

# Current affairs in quantitative targeted proteomics: multiple reaction monitoring–mass spectrometry

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## Abstract

Quantitative targeted proteomics has recently taken front stage in the proteomics community. Centered on multiple reaction monitoring–mass spectrometry (MRM–MS) methodologies, quantitative targeted proteomics is being used in the verification of global proteomics data, the discovery of lower abundance proteins, protein post-translational modifications, discrimination of select highly homologous protein isoforms and as the final step in biomarker discovery. An older methodology utilized with small molecule analysis, the proteomics community is making great technological strides to develop MRM–MS as the next method to address previously challenging issues in global proteomics experimentation, namely dynamic range, identification of post-translational modifications, sensitivity and selectivity of measurement which will undoubtedly further biomedical knowledge. This brief review will provide a general introduction of MRM–MS and highlight its novel application for targeted quantitative proteomic experimentations.

**Keywords:** absolute quantification; quantitative proteomics; mass spectrometry; multiple reaction monitoring; stable isotope dilution; targeted proteomics

## INTRODUCTION

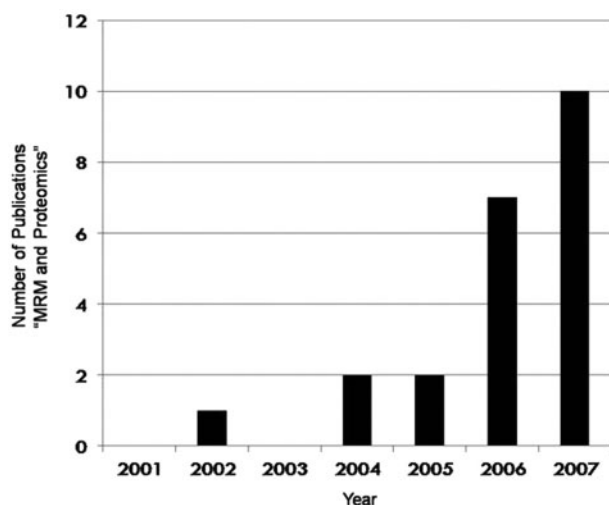
Quantitative targeted proteomics can mean a variety of things depending on a person's perspective, and can include, but is not limited to, any combination of affinity purification strategies followed by mass spectrometry (MS). These techniques are the basis of any targeted or focused, hypothesis driven proteomics experimentation. Simply by selecting and enriching for a compound or group of similar compounds by its very nature is targeted. Therefore, combining these affinity-based methodologies with data-dependent, i.e. global proteomic MS methodologies have been in the past considered targeted. However, recently growing in popularity is the development of a few novel non-data-dependent MS instrumentation methods, e.g. product ion monitoring (PIM), neutral loss scanning (NL), inclusion list scanning, immonium ion detection

and multiple reaction monitoring (MRM). These may or may not require *a priori* information to target a particular class of compounds in the mass spectrometer, the main idea being that the instrument is focused on gathering measurements on a select compound or group of compounds. These instrument methodologies in combination with affinity-based methods can even further target and focus one's proteomic experiments. While this review will touch briefly on a handful of these various methodologies, it will predominantly focus on the development and utilization of multiple reaction monitoring–mass spectrometry (MRM–MS) for targeted quantitative proteomics. This specific method is gaining popularity as indicated by the almost exponential increase in peer-reviewed publications indexed in PubMed, Figure 1. First, this review will provide an introduction to MRM–MS

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**Figure 1:** Number of publications per year indexed in PubMed when searching the terms “MRM and Proteomics”.

and quantification methodologies used in combination with MRM–MS, schematic representation shown in Figure 2, providing examples from the literature of its application to various biomedical research questions. Then, it will briefly introduce some of the other targeted instrumentation methodologies highlighting those primary research articles that provided a comparison of those different methodologies. Then the review concludes with future directions briefly mentioning some of the newer bioinformatic resources available to aid in the optimization and development of MRM–MS methodologies.

## OVERVIEW

MRM–MS is a deviation of selected reaction monitoring (SRM), see Figure 3. While its application is novel in the proteomics community, SRM has been utilized for several decades in the toxicology and pharmacokinetics disciplines. SRM transitions are highly specific scans for detecting specific analytes in complex mixtures utilizing, most predominantly, triple quadrupole-based mass spectrometers. The transitions are designed such that the first mass analyzing quadrupole (Q1) is set to transmit a narrow mass window around the desired parent ion and the third quadrupole (Q3, the second mass analyzing quadrupole) is set to transmit a narrow mass window around the desired fragment ion. Fragmentation via collisional induced dissociation (CID) occurs in the second quadrupole (Q2). Therefore, SRM requires two ions to generate a

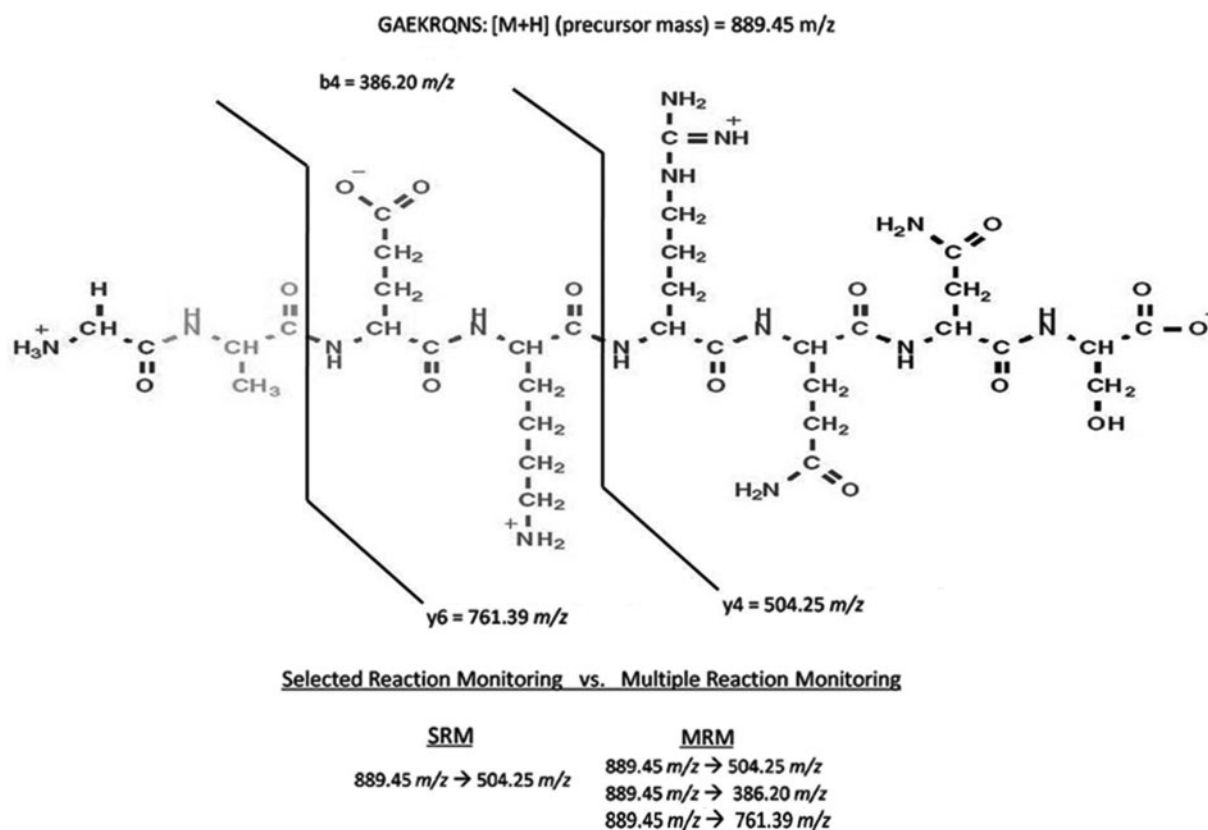
positive result, making it a very specific detection methodology with very low background thereby enhancing sensitivity of detection. Successful SRM transitions depend not only on the ionization efficiency of the parent ion (Q1 transmission) but also the fragmentation efficiency of this parent ion and subsequently the intensity of fragment ion (Q3 transmission). Inputting several different SRM transitions for the same or different analytes, multiple transitions can be monitored within one MS run. This is known as MRM, and is practiced almost as an art balancing productivity against sensitivity.

To increase productivity of the mass spectrometer method, one strives to increase the number of MRMs one can measure in any given experiment. The number of transitions per experiment or time scale is dependent on various factors, including most importantly the mass spectrometer’s cycle-time i.e. the time for the instrument to cycle through separation and detection of each transition. The time to analyze each transition is termed ‘dwell-time’ simply defined as the amount of time where one mass analyzer is detecting and measuring only one ion, and has important consequences for not only the number of transitions one can measure in any given experiment but also the sensitivity of detection. Dwell-time and cycle-time are directly proportional and intimately effect sensitivity. An increase in dwell-time results in a more sensitive measurement. However this more sensitive measurement is at the cost of increasing the cycle-time and therefore decreasing the number of transitions one can measure simultaneously, i.e. the productivity of the MS run. Traditionally, the dwell-time should be optimized such that each transition is being scanned and analyzed at least twice as it is eluted from the column and most importantly that one of the measurements will occur at, or close to, the apex of its elution profile which is important for quantification.

As stated previously, the MRM–MS methodology has been used extensively in the quantitative analyses of small molecules. For protein identification and quantification however, MRM–MS has not been as routinely used due to the additional challenges of method development. Theoretically, it is possible to spike into a sample a standard mixture of proteins in known concentration to perform quantification as is performed in small molecule analyses. However in practice, this does not allow for robust protein quantification because the standard



**Figure 2:** Schematic representative of the Targeted Proteomics Workflow. Traditionally, (shown in the bottom row), biomedical experiments result in an exploratory finding which is either refuted or confirmed in the literature. Those findings that are novel are validated with orthogonal technologies and published. In targeted proteomics workflows (shown in the middle row), discovery global proteomics experiments which may or may not be combined with quantitative measurements are utilized as the foundation for the design of targeted proteomics assay development. This assay development may or may not be quantitative in nature and may very likely be informative to additional discovery work. This targeted assay then leads to validation which may or may not be orthogonal in nature. Overall, different aspects of the work flow can be used simply as research tools or may reach a clinical endpoint with usefulness.



**Figure 3:** Comparison of selected reaction monitoring (SRM), where only one transition is utilized versus multiple reaction monitoring (MRM) where three transitions are monitored. The peptide sequence GAEKRQNS is used as an example. The peptide precursor mass is 899.45 m/z and is utilized for all transitions for both SRM and MRM. After the CID fragmentation reaction, only the following product ion of 504.25 m/z is used with the SRM method in contrast to MRM methods where three product ions 504.25 m/z, 386.20 m/z and 761.39 m/z are used.

proteins and the analyte proteins may not produce similar responses in the mass spectrometer due to ion suppression and differences in fragmentation. Additionally, recovery rates of the different proteins may be different if there are sample preparation steps prior to MS. This is why isotopically-labeled peptides in lieu of proteins are suggested. This technique has been recently utilized to quantify putative peptide and protein biomarkers where a proteotypic peptide is selected as a surrogate for the protein of interest [1–7]. Aptly suited to the multiplex capabilities of this MRM strategy, multiple signature peptides representing a particular protein can then be examined in one experiment allowing for the development of a highly selective, sensitive and high throughput quantitative methodology [1,8].

Quantification methods of peptide MRM–MS are based on the classic isotope–dilution MS. First introduced in the biomedical sciences several decades ago to measure calcium in the blood it became the standard methodology to measure and quantify metabolites, both endogenous and pharmacological. Recently, with regard to proteomics, the term AQUA, first coined by Scott Gerber *et al.*, is utilized for the absolute quantification of proteins and their modifications [9]. A variation of the small molecule isotope–dilution MS techniques, the labeled internal standard peptides are introduced to a protein sample prior to or during the proteolytic step. Both the labeled internal standard and the unlabeled native peptides are analyzed by LC–MRM–MS similar to small molecule isotope–dilution strategies, i.e. the peak areas of the labeled and unlabeled species are related for quantification. The internal standard and the native peptides generated by proteolysis are chemically identical; chromatographically co-eluting, ionization efficiency, and relative distribution of fragment ions, but are different in mass which allows the mass spectrometer to differentiate the two species from one another and from all other peptides in the matrix. Since the concentration of the internal standard is known, the ratio between the internal standard and the native peptide can be related quantitatively thereby deducing the absolute amount of native peptide. Furthermore, the stoichiometric relationship of the peptide or peptides to the protein allows subsequent quantification of the protein, which is the only step that is unlike the classical isotope–dilution experiments on small molecules. Use of peptides synthesized with stable

isotope-labeled amino acids now is a common strategy for creating peptide internal standards for absolute quantification [9,10]. Additionally, chemical labeling of synthetic peptides with the ICAT reagents has been used to create internal standards for accurate quantitation of P450 proteins [11]. Labeling cell lysates with iTRAQ reagents to determine relative differential regulation of proteins has been completed as well [12]. Using the isobaric and/or non-isobaric chemical labeling reagents, one can create unique and individualized internal standards by labeling one cell state with a light reagent which then acts as a control. The other cell state to be quantified relative to the control is labeled with the heavy reagent. Mixing the heavy- and light-labeled samples at a 1:1 concentration ratio creates a sample in which all peptides from the cell can be monitored with specific MRMs to the heavy-labeled peptides compared to the peptides from the control sample (monitored with specific MRMs to the equivalent light-labeled peptides). Computing a ratio of heavy/light for all peptides of interest in the dataset enables relative quantitative comparison. Recently a new labeling reagent is available, the mTRAQ reagent [13]. mTRAQ is derived from the original 4plex iTRAQ reagents. Like its predecessor, the mTRAQ reagent labels the primary amines of peptides (both *N*-terminus and lysine); however, the new mTRAQ light reagent does not contain the  $^{13}\text{C}$  or  $^{15}\text{N}$  isotopes and therefore has a different molecular weight than the original iTRAQ reagent (now termed mTRAQ heavy). These light and heavy mTRAQ reagents create a paired label set with a molecular weight difference of 4 Da, which increases the possible difference in each MRM transition, and can be utilized in the similar manner as iTRAQ with cell lysates.

## METHOD DEVELOPMENT

Still with significant technological developments, the use of MRM–MS for protein quantification is not yet routine. This can be attributed to the tremendous time commitment to method development, optimization and validation for quantification of protein markers. While information regarding *in silico* design is improving, MRM transitions for the peptides yielding the best precursor ion intensities and the most dominant MS/MS fragment ions are most effective if derived from empirical data, when available. The reason for this is because the

accurate prediction of fragmentation patterns for any given instrument and peptide sequence is still difficult, and remains a research question which is discussed briefly below. Furthermore, selection of a fragment ion, the Q3 target, for peptide and protein quantification is even more challenging than small molecule quantification because one is obliged to select a fragment ion that contains the isotopic label to exploit the resolving power and mass difference in Q3. For example, it is common to isotopically label lysine and arginine residues in a sample preparation scheme that includes trypsin as a proteolytic enzyme. Unfortunately, the labeled lysine and arginine residue at the C-terminus will result in the entire b-ion series which will not contain the label and therefore should not be used as Q3 masses as they will not discriminate in Q3 from the native peptide. Thus, careful consideration must be taken in the selection of an MRM transition, as the most abundant fragment ion cannot always be selected. Furthermore, a key requirement of the MRM methodology is the ability to distinguish between correct identifications and false positives. Peptides are much larger and more complex than small molecules, share considerable homology and are measured in very complex matrices. Since each fragmentation product of a peptide only provides information about one position in that peptide, it is highly likely to have interference, i.e. other peptides resulting in the same transition as the peptide analyte in question [14]. A schematic representation of peptide fragmentation is shown in Figure 3 highlighting some possible fragment ions after CID and outlines a comparison of SRM to MRM. By requiring multiple fragmentation product ions in the Q3 which together result in the same chromatographic elution time, the method can be highly specific providing confidence of the peptide identification in the absence of a full-scan tandem mass spectrum.

Different tryptic peptides from the same protein can produce ion currents differing by factors of  $10^3$  in LC-MS/MS experiments, excluding peptides that are not detected at all [15]. This variation is due to multiple factors, including propensity to ionize in the electrospray source, coincidence in elution time with other easily ionizing peptides, efficiency of release during tryptic digestion and the presence of unrecognized post translational modifications (PTM) arising due to biology or artificially during sample preparation [16]. A consistent response is required

across the dynamic range of the analyte measured to ensure proper quantification of both native and isotope-labeled peptide pairs [17]. A potential source of interference is in-source fragmentation of abundant peptides where the fragment ions rather than the precursors are the source of interference. This is caused by coincidence of the primary or secondary fragment of the precursor that has the same or nearly the same mass as the analyte transition of interest [18]. This can be a significant issue for quantification and is dependent on the sensitivity level one is attempting to achieve. It is critical to select transition ions that maximize specificity and potentially minimize interferences from co-eluting species that fall within the mass windows and tolerances of the detector. Decreased mass resolution (1000–3000 FWHH) in the triple quadrupole instruments requires careful consideration of peptide separation so as to ensure accurate measurement of the peptide of choice because of the decreased separation of analytes at the same nominal masses [19]. Therefore, in practice, initial LC-MS/MS experiments are performed on the biological sample to obtain preliminary information on the peptide characteristics, both ionization and fragmentation. Most commonly a full scan MS/MS spectrum in a linear ion-trap or TOF/TOF instrument is gathered. Once the precursor ion and fragmentation characteristics are noted, the precursor ion of interest and multiple fragment ions are chosen for MRM transitions and the sample is transferred to a triple quadrupole instrument. These transitions, based on the experimentally determined fragmentation pattern and CID parameters are further optimized on the triple quadrupole instrument. The sample is then re-analyzed with identical LC conditions to maintain the retention time with the goal of creating a set of MRM transitions with the best qualitative and quantitative properties per peptide of interest.

To address the tremendous time commitment and multiple rounds of analyses, sometimes on different instruments, required for the development of MRM-MS methodologies, a novel hybrid instrument (4000 QTRAP, Applied Biosystems) is now available which is both a triple quadrupole and a linear trap and offers a unique opportunity to design MRM-MS methods [20]. Termed the MIDAS (MRM-initiated detection and sequencing) workflow [21], when significant signal in a specific MRM transition is detected (both the parent mass in Q1 and fragment mass in Q3 are isolated and



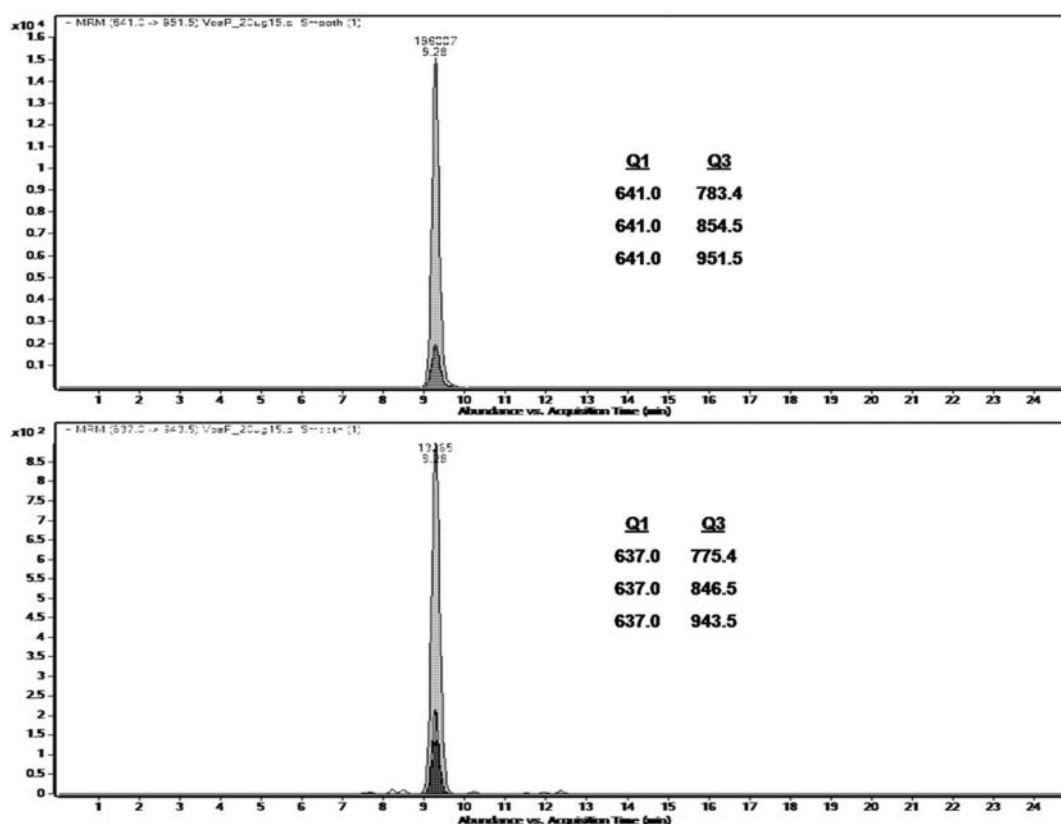
detected), the instrument switches the third quadrupole to ion trap mode and collects a full scan tandem mass spectrum. This full scan ion trap MS/MS obtained from the targeted detection can be submitted to a database search to confirm that the detected peptide is the peptide of interest and be utilized to further optimize the target for Q3. In this way the MIDAS workflow can develop MRM-MS methods in one step, eliminating the need to do preliminary method development confirming selectivity of peptide identification. Like traditional MRM-MS methodology development, the MIDAS workflow is still dependent on two things: (i) that the chosen MRM peptide ionizes efficiently enough to be measured by MS satisfying the first ion, Q1, of the MRM; and (ii) that the peptide fragments well enough and with sufficient intensity to generate the second ion, Q3, of the MRM. If using an *in silico* methodology to predict ionizable peptides such as; a module in MIDAS, P3 (Proteotypic Peptide Predictor) [22], TIQAM (Targeted Identification for Quantitative Analysis by MRM) [23], as discussed below, or other *in silico* prediction methods based on amino-acid sequence such as MDIP (Minimum Acceptable Detectability for Identified Peptides) [24], APEX (Absolute Protein Expression) [25] and those predicted are determined empirically to not ionize, another peptide that does ionize from the same protein can be tested and subsequently used instead. TIQAM is another software-derived workflow similar to MIDAS aimed at reducing the time commitment required for the development and validation of MRM assays [23]. In this workflow, the proteome is extensively mapped utilizing standard global discovery proteomic work-flows. These discover data are then gathered into the PeptideAtlas database or similar user-derived database where those proteotypic peptides uniquely identifying proteins of interest are assembled to establish MRM transitions based on the validated MS2 spectrum. The user can then decide on how many rounds of MRM-triggered quantification experiments for this validated listing of transitions in order to glean biologically useful information. TIQAM also offers a protein/peptide-centered-view to predict proteotypic peptides, perform *in silico* digestions, and view the MRM trace further optimizing the method development.

Whether or not empirical data has been collected, it is recommended that MRM transitions

are designed for chosen peptides approximately conforming to the following rules [1,12]:

- (i) With electrospray ionization, doubly charged parent ions are preferred over singly charged for Q1, however a triply charged parent ion mass is assumed if the peptide sequence contains a histidine residue and can be used if the peptide has a mass greater than 1500 Da.
- (ii) y-ions are most often preferred over b-ions to ensure the transition contains a labeling site. Case in point, with the AQUA labeling strategy, it is common to isotopically label the C-terminal lysine and/or arginine due to trypsin proteolysis thus resulting in all y-ions isotopically-labeled. In contrast, the mTRAQ and iTRAQ reagents label the N-terminal and lysine side chains. Therefore, all b-ions and all y-ions of a C-terminal lysine peptide will be labeled.
- (iii) The Q3 fragment ion should have a greater  $m/z$  than the selected Q1 parent ion, should contain at least four amino acids and not be complimentary unless providing verification on the labeling site. This enhances selectivity and eliminates low mass ion overlap for multiply charged parent ions.
- (iv) If the sequence contains a proline residue, the high abundance y-ion created from fragmentation N-terminal to the proline is selected.

After choosing the most sensitive transition for quantification, it is recommended that two additional transitions are chosen to provide the most selectivity and combined reduced interference from other peptides. The aim being is to assemble in the method at least three transitions per peptide and it is recommended to have three peptides per protein with at least one peptide having a truly unique sequence upon a BLAST search. However, in practice, biomedical considerations and particulars regarding peptide length and sequence homology may not necessitate these recommendations. Additionally, since this methodology is truly measuring a peptide as a stoichiometric representation of the protein in question, one must consider which peptides to use if there are known biological modifications to the protein such as truncation. If using these recommendations, one transition can be used for quantification and two transitions for qualitative identification of the peptide, as shown in Figure 4, thereby collectively identifying the peptide with



**Figure 4:** Extracted ion chromatogram of AQUA MRM spectra for prostate specific antigen (PSA) peptide LSEPAELTDAVK. The heavy-labeled peptide (C-terminal K is labeled with  $^{13}\text{C}_6$  and  $^{15}\text{N}_2$ ) is extracted in the top panel. The bottom panel shows the light, i.e. endogenous peptide found in VcaP whole cell lysate. Three transitions are outlined in each panel and show a 4 Da difference in the doubly charged precursor ion, and an 8 Da difference in the singly charged product ions. As the retention time of all six transitions align chromatographically, one can utilize this data for verification of the identity of the peptide as well as quantification as the amount of spiked heavy peptide is known and can be related to the endogenous peptide by the area under the each curve.

high confidence and allowing optimal cycle time for sensitivity and multiplexing multiple analyses. Alternatively, it is possible to use one charge state for quantitative calculation and the other for verification of identification or use both charge states and average them for quantification of all analyses, however this has yet to be shown in the literature.

Finally, because the MRM methodology is based on a specific peptide or peptides that are used as stoichiometric representation of a protein, and proteins are quantified by comparing the peptide peak area with an internal standard, whether it is a relative or absolute standard, it is imperative that peptides utilized are selective to the particular protein isoform of interest. In the past, measurement of small nuclear polymorphisms (SNPs) and quantitative mRNA expression by PCR technologies have been well established which can easily differentiate between highly homologous isoforms [26–34].

However, there still remains a need to develop similar methodologies for proteins. This is an area where MRM–MS is really gaining interest and momentum. Not only can a targeted approach such as MRM–MS lessen the ever challenging dynamic range issues most commonly encountered during global proteomics experiments thereby digging deeper into the low abundance proteome and low stoichiometric PTM, this methodology is showing tremendous utility in the verification of both global proteomics identification and quantification data [12,23,35–39]. Unfortunately, global proteomics data can be inconclusive. Often these data include one-peptide identifications or multiple peptide identifications which do not discriminate between highly homologous isoforms. Therefore, it is often a requirement to verify global quantitative proteomics results with orthogonal methodologies such as Western blotting. Unfortunately, there may

not be an available antibody with or without PTM specificity needed or discriminatory antibodies which distinguish closely related isoforms. Additionally, quantification is less than absolute and these current immunological methodologies to verify proteomics experiments are low-throughput. Therefore, isotope-labeled peptide and protein standards are particularly useful for focusing instrument detection on low abundant proteins and PTMs (most commonly one-peptide identifications) and the measurement of highly homologous isoforms where multiple signature discriminatory peptides can be examined by MRM in a single experiment [40]. Specificity can be achieved by the selection of sequences with no homology in BLAST, as well chromatographic retention standardization. MRM methodologies are particularly useful for measurement of highly homologous protein isoforms because of the high level of specificity achieved [11,12]. Single amino-acid changes in a peptide sequence can often be distinguished by MRM enabling resolution and quantification of closely related isoforms. Furthermore, targeted detection at higher sensitivity often leads to information on lower abundance isoforms not detected by the intensity driven precursor selection of data dependent global strategies. Therefore, these aspects of MRM methodologies enable precise quantification of protein isoforms and overcome some of the limitations of classical shotgun quantification proteomic strategies thereby obtaining more sensitive, specific and selective information. For instance, Rosalind Jenkins *et al.*, utilized the ICAT labeling methodology to not only discriminate between highly homologous cytochrome P450 enzymes in liver microsomes but also extended their methodology to relatively quantify each of the isoenzymes with MRM-MS [11]. Then utilizing synthesized stable isotopically-labeled peptides, they extended their methodology even further to determine absolute quantification of the isoenzymes.

### Utility of MRM analysis

The confident identification of PTM have always been and still is an enormous challenge to proteomic specialists. The ability to detect phosphorylation, acetylation, glycosylation, and even ubiquitination has become a specialized skill set because each requires knowledge in not only specialized sample preparation but also in MS method development. Because the control of reversible protein

phosphorylation is central to most intra- and inter-cellular signal transduction, it is a most popular topic of interest to molecular biologists and pharmacologists. Unfortunately, development of good antibodies that recognize distinct phosphorylation states is a cumbersome, time-consuming and expensive process that requires *a priori* knowledge of the proteins and its phosphorylation sites [17]. Additionally, due to the high combinatorial possibilities of multiple phosphorylation sites, many different antibodies need to be developed to fully understand the dynamic process [9]. There is also a possibility of antibody cross-reactivity with different versions of multi-phosphorylated peptides leading to misleading quantification data and stoichiometric analysis [5]. Despite these issues, antibody-based approaches have been more favored for the determination of phosphorylation in signal transduction studies. However, by taking advantage of the characteristic fragmentation pattern of phosphopeptides, i.e. the NL of the phosphate group, it is been possible to identify phosphopeptides in the presence of non-phosphorylated peptides in the mass spectrometer. Unfortunately, this method often results in many non-phosphopeptides surreptitiously generating a similar NL, causing false positives that must be reconciled by advanced bioinformatic strategies, and most importantly, causes the mass spectrometer to waste scan time detecting non-phosphorylated peptides. However, by using *a priori* biological knowledge, one can design a targeted MRM-MS-based method to achieve a higher level of selectivity and sensitivity. These MRM-MS-based targeted analyses of phosphorylation and quantification have been conducted [5-7,9,17,21,41-44]. This approach is much different than other MS-based techniques for identifying phosphopeptides, like NL scan or triple play experimentation as it is highly selective and can generate absolute quantification of the dynamic stoichiometric phosphorylation events.

Acetylation is another PTM of great biological interest and is arguably easier to detect in a mass spectrometer than phosphorylation as the modification is not as liable chemically. As such, it is often searched for indiscriminately as a variable modification during database algorithm searching. This however greatly increases the search time and cost. While MRM-MS has not been commonly used to focus on acetylation as a PTM like phosphorylation, Griffiths *et al.*, demonstrated an interesting



approach [8]. Since acetylation can be detected by the characteristic reporter ion of 126.1  $m/z$  after CID, corresponding to the immonium ion of the acetyl-lysine, the authors utilized a MRM-MS approach to detect acetylation in a test case proteins. Using the MIDAS workflow, the authors generated a listing of the proteins' amino-acid sequence and the respective sequences that are purported to contain an acetylated lysine. For the methodology, Q1 precursor scanning was set to dynamically scan over a mass range of 450–1200  $m/z$ . Q3 was held to only transmit the acetyl-lysine immonium ion of 126.1  $m/z$ . If and when a transition was detected, Q3 switched to ion trap and captured the full scan tandem mass spectra thereby allowing database searching and sequencing of the peptide. These authors later compared this MRM-MS-based approach to a typical data-dependent MS approach and found the MRM approach to be approximately 10-fold more sensitive.

Probably one of the most chemically complex PTM of great biological interest, especially in the area of biomarker discovery, is glycosylation. Utilizing a combination of *N*-glycosite isolation and stable-isotope-labeled standard MRM-MS, Stahl-Zeng *et al.*, analyzed and quantified *N*-glycosylated peptides in plasma detecting sub-ng/ml concentrations [45]. These authors also prove that this targeted analysis can accurately quantify over a dynamic range of five orders of magnitude. This is an additional order of magnitude over what can even be detected in a global proteomics experiment, let alone quantified, demonstrating a great power of MRM-MS-based proteomics approaches. Also, utilizing MRM-MS to monitor the asparagine-linked glycosylation, Hulsmeier *et al.* [46] detected several serum glycoproteins to diagnose deficiencies of *N*-glycosylation biosynthetic pathways in congenital disorders of glycosylation. These deficiencies, associated with endocrine and coagulation disorders, manifest themselves with different *N*-glycosylation site occupancies and are hypothesized to correlate to severity of disease. Prior to this publication, the exact degree of protein under-glycosylation had not been determined due to lack of a precise measurement. Utilizing MRM methodologies, Hulsmeier *et al.* was able for the first time, measure precisely *N*-glycosylation and correlate the degree of *N*-glycosylation and severity of disease. Like other PTM measured and analyzed with the targeted

MRM-MS approach, Sahana Mollah *et al.* utilized the MIDAS workflow to identify conclusively four ubiquitinated sites on recombinant receptor interacting protein [43].

### Additional targeted proteomics methods

Other directed MS-based analyses that are used for targeted approaches are inclusion list scanning, NL, immonium ion detection and PIM. Inclusion list scanning, listing precursor masses of interest for detection rather than scanning entire mass ranges [47], NL scanning and immonium ion detection can be used individually or combined with MRM-MS approaches as shown and referenced above and therefore will not be discussed in detail here. PIM, theoretically, is a compilation of different MS methods. In its purest form, PIM is most akin to selected ion monitoring (SIM) which is an older technique that was used to increase sensitivity and dynamic range of detection because instruments did not have the faster scan times found today. In a SIM methodology, an instrument is focused on measuring only one ion at any given time. It is now mostly used to computationally extract a particular ion from complex spectra for ease of visualization. Like SIM, PIM is only measuring one ion at any given time, but it is the product ion after CID that is monitored as opposed to the precursor ion in a SIM method. A newer version of PIM was recently pioneered by Vathany Kulasingam *et al.* [48]. These authors utilized PIM as a technique specifically for ion trap instruments in lieu of triple quadrupoles. Because precursor mass selection, CID and detection of the product ions of interest are all occurring in the same compartment, i.e. space of the instrument it is possible to monitor all product ions from fragmentation of one precursor ion. This will provide additional structural information without increasing the duty cycle of the instrument. Showing proof of principle, in combination with antibody capture, the most sensitive sample purification methodologies, the authors were able to achieve a limit of detection of 10 amol with a coefficient variation of <20% with a linear range of over four orders of magnitude. An interesting compartment of the literature, albeit scant, is the comparisons of these targeted methodologies. All work done in this area concludes that MRM or other MS-based targeted methodologies enhance sensitivity and selectivity over global proteomics methodologies. For example, Hasmik

Keshishian *et al.*, noted that the sensitivity of targeted MS analysis enhances the lower detection limit for peptides by up to 100-fold when compared to unbiased MS analysis [18]. Additionally, Charanjit Sandhu *et al.*, also compared a data-dependent acquisition, where a precursor ion is selected for fragmentation-based on intensities with a targeted peptide monitoring, where predefined  $m/z$  ratios are selected for fragmentation to MRM. They conclude that the targeted peptide monitoring far exceeds the data-dependent acquisition methodology for sensitivity.

These targeted MS-based methodologies are often combined or compared against affinity-based methodologies. While most of these methodologies are well seasoned, some novel approaches have been developed and combined with MRM-MS. It had been shown that two different antibodies used in a sandwich immunoassay can provide extreme sensitivity. However, the limited ability to multiplex immunoassays make it necessary to use an alternative method. SISCAPA (Stable Isotope Standards and Capture by Anti-Peptide Antibodies) was designed and pioneered by Anderson *et al.*, to address this issue. In SISCAPA, specific tryptic peptides are selected as stoichiometric representatives of a protein. Anti-peptide antibodies are generated and immobilized on nanoaffinity columns to enrich these specific peptides along with spiked stable-isotope-labeled internal standards. Both the heavy (internal standards) and light peptides are enriched from a complex matrix such as human serum with the immobilized antibodies. The enriched sample is then introduced into a mass spectrometer for analysis. Both relative and absolute quantification information about the protein of interest is then gathered [49]. This method was further optimized for greater precision and accuracy by Whiteaker *et al.*, reporting a physiologically relevant sensitivity in the ng/mL range [50]. Whiteaker *et al.* also utilized the a combination of antibody and MS approaches to confirm global proteomics results from mouse model of breast cancer while characterizing and transferring a couple of the proteins identified previously to a plasma biomarker [38]. Finally, Kornilayev *et al.*, extended the SISCAPA approach developed by Anderson *et al.* by utilizing polyclonal monospecific anti-peptide antibodies raised against unique cytochrome P450 isoenzymes specific tryptic peptides for qualitative and quantitative proteomic analysis. The point of this approach is that every isoenzyme contains a unique

tryptic peptide(s) that can be used for differential analysis. Chemical proteomics is also a novel affinity-based methodology that is gaining popularity. Pioneered by Bantscheff *et al.*, small molecule non-selective kinase inhibitors were immobilized onto beads (kinobeads) and incubated with whole cell lysate in an effort to bind both known and novel protein targets of the inhibitors. Coupled with iTRAQ labeling, the authors were able to quantitatively profile both known protein targets, assess the binding affinity and map the phosphorylation status of the captured proteome [51, 52].

## INFORMATICS

To conclude, one of the more exciting practical initiatives and developments in targeted MS-based methodologies is within the informatics community. Growing interest in using this technology to quantify proteins has led to a number of new software developments over the last few years. As state above, the MIDAS and TIQAM workflows were developed for targeted detection of proteins [21, 44], their respective PTM [8,41] and for the rapid development of MRM assays [1, 23, 53]. Informatic strategies for the prediction of the most likely peptides (proteotypic peptides) that will be observed for proteins of interest are emerging [14, 15, 23, 54, 55]. Physicochemical properties of the peptide's amino-acid content should provide predictors to enable selection of which synthetic peptides to generate for absolute protein quantification [16, 22, 24, 25, 54–56]. Unfortunately, general proteomic strategies assume that any protein's peptide fragments are observed with equal likelihood, however only a few proteotypic peptides are repeatedly and consistently observed in any given experiment [55]. To address this issue, Kline, KG *et al.*, have developed research grade software, named P3 (Proteotypic Peptide Predictor), that is freely available to predict proteotypic peptides [22]. This software was developed from a large catalog of peptide spectra from human heart [22]. Interrogation of enormous amounts of shot-gun LC-MS/MS data in public repositories containing information on the most commonly observed peptides can assist in the determination of which peptides to use for MRM-MS analyses if annotated correctly [15, 57]. Furthermore, investigations into fragment ion intensities typical in MS/MS spectra has provided useful information on potential tandem MS sequence ions. This information can

also be gleaned from the public repositories of LC–MS/MS data. Some of the public repository databases useful for this type of interrogation are: PeptideAtlas (<http://www.peptideatlas.org>), the Global Proteome Machine Database (GPMDB, <http://gpmdb.thegpm.org>), Protein Identifications Database (PRIDE, <http://www.ebi.ac.uk/pride/>) and Tranche (<http://tranche.proteomecommons.org/>). It is hoped that the combined resources of the many terabytes of data located in these public repositories will offer even faster developments for the analysis of targeted quantitative proteomics. Lastly, the MRMer has been developed to manage MRM–MS-based experiments which extracts and associates precursor and product masses, allows visualization of co-elution and calculates the relative area under the curve for relative and absolute quantification [58]. This is an exciting sub-field of proteomics which is gaining momentum and has the capability to address not only some of the fundamental issues with global proteomics experiments, but may finally realize the promised hype of high-throughput multiplexing biomarker assay development.

### Key Points

- MRM–MS while an older methodology is currently being utilized to expand proteomics experimentation.
- MRM–MS has been shown to have utility distinguishing between highly homologous protein isoforms, verify global proteomics findings and identify PTM.
- MRM–MS can be combined with AQUA or labeling technologies such as ICAT, iTRAQ and mTRAQ for absolute and relative quantification, respectively.
- In combination with additional targeted methodologies such as affinity purification and SISCAPA, MRM–MS is a highly promising methodology to enhance sensitivity and selectivity of the proteomics technology answering some unmet biomedical questions.

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