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Targeted Quantitation of Proteins by Mass Spectrometry

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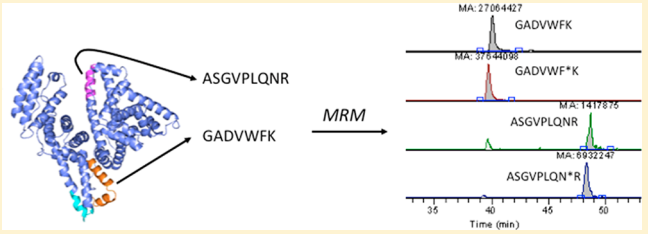
ABSTRACT: Quantitative measurement of proteins is one of the most fundamental analytical tasks in a biochemistry laboratory, but widely used immunochemical methods often have limited specificity and high measurement variation. In this review, we discuss applications of multiple-reaction monitoring (MRM) mass spectrometry, which allows sensitive, precise quantitative analyses of peptides and the proteins from which they are derived. Systematic development of MRM assays is permitted by databases of peptide mass spectra and sequences, software tools for analysis design and data analysis, and rapid evolution of tandem mass spectrometer technology. Key advantages of MRM assays are the ability to target specific peptide sequences, including variants and modified forms, and the capacity for multiplexing that allows analysis of dozens to hundreds of peptides. Different quantitative standardization methods provide options that balance precision, sensitivity, and assay cost. Targeted protein quantitation by MRM and related mass spectrometry methods can advance biochemistry by transforming approaches to protein measurement.



Quantitative measurement of proteins is one of the most commonly performed analyses in biochemistry. These measurements indicate changes in protein expression and posttranslational modifications that help explain the functional states of enzymes, pathways, and networks. For almost 40 years, the mainstay of protein quantitation has been the Western blot, which employs antibodies to detect proteins transferred from polyacrylamide gels to nitrocellulose or polyvinylidene fluoride membranes.[1,2] Western blotting has limitations, mainly related to the performance of the antibodies. Most antibodies used for immunoblotting detect multiple bands in complex protein mixtures, but analysts typically ignore these and focus on the "correct" molecular weight range of the protein. Antibodies also may not distinguish highly similar homologues and sequence variants arising from polymorphisms and mutations. Finally, some antibodies can detect specific modifications (e.g., phosphotyrosine), but few can reliably distinguish between different specific modification sites on proteins.

Mass spectrometry (MS) provides a next-generation platform that overcomes many of the limitations of Western blotting and provides new capabilities for protein analysis. The field of MS-based proteomics has grown tremendously over the past 15 years and has had a broad impact in biochemistry and cell biology (for recent reviews, see refs 3 and 4). Perhaps the most important advance over the past 5 years is the growth of MS-based, targeted protein measurement. A key distinction in proteome analysis platforms is between global profiling and targeted quantitation methods. Global profiling methods employ nondirected analyses (e.g., shotgun proteomics) that yield protein inventories accompanied by quantitative measurements of varying precision. Quantitative estimates in global profiling analyses are achieved through isotope labeling strategies, such as iTRAQ[5] or SILAC,[6] or by label-free strategies, such as spectral counting,[7] and provide a basis for relative quantitative comparisons between samples. The limitations of these strategies arise directly from the semi-random sampling methods used to acquire tandem mass spectra (MS/MS spectra) for peptides in complex proteome digests. Higher-abundance peptides are sampled frequently and yield more precise measurements, whereas lower-abundance peptides are sampled less frequently and yield less precise measurements. Moreover, lower-abundance peptides may be sampled in some global analyses, but not in others, thus creating a "missing data" problem that complicates statistical analyses.[8]

In contrast to global profiling, targeted analyses measure specific peptides. This approach originated with work by Desiderio and colleagues, who used stable isotope-labeled standards to measure peptide hormones by field desorption MS.[9] The application of newer-generation electrospray liquid chromatography−tandem mass spectrometry (LC−MS/MS) instruments allowed the development of targeted peptide quantitation over the past decade. The underlying concept is that proteins may be quantified by measuring their specific constituent peptides following proteolytic digestion. The acquisition of data only for the selected peptides allows measurements with higher precision, sensitivity, and throughput. Protein quantitation by selected measurement of surrogate peptides is the most rapidly growing application of MS in proteome analyses. MS-based targeted protein assays offer two compelling advantages over immunoassays, the first being the ability to systematically configure a specific assay for essentially

Received: January 29, 2013
Revised: March 16, 2013
Published: March 21, 2013

 © 2013 American Chemical Society dx.doi.org/10.1021/bi400110b | *Biochemistry* 2013, 52, 3797−3806

any protein or posttranslationally modified protein form, without the requirement for an antibody. The second is the ability of targeted MS assays to perform multiplexed analysis of many peptides in a single analysis. Targeted peptide-based protein assays allow large-scale, quantitative proteomic analysis projects and have found widespread application in the systematic development of assays for protein biomarker validation studies. The rapid maturation of analysis methods and software for targeted quantitation, together with the widespread accessibility of applicable MS instruments, offers biochemists a transformative platform for systematic, reliable, and essentially universal protein quantitation. Accordingly, our focus in this review is on the application of targeted protein quantitation in biochemistry.

PROTEIN QUANTITATION BY TARGETED ANALYSIS OF PEPTIDES

The concept underlying targeted protein measurements via peptide quantitation is illustrated in Figure 1. Peptides with

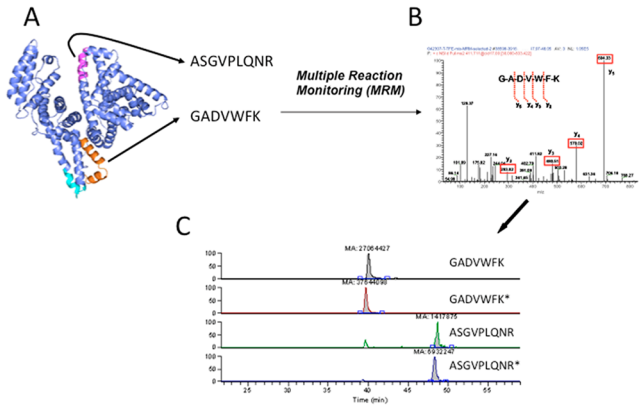


Figure 1. Overview of targeted protein quantitation by MRM. (A) Selection of peptides specific for the target protein. (B) MRM analysis monitors sequence-specific transitions derived from intense product ions in the MS/MS spectrum. (C) Integrated peak areas from the signal corresponding to transitions allow quantitative comparisons. Peak areas for target peptides are compared to peak areas for stable isotope-labeled reference peptide standards.

sequences unique to the target protein are selected as surrogates for the parent protein. Although tryptic peptides typically are selected and offer key advantages (see below), other sequence-specific digestions may be employed. In a tandem MS instrument, each peptide ion undergoes fragmentation to produce characteristic b- and y-ions, which are fragments containing the N- and C-termini of the peptide ions, respectively. Combinations of intact peptide ions (precursors) and resulting specific fragment ions (products) comprise transitions that are specific for the monitored peptide sequences. Although any particular precursor−product transition can map to multiple peptide sequences, the co-occurrence of multiple such transitions indicates the targeted peptide sequence with high specificity. The detection of peptides through the signals created by these transitions is called either selected reaction monitoring (SRM) or multiple-reaction monitoring (MRM). Both terms are used interchangeably in the literature; we will use MRM here to describe the process and method. The peak areas for MRM transitions are integrated as measures of peptide abundance and serve as the basis for quantitative comparisons.

MRM assays can monitor multiple transitions, which allows multiplexed analyses of hundreds of peptides in a single LC−MS run. The numbers of peptides and proteins that can be analyzed in a single run depend on the MS instrument used, the numbers of transitions monitored for each peptide, and the number of peptides monitored for each protein. With LC−MS methods in which MRM transitions are monitored during specific elution time windows for specific peptides, hundreds of peptides representing dozens of proteins can be measured in a single analysis. This capability for multiplexed analyses allows systematic measurement of multiprotein networks and pathways, a task that would be prohibitively expensive and cumbersome with Western blotting.

MS INSTRUMENTATION FOR MRM ASSAYS

Although essentially any tandem MS instrument can be used for targeted quantitation, triple quadrupole and quadrupole-ion trap (Q-Trap) MS instruments are most widely used. Both use the first quadrupole component of the mass analyzer to select targeted peptide precursor ions for fragmentation in a collision cell. Specified fragment ions then are detected either by the third quadrupole or by the linear ion trap. Although both instruments can execute full scan detection of all product ions, the most sensitive and repeatable measurements are achieved when the second mass analyzer targets selected product ions, as in MRM. Because only the specified transitions are recorded, other product ions are not detected. Triple quadrupole and quadrupole-ion trap mass spectrometers provide measurements of a wide dynamic range ($>10^5$), high sensitivity, and low measurement variation for measurement of MRM transitions. A limitation of triple quadrupole instruments is the relatively low resolution of precursor m/z measurements, which may allow interference from nominally isobaric background contaminants in complex mixtures. Triple quadrupole instruments are also limited by their duty cycle, the rate at which transitions can be sampled with an acceptable signal-to-noise ratio. The hydrid configuration of the quadrupole-time-of-flight (QqTOF) instrument can also be used for quantitative applications. This mass spectrometer contains a mass-resolving quadrupole (Q) and a collision cell (q) similar to a triple quadrupole; however, the third quadrupole (Q3) is replaced with a time-of-flight (TOF) mass analyzer offering high sensitivity, mass resolution, and mass accuracy for both precursor and product ion spectra.[10] The ability of both the Q-Trap and QqTOF instruments to acquire full scan product ion data with high mass accuracy allows these platforms to be alternative instrumentation for performing quantitation, in addition to the more traditionally used triple quadrupole mass spectrometer.Variants of MRM can be achieved on ion trap instruments, in which an inclusion (target) list of peptide precursor m/z values is used to direct data acquisition. Instead of monitoring only specified product ions from MS/MS of selected precursors, the process allows the acquisition of full scan MS/MS spectra, from which product ion signals can be extracted during data analysis. The resulting extracted ion chromatograms are used to generate peak areas for transitions of interest and are used for quantitation. This approach has been employed with low-resolution linear ion traps[11,12] and termed pseudoselected reaction monitoring (pSRM) but has been recently demonstrated as a particularly powerful approach on a newer quadrupole-Orbitrap hybrid (Thermo Q-Exactive) instrument. This instrument combines a quadrupole analyzer with the Orbitrap, which actually contains a C-trap, which

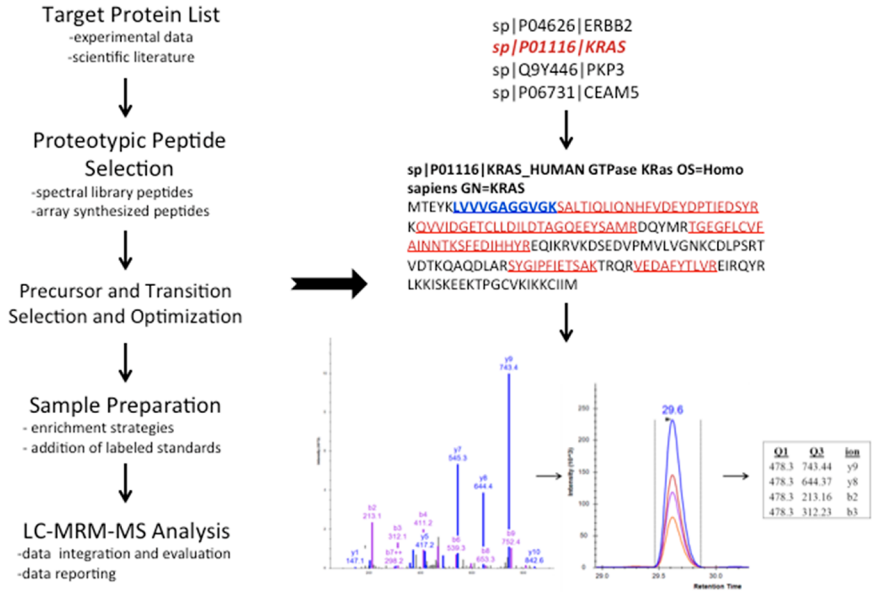


Figure 2. Workflow for the design of an MRM assay. See the text for discussion.

serves to store and shuttle ions between a high-energy collision-induced dissociation (HCD) cell and the Orbitrap analyzer, which performs high-resolution m/z analysis.[13] Recent preliminary reports describe several modes of operation for targeted peptide analysis, the most powerful of which is termed parallel reaction monitoring (PRM), which generates high-resolution, full scan MS/MS data, from which transitions can be extracted as described above.[14,15] A key feature of this approach of fast scanning, high-resolution analyzers is the highly specific extraction of signals for target peptides of interest, thus restricting interference from nominally isobaric contaminants. Preliminary reports suggest that PRM analyses exhibit performance characteristics (dynamic range and measurement variation) comparable to those of MRM analyses performed on triple quadrupole instruments,[14,15] and it appears likely that further development will expand the scope of targeted peptide and protein quantitation.

SELECTION OF TARGET PEPTIDES FOR MRM ASSAYS

Key steps in the workflow for configuring MRM assays for proteins are summarized in Figure 2. The process begins with a list of target proteins inferred from previous experiments or the scientific literature. The first step is to select peptides from the target proteins that will be measured using MRM analysis. The specificity of the assay requires selection of proteotypic peptides, whose sequences are unique to each parent protein. Although essentially any reproducible digestion method can be used, MRM assays most frequently are based on tryptic peptides, which typically range from 5 to 25 amino acids in length and usually form multiply charged positive ions, which provide useful sequence information through MS/MS fragmentation. In addition, selection criteria for proteotypic peptides consider other key characteristics to enhance the specificity of the assay and to minimize interference (for recent reviews, see refs 16−18). One important criterion is selection of peptides that are unique to the target protein and, where necessary, to specific protein isoforms or variants. Tryptic peptides with fewer than eight residues are typically avoided, as these sequences are unlikely to be unique. Sites of known posttranslational modifications (e.g., phosphorylation and acetylation) on peptides are avoided unless the assay is specifically targeted to the modified form.

To reduce potential sources of variability, peptides for MRM are selected for optimal stability. Peptides containing residues susceptible to artifactual modifications during sample preparation, such as methionine (oxidation) and cysteine (carbamidomethylation and oxidation), aspartic acid-glycine pairs (deamidation), and N-terminal glutamine (pyroglutamic acid formation) and asparagine (deamidation), may be avoided. Peptides containing sequences that commonly result in missed cleavages (e.g., Lys-Lys and Arg-Arg) may display variable digestion yields. Ideally, proteotypic peptides are taken from across the full protein sequence. Despite these considerations, peptide selection is an empirical exercise that balances ideal characteristics with practical limitations. Long proteins yield more potential proteotypic peptides than short proteins, and sequence features can greatly constrain peptide selection. In some instances, proteins of interest may have significant sequence homology, making it difficult to adhere to the peptide selection criteria described above. Modification of the inclusion criteria thus may be necessary to define the MRM assay.

An important aspect of peptide selection is identification of peptides that have been previously observed in MS/MS analyses and thus are known to be detectable. Such data are found in online repositories such as PeptideAtlas,[19] the Global Proteome Machine Database,[20] and PRIDE,[21] which contain peptide sequences and spectra. For proteins not found in a database, computational software tools that predict the most likely MS-observable peptides have been developed. Such prediction tools, which include ESP predictor,[22] PeptideSieve,[23] PepFly,[24] and others,[25,26] are trained with MS/MS data sets and fragmentation models to link physicochemical properties of peptides to the likelihood of peptide formation and detection.

OPTIMIZING PEPTIDE DETECTION

Because MRM measurements are based upon the signal from precursor−fragment ion transitions, selection of transitions that optimize selectivity and sensitivity is critical for assay performance. Transitions should be selected to provide a

dx.doi.org/10.1021/bi400110b | *Biochemistry* 2013, 52, 3797−3806

maximal signal while establishing sequence specificity of detection. Transitions can be selected from (1) libraries of previously collected MS/MS spectra in online repositories (see above), (2) computational tools that predict fragmentation, (3) inference from ion trap or Orbitrap CID or HCD spectra, or (4) analysis of synthetic peptide standards. Just as criteria for selecting target peptides exist, characteristic peptide fragmentations empirically found to provide a high signal intensity have been described. Several software tools, such as MRMaid,[27] SRMAtlas,[19] MRMer,[28] and MaRiMba,[29] for facilitating transition selection have been described. Because MRM assays are largely conducted with triple quadrupole instruments (see below), optimization has been directed to this type of mass analyzer. On the other hand, peptide MS/MS spectra in online repositories have been acquired mainly with ion trap, Orbitrap, and tandem time-of-flight (TOF) mass analyzers, all of which produce spectra with somewhat different characteristics. Previous work has systematically explored the inference of triple quadrupole MS/MS fragmentation from ion trap MS/MS data[30,31] and from HCD data.[32] For analyses on triple quadrupole instruments, y-ions having m/z values larger than that of the precursor and high signal intensities are generally favored, as noise (and potential interference) is increased below the precursor.

To ensure the highest selectivity and sensitivity, a systematic refinement of the selected peptides and transitions can be employed.[33,34] Not surprisingly, there is a direct trade-off between the specificity of peptide detection in MRM and the number of transitions monitored per peptide. Assays that monitor a single transition typically detect many signals and have the highest propensity for "false positive" quantitation. Requiring at least three transitions per peptide target to be monitored dramatically decreases the number of detected peptides and increases the level of confidence in assay specificity. Transitions can be optimized and validated experimentally by using synthetic reference peptide standards. This can be done with high-purity, isotope-labeled standards, which are used for stable isotope dilution (SID) analyses. However, libraries of moderately pure (~85%) standards can be generated at a modest cost for high-throughput validation of MRM assays.[35] An alternate approach is the expression and digestion of the target proteins to generate libraries of their constituent peptides.[36] These reference peptides can be used to confirm the selected transitions and relative fragment ion intensity and to determine chromatographic retention times. The use of synthetic standard peptides in assay development provides the highest degree of assurance that measured transitions truly represent the target peptides and proteins.

In a typical MRM analysis, a small set of proteins (typically fewer than five) and their peptides are measured and the corresponding transitions are monitored continuously throughout the entire chromatographic elution program. This approach is relatively inefficient, because the peptide targets each elute during a brief elution window. The signal acquired outside each window is essentially wasted analyzer time. Because the duty cycle of the MS instrument is limited, this inefficiency also limits the number of transitions that can be measured. To increase the efficiency of MRM analyses, a timed acquisition mode, termed scheduled MRM analysis, can be used to increase the number of peptides monitored in a single chromatographic run, while maintaining the highest possible MRM transition duty cycle and degree of sensitivity.[37] In a scheduled MRM analysis, the transitions of a peptide are acquired only during a defined elution time window. To perform a scheduled MRM experiment, the retention times of the target peptides must be known and chromatography must be stable and reproducible. Any shifts in retention time due to instability in the chromatographic performance may cause peptides to elute outside the specific retention time window, resulting in "missed" quantitation events.

SKYLINE: A POWERFUL TOOL FOR MRM ASSAY DESIGN AND DATA ANALYSIS

One of the most important developments in the field of targeted MS assays for peptides and proteins is Skyline, an open-source software platform that provides support not only for experimental design (selecting peptides and optimization of transitions) but also for downstream data analysis[38] (https://brendanx-uw1.gs.washington.edu/labkey/project/home/software/Skyline). Skyline utilizes the ProteoWizard libraries[39] to allow analysis of data from all MS instrument platforms, thus providing a vendor-neutral resource for sharing and creation of both methods and results across instrument platforms. Skyline facilitates the generation and refinement of proteomic peptide lists from protein sequences or database entries, both by utilizing online MS/MS spectral repositories and by supporting the generation of custom-built libraries based upon sets of locally acquired tandem spectral data.

Skyline creates transition lists and vendor-specific instrument methods that can be imported directly into instrument control software for MS instruments from several vendors. Skyline also provides a platform for standardized analysis of MRM result files for peak integration and visualization and data quality assessment across multiple analyses. Finally, Skyline allows the export of processed data in custom report formats compatible with subsequent statistical analyses, publication, and database deposition.

Beyond the capabilities and performance features of the software, Skyline represents an important innovation in proteomics by providing a widely used (>14000 downloads since the initial release in May 2009), community-supported, open-source platform for MRM analyses. Skyline thus helps the analytical community avoid the fragmentation of bioinformatics methods and tools that has hampered standardization of shotgun proteomics and many genomic analyses. This interesting experiment in community-supported software is itself one of the most important innovations in the field of proteomics.

MEASUREMENT NORMALIZATION AND QUANTITATIVE COMPARISONS

The gold standard method for comparison of peptide abundances in MRM measurements is stable isotope dilution (SID), in which a stable isotope-labeled synthetic peptide analogue is used as an internal standard for each target peptide (Figure 3). Typically, standard peptides are labeled by incorporation of [$^{13}C_6{}^{15}N_2$]lysine or [$^{13}C_6{}^{15}N_4$]arginine. Peptide standards for SID should be of high chemical and isotopic purity (>95%) and should be quantitatively standardized by amino acid analysis. The standards usually are spiked into samples immediately after digestion and thus serve to correct for subsequent analytical steps. The heavy-labeled peptide standards co-elute with unlabeled isotopomers in liquid chromatography separations and display identical MS/MS fragmentation patterns but differ only in mass due to the

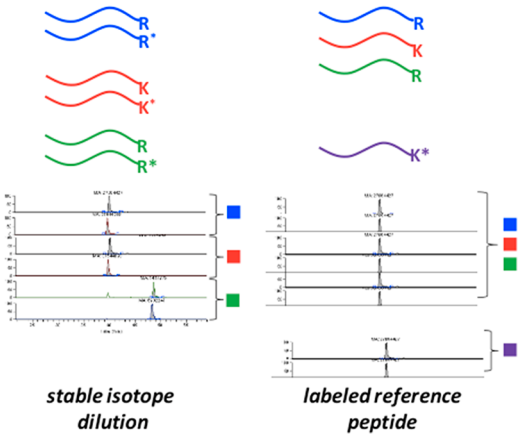


Figure 3. Quantitative comparison by stable isotope dilution (SID) and labeled reference peptide (LRP) methods. In SID, a separate isotope-labeled peptide standard is used for each target peptide. In LRP, a single stable isotope-labeled peptide is used as the reference for all target peptides.

isotope labeling. This resulting mass shift in both precursor and product ions allows the mass spectrometer to differentiate the unlabeled and labeled peptides. Because complex peptide digests often contain multiple sets of co-eluting transitions that may be mistaken for the target peptide, co-elution of the isotopically labeled standard identifies the correct signal and provides the best protection against false positive quantitation.

SID−MRM analyses exhibit the best performance characteristics of all targeted peptide measurements, with typical linearity over 4−5 orders of magnitude, measurement coefficients that typically vary by <10%, and limits of detection in cell and tissue lysates in the range of femtomoles per milligram of protein.[40−43] The one disadvantage of SID assays is the high cost of isotope-labeled standards, which range from $700 to $1000 for ∼1 mg of high-purity isotope-labeled peptides with concentration certification by amino acid analysis. For the development of small numbers of assays, this is a reasonable investment, when compared with the costs of many antibodies.

SID is frequently used to generate a calibration curve from samples containing a fixed amount of labeled standard and varying amounts of unlabeled target peptide. In principle, this approach allows quantitation of absolute protein amounts. However, "absolute quantitation" by SID−MRM with a labeled peptide standard is based on the assumption that enzymatic digestion of the target protein proceeds to completion, which is generally not verified.

The need for a more cost-effective normalization strategy for studies involving larger numbers of proteins and peptides led us to develop a labeled reference peptide (LRP) method, in which a single isotope-labeled peptide standard is used as the reference for all target peptides in an analysis.[43] An isotope-labeled peptide standard is not absolutely required but does reduce the level of interference from any endogenous, unlabeled isotopomer. We have described the use of a human actin peptide, as well as bacterial β-galactosidase and alkaline phosphatase. An advantage of the actin standard peptide is that it allows an SID-based normalization of sample load based on the endogenous actin.[43] The LRP approach does not provide co-elution of a labeled peptide standard with each target peptide but otherwise confers many of the benefits of SID-based quantitation. Measurement CVs with the LRP method range from 10 to 25%, and the linearity is similar to that for SID.[43]

The simplest MRM method is "label-free" quantitative comparison without any reference standard or normalization of the target peptide signal. This approach is subject to higher measurement variation, because of undetected or uncorrected variations in differences in peptide recovery, MS instrument performance, and other factors. The label-free approach has been compared to the SID and LRP methods and actually performs surprisingly well, with measurement CVs ranging from 20 to 30%.[43]

SENSITIVITY AND DYNAMIC RANGE OF MRM ASSAYS

The level of sensitivity that can be achieved using MRM depends on several factors, which include the type of sample being analyzed (e.g., biofluids, tissue, cell lysates, and immunoprecipitates) and whether enrichment or fractionation techniques are incorporated prior to analysis. Proteins that are present in unfractionated plasma in the 0.1−10 μg/mL (fmol/μL) concentration range usually can be detected readily and measured with MRM.[44] The addition of a high-abundance protein immunoaffinity depletion step coupled with peptide fractionation by strong cation exchange chromatography can increase the limits of detection and quantitation of a MRM analysis to low nanogram per milliliter levels in plasma.[42,45] MRM can achieve a broad dynamic range in comparison to global profiling analyses, where peptides from high-abundance proteins directly compete for detection with peptides for low-abundance proteins. In shotgun proteomic analyses of the *Saccharomyces cerevisiae* proteome, shotgun proteome analyses in unfractionated samples can detect proteins over 2−3 orders of magnitude,[46] whereas in fractionated peptide mixtures from *S. cerevisiae*, this range is extended to >4 orders of magnitude.[47] In MRM analyses, abundant peptides do not directly interfere with the selection of lower-abundance peptides, but complex mixtures nevertheless are likely to have co-eluting contaminants with similar precursor m/z values that may interfere with some MRM transitions for a targeted peptide. Nevertheless, MRM analyses can display a much greater dynamic range than global profiling in complex mixtures. Indeed, Picotti et al. described quantitation of *S. cerevisiae* proteins over the entire protein expression range, including measurements at single-digit copy number levels.[48] The same group reported a limit of quantitation of approximately 7500 copies in human cells.[49] Recently, a large-scale study conducted by the Clinical Proteomic Technology Assessment for Cancer network of the National Cancer Institute (NCI-CPTAC) assessed the reproducibility, transferability, and performance characteristics of SID−MRM−MS measurements in plasma performed across multiple laboratories and instrument platforms.[40] With standardized protocols for sample preparation, data acquisition, and analysis, these studies demonstrated that multiplexed MRM-based assays are highly reproducible within and across laboratories, achieving intra- and interlaboratory CV's in the range of 10−25%. LOD and LOQ values observed in unfractionated plasma were in the high hundreds of nanograms per milliliter to low micrograms per milliliter concentration ranges for target proteins and had a linear dynamic range spanning 3 orders of magnitude.

Quantitation of the human epidermal growth factor receptor 2 protein (HER2) was recently demonstrated in fresh and formalin-fixed paraffin-embedded (FFPE) tissue by MRM with

SID, achieving limits of quantitation within a biologically relevant range of 0.1−0.33 fmol/μg of protein.[50] A similar study, which employed a peptide immunoaffinity enrichment strategy, measured both estrogen receptor (ER) and HER2 in breast cancer tissue and cell lines and demonstrated linear ranges covering approximately 4 orders of magnitude and limits of detection in the low-femtomole per milligram of protein range.[51]

SELECTION OF QUANTITATIVE APPROACHES AND ASSAY FITNESS FOR A PURPOSE

The choice of an MS-based approach to protein quantitation should be based on fitness for purpose and cost. The most common use context for protein quantitation is the estimation of expression or posttranslational modification differences for a few proteins in a small number of samples. The LRP method is the best choice in such situations, as these assays can be configured quickly without the cost and delays involved in obtaining labeled peptide standards for SID. MRM analyses easily exceed the performance of typical Western blotting measurements, which yield CVs ranging from 20 to 40%.[45,52] Although the label-free approach mentioned briefly above might be considered, there is little justification for using this approach, when the LRP method shares essentially all of the advantages (ease and speed of configuring assays) and reduces the disadvantages (no means of assessing sample loss or system drift) through the use of reference peptide standards. The LRP method also is ideally suited to multiplexed MRM assay panels for dozens to hundreds of proteins, mainly because of the cost savings associated with the use of a singly labeled peptide standard.

SID is the most appropriate choice for MRM analyses that require the highest analytical precision and in which the analyses will be conducted over an extended period of time or across multiple laboratories. SID provides the greatest protection against system drift and chromatographic instability, which are major contributors to measurement variation in interlaboratory studies.[40]

APPLICATION OF MRM TO QUANTITATIVE PROTEIN MEASUREMENTS IN BIOCHEMISTRY AND CELL BIOLOGY

As noted above, MRM assays for protein quantitation originated with the SID measurements of peptide hormones by Desiderio and colleagues.[9] Gerber et al. first demonstrated in 2003 the versatile application of SID-based MRM analyses to protein quantitation, referring to the method as AQUA (absolute quantification),[41] and quantified low-abundance yeast proteins involved in gene silencing. The literature published over the ensuing decade describes dozens of applications of SID-based MRM for protein quantitation. The following representative examples illustrate the diversity of application of this method.

(1) MRM measurements of Argonaute (Ago) proteins allowed precise measurements of the stoichiometry and dynamics of miRNA−mRNA complexes.[53]

(2) Bennet et al. systematically applied MRM measurements to quantify the stoichiometries of cullin proteins and their associated adaptors to architecture of the cullin−RING ligase network.[54]

(3) Menentret et al. combined MRM measurements with structure analysis by cryo-electron microscopy to define the stoichiometry of *Escherichia coli* ribosomes binding to the SecY protein to form membrane translocation complexes.[55]

(4) Tomazela et al. applied in vivo metabolic labeling and MRM to measure the turnover of human surfactant-B protein in tracheal aspirates from newborn infants.[56]

TARGETED QUANTITATION OF POSTTRANSLATIONAL PROTEIN MODIFICATIONS

Quantitation of specific posttranslational modifications on individual proteins presents a formidable analytical challenge, as development of site-selective antibodies is typically arduous and reagent specificity is difficult to verify. MS/MS-based analyses offer the most specific means of site-specific quantitation of protein posttranslational modifications. MS/MS provides unambiguous detection through specific fragment ions that indicate the sequence position of the modified residue and MS/MS can distinguish sequence isomers, such as phosphorylation at different residues in Ser/Thr-rich sequences. Most reported proteomic analyses of posttranslationally modified proteins involve global profiling of phosphorylation dynamics and other protein modifications by shotgun proteomics, with quantitation by metabolic labeling (e.g., SILAC[3,57]) or isotope tagging (e.g., iTRAQ[58−60]).

MRM assays employing SID with a labeled, modified peptide standard offer the most specific, sensitive, and precise quantitative analyses of modified protein forms. In their seminal paper, Gerber et al. described quantification of cell cycle-dependent phosphorylation of Ser1126 in human seprase, as well as specific phosphorylations generated in vitro in kinase assays.[41] The following representative examples illustrate the utility of MRM for the quantitation of posttranslational modifications.

(1) MRM has been used to observe the dynamics of tyrosine phosphorylation in the epidermal growth factor-regulated signaling network on glioblastoma cells.[61]

(2) MRM with quantitation by stable isotope dilution was used to quantify kinase activity in vivo of leucine-rich repeat kinase 2 (LRRK2).[62] Coding sequence mutations are the most common cause of familial Parkinson's disease, and these investigators were able to directly measure the effect of mutations on the kinetics of autophosphorylation at Ser1292, which reflects the activity of the wild-type and mutant kinase forms in vivo.

(3) Modification by ubiquitin can be detected by MRM analysis of Gly-Gly modifications on ubiquitinated Lys residues (accompanied by missed tryptic cleavage) following tryptic digestion. This approach has been extended to quantify the stoichiometry of different poly-ubiquitin chain modifications by MRM analysis of the corresponding Gly-Gly-modified Lys residues on distinct ubiquitin peptides containing chain branch sites (e.g., Lys11, Lys48, and Lys63).[63,64]

Analysis of protein posttranslational modifications is often complicated by the low stoichiometry of the modifications, which is further complicated by the low abundance of many target proteins. A common solution to this problem for global proteomic analysis is enrichment of the modified peptides by chemistry-based affinity capture, such as immobilized metal affinity chromatography (IMAC) and related affinity chromatography methods for phosphopeptides,[65] or by immunoaffinity capture of Lys-N-acetylated peptides or phosphotyrosine peptides.[58,59] When affinity capture steps are introduced, measurement error due to variation in capture or immunoprecipitation efficiency should be considered. Spiked, modified

peptide standards can be used to assess the efficiency and consistency of recovery, although differences in recovery can be anticipated for different sequences. Differences in measured levels of protein modifications may be due to changes in both the modification stoichiometry and expression levels of the modified protein. Accurate interpretation of modification data thus requires estimates of both protein expression and levels of specific modifications.[66]

A useful strategy for quantitative analysis of protein posttranslational modifications employs internal reference peptides (IRPs) from the target protein for signal normalization without the need for isotope labeling (Figure 4).[11] Ion trap

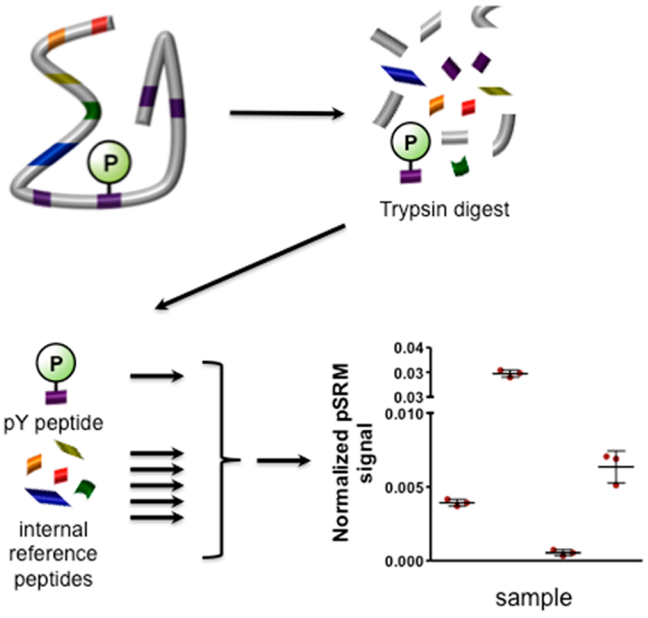


Figure 4. Schematic representation of quantitation of protein modifications with the internal reference peptide (IRP) method. Transitions are monitored for both the peptide bearing the modification and other, unmodified IRPs in the same protein. The signal from the modified peptide is normalized to the signals from one or more of the IRPs.

mass spectrometry and pSRM are used to acquire full MS/MS and MS^3 spectra from target peptides. Transitions are extracted from the full MS/MS or MS^3 spectrum, and peak areas for transitions are summed and normalized to areas for a reference standard. In the IRP method, one or more unmodified proteotypic peptides from the target protein serve as the reference standard for the modified peptides in the analysis. Because the target modified peptides and the reference standard are present in the same protein, the IRP method corrects for variations in the recovery of the protein during immunoprecipitation or during other stages of the analysis. Normalized signals thus reflect an increase or a decrease in the stoichiometry of the modification. Equivalent responses were observed with both IRP and SID methods for quantitation of six site-specific phosphorylations in the epidermal growth factor receptor (EGFR) in epidermal growth factor-stimulated A431 cells.[11] Although pSRM was used in this study, the IRP approach could also be used with MRM or PRM in triple quadrupole, quadrupole-ion trap, or quadrupole-Orbitrap instruments. Analyses using the IRP method typically had higher median CVs (22−31%) than SID (10−20%). The key advantage of the IRP method is the ease of configuring assays for routine quantitation of protein modifications in a biochemistry laboratory setting.

A TARGETED PROTEIN QUANTITATION RESOURCE: WHAT MIGHT IT LOOK LIKE?

Many pharmaceutical companies employ clusters of automated NMR and MS instruments immediately adjacent to synthetic medicinal chemistry laboratories. These instruments allow routine analyses of reaction products or quantitative monitoring of product yields. The relative simplicity of routine quantitation by MRM and related methods, together with the robust performance of newer MS instruments, suggest that an analogous automated protein quantitation resource built around MS methods could be implemented. The simplest variation would employ a triple quadrupole or quadrupole-Orbitrap MS instrument performing MRM or PRM analyses, coupled to a microcapillary LC system and autosampler. Users would digest protein samples according to a standard protocol, introduce a LRP peptide standard, and then analyze it using standardized, automated programs. A triple quadrupole instrument is robust in a multiuser setting with appropriate oversight. If the goal of most analyses was routine quantitation of a few proteins, optimization of peptide selection or MRM transitions would be largely unnecessary and automated experimental design (peptide and transition selection) could be done with Skyline or similar utilities and would allow quantitation of most protein targets. The comparison of protein levels between samples would be based on integrated peak areas for the target peptides and the LRP peptide standard. This basic MS analysis capability could be combined with an automated protein digestion station, which can be purchased or built with a programmable sample handling robot.

CONCLUSION

Targeted protein quantitation by MRM and related methods has emerged rapidly over the past 10 years and has many advantages over immunochemical methods. MRM provides a means of systematically configuring sensitive and specific assays for any protein and an efficient platform for multiplexed assays. The rapid evolution of MS instruments and software for targeted peptide measurement is expected to transform protein measurement in biochemistry.

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Funding

The authors' work in this area has been supported by National Institutes of Health Grants U01CA152647, U24CA159988, and U24CA126479.

Notes

The authors declare no competing financial interest.

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