Chapter 7

Quantitative Mass Spectrometry-Based Proteomics: An Overview

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Abstract

In recent years, mass spectrometry has moved more than ever before into the front line of protein-centered research. After being established at the qualitative level, the more challenging question of quantification of proteins and peptides using mass spectrometry has become a focus for further development. In this chapter, we discuss and review the strategies and problems of the methods currently in use for the quantitative analysis of peptides, proteins, and finally proteomes by mass spectrometry. The common themes, the differences, and the potential pitfalls of the main approaches are presented in order to provide a survey of the emerging field of quantitative, mass spectrometry-based proteomics.

Key words: Mass spectrometry, Proteomics, Absolute quantification, Relative quantification, Label-free, Stable heavy isotope, Isotope label

1. Introduction

The introduction of soft ionization techniques (electrospray ionization, ESI, and matrix-assisted laser desorption/ionization, MALDI), advances in precise and nanoscale liquid chromatography (LC), progress in software development, and increasing computing power have all contributed to making mass spectrometry (MS) the method of choice for the analysis of single proteins or complex protein samples, for dissecting biological pathways and for the identification of hitherto unknown proteins. Following the establishment of the qualitative level of protein analysis by MS, researchers have begun to ask biological questions that require quantitative answers. The transition from "what" to "what and how much" has lain behind much research effort in

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recent years and has engendered a variety of novel MS-based approaches for the quantification of proteins and peptides. Depending on the question and the sample at hand, they can focus, at the one extreme, on the accurate quantification of individual peptides or, at the other, on broad comparisons of (nearly) entire proteomes. They can detect and quantify effects of a specific stimulus ranging from changes in the amounts of a single, defined posttranslational modification to the proteome-wide kinetics of the same modification between different stages of the cell cycle.

The variety of questions being asked has impelled the development of an array of quantitative MS methods. These can be classified into two groups, according to the kind of information that they provide: (a) relative quantification, comparing the amounts of proteins or whole proteomes between samples and yielding a quantitative ratio or relative change (see Chapters 8–16), and (b) absolute quantification (see Chapters 17–20), providing information about the absolute amount or the concentration of a protein within a sample. An alternative classification can be based on the underlying methodology: (a) approaches based on labeling with stable isotopes (see Chapters 8–14, 17–19, and 24–26), involving the artificial labeling of peptides or proteins, and (b) label-free approaches (see Chapters 16, 20, and 22), in which the samples retain their native isotope composition and are compared between separate measurements. The first of the two classifications, along with the quantification methods most commonly used, is summarized in Fig. 1. A brief description of most of the various methods and their most important advantages and disadvantages is given in Table 1.

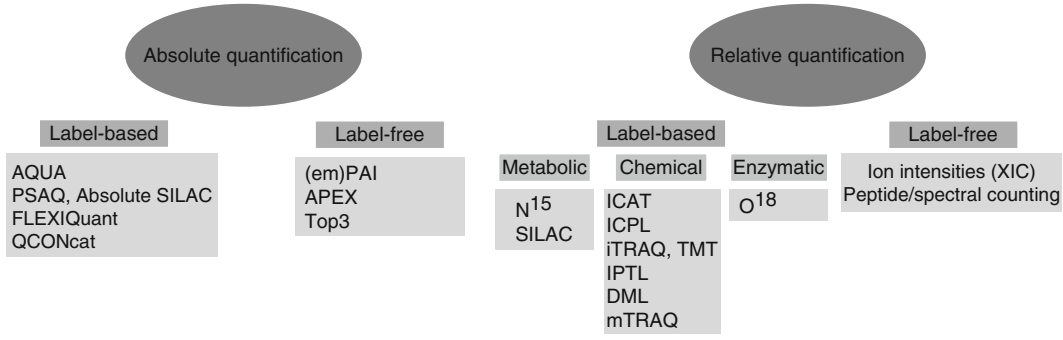

Fig. 1. Overview of the most common label-based and label-free methods for absolute and relative quantification.

Table 1
Description of most of the various methods for relative and absolute quantification

Method	Description	Advantages	Disadvantages	References
Methods for absolute quantification				
AQUA	Based on chemically synthesized peptide standards containing stable isotopes, added in known amounts to the sample	Accurate absolute quantification	High cost; prior information about the quantified peptides is needed; only few peptides per protein are quantified	Desiderio et al. (29), Gerber et al. (38), Kirkpatrick et al. (39)
emPAI	Calculation of the emPAI using the number of observed and observable peptides	Simple; can be applied to every sample as no labeling is required	Low accuracy	Ishihama et al. (50)
APEX	Similar to emPAI, but corrected by background expectation, sampling depth, and confidence in protein identification	Introduces correction factors for more accurate quantification	Correction values based on prior MS results are needed	Lu et al. (51)
Top3	Based on the relationship between the signal response of three most abundant tryptic peptides and the protein concentration	High accuracy; no labeling is required	The sample has to be spiked with a standard protein before tryptic hydrolysis	Silva et al. (52)
Methods for relative quantification				
ICAT	Cysteine-specific chemical labeling	Reduced sample complexity due to affinity enrichment of labeled peptides	Requirement for cysteine-containing peptides; side reactions	Gygi et al. (4)
ICPL	Chemical labeling of free amino groups	Labeling of all peptides within a sample; no side reactions	Peptides resulting from tryptic digests are relatively large	Schmidt et al. (5)

(continued)

Table 1 (continued)

Method	Description	Advantages	Disadvantages	References
Dimethyl labeling	Chemical labeling of free amino groups	Labeling of all peptides; no side reactions; low cost; small size of the label	Toxic reagents	Hsu et al. (7)
iTRAQ and TMTs	Amine-specific labeling with isobaric tags, quantification at MS/MS level	Efficient chemical labeling; multiplexing (up to six or eight samples); enhanced signal intensity in MS and MS/MS; simple data analysis	Requires efficient fragmentation; high cost	Ross et al. (10), Thompson et al. (11)
^{15}N labeling	Metabolic labeling with ^{15}N-enriched media	Efficient labeling	Complex data analysis	Oda et al. (13)
SILAC	Metabolic labeling with amino acids containing stable heavy isotopes	Efficient labeling; one label per (tryptic) peptide; semiautomated data analysis	High cost, especially when applied to whole organisms	Ong et al. (19)
^{18}O labeling	Enzymatic labeling with ^{18}O-labeled water	Simple labeling procedure; relatively cheap	Incomplete labeling complicates data analysis	Mirgorodskaya et al. (30), Scholzer et al. (31)
Spectral/peptide count	Relative comparison of different samples based on the number of identified peptides or acquired MS/MS spectra, respectively	No labeling required; simple data analysis; many samples can be compared	Less robust than label-based approaches, requires high reproducibility	Liu et al. (35)
Ion-current measurements	Based on ion intensities of peptides	No labeling required; many samples can be compared	Requires high reproducibility	Wiener et al. (34), Voyksner et al. (33)

2. General Properties of Protein Quantification by Mass Spectrometry

MS is a technique for the measurement of mass-to-charge ratios of charged particles and does not in itself allow a quantitative statement of the amounts of such particles present. Owing to the different physicochemical properties of different peptides and proteins, their signals in the mass spectrometer cannot be used for quantitative comparisons between different molecular species. Quantification relies mainly on comparison of the same molecules in different experiments, or comparison within a single experiment of molecules that differ only in their isotopic composition and therefore have identical physical and chemical properties. The former includes methods for label-free quantification, where peptides and proteins in their natural states are compared in consecutive experiments. It relies on highly reproducible sample-handling, separation by liquid chromatography and MS measurements, and it has the advantage of allowing the quantification of a virtually unlimited number of samples (multiplexing) without any chemical, metabolic, or enzymatic modification. This helps to keep costs low and to minimize the number of sample-handling steps. On the other hand, poor reproducibility may require analysis of many technical replicates and may lead to low accuracy of the quantitative measurements.

These limitations led to the development of quantification methods based upon labeling with stable isotopes. Owing to the natural occurrence of certain stable heavy isotopes (e.g., ^{13}C, ^{15}N, ^{18}O, ^{2}H) each peptide/protein contains a certain proportion of these; the isotope pattern seen in the mass spectrometer thus reflects the natural abundances of these heavy isotopes within the peptide. Artificial incorporation of heavy isotopes produces a mass shift of the peptide's peaks (including, its largely unchanged isotope pattern) in the mass spectrum. Importantly, additional ^{13}C, ^{15}N, and ^{18}O have little or no impact on the behavior of peptides and proteins during LC or in the mass spectrometer (see Note 1). Thus, intensity ratios of peaks that correspond to different isotopic compositions of the same molecular species reflect quantitatively the isotopic abundance ratios in the molecular species concerned. Labeling techniques take advantage of this feature and afford the opportunity to compare directly two or more samples within the same mass spectrum. Label-based approaches offer a higher accuracy of quantitative measurements, but they require additional steps in sample preparation and usually entail higher costs compared with label-free approaches. Additionally, only a limited number of samples can be quantified within one experiment (from two to eight, depending on the method). Quantitative information can be obtained not only from the MS spectrum comparing intact peptide (or protein) peak intensities, but also from the MS/MS fragment ion spectrum. The advantages of the former are that usually

more than one independent spectrum is available for analysis, and also that the high peak intensity gives statistically more accurate results. Quantification of the fragment ion spectrum benefits from the absence of overlapping precursor ion peaks (as in selected ion monitoring, SIM) and from the identical properties of the precursor ion (as in iTRAQ). Nonetheless, fragment ions are generally detected with lower intensities, and often only a single spectrum leads to quantification. This can impair the accuracy of the quantification (1). At the other end of the scale, too high intensities in the mass spectrometer can lead to detector saturation, again resulting in imprecise measurements (2).

3. Methods for Protein/Peptide Quantification

3.1. Relative Quantification

Relative quantification provides calculation of abundance ratios between peptides and proteins by comparing their signals originating from different samples. Usually performed in "discovery" (nontargeted) mode, it allows quantitative profiling of tens of thousands of peptides from thousands of proteins within a single experiment without a priori information (for an example, see ref. 3). As discussed above and in a manner similar to absolute quantification, it can be based upon heavy isotope labeling or label-free.

3.1.1. Stable Isotope Labeling Methods

Most of the methods for relative quantification make use of labeling by stable heavy isotopes of ^{13}C, ^{15}N, ^{18}O, and ^{2}H. Again, the identical physicochemical properties of labeled and native peptides (e.g., in stable isotope labeling with amino acids in cell culture (SILAC)) (see Chapters 13, 14, 25, and 26), or of peptides labeled with physicochemically identical reagents (e.g., in iTRAQ) (see Chapter 8), are exploited for relative comparison of intensities of mass-shifted peaks within the same mass spectrum. These approaches can be divided into three groups, according to the labeling technique: (a) chemical, (b) metabolic, or (c) enzymatic.

Chemical Labeling

The methods for relative quantification by chemical labeling rely on the chemical reaction (without enzymatic catalysis) between a reagent and the peptides (or proteins) in the sample of interest in vitro (i.e., after isolation of the protein/peptide from the biological sample). The reagent used bears different numbers of stable heavy isotopes and thus produces a mass shift in the MS spectrum (e.g., dimethyl labeling) or MS/MS spectrum (in case of isobaric reagents, e.g., iTRAQ). One of the first chemical labeling approaches is ICAT (isotope-coded affinity tags (4)) (see Chapter 24). The ICAT chemical label consists of three moieties: a sulfhydryl-reactive group for coupling to the analyte cysteines, an affinity group for isolation of the tagged species (peptides), and a linker in light

(with natural isotope distribution) and heavy (containing eight deuterium atoms instead of ^{1}H) form. Two samples to be compared are labeled with light or heavy ICAT reagent and subsequently mixed. After analysis, the peak intensities of identical peptide pairs labeled with the light and the heavy reagent, respectively, are compared, and their ratio is calculated. Significant disadvantages of the approach are the side-reactivity of the tag and its inability to label peptides lacking cysteine.

Another labeling method based on the same principle is ICPL (isotope-coded protein labels (5)) (see Chapter 11). A significant advantage of ICPL is their reactivity towards free amines, allowing labeling of virtually all peptides present in the samples. Dimethyl labeling is a similar approach using simpler chemical reagents (6, 7). Dimethylation of lysine residues by stable-isotope-labeled formaldehyde and cyanoborohydride allows duplex and triplex relative comparison. This approach is a reliable and inexpensive alternative to the common chemical labeling methods, while offering nearly 100% labeling efficiency in a simple chemical reaction. mTRAQ (Applied Biosystems) is a recent addition to the repertoire of amine-reactive labels. It uses double or triple labeling by stable heavy-isotope-labeled chemical reagents and is designed to be used in SRM assays (although full scan MS-based quantification is also possible (8)). It is specifically applied in biomarker discovery experiments as alternative to labeled standard peptides (9).

An important group of reagents used for relative quantification comprises the isobaric chemical labels. These rely on isobaric labeling of peptides from different samples, which upon fragmentation give rise to different reporter ions in the MS/MS spectrum. The iTRAQ (isobaric tags for relative and absolute quantification, Applied Biosystems (10)) (see Chapter 8) labels each contain an amine-reactive group, a balance group, and a reporter group. Overall, different reagents have the same molecular weight and upon labeling produce identical mass shifts. Different samples are labeled with reagents containing different distributions of heavy isotopes between the balance and reporter groups and are subsequently mixed. Identical peptides from the samples to be compared co-elute and are detected as a single precursor ion. The iTRAQ labels are designed in such a way that, upon fragmentation, different reagents give rise to reporter ions with identical chemical composition but different molecular weights, owing to their different isotope compositions. Their intensities are proportional to the relative abundances of the labeled peptide originating from the different samples. A major advantage of this method is that it is capable of "multiplexing"—up to eight samples can be analyzed within a single experiment.

A very similar approach is the labeling with tandem mass tags (TMTs, Thermo Scientific (11)) (see Chapter 9), which allows comparison of up to six samples. Like iTRAQ tags, TMTs also

consist of an amine-reactive, a balance, and a reporter group, which are released upon fragmentation during MS/MS and the intensity of which is used to calculate relative amounts between the samples.

A different approach for quantification at the MS/MS level is IPTL (isobaric peptide termini labeling (12)) (see Chapter 10). This uses isobaric sequential labeling of the C- and N termini of the analyzed peptides with deuterated and non-deuterated reagents. Upon fragmentation, either the N-terminal or the C-terminal label is lost, which results in differentially labeled C- and N-terminal fragment ion series, respectively. These appear as fragment ion pairs in MS/MS and their relative intensities can be used for quantification. An advantage of this strategy is that the quantification is based on several data points per MS/MS spectrum, although this complicates data analysis enormously.

A significant advantage of all chemical labeling methods is that they can be applied to practically any type of sample (cell culture, tissues, body fluids, etc.), in contrast to metabolic labeling as discussed below. However, it is crucial to optimize labeling conditions (see Note 2).

Metabolic Labeling

Metabolic labeling with stable heavy isotope labels introduces the label at the earliest time point in an experiment, i.e., during cell growth and duplication. This is achieved by feeding organisms with special media containing a subset of the metabolites in heavy-labeled form. Metabolic labeling ensures lower deviations in quantification, as the samples to be compared can be mixed at a very early stage during the experiment. Metabolic labeling can easily be achieved in cell culture, but scaling-up to whole organisms such as *Drosophila*, *Caenorhabditis elegans*, and even mice is also possible. Labeling with ^{15}N-containing media (see Chapter 12) has been used successfully for quantification at the level of yeast (13), mammalian cells (14), *C. elegans*, *Drosophila melanogaster* (15), *Arabidopsis thaliana* (16), and rat (17). Very high levels of isotope incorporation can be achieved by this method, but the mass difference between labeled and unlabeled samples depends on the number of ^{15}N atoms present in different peptides and presents a significant challenge for data analysis and quantification. Moreover, highly enriched ^{15}N sources are required in order to avoid complex isotope distributions of partially labeled peptides (18).

A computationally simpler method was developed to address these issues. SILAC (19) (see Chapters 13, 14, 25, and 26) takes advantage of the fact that organisms are naturally (or genetically manipulated to be) auxotrophic for certain amino acids. These amino acids can therefore be provided in labeled and unlabeled form to growth media and would be used by the organism for building proteins in vivo. SILAC experiments usually employ lysine and arginine containing different numbers of the heavy

isotopes ^{13}C, ^{15}N, and ^{2}H. Using trypsin for protein digestion ensures that each resulting peptide will contain at least one labeled amino acid (except for the C-terminal peptide of the protein). By comparison of the intensities of the precursor isotope envelopes of nonlabeled and labeled peptides, quantitative information can easily be obtained. This has been further facilitated by the development of robust and semiautomated computational tools for data analysis, such as the MaxQuant software suite (20) (see Chapters 13 and 29). The SILAC approach can be used to compare simultaneously two or three samples. It has been applied successfully in near-whole proteome quantification profiling (3, 21), in following the kinetic changes of posttranslational modifications (22), in separating background from specific interactors in pull-down approaches (22, 23), and in pulse-labeling to monitor the proteome-wide changes induced by a specific treatment (24). It can be applied not only in cell culture but also to whole organisms such as *Drosophila* (25) or mice (26).

As with most other label-based approaches, when metabolic labeling is applied nearly 100% incorporation of the label should be aimed at. Incomplete labeling results in inaccurate quantification. Additionally, any changes or stress in the experimental organism due to the artificial growth medium should be taken into account (e.g., when using dialyzed fetal bovine serum for mammalian cells). Another important consideration when one is using SILAC is the metabolic conversion of the isotopically labeled amino acids within the cell. This can lead to incorrect quantification if (for example) the pathway leading from arginine to proline is stimulated when the concentration of the added arginine is not carefully adjusted, or if the conversion is not corrected for (27) (see Note 3). In the case of affinity interaction pull-downs using SILAC in vitro, careful adherence to identical conditions for preparation of heavy and light cell extracts is important for obtaining reliable results (23). A significant disadvantage of metabolic labeling methods is their inability to quantify tissues and body fluids from organisms that cannot be labeled easily (e.g., human patients). In a recent approach aimed at circumventing this issue, internal SILAC standards were added; this allowed successful quantification in tumor tissue samples (28).

Enzymatic Labeling

Heavy stable isotopes can be incorporated during enzymatic proteolysis of proteins (see Chapter 15). Performing proteolysis in heavy ($H_2{}^{18}O$) or light ($H_2{}^{16}O$) water incorporates, respectively, two ^{18}O or ^{16}O atoms at the C terminus of the generated peptides, resulting in a mass shift of 4 Da between heavy- and light-labeled peptides (29, 30). This label can also be incorporated after digestion in a second incubation step with a protease. This method ensures near-complete labeling and benefits from the absence of side reactions. Acid-catalyzed back-exchange at extreme pH conditions

can occur (31) (see Note 4); however, the mild conditions used during ESI or MALDI analyses do not influence the stability of the introduced label. Incomplete labeling by incorporation of only one ^{18}O atom can complicate data analysis and needs to be taken account of (32).

3.1.2. Label-Free Methods

Label-based approaches for proteomic quantification usually come at higher cost and require additional steps of sample preparation. Therefore, it is not surprising that the use of label-free methods has been increasing during the last few years. As mentioned above, label-free quantitative approaches rely on the comparison of different features between independent LC-MS or LC-MS/MS measurements. They fall into two general categories: (a) methods that involve comparing peptide signal intensities at the level of LC-MS analysis (see Chapter 16), and (b) methods that involve counting the number of identified peptides or acquired fragment spectra (see Chapter 22).

Peptide Signal Intensities

Signal intensities of ions after electrospray ionization correlate with ion concentrations (33, 34). The extracted peak areas from chromatograms in LC-MS measurements specific for certain ions (extracted ion chromatograms, XIC) can therefore be used for relative quantification of specific peptides and proteins between different samples. The method allows measurements with high precision and wide dynamic range, especially when high-resolution mass spectrometers are used. It can also be applied to MALDI measurements combined with offline-LC separation. However, the following important considerations should be taken into account. First of all, the variation between measurements of the peak intensities of peptides from the same sample (technical replicates) should be recorded and appropriate normalization should be applied. Secondly, and more critically, variation of the LC retention time and/or m/z values of identical peptides between measurement runs should be considered. Any variability in this respect requires alignment of individual ion chromatograms for correct quantification and elimination of any global drift in retention time. Practical normalization strategies may include the addition of identical amounts of standard protein in different sample or normalization, based on a priori information about a protein that does not change quantitatively between the samples compared (1). Reproducibility of LC separation, stability of the electrospray ion source, and the use of computational algorithms for comparison, alignment, and statistical evaluation of several LC-MS datasets in a single procedure are therefore crucial (see Note 5).

Spectral Counting

The second category of label-free quantification methods relies on the practical observation that more abundant peptides are more likely to be observed and detected in an MS experiment.

These approaches use the number of peptides or the number of fragment spectra observed for a particular protein in the analysis. However, Liu et al. found a linear correlation over two orders of magnitude between the number of spectra and the relative protein abundance, whereas no correlation between the relative protein amounts and the number of peptides and the sequence coverage was observed (35). While spectral counting is a relatively simple and reliable technique and is easily implemented, normalization and careful statistical evaluation are still needed for accurate quantification. This accuracy can decrease significantly for proteins with only a few observable peptides, as well as when the quantitative changes between experiments are small (2). Furthermore, since larger proteins give rise to more peptides than do smaller ones, additional normalization factors can be applied to improve the results of quantification (36).

3.2. Absolute Quantification

Absolute quantification is used to determine the absolute amount (mass, mole number, or copy number) of proteins in a mixture or complex. This is very informative, but label-based methods are usually relatively laborious and label-free ones are less accurate. Absolute quantification is generally performed at the peptide level, although top-down absolute quantification has recently been introduced (37).

3.2.1. Stable Isotope Labeling Methods

The arguably most widely used method for absolute quantification (AQUA) employs peptides labeled with heavy stable isotopes (AQUA peptides) as added, internal standards (29, 38) (see Chapter 17). This method can be used for accurate profiling and absolute quantification of proteins within a complex sample, for monitoring changes in posttranslational modification (38, 39), and for determining the stoichiometry of subunits within a protein complex (40). Being a targeted approach, the method requires a priori information about the peptides and proteins that are subject to analysis. The specific characteristics of the targeted precursor ion (elution time, m/z value, charge state), optimum fragmentation conditions (collision energy), and resulting fragmentation pattern are determined in prior measurements. Peptides labeled with heavy stable isotopes (^{13}C- and ^{15}N-labeled amino acids), identical in sequence to the peptides of interest naturally present in the sample, are synthesized chemically. These two peptides have identical physicochemical properties but present a specific mass shift in the mass spectrum. The AQUA peptides are added to the protein digest or peptide sample at known concentrations and analyzed, most commonly on triple-quadrupole instruments operated in selected reaction monitoring (SRM) mode. The co-eluting analytes—i.e., the endogenous and the mass-shifted labeled peptides—are selected for fragmentation on the basis of their (already determined) elution time and m/z value. The intensities of the fragment ions of the

peptide of interest are compared with those of the AQUA peptide, and this reflects directly their quantitative relationship. As the amount of the added peptide is known, the amount of the sample peptide can be deduced.

The AQUA approach allows very specific, targeted detection of the peptides of interest, thereby minimizing the variability and the influence of background noise. Even in complex samples, several hundred peptides can be targeted within a single LC-MS/MS experiment (41). As the method is strictly hypothesis-driven, it allows the selection of peptides with optimal chromatographic performance and ionization efficiency (i.e., good "detectability"), which do not undergo uncontrolled modification in vitro (e.g., oxidation of methionine) and which are unique to the protein of interest. Such peptides are called prototypic peptides and can be identified or predicted for particular proteomic platform using peptide libraries and public databases (42–44). Once established, an SRM quantification assay can be easily and reproducibly repeated for many samples.

There are several critical aspects that should be considered when an AQUA experiment is being planned such as incomplete proteolytic digestion, exact amount of AQUA peptide, application of AQUA peptides, and number of applied AQUA peptides for each protein to be quantified (see Note 6). In order to simplify the quantification of several peptides per protein, heavy-labeled standard proteins can be used instead of individual peptides. Several approaches have been developed in that direction, including PSAQ (protein standard absolute quantification) (45), absolute SILAC (46), and FLEXIQuant (full-length expressed stable isotope-labeled proteins for absolute quantification) (47) (see Chapter 19). QconCAT (concatenated signature peptides encoded by QconCAT genes) uses artificial, labeled standard proteins assembled from diverse peptides belonging to different proteins (48) (see Chapter 18). In all the protein-based approaches, the standard is added to the protein sample and subjected to protease digestion, which gives a mixture of endogenous and heavy-labeled standard peptides.

3.2.2. Label-Free Methods

These approaches to absolute quantification have the typical advantages of label-free methods, namely (a) omitting the time-consuming and often costly step of introducing standard peptides and (b) the opportunity to compare virtually unlimited numbers of samples. On the other hand, they entail the disadvantages of lower accuracy and the requirement for high reproducibility. One of the first label-free approaches used for absolute quantification was variation of the protein abundance index (PAI (49)). The PAI is calculated by dividing the number of observed peptides by the number of theoretically observable peptides. The emPAI (exponentially modified PAI (50)) is defined as $\text{emPAI} = 10^{\text{PAI}} - 1$; it is proportional

to the protein content in a protein mixture and, therefore, can be used for the estimation of absolute amounts of proteins. An approach termed APEX (absolute protein expression (51)) (see Chapter 20) based on spectral counting can also be used for profiling absolute protein quantities per cell. Important features of APEX are the correction factors that it introduces, providing a relationship of direct proportionality between the numbers of observed and expected peptides.

As incomplete digestion is a critical issue when one is performing absolute quantification of peptides or proteins, an alternative approach (generally known as "Top3") has been developed that deals with this problem. In this approach the quantities of the three most abundant tryptic peptides are averaged. It is generally assumed that some parts of the protein are completely digested and, therefore, the three most abundant peptides reflect the protein concentration. The protein sample is therefore spiked with a known amount of standard protein and, after digestion, the average MS signal response of the standard protein is used to calculate a universal signal response factor (ion counts per mole of protein). This factor is then applied to calculate the concentration of the proteins in the sample to be analyzed (52).

Undoubtedly, all methods applied for absolute quantification can also be used for determining relative relations of proteins within or between samples, by comparing the absolute protein amounts in a relative manner.

4. Notes

1. For relative quantification using stable isotopes, the quantitative correspondence does not always apply exactly when deuterium is used as a label, as labeling with deuterium can affect retention time in LC (53).
2. Relative quantification using stable isotope chemical or enzymatic labeling: the labeling procedure has to be optimized ensuring ideal labeling; 100% label incorporation should be aimed at, which might not be achievable for all approaches. Additionally, side reactions should be avoided to prevent erroneous quantification results.
3. Relative quantification using metabolic labeling: In general, large-scale SILAC experiments use both isotope-coded arginine and lysine to obtain labeling of all possible tryptic peptides thereby maximizing quantitative coverage of all potential peptides in a given experiment. Quantification using SILAC may be disturbed by the fact that the isotopically labeled amino acid arginine is a metabolic precursor of proline and as such might

be converted to labeled proline. As with other labeling approaches, complete incorporation of the heavy label should be aimed at (which should be limited only by the isotopic enrichment of the commercially available labeling sources).

4. Relative quantification using enzymatic labeling: Under extreme pH conditions in $H_2{}^{16}O$ buffers, acid-catalyzed back-exchange could result in partial loss of the ^{18}O label. Therefore, it is recommended that the enzymatic reactions are stopped by addition of protease inhibitors or freezing of the reaction mixture, rather than by acidifying with 10% TFA.
5. Label-free quantification: The most crucial parameter in label-free quantification is the consistent reproducibility of the LC separation, ionization, and mass measurements of the peptides. All variations of peptide intensities, as well as LC retention times should be recorded between technical replicates and used for normalization and alignment between runs.
6. Absolute quantification: First of all, when peptides from protease digests are to be quantified, complete digestion of the protein sample must be guaranteed. Missed protease cleavages affecting the targeted peptide will result in an artificial decrease in the amounts observed in quantification. Additionally, AQUA peptides are usually obtained in known absolute amounts in lyophilized form, and therefore have to be dissolved quantitatively. As it is advisable to add standard peptides after rather than before digestion (54), any variability and losses during the prior sample preparation should be minimized. Finally, for reliable quantification results, several peptides per targeted protein should be monitored, in order to provide more than one reference value per protein.

References

1. Wilm M (2009) Quantitative proteomics in biological research. Proteomics 9(20):4590–605
2. Mallick P, Kuster B (2010) Proteomics: a pragmatic perspective. Nat Biotechnol 28(7): 695–709
3. Graumann J, Hubner NC, Kim JB et al (2008) Stable isotope labeling by amino acids in cell culture (SILAC) and proteome quantitation of mouse embryonic stem cells to a depth of 5,111 proteins. Mol Cell Proteomics 7(4):672–83
4. Gygi SP, Rist B, Gerber SA et al (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nat Biotechnol 17(10):994–9
5. Schmidt A, Kellermann J, Lottspeich F (2005) A novel strategy for quantitative proteomics using isotope-coded protein labels. Proteomics 5(1):4–15
6. Boersema PJ, Aye TT, van Veen TA et al (2008) Triplex protein quantification based on stable isotope labeling by peptide dimethylation applied to cell and tissue lysates. Proteomics 8(22):4624–32
7. Hsu JL, Huang SY, Chow NH et al (2003) Stable-isotope dimethyl labeling for quantitative proteomics. Anal Chem 75(24):6843–52
8. Kang UB, Yeom J, Kim H et al (2010) Quantitative analysis of mTRAQ-labeled proteome using full MS scans. J Proteome Res 9(7):3750–8
9. DeSouza LV, Taylor AM, Li W et al (2008) Multiple reaction monitoring of mTRAQ-labeled peptides enables absolute quantification of endogenous levels of a potential cancer marker in cancerous and normal endometrial tissues. J Proteome Res 7(8):3525–34

10. Ross PL, Huang YN, Marchese JN et al (2004) Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. Mol Cell Proteomics 3(12):1154–69
11. Thompson A, Schafer J, Kuhn K et al (2003) Tandem mass tags: a novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. Anal Chem 75(8): 1895–904
12. Koehler CJ, Strozynski M, Kozielski F et al (2009) Isobaric peptide termini labeling for MS/MS-based quantitative proteomics. J Proteome Res 8(9):4333–41
13. Oda Y, Huang K, Cross FR et al (1999) Accurate quantitation of protein expression and site-specific phosphorylation. Proc Natl Acad Sci USA 96(12):6591–6
14. Conrads TP, Alving K, Veenstra TD et al (2001) Quantitative analysis of bacterial and mammalian proteomes using a combination of cysteine affinity tags and 15N-metabolic labeling. Anal Chem 73(9):2132–9
15. Krijgsveld J, Ketting RF, Mahmoudi T et al (2003) Metabolic labeling of *C. elegans* and *D. melanogaster* for quantitative proteomics. Nat Biotechnol 21(8):927–31
16. Nelson CJ, Huttin EL, Hegeman AD et al (2007) Implications of 15N-metabolic labeling for automated peptide identification in *Arabidopsis thaliana*. Proteomics 7(8):1279–92
17. Wu CC, MacCoss MJ, Howell KE et al (2004) Metabolic labeling of mammalian organisms with stable isotopes for quantitative proteomic analysis. Anal Chem 76(17):4951–9
18. Ong SE, Mann M (2005) Mass spectrometry-based proteomics turns quantitative. Nat Chem Biol 1(5):252–62
19. Ong SE, Blagoev B, Kratchmarova I et al (2002) Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Mol Cell Proteomics 1(5):376–86
20. Cox J, Mann M (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol 26(12):1367–72
21. de Godoy LM, Olsen JV, Cox J et al (2008) Comprehensive mass-spectrometry-based proteome quantification of haploid versus diploid yeast. Nature 455(7217):1251–4
22. Oellerich T, Gronborg M, Neumann K et al (2009) SLP-65 phosphorylation dynamics reveals a functional basis for signal integration by receptor-proximal adaptor proteins. Mol Cell Proteomics 8(7):1738–50
23. Nikolov M, Stuetzer A, Mosch K et al (2011) Chromatin affinity purification and quantitative mass spectrometry defining the interactome of histone modification patterns. Mol Cell Proteomics 10(11):M110.005371
24. Selbach M, Schwanhausser B, Thierfelder N et al (2008) Widespread changes in protein synthesis induced by microRNAs. Nature 455(7209):58–63
25. Sury MD, Chen JX, Selbach M (2010) The SILAC fly allows for accurate protein quantification in vivo. Mol Cell Proteomics 9(10):2173–83
26. Kruger M, Moser M, Ussar S et al (2008) SILAC mouse for quantitative proteomics uncovers kindlin-3 as an essential factor for red blood cell function. Cell 134(2):353–64
27. Van Hoof D, Pinkse MW, Oostwaard DW et al (2007) An experimental correction for arginine-to-proline conversion artifacts in SILAC-based quantitative proteomics. Nat Methods 4(9):677–8
28. Geiger T, Cox J, Ostasiewicz P et al (2010) Super-SILAC mix for quantitative proteomics of human tumor tissue. Nat Methods 7(5):383–5
29. Desiderio DM, Kai M (1983) Preparation of stable isotope-incorporated peptide internal standards for field desorption mass spectrometry quantification of peptides in biologic tissue. Biomed Mass Spectrom 10(8):471–9
30. Mirgorodskaya OA, Kozmin YP, Titov MI et al (2000) Quantitation of peptides and proteins by matrix-assisted laser desorption/ionization mass spectrometry using [18]O-labeled internal standards. Rapid Commun Mass Spectrom 14(14):1226–32
31. Schnolzer M, Jedrzejewski P, Lehmann WD (1996) Protease-catalyzed incorporation of 18O into peptide fragments and its application for protein sequencing by electrospray and matrix-assisted laser desorption/ionization mass spectrometry. Electrophoresis 17(5):945–53
32. Johnson KL, Muddiman DC (2004) A method for calculating 16O/18O peptide ion ratios for the relative quantification of proteomes. J Am Soc Mass Spectrom 15(4):437–45
33. Voyksner RD, Lee H (1999) Investigating the use of an octupole ion guide for ion storage and high-pass mass filtering to improve the quantitative performance of electrospray ion trap mass spectrometry. Rapid Commun Mass Spectrom 13(14):1427–37
34. Wiener MC, Sachs JR, Deyanova EG et al (2004) Differential mass spectrometry: a label-free LC-MS method for finding significant differences in complex peptide and protein mixtures. Anal Chem 76(20):6085–96

35. Liu H, Sadygov RG, Yates JR 3rd (2004) A model for random sampling and estimation of relative protein abundance in shotgun proteomics. Anal Chem 76(14):4193–201
36. Zybailov B, Mosley AL, Sardiu ME et al (2006) Statistical analysis of membrane proteome expression changes in *Saccharomyces cerevisiae*. J Proteome Res 5(9):2339–47
37. Waanders LF, Hanke S, Mann M (2007) Top-down quantitation and characterization of SILAC-labeled proteins. J Am Soc Mass Spectrom 18(11):2058–64
38. Gerber SA, Rush J, Stemman O et al (2003) Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. Proc Natl Acad Sci USA 100(12):6940–5
39. Kirkpatrick DS, Gerber SA, Gygi SP (2005) The absolute quantification strategy: a general procedure for the quantification of proteins and post-translational modifications. Methods 35(3):265–73
40. Schmidt C, Lenz C, Grote M et al (2010) Determination of protein stoichiometry within protein complexes using absolute quantification and multiple reaction monitoring. Anal Chem 82(7):2784–96
41. Stahl-Zeng J, Lange V, Ossola R et al (2007) High sensitivity detection of plasma proteins by multiple reaction monitoring of N-glycosites. Mol Cell Proteomics 6(10):1809–17
42. Mallick P, Schirle M, Chen SS et al (2007) Computational prediction of proteotypic peptides for quantitative proteomics. Nat Biotechnol 25(1):125–31
43. Picotti P, Lam H, Campbell D et al (2008) A database of mass spectrometric assays for the yeast proteome. Nat Methods 5(11):913–4
44. Picotti P, Rinner O, Stallmach R et al (2010) High-throughput generation of selected reaction-monitoring assays for proteins and proteomes. Nat Methods 7(1):43–6
45. Brun V, Dupuis A, Adrait A et al (2007) Isotope-labeled protein standards: toward absolute quantitative proteomics. Mol Cell Proteomics 6(12):2139–49
46. Hanke S, Besir H, Oesterhelt D et al (2008) Absolute SILAC for accurate quantitation of proteins in complex mixtures down to the attomole level. J Proteome Res 7(3):1118–30
47. Singh S, Springer M, Steen J et al (2009) FLEXIQuant: a novel tool for the absolute quantification of proteins, and the simultaneous identification and quantification of potentially modified peptides. J Proteome Res 8(5):2201–10
48. Pratt JM, Simpson DM, Doherty MK et al (2006) Multiplexed absolute quantification for proteomics using concatenated signature peptides encoded by QconCAT genes. Nat Protoc 1(2):1029–43
49. Rappsilber J, Ryder U, Lamond AI et al (2002) Large-scale proteomic analysis of the human spliceosome. Genome Res 12(8):1231–45
50. Ishihama Y, Oda Y, Tabata T et al (2005) Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. Mol Cell Proteomics 4(9):1265–72
51. Lu P, Vogel C, Wang R et al (2007) Absolute protein expression profiling estimates the relative contributions of transcriptional and translational regulation. Nat Biotechnol 25(1):117–24
52. Silva JC, Gorenstein MV, Li GZ et al (2006) Absolute quantification of proteins by LCMSE: a virtue of parallel MS acquisition. Mol Cell Proteomics 5(1):144–56
53. Zhang R, Sioma CS, Wang S et al (2001) Fractionation of isotopically labeled peptides in quantitative proteomics. Anal Chem 73(21):5142–9
54. Elliott MH, Smith DS, Parker CE et al (2009) Current trends in quantitative proteomics. J Mass Spectrom 44(12):1637–60