to discover novel biomarkers [3,4]. Quantitative mass spectrometry confers the highly sensitive and reproducible targeted proteomics for the multiplexed quantification of already existing targeted proteins and putative biomarkers [5,6]. These target proteins can be either a single putative protein biomarker or a set of proteins involved in a specific cell signaling or a metabolic pathway.

While conventional antibody-based assays, such as ELISA, offer several benefits for the protein quantification, including ease-of-use and simpler instrumentation, ELISA, however, suffers from cross-reactivities and protein/protein interactions that would alter the quantification results based on the level of carrier proteins and based on the free and conjugated levels of the target molecules [7]. Complementarily, mass spectrometry-based targeted proteomics provides a different mechanism for multiplexed protein quantification, and has indisputable advantages in the analysis of genetic changes, polymorphisms, alternative splicing, protein isoforms, and post-translational modifications [6]. In these circumstances, having an antibody with high resolution and specificity for each of these diversities, even if not impossible, would be very difficult to attain. Hence, mass spectrometry-based targeted proteomics can be of complementary importance for the antibody-based quantifications, in particular, for the instances of validating novel protein biomarkers when the corresponding antibodies are not available, or for the cases of multiplexed interrogation of hypothesis-driven key proteins [6,8].

Unlike the quantification of small metabolites, proteins are complex macromolecules, constituting large masses with multiple charges and various dynamic conformations, preventing them to be effectively separated in the gas phase within a mass analyzer or being detected with high mass accuracies. A general theme in targeted quantification, which is widely known as bottom-up proteomics, is to digest proteins by a proteinase enzyme, usually trypsin, with a high specificity to cleave the basic amino acid residues namely arginine and lysine to generate smaller tryptic peptides for facile separations and comprehensive mass spectrometric analysis [2].

A set of different targeted approaches have been already applied for the targeted quantification of proteins [9]. These approaches are based on the survey of precursor ions, survey of product ions, neutral loss, or a fragmentation pattern using a variety of instruments [9]. The earlier targeted proteomic approach, which was called selected ion monitoring (SIM), was based on the generation of an inclusion list and extraction of the exact ion masses of the targeted molecules for analysis [9]. Though this technique was simple to operate, it suffered from low selectivity as many different ions could have similar masses with low level of sensitivities. In contrast, selected reaction monitoring (SRM) built on a triple quadrupole mass spectrometer generates higher sensitivities and specificities, especially when combined with stable isotope dilution. The SRM technique was built based on the unique fragmentation pattern for each targeted molecule that can be mutually specific and provides high sensitivity.

Triple quadrupole mass spectrometer-based SRM technique has been the most widely used targeted proteomics approach to date, in which targeted analytes are selected in the first quadrupole, fragmented in the collision chamber via collision-induced dissociation (CID), and the produced transitions are further separated by the second quadrupole for detection (See

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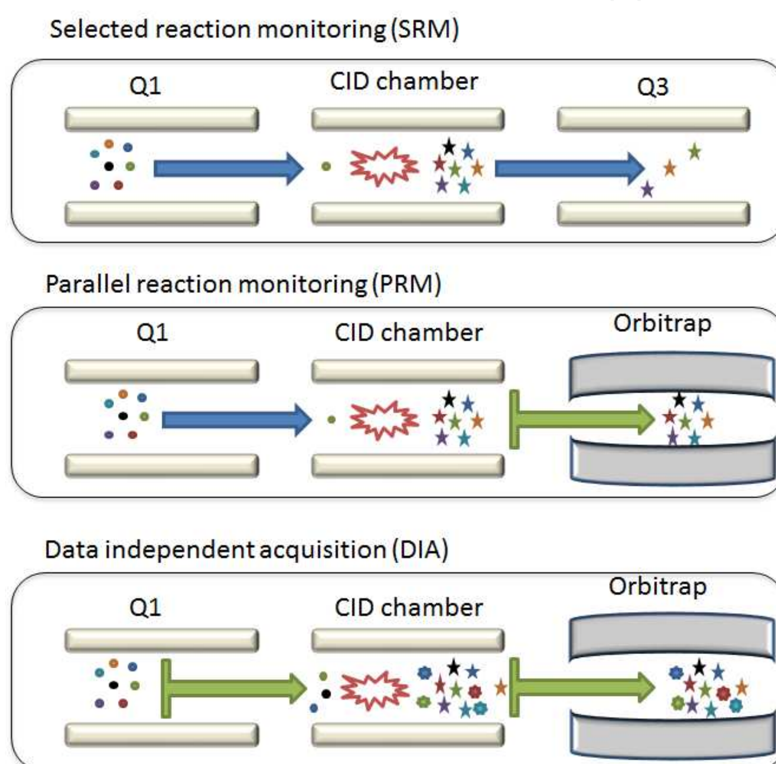
Scheme 1). In such a setting, a combination of selected transitions generated from the corresponding peptide under optimized collision conditions can provide unique identification and accuracy for targeted peptide measurement. The inclusion of multiple product ions makes the SRM analysis more specific in ion selection compared to the inclusion list-based interrogation, and minimizes the interference from a complex background of biological sample via selection of small mass intervals that leads to higher sensitivities. For the optimal use of this technique, there is an immense need to identify the suitable signature peptides that would be highly stable under prolonged digestion and storage and to be highly sensitive through the gas-phase transitions [10].



Sheme 1. Three different approaches in mass spectrometry-based targeted proteomics (a) selected reaction monitoring (SRM) where selected fragment ions from a single precursor are measured for the quantification, (b) parallel reaction monitoring (PRM) where a single precursor ion and entire fragment ions are selected, and (c) data independent acquisition (DIA) where multiple precursors ions are fragmented simultaneously and the entire fragment ions are monitored. The presented PRM and DIA technologies are based on Orbitrap mass spectrometers, such as Q-Exactive.

The concept of stable isotope dilution, which was originally developed for the quantification of metabolites, have been implemented for targeted proteomics analysis using stable isotope-labeled synthetic peptides as internal standards to facilitate mass spectrometric quantifications. Though using different instruments, with different elution, ionization, and collision conditions would impact the intensities of gas-phase transitions, the use of heavy isotope-labeled internal standards can circumvent the variations generated due to the differences in instruments and settings, and provide more robust quantification [11]. Stable isotope-labeled peptides can be used for the absolute quantification of targeted peptides and their post-

translational modifications through the synthesis of different combinations that can contain post-translational modifications [12]. An inter-laboratory study have pointed out that the SRM-based targeted proteomics using common stable isotope-labeled peptide internal standards and calibration approaches can be of high reproducibility and reliability [4].

LC-MALDI-TOF/TOF analysis is a different platform for biomarkers discovery and detection with its own unique characteristics [13,14]. In LC-MALDI-TOF/TOF setting, a peptide separation module is used to generate an array of peptides from complex mixtures in the presence of stable isotope-labeled internal standards on the sample plate; targeted proteomics is then carried out by specific interrogation of selected candidates using MALDI-TOF/TOF mass spectrometer. Such an approach involves detached MS and MS/MS acquisition, allowing repeat interrogation of a wide range of peptide targets with minimal assay development.

Since the fragmentation patterns in SRM-based targeted proteomics can be dependent on the vendor types, parallel reaction monitoring (PRM) is devised to improve the identification and quantification of the targeted peptides. In this technique, as shown in Scheme 1, all the detectable fragmentation ions from the pre-selected substrate are recorded and used for the quantification [15]. The second quadrupole is replaced with a high-resolution and high-frequency Orbitrap mass spectrometer. More recently, the advent of fast and high-resolution mass spectrometers have made a hybrid discovery and targeted proteomics possible through data independent acquisition (DIA) [16], in which, multiple precursor ions are surveyed together (See Scheme 1), rendering a new strategy for targeted mass spectrometric analysis. Using-high resolution mass spectrometer ensures efficient resolving of complex matrices, and higher frequencies enrich quantification profiles.

2. Targeted proteomics in translational and clinical investigations

Genomic and proteomic studies have already introduced a large number of putative protein biomarker candidates for an assortment of diseases [17–22]. This assortment signifies the need for a universal high-throughput targeted proteomics in order to link the putative protein biomarkers with clinical trials and to perform their verification and validation in the large-scale cohort studies [23–25]. The application of the targeted quantitative proteomics in clinical analysis covers extensive objects ranging from the quantification of proteins, multiplexed monitoring of key proteins in a pathway, targeted analysis of post-translational modifications, and examination of the expression of genetic changes.

2.1. Targeted quantification of protein level

Targeted proteomics is widely used for protein quantification. The putative protein biomarkers are designated to be quantified in a large cohort of clinical samples. This form of targeted quantification can bridge the discovery-based proteomics with the pathways analysis through high-throughput quantification of the predefined protein biomarkers. The targeted quantifications of putative protein biomarkers are based on the quantification of signature peptides after exhaustive extraction of the proteins from complex clinical matrixes. For the complex

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clinical biofluids and blood samples, the reduction of complexity is of prime importance (Section 3). The development of highly multiplexed quantitative targeted assays based on the exploration of suitable signature peptides, optimal transitions, and isotope-labeled internal standards are presented in Section 4.

The use of isotope dilution allows absolute protein quantification and improves the analytical accuracy by providing internal standards and compensating the changes occurring through sample preparation and analysis. A variety of studies on targeted quantification of protein biomarkers have been reported, including quantification of C-reactive protein from plasma samples [26,27], quantification of immunoglobulin G and its glycoforms from plasma [28], and multiplexed targeted detection of protein biomarkers in plasma from pancreatic cancer patients [29]. In most of the studies, certain types of sample preparation strategies were applied to reduce the sample complexity or enrich the targeted analytes. Without using prior affinity depletion or enrichment, a study showed the feasibility of absolute quantitation of 45 endogenous proteins, including 31 putative biomarkers of cardiovascular disease, in human plasma using mass spectrometric targeted approach [30].

2.2. Targeted monitoring of key proteins in a pathway network

Targeted proteomics can be utilized for the concomitant quantification of a set of proteins that are involved in a clinical condition, or a biological process [31]. Targeted proteomics has successfully quantified 464 proteins with known or suspected roles in transcriptional regulation at RNA polymerase II transcribed promoters in *Saccharomyces cerevisiae* [32]. A list of 1,261 proteins considered to be differentially expressed in human cancer was compiled from literature and other sources [33]. Some of these cancer-related proteins were analyzed in plasma from cancer patients, and 182 proteins were detected in depleted plasma, spanning five orders of magnitude in abundance and reaching a detection sensitivity of 10 ng/mL [11].

Sentinel proteins report the activation of specific cellular processes. In a study, 570 potentially suitable sentinels for *Saccharomyces cerevisiae* from available biological data were selected for the specific proteins, phosphorylation sites, or protein degradation products that report on four general classes of biological relationships [34]. Quantitative SRM assays were developed for 157 sentinel proteins and 152 sentinel phosphopeptides that could simultaneously probe 188 distinct biological processes in *Saccharomyces cerevisiae* in response to a set of environmental perturbations.

2.3. Targeted quantification of post-translational modifications

Post-translational modifications (PTMs) are playing a significant role in the activation or inhibition of biological processes, and their changes would be indicative for a clinical condition. Among the PTMs that are investigated frequently in clinical studies are phosphorylation and glycosylation. Glycoproteins unequivocally comprise the major biomolecules involved in extracellular processes and found mostly in secretome, such as growth factors, signaling proteins for cellular communication, enzymes, and proteases for on- and off-site processing [35–37]. Glycoproteomics have been used for the discovery of biomarkers in lung and pan-

creatic cancer [38,39]. Phosphorylated secreted proteins of tumor cells have been studied as source of candidates for breast cancer biomarkers in plasma [40].

For some PTMs, such as phosphorylation, methylation, and acetylation, synthetic reference peptides can be prepared with covalent modifications to mimic naturally occurring post-translational modifications [12]. Unlike the total protein quantification, the interrogation of PTMs status relies on the measurement of the targeted peptides that have undergone the desired modification. In this type of quantification, the subproteome is typically enriched using affinity columns or other separation techniques to enhance analytical sensitivity. For example, in glycosylation analysis, enrichment of N-glycosylated peptides coupled with targeted proteomics was applied to quantify the disease-responsive proteins in the sera of prostate cancer patients [41].

2.4. Targeted quantification of genetic changes

Genetic changes may have distinct effects at protein level. It may influence the expression level of proteins, modify their sequences through single nucleotide polymorphisms, the occurrence of allelic variants, or may impact the alternative splicing events [42]. Each individual may carry thousands of nonsynonymous single nucleotide variants in the genome, corresponding to various amino acid polymorphisms in the encoded proteins [43]. In global proteomic analysis, it is challenging to identify and quantify all protein variants in complex biological samples [42]. Targeted proteomics can be used in the quantification of protein isoforms, alternative splicing, SNPs, and other genetic mutations that result in changes in protein sequence. In such studies, the selected signature peptide should be unique and representing the targeted changes and should be suitable for mass spectrometric analysis [44].

In a study, which utilized targeted proteomics to quantify single amino acid polymorphisms, the absolute concentrations of three selected single amino acid polymorphism-peptides were measured in plasma from multiple individuals using SRM with the aid of heavy isotope-labeled peptide internal standards [44]. In a different study, a strategy for the comparative analysis of single amino acid polymorphism was developed by integration of stable isotope dimethyl labeling with a variation-associated database search approach. The technique could discover as many as 282 unique variation sites and quantify them in the human liver tissues. Although the identifications were restricted to the known genomic sequence variations, the use of a concise database improved the identification of variants at the protein level [45].

3. Reducing sample complexity — Blood analysis

Blood is a highly informative clinical matrix, which has been widely used in clinical analysis. In proteomics, the major challenge associated with the plasma or serum analysis is not only the sample complexity but also the enormous dynamic range (more than 11 orders of magnitude) in protein concentration [46]. The presence of high abundance proteins, such as albumin and IgG, can significantly mask the detection of low abundance proteins. Without prior sample treatment, the reported lower detection limit for plasma analysis using targeted proteomics

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was at μg/mL level [27], which is not suitable for measuring the majority of low and medium abundant proteins.

The depletion of high abundance proteins, fractionations at either protein or peptide levels, enrichment of target proteins, peptides, or sub-proteomes are among the suitable techniques that can be used to reduce the complexity of blood samples. A useful and convenient reduction of blood complexity should be performed based on the purpose of study and target molecules that are needed to be quantified [5].

3.1. Immuno-depletion of the high-abundance proteins

Immuno-depletion of the high-abundance proteins has been widely used to reduce the blood sample complexities. By depletion of the major plasma proteins, targeted mass spectrometric analysis could reach the lower limit of detection between 1 and 10 ng/mL [5]. The number of high-abundance proteins to be depleted varies and depends on the purpose of studies. Potential loss of non-target binding proteins associated with immuno-depletion may be a concern in some cases [47,48]. It has been proven that such a simple treatment of sample is an effective way to reduce complex matrix background and to highlight the candidate analytes for targeted analysis in a high-throughput manner [29,47,49].

Candidate protein biomarkers at low ng/mL to pg/mL levels were detected in serum after removing the 12 most abundant and 77 moderately abundant proteins from serum samples using antibody affinity columns [50]. Using immuno-depletion approach, proteins with 100 ng/ml or higher concentrations are readily accessible by targeted MS in plasma without antibody enrichment [51].

3.2. Fractionation of the plasma samples at protein or peptide levels

Besides immuno-depletion of high-abundance proteins, the fractionation of the proteins by size exclusion chromatography or using 2D electrophoresis can reduce blood complexity. On the other hand, tryptic peptides from the shotgun proteomics can be separated at peptide level using orthogonal separations, such as ion exchange chromatography coupled with reversed phase LC separation (e.g. MudPIT – multidimensional protein identification technology [52]), to obtain a better resolution of the eluting peptides. Online peptide fractionation strategies were also introduced to enhance quantitative analysis [53].

3.3. Targeted enrichment of proteins, peptides, or sub-proteome

Besides the fractionation practices and immuno-depletion, target proteins or peptides or sub-proteome can be enriched from the complex matrices using affinity or chemical methods to facilitate targeted analysis.

The method of stable isotope standards and capture by anti-peptide antibodies (SISCAPA) can reach a LOD as low as 0.1 ng/mL for plasma detection [54,55]. Rabbit polyclonal antibodies raised against the selected peptide sequences were covalently immobilized on POROS supports for enrichment of target peptides along with their heavy isotope labeled counterparts,

which were spiked in as internal standards for absolute quantification [54]. The technique has proven to enrich the target peptides against the background peptides by more than 100 times, and can be used to achieve high-throughput analysis using SPE-MS/MS technique [56].

Similar to the enrichment of specific target proteins or peptides, a sub-proteome at protein or peptide level can be enriched from the complex blood samples. Among the sub-proteome that are widely enriched from blood samples are N-linked glycoproteins/glycopeptides and phosphorylated residues. For N-glycoproteome analysis, lectin affinity and hydrazide chemistry have been the most widely utilized methods for the enrichment of glycoproteins or glycopeptides. TiO_2 columns are able to selectively purify phosphorylated peptides and sialic acid-containing N-glycopeptides [57].

Lectins are glycan-binding proteins that can bind to their target glycan moiety with high specificity [58,59]. The lectin affinity of sugar-containing residues can help their affinity enrichment at protein or peptide levels [60], which may be followed with the site-directed tagging of N-glycosylation sites by ^{18}O during the elution with N-glycosidase [61]. Hydrazide chemistry occurs when certain sugars of the glycoproteins are oxidized to form reactive carbonyl groups. These carbonyls can then be conjugated to hydrazide-activated cross-linkers. The conjugated peptides/proteins are digested by PNGase F enzyme to cleave glycans from protein N-glycosylated sites, causing a mass shift of 0.98 Da due to the conversion of asparagine to aspartic acid [62,63]. This specific mass shift can be used for targeted interrogation of N-glycopeptides to identify N-glycosylation sites [64] and to monitor the glycosylation levels associated with the corresponding N-glycosylation sites [65]. Using such an approach, studies have demonstrated a LOD in the low ng/mL range and an analytical dynamic range over 5 orders of magnitude for plasma detection [66].

TiO_2 column is able to selectively purify phosphorylated peptides and sialic acid-containing N-glycopeptides. A method that combines an optimized TiO_2 protocol and hydrophilic interaction liquid chromatography to simultaneously enrich, identify, and quantify phosphopeptides and formerly N-linked sialylated glycopeptides to monitor changes associated with cell signaling in brain tissues has been reported [57]. Head-to-head comparison of several serum fractionation schemes, including N-linked glycopeptide enrichment, cysteinyl-peptide enrichment, magnetic bead separation, size fractionation, and immuno-depletion of abundant serum proteins have been performed. The analysis showed that immuno-subtraction was the most effective way to simplify the serum proteome while maintaining reasonable sample throughput [67].

3.4. Other instrumental innovations in reducing the blood complexity

High-pressure, high-resolution separations coupled with intelligent selection and multiplexing (PRISM) is an antibody-free strategy to reduce the plasma complexity for SRM analysis [68]. The strategy capitalizes on high-resolution reversed-phase liquid chromatographic separations for analyte enrichment, intelligent selection of target fractions via online SRM monitoring of internal standards, and fraction multiplexing before nano–liquid chromatography–SRM quantification. With the aid of the depletion of the 14 most abundant proteins, a study demonstrated that this method could detect AGR2 protein in human serum with the

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concentration in the range of 50–100 pg/mL [69]. It is also reported that without the upfront immuno-depletion of the high-abundance proteins, the PRISM technique can reach limit of detections at low ng/mL range [70]. In addition to sample preparation strategies, ion mobility separation has been used for analyzing plasma samples, capitalizing on the gas phase separation of the co-eluting ions [71].

4. SRM assay development

Targeted quantitative proteomics requires development of high-throughput assays [72] to effectively detect a wide range of proteins in a biological sample with high reproducibility and robustness [73]. SRM-based methods have been the gold standard for MS-based protein quantification and have been widely applied in various studies. The development of an SRM assay typically involves an appropriate sample preparation, an optimal selection of signature peptides, and a well-calibrated MS protocol [74–76]. In the analysis of blood and other biofluids, especially when targeting low-abundance proteins, an effective sample preparation is almost mandated to reduce sample complexity or/and enrich targeted analytes, as aforementioned in Section 3.

4.1. Exploration of the most suitable signature peptide

An optimal assay should include the most sensitive and the most stable unique signature peptides to represent the target proteins. Ideally, multiple signature peptides that are belonging to different domains of the protein are preferred to quantify the target protein for the reasons of reliability of the quantifications. This is because various domains may have different efficiencies for trypsin digestion. The results of quantification for each signature peptide may differ, which in this case might be indicative for the truncation of the target protein or degradation besides the different digestion rates from different domains [10].

Evaluation of candidate signature peptides from the target proteins is of importance to obtain a sensitive and reliable quantification. The uniqueness of the signature peptides can be verified by comparison with the protein databases using alignment software such as protein BLAST, and empirically verified from matrix. Moreover, the unique peptides should be evaluated for their stability, the absence of labile residues, and the risk of incomplete digestion, PTMs, having appropriate length, hydrophilicity, and other relevant parameters, such as their chromatographic and mass spectrometric characteristics [77]. Human plasma proteome project have already identified 20,433 distinct peptides, from which a highly nonredundant set of 1,929 protein sequences at a false discovery rate of 1% are inferred [78]. In addition, collections of peptide spectral libraries, such PeptideAtlas [79] and SRMAtlas [80], provide empirical data to facilitate signature peptide selection.

4.2. Optimization of collision energy and most sensitive transitions

Selection of the most robust transitions is essential for quantification of signature peptides. Usually, multiple transitions are selected for the verification of a same signature peptide. In

the case of presence of pure signature peptides, the fragmentation pattern for each of them can be performed empirically and the most stable and sensitive transitions can be selected for assay development. But in the absence of pure signature peptides, using the existing spectral libraries such as PeptideAtlas [79,81] or SRMAtlas [80] can provide useful information in evaluating the mass spectral characteristics of a peptide. These libraries are based on the computational or experimental data resulted from the collision fragmentation of a large number of synthesized peptides. However, it should be noted that the fragmentation patterns depend on the instrument types and may differ by different vendors. One possible solution can be the use of on-the-fly orthogonal-array optimization of the collision energies and transitions for any given signature peptide, especially in the absence of the pure signature peptides [82]. Another approach is using PRM, in which all the fragmented ions obtained from the same substrate are monitored together to obtain a more reliable quantification result [15], as illustrated in Scheme 1.

To assist transition selection, a novel algorithm was presented to allow the construction of SRM assays from the sequence of the targeted proteins alone. This approach relies on combinatorial optimization with machine learning techniques to predict proteotypicity, retention time, and fragmentation of peptides, enabling rapid development of a targeted SRM experiment [83]. Using the contemporary MS capabilities, instrument parameters can be optimized for each peptide for any given retention time and transition. A study has shown that the optimal collision energies for each respective charge-state can be predicted using linear equations based on the peptide precursor mass. These charge-state-dependent equations for predicting the optimal collision energies are embedded within Skyline software [84].

It is also worthy to mention that in triple Q based SRM methods, there is a reverse relation between increased dwell time to obtain higher sensitivities, and a reliable peptide profiling. Spending longer times for each analysis means less number of quantified points, and poor peptide time profiles. This issue can be partly addressed by using scheduled SRM acquisition and restricted time window for the known peptides expected to elute in the corresponding time interval [85]. Higher resolution separation with high reproducibility and longer gradient times would increase the number of target peptides to be quantified with high sensitivity and reliability within a single run.

4.3. Exploration of the most suitable internal standards

Targeted proteomics can be used for either relative or absolute quantification. In the case of absolute quantification, there is a need for appropriate calibration set up next to the isotope dilution mass spectrometry. Individual heavy isotope-labeled internal standards, which are spiked in a sample with known amounts, would serve as internal standards for the corresponding endogenous peptides for specific quantification. On the other hand, with less quantitative accuracy, a single internal standard or fluorinated internal standards can be used. In addition, stable isotope-labeled proteins, such as QconCATs, can be used as internal standards, having the advantage of circumventing the variations caused during digestion [86]. With optimal settings and a stringent quality control, SRM-based targeted proteomics can be highly reproducible within and across laboratories [4]

5. Data Independent Acquisition (DIA) for targeted analysis

Advent of high-frequency and high-resolution mass spectrometry has provided the potential for data independent acquisition (DIA) [16,87]. While conventional data dependent analysis precludes the analysis of some eluted peptides [88], in DIA acquisition, MS generates virtually all the MS/MS fragmentation spectra from all precursor ions that are falling into a predefined *m/z* range. Hence, each recorded MS/MS fragmentation spectrum is a multiplexed recording of the fragment ions derived from all peptides eluting in real time within the predefined *m/z* range of the precursor window [87]. Scheme 1 illustrates the main elements in DIA technique. Due to the unbiased fragmentation of precursor ions, DIA approach provides a high multiplexing capability, high reproducibility, and wide analytical scope. Conceptually, DIA-based mass spectrometric analysis can be viewed as an SRM assay on all peptides detected, allowing extraction of pseudo SRM data for any peptide of interest within the mass spectrometric detection limit. The design of a DIA method may be dependent on study purpose and sample type, and requires an optimal balance of multiple instrument parameters, including targeted mass range, DIA window width, duty cycle time, and automated gain control, etc.

DIA is a generic term encompassing a wide range of recently developed techniques that are built on the analysis of a non-predefined set of precursor ions. The early DIA technique, PAcIFIC, was based on the multiple LC/MS runs at limited mass ranges [89,90]. The technique suffered from prolonged analysis times. Recently, a variety of DIA techniques have been explored and implemented using different mass spectrometers, including triple TOF based sequential window acquisition of all theoretical fragment ion spectra (SWATH), Q/TOF based MS^E, and Orbitrap based multiplexing strategy (MSX) [87]. These DIA techniques differ in the instrument platforms and using isolation windows of various widths, depending on different study purposes and instrument settings [91,92].

Coupled with hydrazide-based solid phase extraction for N-glycosylation enrichment, SWATH has been applied to analyze deglycosylated N-glycopeptide in human plasma. While the sensitivity of SWATH was slightly less than SRM, the study demonstrated that SWATH could reach a detection limit of 5 ng/mL in plasma and quantify N-glycopeptides with a concentration range of 4 orders of magnitude [93]. The same approach (using N-glycopeptide enrichment) was successfully applied to analyze prostate cancer tissues and identified 1,430 N-glycosylation sites from each sample in average, including 220 proteins that showed quantitative changes associated with tumor aggressiveness [94].

A recent study has suggested that more than 10,000 human proteins (the majority of human proteins from UniProt database) could potentially be covered using SWATH-MS technique that can be of high value for clinical studies [95]. In this study, a variety of human cell types and depleted human plasma samples were analyzed with the aid of various sample preparation techniques, including affinity purification, size exclusion chromatography, strong anion exchange, and gel electrophoresis [95]. In the quantitative study of human twin population, the plasma samples from twins are used to explore the impact of longitudinal factors in blood proteomic changes. This study included the identification of some genetic changes that occurred by time [96].

For phosphoproteomics, SRM and SWATH have shown similar performance in the determination of changes of phosphopeptide levels extracted from human plasma [97]. The general theme in the DIA analysis of phosphorylation and glycosylation is the selective enrichment of the corresponding sub-proteome [15,34,94]. A DIA method, namely combination hyper-reaction monitoring (HRM), used retention time normalized (iRT) spectral libraries for spectral identification. Using a controlled sample set, the HRM outperformed shotgun proteomics both in the number of consistently identified peptides across multiple measurements and quantification of differentially abundant proteins when it profiled acetaminophen (APAP)-treated 3D human liver microtissues [98].

6. Software used in targeted proteomics

A variety of software has been developed to assist targeted proteomic data analysis. MRMer is an interactive open source and cross-platform system for data extraction and visualization of multiple reaction monitoring experiments [99]. MRMer parses and extracts information from MS files encoded in the platform-independent mzXML data format. mProphet is an automated data processing and statistical validation tool for large-scale SRM experiments [100]. Skyline can be used for analyzing a variety of targeted proteomic data, including SRM- and DIA-based data [101]. The extraction of pseudo-SRM profiles from DIA data requires a spectral library, which can be built using global profiling data for peptide and protein identification. Skyline is also capable of analyzing MSX (Multiplexed MS/MS) based DIA data [102]

DIA-Umpire is a software program that has been recently developed and performs the data extraction based on the co-elution of the substrate and its corresponding fragmentation to build a pseudo-MS/MS library, which later can become useful in identification and targeted quantification [103]. Spectronaut extends the limits of quantitative proteome profiling with DIA [98].

7. Current status and further research

Currently, SRM is the gold standard for mass spectrometry-based targeted analysis and has been widely applied in a broad range of translational and clinical studies. While SRM provides high sensitivity, one major drawback from using SRM is that a SRM assay is dependent on the geometry of instruments and the instrumental settings. Thus, it would require an extensive effort in assay development for each specific group of analytes on a particular instrument. The number of SRM assays is also limited as there is a reverse correlation between the number of transitions (selectivity) and the quality of quantification (sensitivity). On the other hand, the advent of DIA has introduced a virtually unlimited pseudo-SRM analysis that can be run once and used for the extraction of any given data within the detection limit. The technique, which, in a way, hybrids the technical characters of discovery-based proteomics and targeted analysis, is undergoing a rapid progress and represents a paradigm shift in targeted proteomics. The

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non-biased nature and the highly multiplexing capacity that is enhanced by DIA will render a universal approach for targeted proteomics in translational and clinical investigations.

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