Chapter 17

Absolute Quantification of Proteins Using Standard Peptides and Multiple Reaction Monitoring

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Abstract

Mass spectrometry (MS) is a powerful tool for protein identification and has therefore become indispensable for proteome studies. In recent years, simple protein identification by MS has become routine, and more attention has been devoted to the MS-based investigation of posttranslational modifications and the quantification of proteins and peptides. Numerous methods and techniques for relative quantification of proteins by MS have emerged and have been applied successfully to answer various questions of protein abundance. Absolute quantification is often applied in clinical research and biomarker discovery, but has also been used to determine protein stoichiometries in protein complexes. However, the number of methods available for absolute quantification is still restricted and often requires the generation of standard peptides containing amino acids labeled with stable isotopes, although label-free approaches are also gaining importance. Complete hydrolysis of the proteins to be quantified is known to be one of the prerequisites for reliable absolute quantification, and selection and suitability of the standard peptides are critical factors in the planning of a quantitative study. Along the different methods to read out quantitative signals by MS, multiple reaction monitoring (MRM) has proven to be most suitable, with a wide linear range. However, analysis by MRM is a targeted approach and each case requires the individual design of suitable assays, which is a time-consuming step during the preliminary analysis. In this chapter, we present various protocols for in-solution hydrolysis, manual selection of suitable standard peptides, and design of MRM transitions.

Key words: Absolute quantification, Multiple reaction monitoring, Standard peptides, Protein hydrolysis

1. Introduction

1.1. Quantitative Mass Spectrometry

MS is not a quantitative method per se. To overcome this problem, incorporation of heavy stable isotopes, such as ^{13}C, ^{15}N, ^{18}O, and ^{2}D, can be used for comparison of relative amounts of peptides or proteins in different samples (for summary see Chapter 7). Artificial incorporation of heavy stable isotopes induces a mass shift of the

Katrin Marcus (ed.), *Quantitative Methods in Proteomics*, Methods in Molecular Biology, vol. 893,
DOI 10.1007/978-1-61779-885-6_17,

peptide's (or protein's) isotopic envelope in the mass spectrum. Importantly, the endogenous ("light") and the labeled ("heavy") peptide or protein shows the same behavior in the mass spectrometer, and the intensities of the peaks from the differently labeled peptides (or proteins) reflect their relative quantities.

Incorporation of stable isotopes is usually performed by chemical (see Chapters 8–11), metabolic (see Chapters 12–14, 25 and 26), or enzymatic labeling (see Chapter 15), or by providing standard peptides (see Chapter 18) or proteins (see Chapter 19) harboring amino acids that are labeled with stable heavy isotopes. Chemical labeling is performed at the peptide or protein level, whereas metabolic labeling takes place during cell growth and enzymatic labeling during protein hydrolysis. In contrast, standard peptides are synthesized chemically, by the incorporation of amino acids labeled with stable isotopes. In addition to methods employing stable isotopes, label-free approaches are gaining in importance for both relative (see Chapters 16 and 22) and absolute quantification (see Chapter 20).

In the last decade, numerous MS-based quantification techniques have emerged, and in proteomic research there is a clear trend towards MS-based quantification (1–4). However, most of the techniques developed so far are only capable of relative quantification, and not many methods are available for absolute quantification. Most of the latter approaches are based on the analysis of peptides in shotgun proteomic studies; in such studies, peptides are generated by hydrolysis of the proteins using a specific endoproteinase and, in most cases, they are then separated by reversed-phase liquid chromatography and analyzed directly in the mass spectrometer (LC-online ESI-MS/MS). The peptide masses are measured and suitable precursors are selected for fragmentation. The proteins are identified by comparing the masses of the peptides (precursors) and the corresponding fragments with the theoretical values in a database. Finally, quantification of the peptides (proteins) is performed by comparison of the MS peak intensities of the differently labeled peptides (e.g., in SILAC (5)) (see Chapters 13, 14, 25, and 26) or of the reporter ions generated in MS/MS [e.g., in iTRAQ (6) (see Chapter 8) or TMTs (7) (see Chapter 9)].

An alternative to shotgun proteomics is targeted proteomics, which uses prior information to generate MS-based assays for the detection and quantification of predetermined target peptides. Quantification of the target peptides is usually performed by spiking samples with stable-isotope-labeled standard peptides (see Chapter 18) and then comparing the intensities of the light (endogenous) and heavy (standard) peptides. Both strategies are based on the analysis of peptides generated from the proteins under investigation. The advantages and disadvantages of peptide-based proteome analyses have recently been discussed elsewhere (8).

Alternatively, although not widely used, absolute quantification of intact proteins by labeling standard proteins with stable isotopes has also been described (9).

1.2. Absolute Quantification by Mass Spectrometry

1.2.1. Absolute Quantification Using Standard Peptides

The use of standard peptides for absolute quantification was first described in 1983, where standard peptides were labeled enzymatically with deuterium (10). This approach was later named AQUA by Gygi and coworkers, whereby ^{13}C and/or ^{15}N-labeled amino acids were incorporated into standard peptides during chemical peptide synthesis (11, 12). Importantly, the heavy-labeled standard peptide and the endogenous peptide have identical physicochemical properties but can be distinguished in the mass spectrum by a defined mass shift. The peak intensities of the light (endogenous) and the heavy (standard) peptides thus reflect their relative amounts, and the addition of known quantities of standard peptides thus allows absolute quantification of the peptides and, ultimately, the proteins. Absolute quantification with the help of standard peptides is often applied to biomarkers in clinical studies (e.g., ref. 13). Furthermore, it has been used to measure the level of certain peptide modifications, such as phosphorylation (11) or ubiquitinylation (12).

As the procedure for absolute quantification is based on the analysis of peptides, it is highly recommended that several peptides be added for each protein, to increase confidence in the result. This can be simplified by using labeled standard proteins, which provide several standard peptides for absolute quantification after their hydrolysis. For this purpose, heavy-labeled amino acids are incorporated into entire proteins resulting in heavy-labeled standard proteins, which are then added to the sample under investigation. Cohydrolysis of the endogenous and the labeled standard proteins generates standard peptides for all generated peptides. Several approaches using labeled standard proteins have recently been introduced [PSAQ, Protein Standard Absolute Quantification (14); Absolute SILAC (15); FLEXIQuant, Full-Length EXpressed stable Isotope-labeled proteins for Quantification (16) (see Chapter 19)). Very similar is the use of artificial QconCAT proteins, which are assembled from different standard peptide sequences [concatenated signature peptides encoded by QconCAT genes (17) (see Chapter 18)]. During hydrolysis of QconCAT proteins, several standard peptides belonging to different proteins are generated, allowing the quantification of more than one protein in a sample.

To bypass the effort and the costs of labeling standard peptides with several isotopes for absolute quantification studies, other peptide-based approaches have recently been introduced. A first study has described the use of synthetic peptides that were chemically labeled with the so-called ICPL (isotope-coded protein label (18)) reagents. The labeled peptides were added to the hydrolyzed protein sample as internal standards, and quantification was performed by

using the signal intensities of the labeled and the nonlabeled peptides (19). Wepf et al. incorporated an additional amino-acid sequence into a common affinity tag, which served—after trypsin digestion—as a standard peptide. Addition of the isotope-labeled version of the peptide allowed absolute quantification of the tagged protein (20). A different approach involved the synthesis of isotope-labeled standard peptides including a so-called N-terminal equalizer peptide, which is an artificial tryptic peptide sequence. After trypsin digestion of the standard peptide mixture, the standard and equalizer peptides generated are chemically labeled with the heavy mTRAQ reagents. The standard peptide mixture is then "equalized," i.e., normalized to the absolute amount of the light labeled (mTRAQ) equalizer peptide, the concentration of which has been determined by amino-acid analysis. The equimolar mixture of standard peptides is used to spike a trypsin digest of the proteins to be analyzed (21). However, although different alternative peptide-based approaches are available, AQUA is the method most frequently used for the absolute quantification of proteins in different samples.

1.2.2. The AQUA Strategy

The AQUA strategy involves absolute quantification of proteins with the help of standard peptides using the following workflow: (a) definition of the target proteins, either by preliminary qualitative analysis of the sample to be analyzed or by other methods, (b) selection of suitable standard peptides, (c) chemical synthesis of the standard peptides by incorporation of heavy stable isotopes, (d) standardization of the sample with known amounts of the standard peptide(s), (e) analysis of the sample containing endogenous and standard peptides by MS (mostly by multiple reaction monitoring, MRM), and (f) data analysis (Fig. 1).

Absolute quantification of proteins using synthetic standard peptides requires prior information on the analytes. In contrast to relative quantification, the target proteins have to be defined before the quantification study. Selection of the target proteins is based either on previous experiments or on the literature (22). Selection of the standard peptides is often empirical (1), and is based upon previous analyses of the endogenous peptides generated from the proteins under investigation. There are several factors that have to be taken into account when one is selecting standard peptides; these include ionization efficiency, possible modification of amino-acid residues (e.g., oxidation of methionine), chromatographic elution, etc. A useful tool is a list of computationally predicted tryptic peptides that are frequently detected during MS studies (so-called proteotypic peptides) (23); this can help in the selection of peptide sequences for absolute quantification. In addition, several databases such as *PeptideAtlas* (24, 25) provide useful sources for MS targets. Selected peptide sequences are then synthesized chemically by incorporation of amino acids labeled with stable heavy isotopes (typically ^{13}C and/or ^{15}N). However, it must be pointed out that

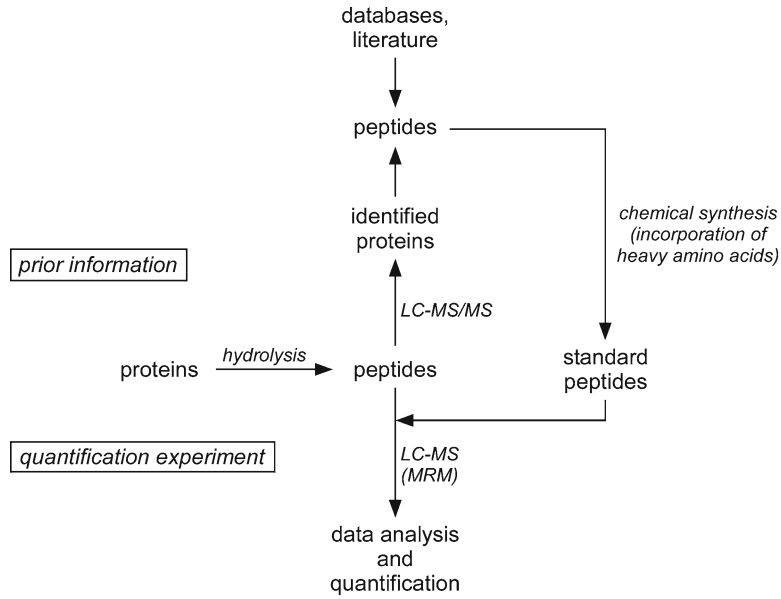

Fig. 1. AQUA workflow. The AQUA method is a targeted approach and, therefore, requires prior information on the target proteins. Prior information on the targets is achieved from hydrolysis of the proteins and subsequent LC-MS/MS analysis of the peptides generated. Standard peptides are selected from the analysis or, alternatively, from databases or the literature. After chemical synthesis of standard peptides labeled with stable isotopes, the sample is spiked with known amounts of standards and is analyzed by LC-MS (usually applying multiple reaction monitoring (MRM)). Quantification is performed by comparison of the signal intensities of the peptides and the corresponding standard peptides.

the protein concentration to be determined relies on accurate determination of the standard peptide concentrations. The most accurate procedure is amino-acid analysis (26), which should be chosen when the concentrations of standard peptides are to be determined.

The next step in an absolute quantification study involves the standardization of the sample under investigation with the stable-isotope-labeled standard peptides. The sample is spiked with standard peptides either before or after trypsin hydrolysis. As the amount of proteins is not accurately known before absolute quantification, the operator has to estimate the amount of standard peptides to spike the sample with. Ideally, the amounts of standard peptides added are derived from preliminary experiments. The recommended amount, as a starting point, is 10–50 fmol per peptide (22). The spiked sample can in principle be analyzed by any MS method, but owing to several limitations of shotgun MS analysis (e.g., coelution of peptides), targeted MS using selected or multiple reaction monitoring (SRM/MRM) is most often used for absolute quantification, calling for the design of an SRM/MRM assay in each situation. (However, various studies with MALDI-TOF-MS (19) and LC-ESI-MS (27) have been published showing

that quantitative information can be obtained when these experimental setups are used.) The final step is the data analysis, which is usually performed by using appropriate computer software, but can also be performed manually for samples of low complexity.

1.2.3. Label-Free Absolute Quantification

In addition to use of standard peptides, several label-free approaches for absolute quantification have recently been described. One of the first label-free methods for approximate absolute quantification was introduced by Ishihama et al. (28). The exponentially modified Protein Abundance Index (emPAI) is calculated from the experimentally observed and the theoretically possible numbers of peptides of a protein in a mixture after trypsin digestion and subsequent MS analysis. A similar approach is Absolute Protein EXpression profiling (APEX), which is also based upon the observed and expected numbers of peptides (29) (see Chapter 20); the authors who described this method introduced correction by the background expectation, the total sampling depth and the confidence in protein identification (29). Another widely used nondirected LC-MS/MS method for the determination of protein abundance has been introduced by Silva et al. (30). This method (called top3) is based on the relationship between the average MS signal response for the three most intense tryptic peptides on the one hand and the protein concentration on the other. The sample under investigation is spiked with a known amount of standard protein before tryptic hydrolysis, and the average signal response for the three most intense tryptic protein is used to calculate a universal signal response factor (count/mole of protein). The universal response factor is then used to calculate the concentration of the other proteins in the sample.

1.3. MRM for Absolute Quantification

MRM is used mainly in targeted proteomics, i.e., prior information about the sample is used, and only target peptides (or proteins) are analyzed. The use of triple-quadrupole mass analyzers allows detection of a target-specific reaction of a precursor ion in the mass spectrometer to give a user-defined fragment ion (MRM transition). For this, the precursor mass is selected in quadrupole 1 and, after fragmentation in quadrupole 2, the specific fragment ion is detected in quadrupole 3. MRM allows for detection of several fragment ions per precursor. Absolute quantification can be performed by spiking the sample with known amounts of stable-isotope-labeled standard peptides and comparing the signal intensities from MRM transitions of the endogenous (light) and the standard (heavy) peptides.

In MRM, the required prior information on the proteins under investigation is used to set up sensitive MRM assays. First, MRM transitions for the target peptides need to be chosen. The m/z value of Q1 is defined by the mass and the charge state of the endogenous and the standard peptide, respectively. The precursor-specific fragment ion of the MRM transition (m/z value of Q3) should be the most intense fragment ion, to ensure high sensitivity

of the assay. This might be based on data from shotgun experiments; ideally, as the fragment-ion intensities are dependent on the instrument type, they are determined experimentally on same the triple quadrupole mass spectrometer as will be used for the determination. The total number of transitions per LC run is limited by the number of target peptides and, therefore, only a few of the best transitions (typically 2–4) should be chosen. However, the total number of transitions can be increased by scheduled MRM, whereupon peptide transitions are only analyzed during a time window around its elution time (31). When one is using heavy-labeled standard peptides for absolute quantification, the MRM transitions of the corresponding peptides can be calculated from the known mass difference between the endogenous and the standard peptides.

Once the transitions of the MRM assay have been selected, various parameters can be optimized to maximize sensitivity. Together with the total number of transitions, the dwell time on each transition defines the cycle time, i.e., the time that is needed to acquire one intensity value for each transition. The dwell time needs to be long enough to obtain sufficient signal, but conversely the cycle time needs to be as short as possible to obtain enough data points per peptide. For complex samples, which require a large number of transitions, the dwell time can be adjusted to the abundance of the target peptides in the sample. Shorter dwell times are sufficient for highly abundant peptides, whereas longer dwell times can be applied for low-abundance peptides. Another parameter that influences the signal intensity of the transitions is the collision energy. This parameter (and also others) can be optimized for every specific transition by ramping the value and selecting the one that yields the highest signal intensity. As the standard and the endogenous peptides have the same physicochemical properties, all optimized parameters can be adopted.

A good check on the specificity of the designed MRM assay is the analysis of the same sample but with no standard peptides added or analysis of the mixture of standard peptides alone. In both cases, only signal intensities for the endogenous or the standard peptides, respectively, should be detected. In addition, signal intensities for transitions of the same peptide have to be determined at the same time and only once per LC run.

For absolute quantification of the target proteins/peptides, the sample is then spiked with known amounts of standard peptides and the designed MRM assay (including transitions for endogenous and standard peptides) is conducted (Fig. 2). The target peptides are then quantified by comparing the signal intensities of the endogenous and the standard peptides (for an example see Fig. 3) and absolute amounts of the endogenous peptides can be calculated. Data analysis can be performed manually or using commercial software, which are given for all available triple quadrupole instruments.

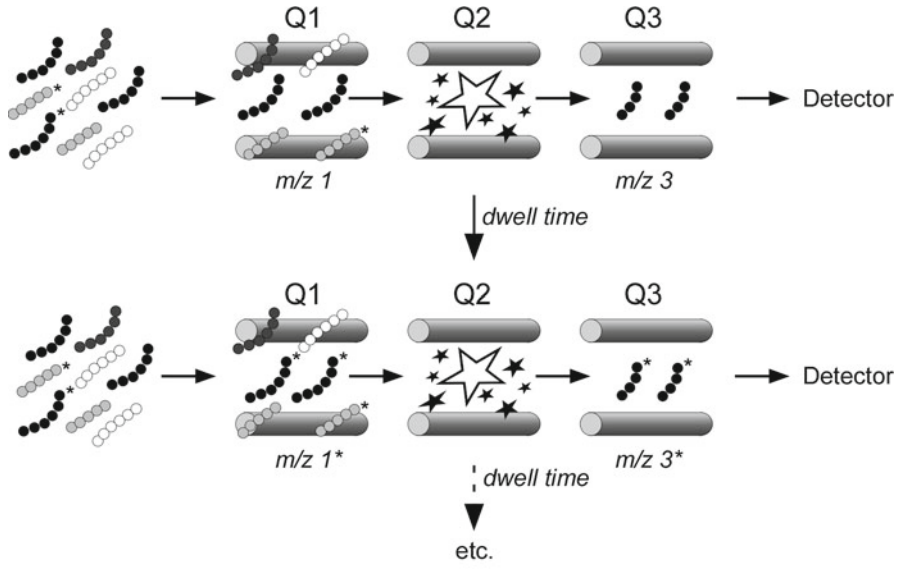

Fig. 2. Multiple reaction monitoring (MRM) analysis of endogenous and standard peptides. The sample containing endogenous (*unlabelled*) and standard (*labeled with asterisk*) peptides is analyzed by MRM on a triple quadrupole mass spectrometer. In the first quadrupole (Q1) the *m*/*z* of the unlabelled precursor (*m/z 1*) is selected and, after fragmentation in Q2, a specific fragment ion of the precursor (*m/z 3*) is selected in Q3 and passes on to the detector (*upper panel*). After a defined time period (dwell time), the next MRM transition is monitored, with the selection parameters altered so that the MRM transition of the corresponding standard peptide (*m/z 1**) to its corresponding fragment (*m/z 3**) is observed (*lower panel*). The duration of the duty cycle (i.e., monitoring all MRM transitions) is thus defined by the number of transitions to be monitored and the dwell time for each transition.

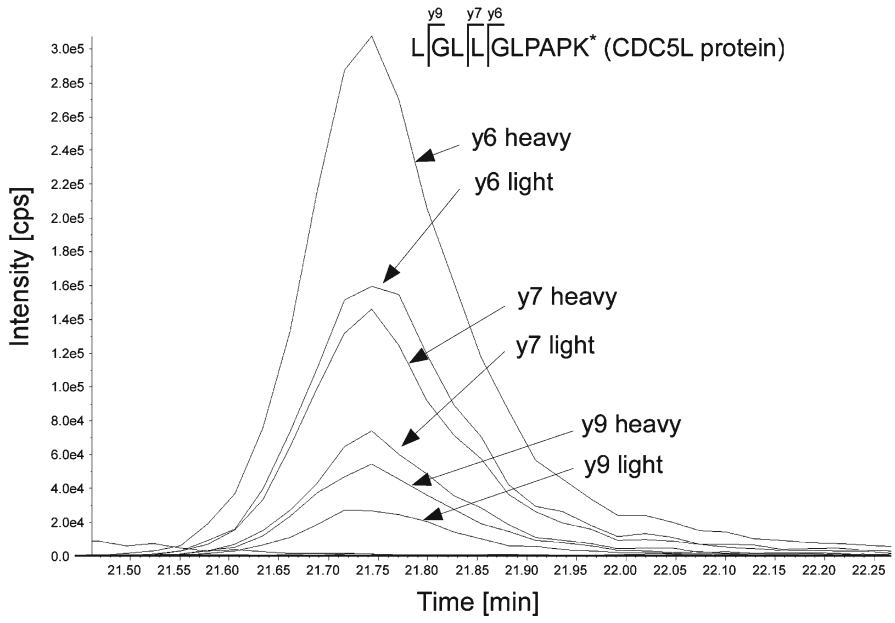

Fig. 3. Multiple reaction monitoring (MRM) signals from LGLLGLPAPK and the corresponding standard peptide. Three MRM transitions were monitored for the endogenous and the standard peptides. As they have the same physicochemical properties, the peptides coelute during LC. Fragment ions y6, y7, and y9 were chosen as *m*/*z* values detected in Q3. Note that the signal intensity ratio of the peptide pair (heavy/light) is the same for the different MRM transitions.

2. Materials

2.1. In-Solution Hydrolysis of Proteins

2.1.1. Ethanol Precipitation

1. Absolute ethanol, stored at –20°C.
2. 80% (v/v) ethanol, stored at –20°C.
3. 3 M sodium acetate (NaOAc), pH 5.3, stored at room temperature (RT).

2.1.2. In-Solution Hydrolysis in the Presence of Urea

1. 25 mM Tris–HCl, pH 7.9.
2. 8 M urea (dissolved in 25 mM Tris–HCl, pH 7.9).
3. 10 mM dithiothreitol (DTT, dissolved in 8 M urea).
4. 60 mM iodoacetamide (IAA dissolved in 8 M urea).
5. 100 mM ammonium bicarbonate (ABC), pH 7.9.
6. Lys-C (Roche Diagnostics GmbH, Mannheim, Germany), stored at –20°C.
7. Trypsin (Promega Corp., Madison, WI), stored at –20°C.
8. See also Notes 1 and 2.

2.1.3. In-Solution Hydrolysis in the Presence of Acetonitrile

1. 100 mM ABC, pH 7.9.
2. 80% (v/v) acetonitrile (ACN, diluted with 100 mM ABC).
3. 10 mM DTT (dissolved in 80% (v/v) ACN).
4. 60 mM IAA (dissolved in 80% (v/v) ACN).
5. Lys-C (Roche Diagnostics GmbH), stored at –20°C.
6. Trypsin (Promega Corp.), stored at –20°C.
7. See also Notes 1 and 2.

2.1.4. In-Solution Hydrolysis Using RapiGest

1. 25 mM ABC, pH 7.9.
2. 1% (m/v) RapiGest SF surfactant (Waters Corp., Manchester, UK), stored at –20°C.
3. 50 mM DTT (dissolved in 25 mM ABC).
4. 25 mM IAA (dissolved in 25 mM ABC).
5. Trypsin (Promega Corp.), stored at –20°C.
6. 5% (v/v) trifluoric acid (TFA).
7. See also Notes 1 and 2.

2.2. Dissolving Standard Peptides

1. Dimethylformamide (DMF).
2. 100% (v/v) ACN.
3. 20% (v/v) ACN/0.1% (v/v) FA.

2.3. Selection and Optimization of MRM Transitions

1. 40% (v/v) ACN/0.1% (v/v) FA.
2. See also Note 3.

2.4. Internal Standardization with the Selected Standard Peptides

1. 2% (v/v) ACN/0.5% (v/v) FA.
2. See also Note 4.

2.5. Absolute Quantification by MRM

1. 2% (v/v) ACN/0.5% (v/v) FA (loading buffer).
2. 2% (v/v) ACN/0.1% (v/v) FA (mobile phase A).
3. 98% (v/v) ACN/0.1% (v/v) FA (mobile phase B).
4. See also Note 5.

3. Methods

3.1. In-Solution Hydrolysis of Proteins

Complete hydrolysis of the proteins to be determined is the major prerequisite for absolute quantification when using standard peptides. In our previous studies, we found that denaturing conditions during in-solution hydrolysis are crucial for reliable absolute quantification (27). For this reason, it is highly recommended to test (and eventually optimize) different hydrolysis protocols to achieve complete hydrolysis of the proteins. As there is no effective method to check whether a protein is hydrolyzed completely, the analysis of the number of identified peptides, the sequence coverage and the number of missed cleavages of the identified peptides after LC-MS/MS analysis can give information about the quality of the protein hydrolysis.

Below we present three different hydrolysis protocols that are frequently used in our laboratory. As tryptic peptides are well suited for MS analysis and tryptic hydrolysis is most frequently applied in proteome studies, the hydrolysis protocols presented here involve only tryptic hydrolysis and, when indicated, Lys-C prehydrolysis. To remove buffers from protein purification and to concentrate the sample, we usually perform ethanol precipitation before protein hydrolysis.

3.1.1. Ethanol Precipitation

1. Precipitate the proteins with 3 volumes ice-cold 100% (v/v) ethanol and 1/10 volume 3 M NaOAc, pH 5.3.
2. Incubate for at least 2 h at –20°C.
3. Spin down for 30 min, 16,200 ×*g* 4°C.
4. Remove the supernatant and wash the protein pellet with ice-cold 80% (v/v) ethanol.
5. Spin down for 30 min, 16,200 ×*g* 4°C.
6. Remove the supernatant and dry the protein pellet in a vacuum centrifuge.

3.1.2. In-Solution Hydrolysis in the Presence of Urea

1. Dissolve the protein pellet in 20 µl 8 M urea; incubate at RT for approx. 30 min.
2. Add 20 µl 10 mM DTT and incubate for 30 min in a thermomixer at RT.
3. Add 20 µl 60 mM IAA and incubate for 30 min in a thermomixer at RT.
4. Add Lys-C (1:10 enzyme–protein) and incubate for 3 h in a thermomixer at RT.
5. Dilute the sample to 2 M urea by addition of 180 µl 100 mM ABC, pH 7.9.
6. Add trypsin (1:5 enzyme:protein) and continue hydrolysis overnight at RT.
7. Store the tryptic peptides for future analysis at –20°C.
8. See also Note 6.

3.1.3. In-Solution Hydrolysis in the Presence of Acetonitrile

1. Dissolve the protein pellet in 50 µl 80% (v/v) ACN; incubate at RT for approx. 30 min.
2. Add 50 µl 10 mM DTT and incubate for 30 min in a thermomixer at RT.
3. Add 50 µl 60 mM IAA and incubate for 30 min in a thermomixer at RT.
4. Add Lys-C (1:10 enzyme:protein) and incubate for 3 h in a thermomixer at RT.
5. Add trypsin (1:5 enzyme:protein) and continue hydrolysis overnight at RT.
6. Dry the tryptic peptides in a vacuum centrifuge.
7. Store the sample for future analysis at –20°C.

3.1.4. In-Solution Hydrolysis Using RapiGest

1. Dissolve the protein pellet in 10 µl 1% (m/v) RapiGest; incubate at RT for approx. 30 min.
2. Add 10 µl 50 mM DTT and incubate for 1 h in a thermomixer at 37°C.
3. Add 10 µl IAA and incubate for 1 h in a thermomixer at 37°C.
4. Add 70 µl trypsin solution (diluted with 25 mM ABC, 1:20 enzyme: protein) and incubate overnight in a thermomixer at 37°C.
5. Add 20 µl 5% (v/v) TFA and incubate for 2 h in a thermomixer at 37°C.
6. Centrifuge the sample for 30 min, 16,200 ×*g*.
7. Discard the pellet and transfer the supernatant to a new tube.

8. Dry the tryptic peptides in a vacuum centrifuge.
9. Store the sample for future analysis at –20°C.

3.2. Selection of Standard Peptides

1. Analyze the hydrolyzed proteins (tryptic peptides) qualitatively by LC-MS/MS to detect suitable standard peptides.
2. Search the MS and MS/MS spectra against a database to identify suitable peptide sequences.
3. Select standard peptides from qualitative analysis according to the following selection criteria (see also Note 7):
   (a) The peptide should resolve well by HPLC.
   (b) The peptide should not be too hydrophobic. (A peptide is classed as hydrophobic if more than 50% of its amino acids are Ile, Leu, Val, Phe, Trp, and Met.)
   (c) The peptide should not be too hydrophilic.
   (d) The peptide should ionize well to ensure detection in the mass spectrometer.
   (e) The peptide should not contain chemically reactive amino acids (Cys, Met, and Trp).
   (f) The peptide should not contain chemically unstable sequences (N-terminal Asn, N-terminal Gln, and Asp-Gly).
   (g) The peptide's length should be limited to 15 amino acids.
   (h) The peptide should contain amino acids well suited to labeling with stable isotopes.
   (i) If possible, the peptides should be proteotypic (see Note 8).
4. Check the sequence of the protein to be analyzed for additional tryptic cleavages sites near/next to the selected peptide sequences. Avoid peptide sequences with additional cleavage sites. See also Note 9.
5. See also Notes 10–12.

3.3. Dissolving/ Handling Standard Peptides

1. Standard peptides are delivered as lyophilized peptides (Sigma Genosys) or dissolved in 5% (v/v) ACN (Thermo Fisher Scientific).
2. Dissolve one nanomole of lyophilized peptide in 20 µl DMF (see Note 13). To ensure complete dissolution vortex and sonicate extensively. Dilute the dissolved peptides with 180 µl of 20% (v/v) ACN/0.1% (v/v) FA to give a concentration of 5 pmol/µl. Store aliquots at –20°C.
3. Dissolved peptides are delivered in 5% (v/v) ACN at a concentration of 5 pmol/µl. Store the entire vial at –20°C or prepare smaller aliquots and store at –20°C (see Note 14).
4. See also Note 15.

3.4. Design of MRM Transitions/MRM Assays

In our studies, we quantified peptides/proteins absolutely on a 4000 QTRAP hybrid Triple Quadrupole/Linear Ion Trap Mass Spectrometer (ABSciex). The following section, therefore, refers to this specific instrument. In general, MRM analyses can be performed on any triple quadrupole instrument. The details for parameter optimization and MRM assay setup might then differ.

1. To design MRM transitions, each standard peptide is analyzed by nanoSpray direct-infusion MS.
2. Dilute each standard peptide with 40% (v/v) ACN/0.1% (v/v) FA to a final concentration of 200 fmol/μl.
3. Analyze peptides by enhanced resolution (ER) single MS and enhanced product ion (EPI) MS/MS scans to establish molecular weight and fragmentation pattern.
4. Choose the monoisotopic m/z of the doubly or triply charged precursor as Q1 mass.
5. Choose the three or four most abundant fragments with an m/z above that of the precursor.
6. Optimize the declustering potential (DP), the entrance potential (EP), the collision energy (CE), and the collision cell exit potential (CXP) by ramping the parameters and choosing the values displaying the highest signal intensity.
7. Calculate the MRM transitions for the corresponding endogenous peptides using the expected mass differential from the standard peptides.
8. Test the optimized MRM assay for interfering signals by analyzing the standard peptides and the endogenous peptides (i.e., the hydrolyzed sample) separately (Note 16).
9. See also Note 17.

3.5. Internal Standardization and Absolute Quantification by MRM

1. Dilute the dissolved (Sigma Genosys) and redissolved (Thermo Fisher Scientific) peptides twofold with 2% (v/v) ACN/0.5% (v/v) FA or the loading buffer used during LC-MS/MS (resulting concentration 2.5 pmol/μl).
2. Prepare a peptide mixture containing all standard peptides at the desired concentration(s) (e.g., 100 fmol/μl each) in loading buffer (e.g., 2% (v/v) ACN/0.5% (v/v) FA).
3. Spike the hydrolyzed sample with standard peptides at the desired concentrations.
4. Prepare dilutions of the hydrolyzed sample and the peptide mixture. Spike different concentrations of sample with constant amounts of standard peptides and vice versa.
5. Analyze the sample containing endogenous and standard peptides by targeted LC-MS/MS and monitor the MRM transitions for endogenous and standard peptides.

3.6. Data Analysis

1. Extract the peak area of each MRM transition and calculate the ratios of the MRM transitions for the endogenous and the corresponding standard peptides.
2. Calculate the amount of peptide from the ratios obtained, by using the known amount of standard peptides.
3. See also Note 18.

4. Notes

1. Buffers for in-solution hydrolysis should be prepared fresh and filtered before use.
2. Lys-C and trypsin should be reconstituted according to manufacturer's protocols and stored at –20°C.
3. For analysis of the standard peptides by direct-infusion MS, a buffer containing a high percentage of ACN should be used.
4. For dilution of the standard peptides to the final peptide concentration, the loading buffer used in the laboratory's individual LC-MS/MS setup should be used.
5. All buffers can be replaced by the buffers for the laboratory's individual LC-MS/MS setup.
6. As the use of urea always entails the risk of carbamylation of the peptides/proteins, all incubation steps should be performed at RT to minimize the degree of carbamylation.
7. Selection criteria and additional information about peptide synthesis (e.g., availability of stable isotope labeled amino acids, concentration, etc.) are available on the manufacturer's homepage. There are two main suppliers: (1) Sigma Genosys (www.sigma.com/aqua) and (2) Thermo Fisher Scientific (www.thermo.com/heavypeptide).
8. A peptide is assigned to be proteotypic if it is repeatedly detected during LC-MS/MS analyses. For further information, see ref. 23.
9. To circumvent problems during absolute quantification, it is highly recommended to check carefully the sequence of the protein to be analyzed for additional tryptic cleavage sites near or next to the selected peptide sequences. Additional cleavage sites near the selected peptide sequences increase the possibility of generating peptides with missed cleavage sites during hydrolysis and thus altering the peptide's concentration after protein hydrolysis.
10. To guarantee reliable quantification of the proteins of interest it is recommended that more than one standard peptide be selected for absolute quantification. A reasonable number of

standard peptides per protein is three, while two standard peptides can also deliver sufficient information.

11. To ensure accurate quantification, it is highly recommended that standard peptides be used, with accurately known concentration, preferably as determined by amino-acid analysis.
12. Suitable standard peptides for absolute quantification can be selected manually from quantitative analysis of the proteins to be analyzed (see above) or can be selected automatically by utilizing the relevant computer software. In the latter case, data from previous analyses is provided for selection of standard peptides or suitable peptide sequences are predicted from hydrolysis in silico of the target proteins. Furthermore, information about suitable target peptides can be obtained from spectral libraries such as PeptideAtlas (www.peptideatlas.org). Available software packages for MRM assay setup and optimization including target peptide selection are, for example, MRMPilot (QTRAP systems, ABSciex) and the Pinpoint software (TSQ instruments, Thermo Fisher Scientific).
13. The lyophilized peptides can be dissolved in various solvents. In our experience, DMF has proven to be well suited for the complete dissolution of lyophilized peptides. Other recommended solvents are the following: (1) for hydrophobic peptides, dimethyl sulfoxide (DMSO), ACN, or acetic acid, (2) for hydrophilic peptides, 10% (v/v) aqueous FA.
14. When storing relatively small aliquots of the peptides in 5% (v/v) ACN, we found that the peptides might not be reconstituted completely after thawing (27). In such cases, the peptides were dried in a vacuum centrifuge, redissolved in ACN, and diluted to the final concentration with the loading buffer used during LC-MS/MS.
15. Avoid repeated freeze–thaw cycles. One possibility to check if the peptide is completely redissolved after storage at −20°C is analysis by nanoLC. If the peptides do not reveal a sufficient signal in nanoLC, dry them in a vacuum centrifuge and redissolve them in 100% (v/v) ACN. Compare the signal intensities in nanoLC before and after redissolving (see also Note 13). Other possible solvents for redissolving the peptides are: DMF, 10% (v/v) FA or 100% (v/v) FA.
16. MRM transitions should be well separated and should show sufficient intensity. Analysis of the endogenous peptides should show no MRM transitions of the heavy standard peptides and vice versa.
17. In case where samples are complex, automated assay design can be very advantageous, as selection and optimization of MRM transitions is very time-consuming. Most software packages that are available for automated target peptide selection

(see Note 12) also include MRM assay setup and optimization. In addition, spectral libraries containing targeted proteomics assays can be consulted (e.g., MRM Atlas; www.mrmatlas.org). Parameters to be optimized are computed but can also be varied by the user.

18. Peak integration of MRM signals and data evaluation is at the best performed automatically. Different software for MRM data analysis are available (e.g., MultiQuant, ABSciex) and described software packages for MRM assay design (see Notes 12 and 17) are also applicable for analysis of MRM transitions and absolute quantification.

Acknowledgments

We thank Christof Lenz for help with MRM assay setup and Johanna Lehne, Uwe Plessmann, and Monika Raabe for excellent technical assistance and maintenance of the instruments.

References

1. Bantscheff M, Schirle M, Sweetman G et al (2007) Quantitative mass spectrometry in proteomics: a critical review. Anal Bioanal Chem 389(4):1017–1031
2. Ong SE, Foster LJ, Mann M (2003) Mass spectrometric-based approaches in quantitative proteomics. Methods 29(2):124–130
3. Ong SE, Mann M (2005) Mass spectrometry-based proteomics turns quantitative. Nat Chem Biol 1(5):252–262
4. Wilm M (2009) Quantitative proteomics in biological research. Proteomics 9(20): 4590–4605
5. 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):376–386
6. 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):1154–1169
7. 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): 1895–1904
8. Duncan MW, Aebersold R, Caprioli RM (2010) The pros and cons of peptide-centric proteomics. Nat Biotechnol 28(7):659–664
9. Waanders LF, Hanke S, Mann M (2007) Top-down quantitation and characterization of SILAC-labeled proteins. J Am Soc Mass Spectrom 18(11):2058–2064
10. 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):471–479
11. 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): 6940–6945
12. 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):265–273
13. Pan S, Zhang H, Rush J et al (2005) High throughput proteome screening for biomarker detection. Mol Cell Proteomics 4(2):182–190
14. Brun V, Dupuis A, Adrait A et al (2007) Isotope-labeled protein standards: toward absolute quantitative proteomics. Mol Cell Proteomics 6(12):2139–2149
15. 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):1118–1130

16. 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):2201–2210
17. 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):1029–1043
18. Schmidt A, Kellermann J, Lottspeich F (2005) A novel strategy for quantitative proteomics using isotope-coded protein labels. Proteomics 5(1):4–15
19. Hochleitner EO, Kastner B, Frohlich T et al (2005) Protein stoichiometry of a multiprotein complex, the human spliceosomal U1 small nuclear ribonucleoprotein: absolute quantification using isotope-coded tags and mass spectrometry. J Biol Chem 280(4): 2536–2542
20. Wepf A, Glatter T, Schmidt A et al (2009) Quantitative interaction proteomics using mass spectrometry. Nat Methods 6(3):203–205
21. Holzmann J, Pichler P, Madalinski M et al (2009) Stoichiometry determination of the MP1-p14 complex using a novel and cost-efficient method to produce an equimolar mixture of standard peptides. Anal Chem 81(24): 10254–10261
22. Lange V, Picotti P, Domon B et al (2008) Selected reaction monitoring for quantitative proteomics: a tutorial. Mol Syst Biol 4:222
23. Mallick P, Schirle M, Chen SS et al (2007) Computational prediction of proteotypic peptides for quantitative proteomics. Nat Biotechnol 25(1):125–131
24. Desiere F, Deutsch EW, King NL et al (2006) The PeptideAtlas project. Nucleic Acids Res 34(Database Issue):D655–D658
25. Deutsch EW, Lam H, Aebersold R (2008) PeptideAtlas: a resource for target selection for emerging targeted proteomics workflows. EMBO Rep 9(5):429–434
26. Macchi FD, Shen FJ, Keck RG et al (2000) Amino acid analysis, using postcolumn ninhydrin detection, in a biotechnology laboratory. Methods Mol Biol 159:9–30
27. 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):2784–2796
28. 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):1265–1272
29. 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): 117–124
30. 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):144–156
31. 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):1809–1817