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Labeling Methods in Mass Spectrometry Based Quantitative Proteomics

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1. Introduction

Proteomics is loosely defined as the description of sets of proteins from any biological source, which have in most cases been identified by using mass spectrometry. However, only the mere identity of proteins present in a certain sample does not give any information about the dynamics of the proteome, involving relevant cellular events such as protein synthesis and degradation, or the formation of protein assemblies. In order to retrieve information on proteome dynamics, relative protein abundances between different protein samples should be assessed. Comparative or differential proteomics aims to identify and quantify proteins in different samples, to study e.g. differences between healthy and diseased states, mutant and wildtype cell lines, undifferentiated and differentiated cells, etc. Since mass spectrometry is in itself only a qualitative technique, various methods to obtain quantitative information of the proteome have been developed over the past decade and will be described in this Chapter.

We will focus on post-digestion labeling methods in the field of functional proteomics. Functional proteomics focuses on characterizing the composition of protein complexes, and generally involves the affinity purification of a protein of interest followed by the identification of co-purifying proteins by mass spectrometry (AP-MS). Generally, proteins in a negative control sample and those identified in the sample containing the protein of interest and its interacting partners are directly compared to determine which of the proteins interact in a specific manner. However, the mere presence or absence of a certain protein in protein data sets as a measure for either overlap or specificity is generally not sufficient, as this gives no information about the relative abundances of the present proteins. A generally recognized problem is the presence of contaminating proteins that are identified in the mass spectrometric screen, but do not really make part of the protein complex. Often, these background proteins are highly abundant proteins that stick to the complex or to beads to which the antibody is conjugated in a non-specific manner. A more accurate and correct approach would therefore involve a strategy in which protein abundance differences between sample and control can be assessed in a quantitative manner and which helps in discriminating bona fide interaction partners from such background proteins. Ideally, a differential mass spectrometric method would allow for an unbiased, sensitive, and high-throughput screening for protein-protein interaction networks.
1.1 SDS-PAGE based methods for protein quantitation

Two-dimensional sodium dodecylsulfate polyacrylamide gel electrophoresis (2D-SDS-PAGE) has traditionally been a popular method for differential-display proteomics on a global scale, although recently the popularity and applicability of stable isotope LC-MS based methods has exceeded those presented by gel based methods. 2D-SDS-PAGE based methods enable the separation of complex protein mixtures on a single gel. Proteins are separated in two dimensions: in the first dimension, they are fractionated according to their isoelectric point using a pH gradient gel, which is subsequently placed on a polyacrylamide gel slab for further separation based on their molecular weight using SDS-PAGE. Proteins are then visualized by staining the gel with a dye such as Coomassie, silver or Sypro Ruby. In principle, for comparative issues, samples are loaded on separate gels and protein spot patterns are compared visually. Proteins that differ in abundance can then be punched out of the gel, digested with a suitable protease and analyzed by mass spectrometry. In a variation of this technique, difference gel electrophoresis (DIGE), proteins from two samples are first labeled with different fluorescent dyes and then mixed, making it possible to compare two different samples on a single gel. Two fluorescence images are recorded and overlayed, and differentially expressed proteins appear in only one of the images (Unlu et al., 1997). Limitations of this method include the manual selection of proteins to be analyzed, making it a time-consuming technique, as well as the limited sensitivity, as a consequence of which that proteins with a low concentration may be failed to be selected. Nowadays, in many laboratories there is a tendency to replace 2D-SDS-PAGE based methods by more powerful, LC-MS based methods for relative protein quantitation.

1.2 Protein and peptide quantitation using LC-MS based methods

Rather than by comparing protein spot intensities on a gel, quantification of proteins in LC/MS based methods is based on the peak height or area of the proteolytic peptide peaks in the mass spectrum and/or chromatogram. As mentioned before, mass spectrometry is not an inherently quantitative analytical technique, meaning that the peak height or area in a mass spectrum in itself does not accurately reflect the abundance of a peptide in the sample. The main reasons for this are the differences in ionization efficiency and detectability of peptides because of their different physicochemical characteristics, as well as the limited reproducibility of an LC-MS experiment. Altogether, this makes it difficult to compare peptide peak intensities between different mass spec runs. In principle however, peak intensity differences of the same analyte within one LC-MS run do accurately reflect the abundance difference. One way to distinguish the same analyte from different sample sources within one LC-MS experiment is by using stable heavy isotope labeling. When different stable isotope labels are used for proteins or peptides which are derived from different samples, the same analyte can in principle be quantified in one experiment. Such heavy stable isotope labels should in principle not affect the biophysical and chemical properties of peptides and proteins, but solely the mass, designating one of the samples as ‘light’ and the other sample ‘heavy’ according to the mass introduced by the label. The heavy and light peptides co-elute from the LC column at the same retention time and the heavy stable isotope leads to a mass shift in the mass spectrum, resulting in the observation of peak pairs. The peak heights or areas of such pairs can be compared and give an accurate reflection of the difference in abundance of this peptide between both samples. Heavy stable isotope labels can be introduced at different stages in the sample treatment protocol. Below, we will give an overview of the most widely used labeling techniques.
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1. In a differential labeling AP-MS experiment, proteins in a control sample are labeled with a heavy stable isotope label, whereas proteins in the experimental sample are labeled with a light label. Incorporation of the heavy label results in a shift of the m/z value and allows one to differentiate between the sources of the protein of interest.

2. LC-MS-based quantitation methods

2.1 Incorporation of stable isotopes by metabolic labeling

Heavy stable isotope labels can be introduced in vivo by growing cells or even whole organisms in the presence of amino acids or nutrients carrying such stable isotopes. Metabolic labeling is often the preferred labeling technique, since incorporation occurs at the earliest possible moment in the sample preparation process, thereby minimizing the error in quantification (see Figure 2). Several methods based on metabolic labeling have been developed and here we will give a brief overview. The first metabolic labeling studies were performed utilizing ^15N-enriched media to grow *S. cerevisiae* (Oda et al., 1999) and *E. coli* (Conrads et al., 2001). Next, the method was extended towards multicellular organisms which were ^15N labeled, such as *D. melanogaster* and *C. elegans* by feeding them on labeled yeast or bacteria, respectively (Krijgsveld et al., 2003). Even a higher eukaryote like a rat has been labeled with ^15N (McClatchy et al., 2007). Plants, as they are autotrophic organisms, can easily be labeled metabolically through feeding of labeled inorganic compounds in the form of ^15N-nitrogen-containing salts, as first demonstrated in NMR studies (Ippel et al., 2004), and later in MS-based proteomics (Engelsberger et al., 2006; Lanquar et al., 2007). ^15N atoms are incorporated into the sample during cell growth, eventually replacing all natural isotopic (i.e., ^14N) nitrogen atoms. The corresponding mass shift depends on the number of nitrogen atoms present in each of the resulting proteolytic peptides. However, this variable mass shift complicates data analysis to a large extent and requires high resolution mass spectrometry for the analysis (Conrads et al., 2001). Specific software for the analysis of ^15N labeled samples has been developed (Mortensen et al., 2010).

Stable isotope labeling in cell culture (SILAC) is a metabolic labeling approach first published in 2002 by the lab of Matthias Mann (Ong et al., 2002). During cell growth, essential amino acids that carry heavy stable isotopes and which have been added to the...
Fig. 2. Stages of incorporation of stable isotope labels in typical labeling workflows in quantitative proteomics. The light and dark grey diamonds represent the two protein samples to be differentially labeled and compared. Figure adapted from (Ong & Mann, 2005).

culturing medium are introduced in all newly synthesized proteins. After several cell doublings, the complete cellular proteome will have incorporated the supplied labeled amino acid(s). This results in a shift of the proteolytic peptide mass after protein digestion and subsequent MS analysis. When labeled and non-labeled cell cultures are now mixed and analyzed in the same experiment, peptides will be represented by peak pairs in the mass spectrum, where the mass difference will depend on the number and nature of the labeled amino acid(s). Usually, labeled lysine and arginine are used, with the result that every peptide will carry a label except for the carboxyl-terminal peptide of the protein, when digested with trypsin, as does labeling with lysine when digested with Lys-C (Ibarrola et al., 2003). In contrast to $^{15}$N labeling, the number of incorporated labels in SILAC is defined and not dependent on the peptide sequence, thus facilitating data analysis. SILAC has been successfully applied in global proteome studies (de Godoy et al., 2006), for functional proteomics assays, as well as for the study of post-translational modifications (Blagoev et al., 2003; Blagoev & Mann, 2006).

Because of the label incorporation at early stages in the sample preparation protocol, SILAC is generally the preferred choice of labeling method. However, SILAC is limited in sample applicability, for example, not every cell line can grow in an efficient manner in media optimized for SILAC, often due to the requirement of dialyzed serum in the medium to prevent contamination with natural amino acids. Besides, the method may be hampered by in vitro conversion of labeled arginine to proline (Van Hoof et al., 2007). SILAC has been used to label higher organisms, for instance flies (Sury et al., 2010) and mice (Kruger et al., 2008), by feeding them with labeled food. In general though, this is a time consuming and expensive process. In the plant, SILAC has only yielded label incorporation of approximately 70% (Gruhler et al., 2005), which is not satisfying for many proteomics applications. Moreover, there are practical and moral limitations to SILAC labeling of human tissue. For these cases, methods for stable isotope label incorporation at a later stage in the sample preparation protocol are required. Chemical and enzymatic labeling techniques have been developed that can introduce the heavy stable isotope label only after sample collection and proteolytic digestion at the peptide level.
2.2 Incorporation of stable heavy isotope labels by chemical or enzymatic labeling

In general, the advantage of chemical labeling over metabolic labeling is the possibility to label a wide range of different sample types, since incorporation of the label is performed only after harvesting cells and subsequent purification of proteins. Chemical labeling is essentially based on similar mechanisms as metabolic labeling, except that the label is introduced into proteins or peptides by a chemical reaction, e.g., with sulfhydryl groups or amine groups, or through acetylation or esterification of amino acid residues. Alternatively, the heavy stable isotope label can be introduced into the peptide during an enzymatic reaction with heavy water (H$_{2}^{18}$O). Below, several of the most widely applied chemical and enzymatic labeling approaches are described.

2.2.1 Isotope-Coded Affinity Tags (ICAT)

Isotope-Coded Affinity Tagging (ICAT) is a chemical labeling method that was first described by the Aebersold lab in 1999 (Gygi et al., 1999). In chemical modification-based approaches, stable isotope-bearing chemical reagents are targeted towards reactive sites on a protein or peptide. The ICAT reagent consists of a reactive group that is cysteine-directed, a polyether linker region with eight deuteriums, and a biotin group that allows purification of labeled peptides. In an ICAT experiment, two pools of proteins are denatured and reduced, and the cysteine residues of the proteins are subsequently derivatized with either the ‘heavy’ or ‘light’ ICAT reagent. The labeled pools are then combined, cleaned up to remove excess reagent, and digested with an appropriate protease. The cysteine-containing peptides, carrying ‘heavy’ and ‘light’ isotope tags, are then captured on an avidin column via the biotin moiety present at the incorporated label. Peptides are then eluted from the column and analyzed by mass spectrometry. Since only cysteine-containing peptides are isolated, the peptide mixture complexity is in general limited, which in principle would enable identification of lower abundant proteins. On the other hand, some proteins contain no cysteines, while others would have to be quantified on the basis of just a single peptide. Additionally, the large biotin tag significantly increases the complexity of fragmentation spectra, complicating peptide identification, and, besides that, it has been demonstrated that deuterium atoms that are associated with the tag can cause a shift in retention time between the light and heavy peptides in reverse phase chromatography (Zhang et al., 2001). Subsequent iterations of the ICAT approach by substituting a cleavable and co-eluting tag have improved the method (Hansen et al., 2003; Li et al., 2003).

2.2.2 Dimethyl labeling

An alternative method based on chemical labeling is dimethylation of peptides. In this workflow, samples are first digested with proteases such as trypsin and the derived peptides of the different samples are then labeled with isotopomeric dimethyl labels. The labeled samples are mixed and simultaneously analyzed by LC-MS whereby the mass difference of the dimethyl labels is used to compare the peptide abundance in the different samples. Stable isotope labeling by dimethylation is based on the reaction of peptide primary amines (peptide N-termini and the epsilon amino group of lysine residues) with formaldehyde to generate a Schiff base that is rapidly reduced by the addition of cyanoborohydride to the mixture. These reactions occur optimally between pH 5 and 8.5. Dimethyl labeling can be used as a triplex reagent, making it possible to quantitatively analyze three different samples in a single MS run. Labeling with the light reagent generates
a mass increase of 28 Da per primary amine on a peptide and is obtained by using regular formaldehyde and cyanoborohydride. Using deuterated formaldehyde in combination with regular cyanoborohydride generates a mass increase of 32 Da per primary amine; this is referred to as the intermediate label (Hsu et al., 2003). Incorporation of the heavy label can be achieved through combining deuterated and $^{13}$C-labeled formaldehyde with cyanoborodeuteride, resulting in a mass increase of 36 Da (Boersema et al., 2008). These reactions are visualized in Figure 3.

![Fig. 3. Labeling schemes of triplex stable isotope dimethyl labeling. R: remainder of the peptide. Figure adapted from (Boersema et al., 2008).](image)

One drawback of the incorporation of deuterium is that deuterated peptides show a small but significant retention time difference in reversed phase chromatography compared to their non-deuterated counterparts (Zhang et al., 2001). This complicates data analysis because the relative quantities of the two peptide species cannot be determined accurately from one spectrum but requires integration across the chromatographic time scale. As the stable isotope dimethyl labeling is performed at the peptide level, the method is not subjected to restrictions on the origin of the biological sample. Stable isotope dimethyl labeling can be performed in up to 8M urea, as well as after in-gel digestion protocols. It should be noted that during the sample preparation workflow, no buffers and solutions containing primary amines (such as ammonium bicarbonate and Tris) ought to be used, as formaldehyde would react with these, which would affect the labeling efficiency. This can be circumvented by desalting the peptide sample before the labeling reaction or by performing the digestion in buffers without primary amines (e.g., triethyl ammonium bicarbonate (TEAB)). Since both the peptide N-termini and lysine side chain amino groups are labeled in this protocol, it is compatible with the peptide products of virtually any protease, such as trypsin, Lys-C, Lys-N, Arg-C, and V8 (Boersema et al., 2008). Typically, for proteomics experiments trypsin is used, which cleaves C-terminal of lysine and arginine residues. Labeling of tryptic peptides using the method described here results in a mass shift of either 4 Da (when cleaved after an arginine residue) or 8 Da (when cleaved after a lysine residue) between the light and intermediate and between the intermediate and heavy label.
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Differential labeling of peptides resulting from digestion with Lys-C or Lys-N (cleaving respectively C- and N-terminal of lysine residues) will result in a mass difference of mainly 8 Da (both the N-terminus and the lysine residues are labeled), whereas peptide products from Arg-C and V8 will result in varying mass differences as the number of lysine residues per peptide will typically vary. After proteolytic digestion, the samples are labeled separately by incubation with CH$_2$O and NaBH$_3$CN (light), CD$_2$O and NaBH$_3$CN (intermediate) or $^{13}$CD$_2$O and NaBD$_3$CN (heavy).

Boersma and co-workers have described three different experimental protocols for dimethyl labeling, i.e. in-solution, online, and on-column (Boersema et al., 2009). In-solution labeling (Boersema et al., 2008; Hsu et al., 2003) can be used for sample amounts from 1 µg to several milligrams of sample and is most suitable for experiments in which large sample numbers have to be labeled since labeling can be performed in parallel here. Online stable isotope labeling is the optimal method for the labeling of small quantities (<< 1 µg) of sample, because the sample loss is diminished by combining sample clean-up and labeling and by performing LC-MS directly after labeling. Finally, the on-column stable isotope labeling method is most suited for larger (up to milligrams) sample amounts, as sample clean-up and labeling steps are combined and the quenching step is avoided. After labeling, the samples are mixed and analyzed by mass spectrometry. Finally, quantification is performed by comparing the signal intensities of the differentially labeled peptides (see section on Data Analysis).

Protein quantitation by dimethyl labeling has been applied in a variety of studies, e.g. for the investigation of tyrosine phosphorylation sites in Hela cells upon EGF stimulation (Boersema et al., 2010). Proteins in a HeLa cell extract were dimethyl labeled and subsequently enriched for phosphorylated-tyrosine-containing peptides using immunoaffinity assays. Several tens of unique phosphotyrosine peptides were found to be regulated by EGF, illustrating that such a targeted quantitative phosphoproteomics approach has the potential to study signaling events in detail. Furthermore, the method has been applied to unravel differences in composition between highly related protein complexes, such as tissue-specific bovine proteasomes (Raijmakers et al., 2008) and the yeast nuclear and cytoplasmic exosome protein complex (Synowsky et al., 2009).

In conclusion, dimethyl labeling is a reliable, cost-effective and undemanding procedure that can be easily automated and applied in high-throughput proteomics experiments. It is applicable to virtually any sample, including tissue samples derived from animals or humans and up to three samples can be analyzed simultaneously. Like other chemical labeling methods though, stable isotope dimethyl labeling is performed in one of the final steps of a typical proteomics workflow and is therefore more prone to errors in the quantitative analysis as compared to workflows in which the label is added at an earlier stage.

2.2.3 $^{18}$O labeling

$^{18}$O labeling relies on class-2 proteases, such as trypsin, to catalyze the exchange of two $^{16}$O atoms for two $^{18}$O atoms at the C-terminal carboxyl group of proteolytic peptides, resulting in a mass shift of 4 Da between differently labeled peptides, as illustrated in Figure 4.

Hydrolysis of a protein in H$_2$O by a protease results in the incorporation of one $^{18}$O atom into the carboxyl terminus of each proteolytically generated peptide. This mechanism involves a nucleophilic attack by a solvent water molecule on the carbonyl carbon of the scissile peptide bond (reaction 1). Following this hydrolysis reaction, the protease
Fig. 4. Principle of trypsin catalyzed $^{18}$O labeling. Incorporation of two $^{18}$O labels at the C-terminus of a tryptic peptide takes place in a two-step reaction.

Incorporation of one more $^{18}$O atom into the carboxyl terminus of the proteolytically generated peptide. This second incorporation results in two $^{18}$O atoms being incorporated into the carboxyl terminus of the peptides (reaction 2). The second $^{18}$O atom-incorporation is essentially the reverse reaction of peptide-bond hydrolysis or the peptide-bond formation reaction (Miyagi & Rao, 2007).

Proteolytic $^{18}$O labeling has shown to be a useful tool in the field of comparative proteomics. A number of studies have been published, involving among others relative protein quantitation of the virus proteome (Yao et al., 2001), proteomes of cultured cells (Blonder et al., 2005; Brown & Fenselau, 2004; Rao et al., 2005) and proteins in serum (Hood, Lucas et al., 2005; Qian et al., 2005) and tissues (Hood, Darfler et al., 2005; Zang et al., 2004). In addition, $^{18}$O labeling has been used for the relative quantitation of post-translational modification, e.g. changes of protein phosphorylation in response to a stimulus (Bonenfant et al., 2003). In the latter study, pools of differentially labeled phosphorylated proteins were enriched by using immobilized metal-affinity chromatography. Peptides were then dephosphorylated by alkaline phosphatase in order to quantify the changes in phosphorylation by mass spectrometry. A similar approach has been used for the global phosphoproteome analysis of human HepG2 cells (Gevaert et al., 2005).

Despite its relatively simple mechanism and low costs, $^{18}$O labeling has not become the preferred method for differential proteomics based on heavy stable isotope labeling. The practical difficulties involved, most importantly the occurrence of incomplete incorporation of two $^{18}$O atoms into the proteolytic peptide, and, as a consequence, the difficulties in data analysis and interpretation are the most likely reasons for this. Several factors are responsible for the variable degree of $^{18}$O incorporation, including variable enzyme substrate specificity, oxygen back exchange, pH dependency and peptide physicochemical properties. To overcome inefficient labeling, algorithms for the correction of $^{18}$O labeling efficiency have been developed (Ramos-Fernandez et al., 2007), while other studies have focused on minimizing back exchange of $^{18}$O to $^{16}$O. It was found that the latter can be achieved by either decreasing the pH value for trypsin catalyzed incorporation reactions.
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(Hajkova et al., 2006; Staes et al., 2004; Zang et al., 2004), or by using immobilized trypsin for the exchange reaction (Chen et al., 2005; Fenselau & Yao, 2007; Sevinsky et al., 2007). Trypsin immobilization allows the investigator to significantly increase the molar ratio of protease-to-substrate ratio, which subsequently increases the labeling efficiency. Another advantage of using immobilized proteases is that no protease-catalyzed oxygen back exchange reaction occurs, because the immobilized proteases are completely removed from the peptides after the labeling reaction.

Our lab has developed a two-step approach in order to completely label all proteolytic peptides (Bezstarosti et al., 2010). In this method, proteins are first digested with soluble trypsin. Subsequently, proteolytic peptides are incubated with $\text{H}_2^{18}\text{O}$ at pH 4.5 in the presence of immobilized trypsin. Clearly, no singly $^{18}\text{O}$ labeled variants were observed in any of the peptide mass spectra (see Figure 5), indicating that no partial labeling whatsoever occurred, nor did any back exchange from $^{18}\text{O}$ to $^{16}\text{O}$ take place during sample treatment or analysis. Thus, complete incorporation of two $^{18}\text{O}$ labels into each of the tryptic peptides in a mixture can be achieved routinely.

![Fig. 5. Doubly charged tryptic peptide FLEQQNQVLQTK A) in the absence of $^{18}\text{O}$ label and B) after incorporation of the label. The two-step labeling reaction in the presence of immobilized trypsin as described here ultimately results in the complete incorporation of two $^{18}\text{O}$ labels, with no intermediary products present. The peptide isotope peaks in B) at m/z 739.39 and 739.90 are due to impurities of commercial $\text{H}_2^{18}\text{O}$, containing only 97% $^{18}\text{O}$.

It was shown in this study that $^{18}\text{O}$ labeling can be applied in a functional proteomics assay to discriminate background proteins from specific interactors of a protein of interest. Generally, controls are heavy labeled and the coimmunoprecipitation (co-IP) sample is labeled light. Specific interactors are expected not to be present in the control and would thus have a ratio of (close to) zero, whereas background proteins would show heavy-to-light (H:L) ratios of (close to) 1. $^{18}\text{O}$ labeling was used in order to differentiate between non-specific background proteins and specific, bona fide interactors of the Cyclin dependent kinase 9

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(Cdk9) purified from nuclear extracts of murine erythroleukemia (MEL) cells. Biotinylated Cdk9 was expressed in MEL cells and purified using streptavidin beads under relatively mild conditions (de Boer et al., 2003). The proteins that co-purified with Cdk9 were washed and digested with trypsin while still bound to the beads and subsequently identified by tandem mass spectrometry. A control sample was taken following the same procedure from an equal number of cells, but using non-transfected MEL cells. Proteolytic peptides from the control sample were then labeled using $\text{H}_2^{18}\text{O}$ in the two-step approach mentioned earlier, while proteolytic peptides from the Cdk9 pulldown sample underwent the same procedure with unlabeled $\text{H}_2\text{O}$. The peptide mixtures were dissolved in equal volumes of buffer, mixed in a 1:1 volume ratio and identified by LC-MS/MS. H:L ratios were calculated for all proteins identified from the mixed sample.

As expected, H:L ratios of close to 1 were observed for typical background proteins, such as ribosomal, housekeeping, and structural proteins, which were present as non-specific background proteins (see Figure 6). In contrast, among the proteins that were quantified with H:L ratios close to 0, indicating specificity for the Cdk9 co-immunopurification sample, the far majority of interacting proteins that have been described in different studies in the literature were identified in a single experiment, as well as several novel interaction partners of diverse functionalities, suggesting putative additional roles for Cdk9 in various nuclear events such as transcription and cell cycle control (Bezstarosti et al., 2010). It was shown in this study that complete $^{18}$O labeling of peptides in complex mixtures can be routinely achieved. This greatly simplifies the analysis of peak intensity ratios, since only two components (i.e., ‘light’ and ‘heavy’) need to be considered and no correction algorithms have to be applied to convert peak intensities of intermediate labeled peptide species.

![Fig. 6. MS spectra of two tryptic peptides from a 1:1 mixture of a digest of a Cdk9 co-IP experiment ($\text{H}_2^{18}\text{O}$) and a control sample (in $\text{H}_2\text{O}$). (A) Doubly charged peptide LGTPELSPTER of the contaminant acetyl-CoA carboxylase shows both the “light” and “heavy” forms and is therefore marked as a nonspecific protein. (B) Triply charged peptide GPPEETGAAVFDHPAK of cyclin T1 is only present in the “light” form and is therefore specific for the Cdk9 sample (see (Bezstarosti et al., 2010)).](www.intechopen.com)
2.2.4 Labeling with isobaric tags

Metabolic labeling, ICAT, enzymatic labeling and most other chemical labeling approaches for relative quantification are based on the mass difference between differentially labeled peptides. There are, however, some limitations imposed by mass difference labeling. The mass difference concept of many practical purposes is limited to a binary (2-plex) or ternary (3-plex) set of reagents; higher order multiplexing would increase the complexity of MS\textsuperscript{1} spectra too much. This limitation makes comparison of multiple states difficult to undertake. Therefore, multiplexed sets of reagents for quantitative protein analysis have been developed. The isobaric tag for relative and absolute quantitation (iTRAQ) (Ross et al., 2004) and tandem mass tag (TMT) (Thompson et al., 2003) technologies are commercially available isobaric mass tagging reagents and protocols (Figure 7).

![Fig. 7. A) Chemical structure of the TMT tag. The 6-plex tags have different distributions of the stable heavy isotopes of carbon and nitrogen in the molecule, resulting in different fragmentation spectra. B) A peptide that is present in 6 different samples is differentially labeled with a 6-plex TMT tag, containing reporter-balancer combinations, resulting in all conjugated peptides having the same m/z value. Upon high energy collision dissociation (HCD), the differentially labeled peptides show identical b and y fragment ions, but the reporter ion masses in the low m/z region are different. C) As an example, a protein was labeled in a 6-plex (TMT-126 through TMT-131) protocol and mixed in a 2:2:1:1:3:3 ratio. The resulting reporter ion intensity ratios show an excellent correlation with the mixing ratios. Panels A and B were adapted from (Thompson et al., 2003).](www.intechopen.com)
In these procedures, both N-termini and lysine side chains of peptides in a digest mixture are labeled with different isobaric mass reagents in such a way that all derivatized peptides are isobaric and chromatographically indistinguishable. Only upon peptide fragmentation can the different mass tags be distinguished. As each tag adds the same total mass to a given peptide, each peptide species produces only a single peak during liquid chromatography, even when two or more samples are mixed. Thus, there will be only one peak in the MS\textsuperscript{1} scan, and, therefore, only a single \textit{m/z} will be isolated for fragmentation. The different mass tags only separate upon fragmentation, when reporter ions that are typical for each of the different labels are generated. These reporter ions are in the low mass range, which usually is not covered by typical peptide fragment ions. The intensity ratio of the different reporter ions is used as a quantitative readout. Thus, quantitation in combination with isobaric mass tagging is based on peptide fragmentation (MS\textsuperscript{2}) spectra rather than on the survey scans and quantitative accuracy will depend on the isolation width of precursor ions for fragmentation, since all ions isolated in that window will contribute to fragments in the reporter ion mass ranges. One drawback of such a method is that often only a single fragmentation spectrum per peptide is available, while in quantitation based MS\textsuperscript{2} scans, usually several data points across the eluting peptide peak are sampled, which may result in a lower overall sensitivity.

2.3 Label free quantitation

Over the past few years, mainly as a result of constantly improving LC-MS equipment, there has been growing interest in the use of label-free approaches for quantitative proteomic analysis (see (Neilson et al., 2011) for a recent review). In a label free quantitative proteomic analysis, protein mixtures are analyzed directly and samples are compared to each other after independent analyses. As a result, there is no mixing of samples, so that higher proteome coverage can be achieved and there is no limit to the number of experiments that can be compared (Bantscheff et al., 2007). The disadvantage of this approach is a lack of a formal internal standard, which can lead to greater error in individual datasets but is minimized through the analysis of several biological replicates.

Label-free approaches may be divided into two main groups by the way that the abundance of a peptide is measured. The first group comprises methods that are based on the ion count and compare either maximum abundance or volume of ion count for peptide peaks at specific retention times between different samples (Chelius & Bondarenko, 2002; Listgarten & Emili, 2005; Silva et al., 2005; Wiener et al., 2004). As ionized peptides elute from a reversed-phase column into the mass spectrometer, their ion intensities can be measured within the given detection limits of the experimental setup. Although this method is relatively straightforward conceptually, several considerations must be taken into account to ensure reproducible and accurate detection and quantitation between individual sample runs. Concerns with LC signal resolution can arise when peptide signals are spread over a large retention time range causing overlap with co-eluting peptides. Similar concerns include biological variations resulting in multiple signals for the same peptide as well as technical variations in retention time, MS intensity, and sample background noise from chemical interference. These aspects of quantitation based on ‘area under the curve’ necessitate a computational ‘clean up’ of the raw LC-MS data (Neilson et al., 2011).

The second group is based on the identification of peptides by MS/MS and uses sampling statistics such as peptide count, spectral counts (Lundgren et al., 2010), or sequence coverage to quantify the differences between samples (Choi et al., 2008; Liu et al., 2004; Old et al.,
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2005; Rappsilber et al., 2002). For protein quantification based on spectrum counting, the data processing steps are basically identical to the general protein identification workflow in proteomics, which is one of the reasons why this approach has become so popular. The rationale behind this quantitation method is that more abundant proteins are sampled more often in fragment ion scans than are low abundance peptides or proteins. Obviously, the outcome of spectrum counting depends on the settings of data-dependent acquisition on the mass spectrometer. In particular the linear range for quantitation and the number of proteins to be quantified are influenced by different settings for dynamic exclusion (Wang & Li, 2008); the optimal settings will depend on sample complexity. The most significant disadvantage of spectrum counting is that it behaves very poorly with proteins of low abundance and few spectra. The accuracy of the spectrum count method, especially for low abundance proteins, suffers from the fact that each spectrum is scored independently of its ion intensities.

3. Comparison of different methods for quantitation

With the existence of a wide variety of LC-MS based quantitation methods, it may be hard to decide which approach to utilize for a certain application. As described earlier, each approach has its own strengths and limitations, and, additionally, other factors may play a role, such as available equipment, level of experience and budget. In the following section we summarize the pros and cons of earlier described quantitation methods which might serve as a guidance to decide which approach is most suitable in a specific situation.

3.1 Metabolic labeling

If it is possible to label samples metabolically, this would be the most advantageous option to quantitate proteins. The most important reason for this is that different samples can be combined at the level of intact cells, which, as a result, excludes all sources of quantitation error introduced by biochemical and mass spectrometric procedures, as these will affect both protein populations in the same way. Metabolic labeling is therefore the most sensitive MS based labeling technique to date, making it possible to study small protein abundance differences as small as 1.5-fold changes or even smaller. Despite a number of cases that demonstrate the feasibility of metabolic labeling of higher organisms using $^{15}$N sources in vivo, such as C. elegans, D. Melanogaster (Krijgsveld et al., 2003) and the rat (Wu et al., 2004), it is not practical to apply this strategy routinely. The most important reason for this is that labeling with $^{15}$N complicates data analysis to a large extent, as discussed in section 2.1. Nowadays, the most widely applied method to metabolically label material of eukaryotic origin is SILAC in immortalized cell lines. SILAC based MS has been extensively applied for the study of global proteomes, in the field of functional proteomics, and for the analysis of post-translational modifications. Additionally, SILAC can be applied to whole organisms, such as E. coli, S. cerevisiae, and D. melanogaster. Even metabolic labeling of higher eukaryotes like the mouse (Kruger et al., 2008) has shown to be possible. Although SILAC is the most accurate MS based quantitation approach, it might not always be possible or preferable to use SILAC. As mentioned earlier, not every cell type might grow well in the SILAC medium. Some cell lines readily convert arginine to proline, which complicates data analysis, and require adaptation of the protocol such as titration of arginine in the medium (Ong et al., 2003). Otherwise, computational approaches to correct
arginine-to-proline conversion may be applied (Park et al., 2009). Finally, cell lines that are sensitive to changes in media composition or are otherwise difficult to grow or maintain in culture may not be amenable to metabolic labeling at all. When it is not possible to label a cell culture in SILAC medium, post-digestion incorporation methods may serve as an alternative. Moreover, post-digestion labeling might be the preferred method for affinity purification mass spectrometry (AP-MS) applications, as the starting material for co-IP assays is typically several milligrams of proteins. The use of stable isotope labeling by SILAC can be cost-prohibitive, whereas post-digestion labeling approaches such as stable isotope dimethyl labeling and $^{18}$O labeling are performed with inexpensive generic reagents and do not pose severe financial restrictions to the amount of sample to be labeled.

In conclusion, SILAC can be applied in almost all sorts of proteomic applications since it is very sensitive, and limitations are mainly biological applicability or involve practical issues such as time, cost, or available equipment.

### 3.2 Chemical and enzymatic post-digestion labeling

One of the advantages of a chemical modification approach over metabolic labeling is the ability to label proteins after cell lysis and in a post-digestion manner. This makes the approach generally applicable, since it allows the quantitative analysis of biological samples that cannot be grown in culture, such as human body fluids or human tissue. ICAT was one of the first chemical labeling methods introduced for quantitative mass spectrometry. Although often and successfully applied, its main drawbacks are adverse side reactions and its inability to label peptides that do not contain cysteine residues. As a result, in many laboratories, ICAT has been substituted by other approaches, such as chemical dimethyl labeling or enzymatic $^{18}$O labeling. Compared to ICAT, both $^{18}$O labeling and dimethyl labeling are simple, free of extensive sample manipulations, virtually free of side reactions, and amenable to all protein species (i.e., proteins that contain no cysteine residues). In contrast to ICAT, there is no lower limit of the protein amount that can be labeled for $^{18}$O and dimethyl. Another advantage of the latter two labeling approaches is that they are cost-effective. This, together with the fact that proteins for any species can be labeled and the ease of sample preparation, makes chemical labeling the preferred method for the quantitative analysis of for instance size-limited human tissue specimens. Also, post-digestion labeling is practical for tissue samples of higher organisms such as mice, or cell lines that cannot be metabolically labeled.

One drawback of dimethyl labeling is that deuterated peptides show a small but significant retention time difference in reversed-phase HPLC compared to their non-deuterated counterparts (Zhang et al., 2001). This complicates data analysis because the relative quantities of the two peptide species cannot be determined accurately from one spectrum but requires integration across the chromatographic time scale. Retention time shifts are far less pronounced for labels such as $^{13}$C, $^{15}$N, or $^{18}$O isotopes (Zhang & Regnier, 2002), so that the additional signal integration step over retention time can generally be omitted in approaches based on incorporation of these labels. However, compared to iTRAQ and TMT, dimethyl labeling is performed with inexpensive generic reagents and do not pose severe financial restrictions to the amount of sample to be labeled.

Multiplex labeling using TMT or iTRAQ has turned out to be particularly useful for following biological systems over multiple time points or, more generally, for comparing multiple treatments in the same experiment. With dimethylation labeling, iTRAQ and TMT
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Labeling, multiplexing can be achieved, which is not possible for $^{18}$O labeling. iTRAQ is capable of simultaneously analyzing eight samples (Pierce et al., 2008), whereas with TMT labeling, six samples can be measured together (Thompson et al., 2003). It should be noted that the use of commercial isobaric iTRAQ or TMT labels can be cost-prohibitive. In terms of equipment, TMT and iTRAQ labeling approaches are limited to mass spectrometers which are capable of efficiently detecting ions that are present at a relatively low $m/z$ and peptide quantification is based on a single fragmentation mass spectrum. An advantage of isobaric tags is that the labeled peptides co-elute from the chromatographic column which means that the MS signal is not split into different peaks, as in conventional isotope labeling, improving sensitivity in the MS mode.

In conclusion, chemical and enzymatic labeling can be applied to virtually any biological sample since incorporation is performed after cell lysis and generally also after digestion. Therefore, post-digestion labeling is specifically useful for the study of mammalian and human tissue or body fluids. Importantly, compared to metabolic labeling, label incorporation in chemical and enzymatic labeling approaches takes place at a later stage in the sample treatment protocol and are therefore in general less accurate. For absolute protein quantitation, peptides have to be labeled with stable heavy isotopes, which is usually done by synthesizing them with labeled amino acids, in order to serve as an internal standard.

3.3 Label free approaches

Since no labels are used whatsoever in label free quantitative proteomics, these approaches are inexpensive, they can be applied to any kind of biological material and the proteome coverage of quantified proteins is high because basically every protein that is identified by at least one peptide spectrum can in principle be quantified. In addition, the complexity of the sample is not increased by mixing different samples. Label free methods therefore usually have a high analytical depth and dynamic range, giving this method an advantage when large, global protein changes between treatments are expected. Also, since the samples are not mixed and quantification is done after MS analysis, the obtained data is not fixed and can be used in other contexts as well. These advantages make label free quantification an attractive approach for e.g. clinicians who have large patient material-derived datasets and want to compare multiple datasets, and have no wet lab available.

Despite the many advantages of label free quantitation, it is probably the least accurate among the mass spectrometric quantification methods when considering the overall experimental process because all the systematic and non-systematic variations between experiments are reflected in the obtained data. Consequently, the number of experimental steps should be kept to a minimum and every effort should be made to control reproducibility at each step.

There has been growing interest in the use of label-free approaches for quantitative proteomic analyses over the recent years, particularly because of ever increasing accuracy and reproducibility of high-resolution LC-MS equipment. Most MS analysis is performed with data dependent analysis (DDA) where the mass spectrometer runs a parent ion scan and selects the most abundant ions on which to conduct fragmentation scans, typically 4-10 scans, before returning to a parent ion scan. There may be a bias in this type of data for co-eluting peptides towards omitting the lower abundant peptides from MS/MS (Venable et al., 2004). This bias creates a subset of proteins effectively unseen due to the resultant level
of detection limit. An experimental setup has been developed in which the mass spectrometer no longer cycles between MS and MS/MS mode but aims to detect and fragment all peptides in a chromatographic window simultaneously by rapidly alternating between high- and low-energy conditions in the mass spectrometer (Silva et al., 2006). Obviously, there are challenges with analyzing such data from complex samples as many fragmentation spectra will be populated with sequence ions from multiple peptides each contributing differently to the overall spectral content. Also, there is evidence that label-free methods provide higher dynamic range of quantification than any stable isotope labeling approach (i.e., 2-3 orders of magnitude) and therefore may be advantageous when large and global protein changes between experiments are observed (Old et al., 2005).

4. Data analysis

No matter the choice of quantitative method, quantitative proteomic data are typically very complex and often of variable quality. The main challenge stems from incomplete data, since even today’s most advanced mass spectrometers cannot sample and fragment every peptide ion present in complex samples. As a consequence, only a subset of peptides and proteins present in a sample can be identified. Over the past years, a series of experimental strategies for mass spectrometry based quantitative proteomics and corresponding computational methodology for the processing of quantitative data have been generated (reviewed in (Matthiesen et al., 2011; Mueller et al., 2008). Conceptually different methods to perform quantitative LC-MS experiments demand different quantification principles and available software solutions for data analysis. Quantification can be achieved by comparing peak intensities in differential stable isotopic labeling, via spectral counting, or by using the ion current in label-free LC-MS measurements. Numerous software solutions have been presented, with specific instrument compatibility and processing functionality and which can cope with these basically different quantitation methods. It is important for researchers to choose an appropriate software solution for quantitative proteomic experiments based on their experimental and analytical requirements. However, it goes beyond the scope of this Chapter to discuss all of the available software tools separately. For an extensive and up-to-date overview of software solutions including links to websites for downloads, the reader is referred to http://www.ms-utils.org.

5. Concluding remarks

As we have discussed in this Chapter, all of the mass spectrometry based quantification methods have their particular strengths and weaknesses. The researcher has to choose the best method from the multitude of methods that have emerged for the analysis of simple and complex (sub-)proteomes using quantitative mass spectrometry for his or her specific research; a choice that depends on the financial aspects involved, the availability of high-resolution mass spectrometer and LC equipment and the available expertise present in the lab. Quantitative proteomics methods are now starting to mature to an extent that they can be meaningfully applied to the study of proteomes and their dynamics. Using the labeling methods described in this Chapter, it is now possible to identify and quantitate several thousands of proteins in a single experiment. However, there is still room for significant improvements to the experimental strategies that are required for the quantitative analysis
of very complex mixtures and of post-translational modifications, with the ultimate aim to generate quantitative proteomic data at a scale which would allow the comprehensive investigation of a biological phenomenon. At the same time, the recent exponential increase in data volume and complexity demands the development of appropriate bioinformatic and statistical approaches in order to arrive at meaningful interpretations of the results. This can only be achieved if the influence of the employed technologies on the results obtained is well understood.

6. References


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Ross, P. L., Huang, Y. N., Marchese, J. N., Williamson, B., Parker, K., Hattan, S., Khainovski, N., Pillai, S., Dey, S., Daniels, S., Purkayastha, S., Juhasz, P., Martin, S., Bartlet-


Proteomics was thought to be a natural extension after the field of genomics has deposited significant amount of data. However, simply taking a straight verbatim approach to catalog all proteins in all tissues of different organisms is not viable. Researchers may need to focus on the perspectives of proteomics that are essential to the functional outcome of the cells. In Integrative Proteomics, expert researchers contribute both historical perspectives, new developments in sample preparation, gel-based and non-gel-based protein separation and identification using mass spectrometry. Substantial chapters are describing studies of the sub-proteomes such as phosphoproteome or glycoproteomes which are directly related to functional outcomes of the cells. Structural proteomics related to pharmaceutics development is also a perspective of the essence. Bioinformatics tools that can mine proteomics data and lead to pathway analyses become an integral part of proteomics. Integrative proteomics covers both look-backs and look-outs of proteomics. It is an ideal reference for students, new researchers, and experienced scientists who want to get an overview or insights into new development of the proteomics field.

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