Chapter 12

iTRAQ-Labeling of In-Gel Digested Proteins for Relative Quantification

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Summary

In addition to standard MS-based protein identification, quantification of proteins by mass spectrometry (MS) is rapidly gaining acceptance in proteomic studies. MS-based quantification involves either the incorporation of stable isotopes or can be performed label-free. Recently, more attention has been devoted to label-free quantification; however, this approach has not been fully established among the proteomic community yet. More common is still the introduction of stable isotopes, which can be done by metabolic (e.g., SILAC) or by chemical (e.g., ICAT, iTRAQ, etc.) labeling. Here, we present an overall quantification strategy for chemical labeling of in-gel digested proteins using iTRAQ reagents. This includes (1) protein separation by gel electrophoresis, (2) excision of protein bands, (3) in-gel digestion and extraction of peptides, (4) labeling of peptides, (5) pooling the samples to be compared, (6) LC-MS/MS of labeled peptides, and (7) database search. The presented workflow is well suited for protein samples of moderate complexity (i.e., protein samples of 300–400 components), and it is exemplified by using different amounts of 25S [U4/U6.U5] tri-snRNPs.

Key words: PAGE, iTRAQ, Mass spectrometry (MS), Liquid chromatography (LC)

1. Introduction

A typical proteomics workflow comprises protein separation using polyacrylamide-based gel electrophoresis or liquid chromatography (LC), digestion of proteins, and finally identification by mass spectrometry (MS) and database search. This workflow is widely accepted in the diverse field of proteomic research. In addition, much attention is currently being devoted to the relative quantitative comparison of different samples by chemical or metabolic labeling of samples and subsequent analysis of the labeled species in the mass spectrometer. By combining the protein identification

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with quantitative analysis, reliable information about the abundance of a certain protein component in, e.g., different functional stages of a cell or in different cellular complexes are obtained.

Nowadays, many and diverse techniques are available for separation of proteins, and for their relative quantification by MS *(1)*. However, the choice of application depends on the biological/biochemical problem to be solved, the cost of the analysis, the time it is expected to require, and, last but not the least, on the individual experimental setup for proteomics in the laboratory concerned.

In this chapter, we describe in detail the proteomic workflow generally followed in our laboratory. It comprises sample separation by 1D gel electrophoresis, digestion of the proteins within the gel, chemical labeling of the extracted peptides by isotope-labeled reagents, and the ESI and MALDI techniques for MS analysis of the labeled peptides with a view to protein identification and subsequent quantification.

The protocols listed in this chapter should prove useful for the analysis of protein samples of moderate complexity, e.g., isolated protein complexes, or purified cellular compartments with a protein composition that does not exceed 300–400 components.

The approach is illustrated by the in-gel-digestion of different amounts of 25S [U4/U6.U5] tri-snRNPs *(2, 3)*, labeling of the extracted peptides with iTRAQ reagents *(4)*, and analysis of the differentially labeled peptides for quantification of the corresponding proteins by LC-coupled tandem MS (LC-MS/MS).

1.1. Protein Separation – General Remarks

The final step in a proteomics workflow is the identification of proteins by mass-spectrometric sequencing of the peptides derived from hydrolysis of the proteins in the sample to be analyzed. The more proteins are present in a given sample, the more peptides are generated by hydrolysis. However, even state-of-the-art Mass Spectrometers can only analyze a limited number of peptides at a given time (see below). Therefore, apart from the strategic design of proteome experiments (analysis of entire cells, cellular compartments, isolated protein complexes), sample preparation and protein/peptide separation is one of the major prerequisites for a successful proteome analysis using MS. During this initial step, the complexity of the sample is reduced, which in turn leads to the acquisition of more and better-evaluable data in the MS analysis.

There are two well-established general strategies for reducing the complexity of a protein sample in preparation for MS: (1) Hydrolysis of the protein sample in situ, followed by separation of peptides by one- or multidimensional LC and subsequent elution of peptides via reversed-phase chromatography (RP-LC) with organic solvents into the tandem mass spectrometer (RP-LC-MS/MS) and (2) separation of proteins by gel electrophoresis

(e.g., 1D *[5]*, 2D *[6, 7]*, Blue Native *[8, 9]*, 16BAC gel electrophoresis *[10, 11]*), followed by hydrolysis of proteins within the gel, and subsequent elution of peptides from the gel through a RP-LC column with organic solvents into the tandem mass spectrometer (LC-MS/MS).

The first approach has the advantage that samples are hydrolyzed into peptides *in situ*, which bypasses the (more time-consuming) protein-separation step. Owing to the great complexity of the peptide mixture thus generated, a further peptide-separation step is recommended. For this purpose, it is widely accepted that two-dimensional liquid chromatography – using strong cation-exchange (SCX) chromatography in the first dimension and RP-LC in the second dimension – is the best option. The two LC steps can be coupled directly to each other (online), or they can be separated from each other (offline). The former configuration has been successfully introduced in multidimensional protein identification technology (MuD-PIT), where the generated peptides are loaded onto a biphasic microcapillary column packed with SCX and RP material. The eluted peptides are detected and identified on a tandem mass spectrometer *(12)*. Although highly sensitive and extremely fast, such a workflow requires much experience in handling the samples, the multidimensional LC system and the mass spectrometer. Very recently, an interesting alternative approach has been described, which uses isoelectric focusing (IEF) on IPG strips to separate complex peptide mixtures. The IPG strips are cut into pieces, and peptides are eluted from them and subsequently analyzed by RP-LC-coupled MS *(13)*.

Separation of the protein mixtures by gel electrophoresis before digestion and LC-MS/MS has two advantages: (1) proteins are separated according to their size (using one-dimensional gel electrophoresis) and charge (two-dimensional gel electrophoresis) and (2) they can be visualized by staining. Both of these features make the subsequent MS analysis more reliable by allowing a rough check of molecular weights inferred from the proteins' migration behavior in gel electrophoresis.

The combination of IEF and sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE) is currently the most common technique in proteomic analysis, with a resolution of up to 10,000 protein spots per gel *(14)*. IEF separates proteins according to their isoelectric point. Most often, IEF is performed with immobilized pH gradients *(15)*. Alternatively, carrier ampholytes are used to establish the pH gradient during the IEF process *(6, 16)*. In the second dimension, the focused proteins are separated by SDS-PAGE (see below) according to their apparent molecular weight. A drawback of 2D PAGE is the fact that it is less suitable for the separation of hydrophobic proteins, such as integral membrane proteins. Hydrophobic and membrane proteins exhibit incomplete solubility in the buffers

used for 2D PAGE. Moreover, such proteins can precipitate, resulting in less efficient transfer of material from the first to the second dimension.

1.1.1. 1D-page

When working in experimental systems that investigate protein samples with moderate complexity (e.g., purified cell compartments, purified protein complexes, or interaction between proteins– the so-called subcellular proteomics), a simpler alternative for 2D gel-based analysis of proteins is protein separation by 1D PAGE. The most common technique for 1D PAGE is the Laemmli system *(5)* that contains SDS as detergent and Tris-glycine as buffer. Although widely used, it has some drawbacks. The highly alkaline pH of the Laemmli system may cause band distortion, loss of resolution, or artifact bands. Laemmli-gels exhibit a short shelf life of 4–6 weeks that is limited by hydrolysis of polyacrylamide at the high gel pH. In addition, chemical modification of proteins, such as deamidation and alkylation, can occur. Since the redox state of the Laemmli-gels is not constant, reoxidation of reduced disulfides of cysteine-containing proteins is possible, and because of the need to heat the samples at 100°C in Laemmli sample buffer, cleavage of Asp–Pro bonds has been observed *(17)*.

Alternatively, pre-cast 1D polyacrylamide gels (e.g., the NuPAGE® system by Invitrogen, Carlsbad, USA) can be used for protein separation prior to MS analysis. NuPAGE® pre-cast gels have several advantages over Laemmli SDS gels *(18)*: (1) the neutral operating pH of pre-cast gels provides a longer shelf life of the gels (8–12 months) and improves protein stability during electrophoresis, resulting in sharper band resolution. (2) Manufacturers guarantee complete reduction of disulfides, and no cleavage of Asp–Pro bonds is observed. (3) Importantly, pre-cast gels are small in size and yield highly reproducible results. The commercially available polyacrylamide-gradient gels offer a separation range (i.e., from 1 to 120 kDa, or from 36 to 400 kDa) that is difficult to achieve with in-house-prepared polyacrylamide gels. (4) Separation of proteins, staining, and destaining can be achieved within a couple of hours. (5) The small dimensions and high resolution of the gels make them ideal for cutting the entire lanes into pieces of exactly the same size for comprehensive analysis and/or relative quantification of samples in two (or more) different gel lanes.

Pre-cast gels are compatible with silver-staining protocols *(19)*, with all the standard Coomassie staining procedures and with alternative staining methods such as copper or zinc and fluorescence staining (all reviewed, e.g., by Westermeier & Marouga *[20]*).

Staining is generally recommended for visualizing protein bands of interest, which can then be excised and hydrolyzed within the gel for MS analysis (see below). Alternatively, entire lanes can be cut into pieces and the proteins hydrolyzed. However,

when this approach is used, staining is not necessary, or it should be restricted to Coomassie staining to guarantee a maximum yield of extracted peptides after in-gel digestion.

1.2. Protein Quantification Methods – General Remarks

The power of proteomics has been greatly enhanced by the development of relative and absolute quantitative proteomic methods. This has been demonstrated by using traditional quantitative approaches such as 2D PAGE combined with differential staining techniques, such as DIGE *(21, 22)*. More recently, quantitative methods – including chemical, enzymatic, and metabolic labeling of specific protein populations with different isotopes and subsequent quantification by MS – were introduced, and they show increased sensitivity when compared with gel-based methods. They include cleavable isotope-coded affinity tags (cICAT) *(23)*, isobaric tags for relative and absolute quantification (iTRAQ) *(4)*, enzymatic labeling with protein hydrolysis in the presence of heavy (i.e., ^{18}O-containing) water *(24, 25)* ($^{18}O/^{16}O$ proteolytic labeling), and stable isotope labeling with amino acids in cell culture (SILAC) *(26)*. Basically, in all chemical labeling approaches, proteins derived from various samples are hydrolyzed, and the corresponding peptide pool is chemically labeled with reagents that differ in mass because of incorporation of different heavy stable isotopes, such as ^{13}C, ^{15}N, or ^{2}D. The samples thus labeled are mixed and then analyzed by MS. Importantly, the peak's signal intensity in the mass spectrum of the same, but differently labeled, peptides derived from the various samples can be directly compared, and this reflects the relative quantities of the particular peptide and therefore of the particular protein in the different samples. Chemical labeling is used most frequently at the peptide level, i.e. labeling takes place after the proteins have been digested with specific endoproteinases. cICAT was originally reported for labeling at the protein level and, in principle, all its reagents should also work at the protein level. The advantage of the chemical labeling strategy is the fact that it can be used for MS-based quantification approaches in tissues (e.g., brain). SILAC, although a highly efficient labeling approach, as ^{13}C- and ^{15}N-labeled amino acids (of which [^{13}C]-lysine and [^{13}C]-arginine are especially relevant on account of the specificity of trypsin for these residues) are incorporated into proteins to nearly 100% in cell cultures, is restricted to the investigation of cells growing in culture (e.g., HeLa cells).

More recently, label-free quantification by MS has also shown great promise; this uses techniques, such as spectral counting *(27)* and ion-current measurement *(28)*.

1.2.1. Isobaric Tags for Relative and Absolute Quantification

The iTRAQ method *(4, 29)* involves protein reduction and alkylation, enzymatic digestion, labeling up to four different samples (or nowadays eight samples) with heavy and light isotope-labeled rea-

gents, sample pooling, HPLC separation, and finally detection and quantification by tandem MS. The iTRAQ reagent is an amine-specific reagent that labels peptide amino termini and lysine side chains with multiplex (4-plex or 8-plex) mass tags: i.e., tags with identical masses. These tags are isobaric (i.e., they have the same mass of 144.1 Da), but their fragmentation patterns differ, allowing species bearing them to be distinguished after fragmentation. Upon fragmentation, every isobaric tag produces a specific marker ion (114.1, 115.1, 116.1, and 117.1 Da, respectively) and a corresponding neutral fragment, which is not detectable (28, 29, 30, and 31 Da, respectively). Thus, in this method (in contrast to other chemical labeling methods described above) differentially labeled peptides show no difference in the mass spectrometer when intact: i.e., in MS mode of the instrument. Only after fragmentation of the peptides, and thus of the tag attached to the peptides, a low-molecular-mass reporter ion is generated, the mass of which is specific to the iTRAQ tag used to label the sample. Therefore, quantification of the differently labeled samples is achieved solely upon fragmentation of the correspondingly labeled peptides in the mass spectrometer. The MS measurement of the intensity of these reporter-ion peaks allows the relative quantification of the peptides in each sample.

iTRAQ has several advantages: (1) Multiplexing (i.e., mixing of more than two differentially labeled species) per se can save time during the quantification of samples taken at several time points or from different stages. (2) It offers the unique possibility to create an internal standard that contains a mixture of all samples, so that more than four (or eight) samples can be investigated and quantified in relation to the internal standard. (3) Since the mixed peptides carry an isobaric tag, the overall signal intensity of the peptide/precursor ions derived from different samples is enhanced in the MS mode (i.e., before fragmentation). (4) Importantly, the signal intensity of the peptide fragment ions in the MS/MS is enhanced as well, since the tag is completely cleaved from the labeled NH_2 group upon fragmentation.

For reliable quantification by chemical labeling, the following practical aspects need to be considered carefully: (1) (Exact) knowledge of the sample amount subjected to quantitative analysis. This is the prerequisite for a reliable analysis. However, differences in sample amounts can be monitored by the ratio of, e.g., marker proteins that are known to be present in equal amounts in the different samples; appropriate correction can be applied later by evaluation software. (2) Digestion efficiency. When sample labeling with iTRAQ is performed after digestion, reliable quantification is only possible when proteins in both samples are digested either completely or exactly to the same degree. (3) Labeling efficiency. This should be as high as possible (i.e., <90%). With iTRAQ, the labeling efficiency can be monitored by searching the fragment spectra of the labeled peptides against

a database (using a search engine, see below) with the iTRAQ as variable (optional) extra mass. In this manner, the number of non-labeled peptides is also displayed. (4) The number of labeled peptides per identified protein by MS and subsequent database search should be high enough to ensure significant statistics on the labeling/quantification. (5) As the iTRAQ method does not reduce the sample complexity (in contrast to, e.g., cICAT, where only peptides that carry cysteine residues are labeled and quantified), and this places greater demands upon the separation step before tandem MS.

1.3. LC-Coupled Tandem MS

MS uses two main methods to ionize "softly" biomolecules, such as proteins and peptides: matrix-assisted laser desorption/ionization *(30–32)* and electrospray ionization *(33)*. Both techniques, when implemented with a state-of-the-art mass spectrometer, allow (1) accurate determination of the mass-to-charge ratio (m/z) of a peptide within a mixture (the so-called MS experiment) and (2) isolation of peptides (precursor) within the mass spectrometer and subsequent fragmentation of the isolated precursor in the mass spectrometer (the so-called MS/MS experiment). Mass analysis of the fragments (giving the sequence of the peptide) combined with the accurate monoisotopic precursor (peptide) mass allows the identification of corresponding protein sequence in the database. For reliable identification of proteins by tandem MS, several peptides from one protein should be selected, fragmented, and searched against a database (using search engines, e.g., Mascot *[34]*).

Coupling of nanoLC to an ESI mass spectrometer is a fast and powerful way to obtain peptide sequence data. The chromatography system greatly reduces the complexity of the sample, and the direct coupling to the source ensures that the peptides eluted are immediately ionized and analyzed in the instrument.

State-of-the-art ESI mass spectrometers use data-dependent acquisition (DDA) to analyze the peptide mixture that is eluted from the LC system. The DDA duty cycle consists of two main steps: First, an MS scan over a certain mass range (typically 350–1,500 m/z) is performed to detect the charge state of the precursor (peptide). In a routine online-coupled ESI MS approach only doubly, triply, and quadruply charged peptide precursors/peptides are selected for further MS/MS analysis (fragmentation). Importantly, after the initial MS scan, several peptide precursors can be selected for subsequent MS/MS analysis, which allows the most intense precursor signals in the MS spectrum to be sequenced. Note that while the instrument is occupied with MS/MS experiments, usually no MS scans can be performed (except for Orbitrap-FT mass analyzer); therefore, during this time, peptides that are eluted from the column into the instrument cannot be detected and sequenced. Consequently,

instruments with a short duty cycle can sequence more peptides in a given time than instruments with a long duty cycle.

Despite the separation power of modern LC techniques, in complex samples, a multitude of peptides are still eluted every second from the column, and even modern mass spectrometers cannot register all of these in the time available. Therefore, an important factor is the acquisition speed (duration of the duty cycle) of the mass spectrometer. In general, ion-trap instruments have a shorter duty cycle than with qQ-ToF instruments, and the former are therefore frequently used for the analysis of very complex mixtures. On the other hand, the quality of the MS/MS spectra from qQ-ToF instruments is considerably better, so that the number of MS/MS spectra that can be used to assign a peptide sequence correctly is higher *(35)*. Importantly, in relative quantification with the iTRAQ reagent, a reliable result depends on the spectral quality in the low m/z range: i.e., between 110 and 120 m/z. However, 3D and/or linear ion-trap instruments have no – or only a limited – capability to monitor ions within this mass range and are thus not recommended for iTRAQ quantification approaches. In preference, qQ-ToF instruments, the Orbitrap mass-analyzer *(36)* or MALDI instruments should be chosen.

1.4. Combining 1D-PAGE with iTRAQ Labeling for Relative Quantification of Protein (RNA) Complexes

Because of the above-mentioned restrictions and drawbacks of a quantitative proteome study using iTRAQ, we designed an approach to reduce the complexity of the sample that combines 1D PAGE of protein samples with iTRAQ labeling of the extracted peptides derived from in-gel digested proteins. We use the following workflow to monitor quantitative differences in protein complexes (with or without bound RNA) derived from different functional stages of a cell:

1. Separation of purified protein (-RNA) complexes by 1D PAGE on pre-cast gels (8 × 8 cm × 1 mm); *see* **Subheading 3.1**
2. Cutting the entire sample lanes into pieces of exactly the same size; *see* **Subheading 3.2.1** and **Fig. 2**
3. Digestion of proteins with the endoproteinase trypsin; *see* **Subheading 3.2.2**
4. iTRAQ labeling of extracted peptides; *see* **Subheading 3.3**
5. Pooling of the differentially labeled samples and subsequent LC-MS/MS analysis (ESI MS); *see* **Subheading 3.3** paragraph 8, **Fig. 3**

The overall workflow is illustrated in **Fig. 1**.

On the one hand, the workflow – although time-consuming, owing to labeling of numerous separate gel pieces – showed several advantages over *in situ* digestion of samples and subsequent labeling: (1) The overall analysis yield is greatly enhanced. We obtain more labeled peptides, higher sequence coverage, and thus

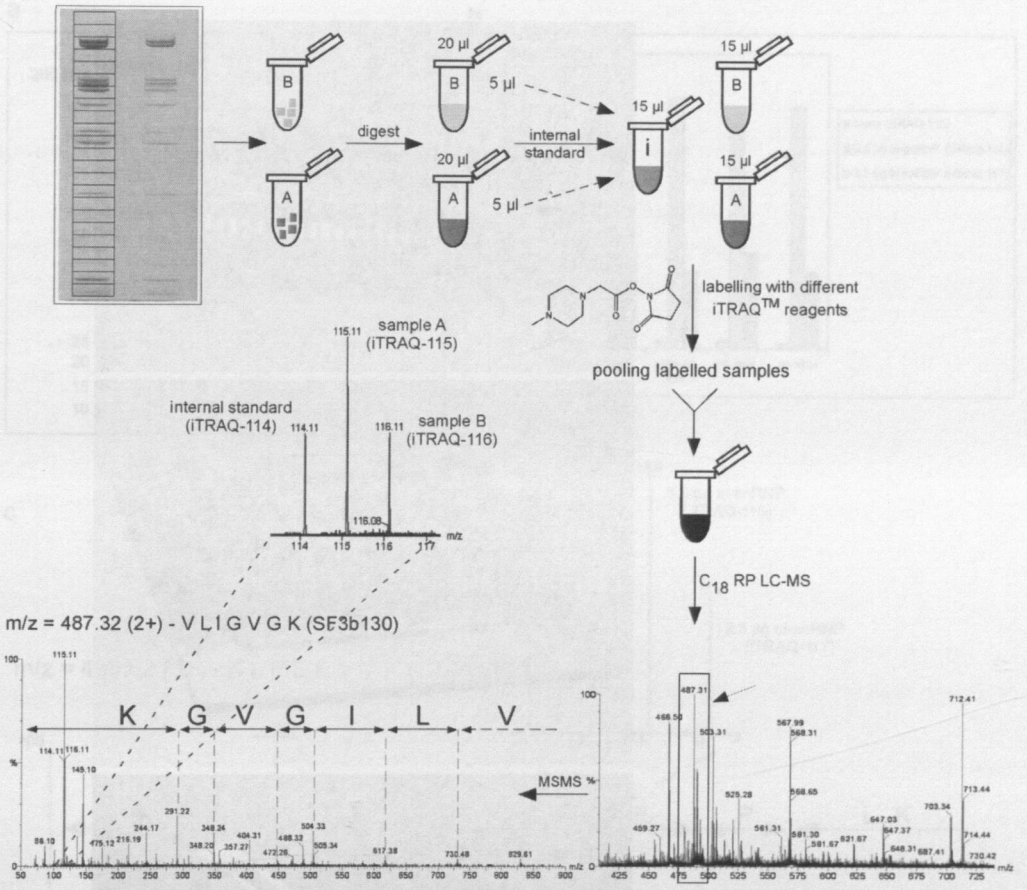

Fig. 1. Overall workflow of in-gel digestion and subsequent labeling with iTRAQ™ reagents. Entire gel lanes of different samples to be analyzed and quantified are cut into gel slices of equal size resulting in 23 samples per lane. Each gel slice further manually cut into small pieces and proteins within gel pieces are digested with trypsin as described in **Subheading 3.2.2**. Extracted peptides are re-dissolved in 20 µL 100 mM TEAB and an internal standard is prepared by pooling 5 µL of each sample. Samples and internal standard are labeled with iTRAQ™ reagents 114, 115, 116, respectively (*see* **Subheading 3.3**). After pooling, the samples are analyzed by LC-MS/MS and quantitation is done by calculating the peak areas of individual reporter ions (114, 115, 116, and 117) in MS/MS.

a higher confidence in quantification when compared with digestion of the samples *in situ* under semi-denaturing conditions followed by labeling of the peptides. We assume that the protein/peptide complexity is less within the single gel regions and, therefore, labeling of peptides is more efficient. On the other hand, the digestion efficiency might be enhanced as well. (2) Digestion, extraction, and labeling can be performed using the same buffer (*see* **Subheadings 3.2.2** and **3.3**), the volume can be kept much smaller and labeled species can be injected directly into the LC system coupled to the mass spectrometer without any further chromatography step. (3) If desired, the gel regions of interest can be investigated and compared selectively. (4) Importantly,

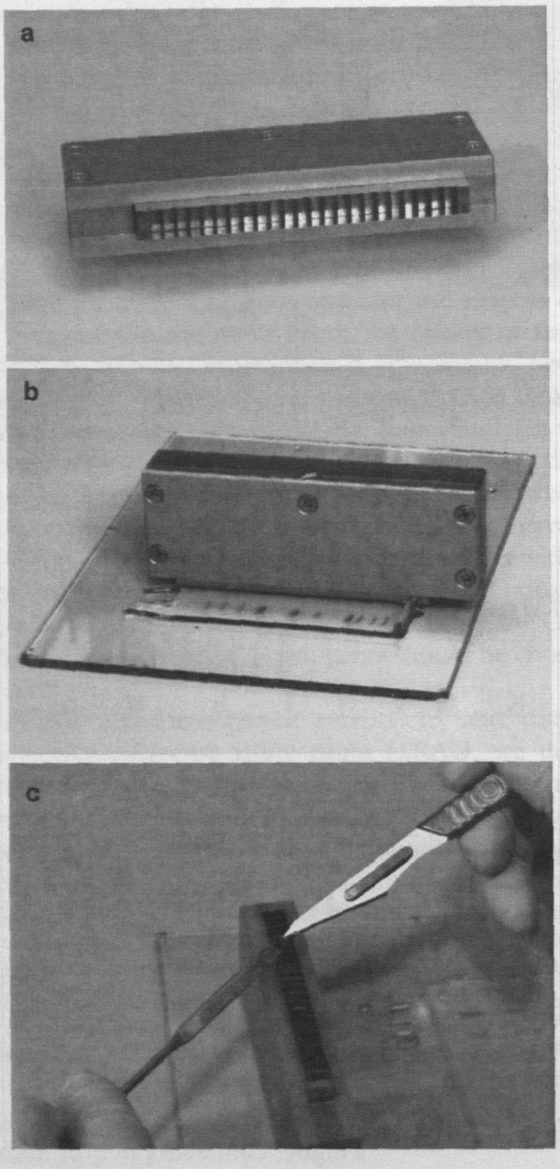

Fig. 2. Gel lane cutting device: (**a**) Site view of in-house manufactured gel-lane cutter for cutting lanes of pre-cast gels (e.g., NuPAGE® 10 cm in length) into 23 slices of equal size (3 × 7 mm). (**b**) Gel cutter attached to the gel. (**c**) Additional manual cutting of slices into gel pieces of approximately 1 × 1 mm by using spatula and scalpel.

the relative amounts of sample can be compared directly with the relative amounts seen in the staining of the samples after gel electrophoresis. Although the latter is not highly accurate, the investigator can at least estimate whether the samples drastically differ in amount by comparing the staining intensity of marker proteins (see also above).

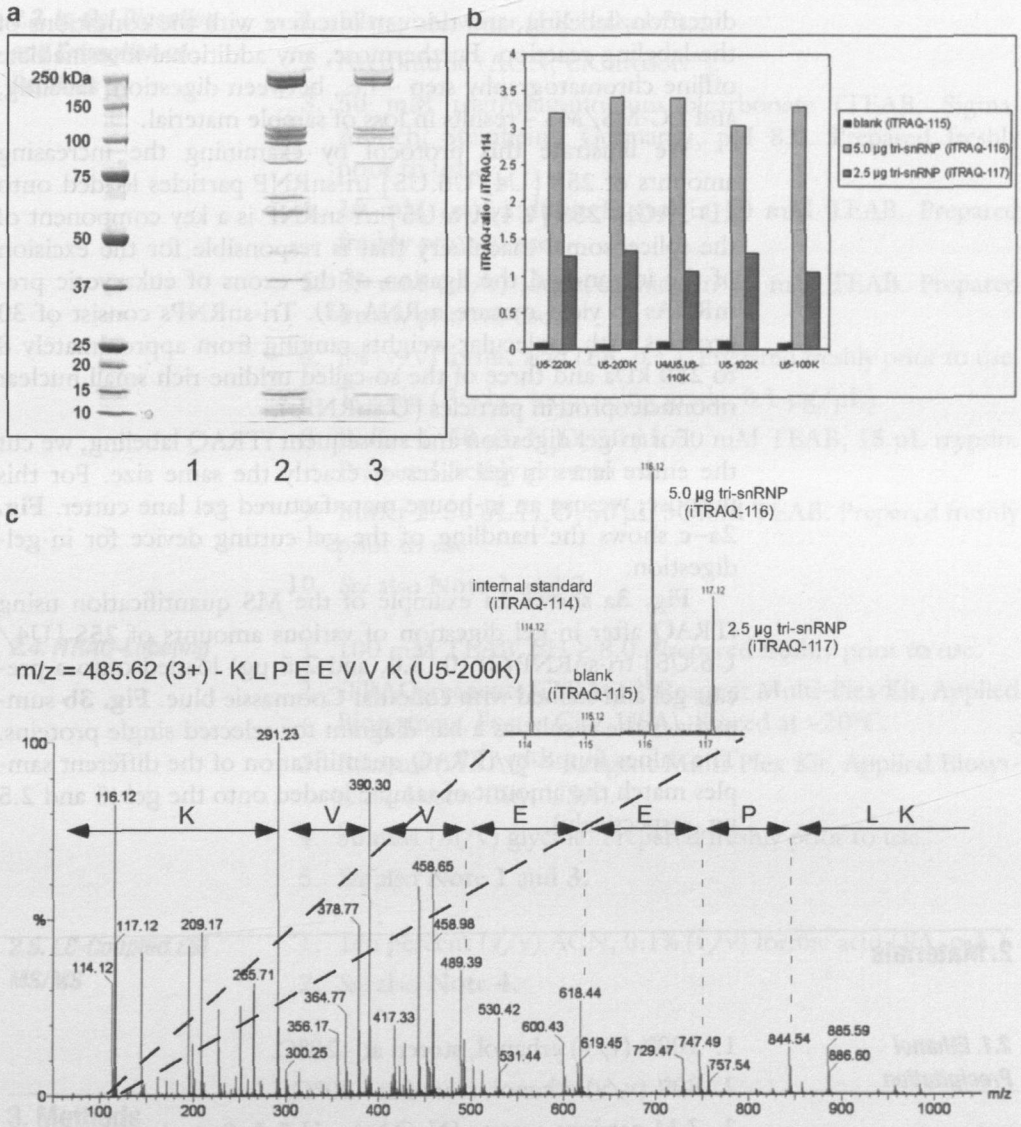

Fig. 3. Quantification of various amounts of tri-snRNP particles loaded onto a pre-cast gel, digested within gels, and subsequently labeled with iTRAQ. (**a**) Colloidal Coomassie stained NuPAGE® 4–12% Bis–Tris Gel; lane 1: blank, lane 2: 5.0 µg tri-snRNPs, lane 3: 2.5 µg tri-snRNPs. (**b**) Example of iTRAQ ratios for various identified proteins. Ratios are calculated by dividing the peak area of reporter ions of the blank sample (iTRAQ-115), 5.0 µg (iTRAQ-116), and 2.5 µg tri-snRNP (iTRAQ-117), respectively, by the peak area of the internal standard (iTRAQ-114). (**c**) Example of MS/MS spectrum of a labeled peptide (KLPEEVVK, U5-200K) derived after in-gel digestion and labeling procedure. The reported region (m/z = 114–m/z = 117) is magnified and shows the individual reporter ions used for quantification.

In contrast, when digestion is performed *in situ*, most protein (-RNA) complexes must be completely denatured before digestion, using a higher concentration of detergents/denaturants than is compatible with endoproteinase digestion and labeling. Consequently, the sample volume has to be adjusted before

digestion/labeling, and this can interfere with the conditions of the labeling reaction. Furthermore, any additional intermediate offline chromatography step – i.e., between digestion, labeling, and LC-MS/MS – results in loss of sample material.

We illustrate this protocol by examining the increasing amounts of 25S [U4/U6.U5] tri-snRNP particles loaded onto 1D-PAGE. 25S [U4/U6.U5] tri-snRNP is a key component of the spliceosomal machinery that is responsible for the excision of the intron and the ligation of the exons of eukaryotic pre-mRNAs to yield mature mRNA *(3)*. Tri-snRNPs consist of 30 proteins with molecular weights ranging from approximately 8 to 280 kDa and three of the so-called uridine-rich small nuclear ribonucleoprotein particles (U snRNPs).

For in-gel digestion and subsequent iTRAQ labeling, we cut the entire lanes in gel slices of exactly the same size. For this purpose, we use an in-house manufactured gel lane cutter. **Fig. 2a–c** shows the handling of the gel-cutting device for in-gel-digestion.

Fig. 3a shows an example of the MS quantification using iTRAQ after in-gel digestion of various amounts of 25S [U4/U6.U5] tri-snRNP *(2)* (0, 5.0, and 2.5 µg) loaded onto a pre-cast gel and stained with colloidal Coomassie blue. **Fig. 3b** summarizes the results as a bar diagram for selected single proteins. The values found by iTRAQ quantification of the different samples match the amount of sample loaded onto the gel (5 and 2.5 µg, respectively).

2. Materials

2.1. Ethanol Precipitation

1. 100% (v/v) ethanol, stored at –20°C.
2. 80% (v/v) ethanol, stored at –20°C.
3. 3 M natrium acetate (NaOAc), pH 5.3. Stored at room temperature.

2.2. NuPAGE® Electrophoresis System Components

1. NuPAGE® Novex Bis-Tris pre-cast gels (4–12%, 1.0 mm, 8 × 8 cm). Stored at 4°C–25°C.
2. NuPAGE® LDS sample buffer (4×). Stored at 4°C.
3. NuPAGE® reducing agent (10×). Stored at 4°C.
4. NuPAGE® antioxidant. Stored at 4°C.
5. NuPAGE® MES SDS or MOPS SDS running buffer (20×). Stored at room temperature.
6. Gel chamber (Xcell *SureLock™* Mini-Cell).
7. *See* also **Note 2**

2.3. In-Gel Digestion and Extraction of Peptides

1. Ultrapure water (LiChrosolv®).
2. Acetonitrile (ACN, LiChrosolv®).
3. 50 mM triethylammonium bicarbonate (TEAB, Sigma-Aldrich, Steinheim, Germany), pH 8.0. Prepared freshly prior to use.
4. 10 mM (m/v) dithiothreitol in 50 mM TEAB. Prepared freshly prior to use.
5. 55 mM (m/v) iodoacetamide in 50 mM TEAB. Prepared freshly prior to use.
6. 5% (v/v) formic acid (FA, p.a.). Prepared freshly prior to use.
7. Trypsin (Roche, sequencing grade, 0.1 µg/µL).
8. Buffer 1: 50 µL H_2O, 50 µL 50 mM TEAB, 15 µL trypsin. Prepared freshly prior to use.
9. Buffer 2: 50 µL H_2O, 50 µL 50 mM TEAB. Prepared freshly prior to use.
10. *See* also **Note 1** and **3**.

2.4. iTRAQ-Labeling

1. 100 mM TEAB, pH ≥ 8.0. Prepared freshly prior to use.
2. iTRAQ-reagents (iTRAQ™ Reagent Multi-Plex Kit, Applied Biosystems, Foster City, USA). Stored at −20°C.
3. Ethanol (iTRAQ™ Reagent Multi-Plex Kit, Applied Biosystems, Foster City, USA).
4. 50 mM (m/v) glycine. Prepared freshly prior to use.
5. *See* also **Note 1** and **3**.

2.5. LC-Coupled ESI MS/MS

1. Ten percent (v/v) ACN, 0.1% (v/v) formic acid (FA, p.A.).
2. *See* also **Note 4**.

3. Methods

3.1. Sample Separation by 1D Gel Electrophoresis Using Pre-cast Gels

All steps necessary for running the gels including the recommended sample buffer are according to the manufacturer's protocols (e.g., Invitrogen, Carlsbad, USA). Protocols are delivered on purchase. Note that all pre-cast gel systems require gel chambers from the same manufacturer.

Most pre-cast gels are available in different thicknesses (e.g., 1.0, 1.5 mm). It is advisable to use 1-mm gels for in-gel digestion (*see* Chapter 3.1), as the amount of gel material should be kept as small as possible. The sample amount depends on the gel's thickness. On 1-mm gels approximately 40 µL of sample (including 4 × sample buffer, e.g., NUPAGE® (Invitrogen, Carlsbad, USA)) can be loaded. Therefore, most samples should be concentrated

before loading, either by precipitation (e.g., TCA, EtOH, and $CHCl_3$/MeOH) or by ultrafiltration (e.g., Sartorius stedim biotech, Aubagne Cedex, France, or Millipore Corporation, Billerica, USA).

3.1.1. Sample Preparation for Gel Electrophoresis

25S [U4/U6.U5] tri-snRNP particles (harboring 30 proteins *[2]*) were obtained after glycerol-gradient centrifugation of immunoisolated total snRNPs as described elsewhere *(37–39)*. Protein concentration is determined according to Bradford *(40)*.

1. Precipitate glycerol-gradient fraction containing 2.5 and 5.0 µg, respectively, tri-snRNP particles with three volumes ice-cold EtOH and 1/10 volume 3 M NaOAc, pH 5.3.
2. Spin down for 30 min, 13,000 rpm, 4°C.
3. Wash with ice-cold 80% (v/v) EtOH.
4. Spin down for 30 min, 13,000 rpm, 4°C.
5. Dry pellets in vacuum centrifuge.
6. Dissolve samples in 30 µL sample buffer (mix of 4 × NuPAGE® LDS sample buffer, 10 × NuPAGE® reducing agent, and double-distilled water).
7. Heat samples to 70°C for 10 min.
8. Load sample onto Bis-Tris pre-cast 4–12% gradient gels (NuPAGE®, Invitrogen).

3.1.2. Gel Electrophoresis

Electrophoresis is carried out constantly at 200 V (Bis-Tris Gels) using the appropriate gel chamber (Xcell *SureLock™* Mini-Cell) according to the manufacturer's protocols *(18)*.

3.1.3. Staining

Pre-cast gels were stained with silver *(19)* or colloidal Coomassie *(41)* and stored in water (LiChrosolv®).

3.2. In-Gel Digestion

3.2.1. Excision of Bands/ Lanes

After resolution of proteins by gel electrophoresis, the protein spots/lanes of interest are excised from the gel, and the proteins within them are digested with endoproteinases.

Proteins can be excised from 1D PAGE by two different methods, i.e., excision of single bands or cutting entire lanes into equal-sized pieces.

In our laboratory, the routine method is analysis of entire lanes cut from pre-cast gels. For this purpose, we use a gel-cutter made in-house (**Fig. 2**) that cuts an entire lane from a NuPAGE® pre-cast gel into exactly 23 pieces. Cutting the whole lane ensures that the entire sample will be analyzed. Furthermore, owing to the identical sizes of the gel pieces, higher reproducibility and more confidence in relative quantification between samples in different lanes is achieved. **Fig. 2** shows the gel-cutter together with the stained protein samples on NuPAGE®-gels.

The gel pieces have a size of 3 × 7 mm. Slices are further cut manually into pieces of 1 × 1 mm using a scalpel and a spatula, and the pieces are transferred into 0.5-ml Eppendorf tubes for in-gel digestion.

3.2.2. In-Gel Digestion

Trypsin is normally the preferred endoproteinase for the in-gel digestion of proteins, for several reasons: (1) it cleaves proteins specifically at the C-terminal side of arginine and lysine residues. The peptides (precursors) generated thus always contain a charged amino acid that favors their ionization under acidic conditions. (2) Tryptic peptides have mass-to-charge ratios that are well suited for MS analysis (m/z 350–1,400). (3) Trypsin is active in many buffers containing denaturing agents (urea, guanidinium chloride, and SDS).

The protocol is modified from Shevchenko et al. *(42)* for direct iTRAQ labeling. Note that during iTRAQ labeling, the presence of primary amines should be avoided. TEAB is the preferred buffer.

Digestion

1. Wash the gel pieces with 150 µL H_2O and incubate for 5 min in a thermomixer at 26°C and 1,050 rpm.
2. Spin the gel pieces down and remove all liquid with thin gel-loader tips.
3. Add 150 µL ACN to shrink the gel pieces and incubate for 15 min in a thermomixer at 26°C and 1,050 rpm.
4. Spin the gel pieces down and remove all liquid with thin gel-loader tips.
5. Dry the gel pieces in a vacuum centrifuge for approximately 10 min.
6. Add 100 µL 10 mM dithiothreitol (in 50 mM TEAB) to reduce the disulfides and incubate for 50 min in a thermomixer at 56°C.
7. Spin the gel pieces down and remove all liquid with thin gel-loader tips.
8. Add 150 µL ACN to dehydrate and incubate for 15 min in a thermomixer in the dark at 26°C and 1,050 rpm.
9. Spin the gel pieces down and remove all liquid with thin gel-loader tips.
10. Add 100 µL 55 mM iodoacetamide (in 50 mM TEAB) to alkylate the reduced cysteines and incubate for 20 min in a thermomixer in the dark at 26°C and 1,050 rpm.
11. Wash the gel pieces with 150 µL 50 mM TEAB and incubate for 15 min in a thermomixer at 26°C and 1,050 rpm.
12. Add 150 µL ACN and incubate for 15 min in a thermomixer at 26°C and 1,050 rpm.

13. Spin gel pieces down and remove all liquid with thin gel-loader tips.
14. Add 150 µL ACN to dehydrate and incubate for 15 min in a thermomixer at 26°C and 1,050 rpm.
15. Spin gel pieces down and remove all liquid with thin gel-loader tips.
16. Dry the gel pieces in a vacuum centrifuge for approximately 10 min.
17. Rehydrate the dried gel pieces with buffer 1 (*see* Subheading 2.3) on ice; if necessary add more buffer 1 after approximately 30 min.
18. Cover the rehydrated gel pieces with buffer 2 (*see* Subheading 2.3) on ice; if necessary add more buffer 2 after approximately 30 min.
19. Incubate overnight at 37°C.

Extraction of the Tryptic Peptides

1. Add 25 µL H_2O to the gel pieces and incubate for 15 min in a thermomixer at 37°C and 1,050 rpm.
2. Add 50 µL ACN and incubate for 15 min in a thermomixer at 37°C and 1,050 rpm.
3. Transfer the supernatant with thin gel-loader tips to new tubes.
4. Add 50 µL 5% (v/v) FA to the gel pieces and incubate for 15 min in a thermomixer at 37°C and 1,050 rpm.
5. Add 50 µL ACN and incubate for 15 min in a thermomixer at 37°C and 1,050 rpm.
6. Remove the supernatant and pool with the first supernatant.
7. Add 50 µL ACN and incubate for 15 min in a thermomixer at 37°C and 1,050 rpm.
8. Remove the supernatant and pool supernatants.
9. Dry the supernatant in a vacuum centrifuge for approximately 1.5 h.
10. The dried samples could be prepared for MS analysis or stored at −20°C for future labeling.

3.3. iTRAQ-Labeling

Labeling of peptides with iTRAQ™ reagents

1. Dissolve the extracted peptides in 20 µL 100 mM TEAB (sonication).
2. Prepare an internal standard by pooling 5 µL of each sample to a final volume of 15 µL, if there are fewer than three samples fill up with a corresponding volume (e.g., 5 µL) of 100 mM TEAB.

3. Reconstitute each iTRAQ™ reagent in 70 µL ethanol (vortex).
4. Add to each sample 5 µL of the different iTRAQ™ reagents resulting in a final concentration of 25% (v/v) ethanol; use iTRAQ™-114 for labeling the internal standard.
5. Incubate for 1 h in a thermomixer at 25°C and 550 rpm.
6. Quench excess of reagent by adding 5 µL 50 mM glycine resulting in a final concentration of 10 mM glycine.
7. Incubate for 30 min in a thermomixer at 25°C and 550 rpm.
8. Pool the labeled samples and dry down in a vacuum centrifuge.
9. The dried samples could be prepared for MS analysis directly or stored at −20°C.
10. For MS analysis, dissolve samples in appropriate volume (e.g., 20 µL) of 10% (v/v) ACN, 0.1% (v/v) FA.

3.4. Standard LC-Coupled ESI MS/MS of iTRAQ-Labeled Peptides and Data Evaluation

Since every laboratory has its own very individual setup for LC-coupled MS/MS and uses different software, we do not provide any specific protocol but rather a view comments on more general aspects.

1. As mentioned above, Q-ToF, the Orbitrap-FT mass-analyzer, and MALDI-MS/MS instruments are most suitable for iTRAQ quantification.
2. Labeled samples can be analyzed either by LC-coupled ESI-MS/MS using qQ-ToF or with an Orbitrap-FT mass-analyzer or off-line LC MALDI-MS/MS instruments.
3. LC systems should be equipped with a trapping column (RP C18) on which the sample is extensively desalted (several minutes with elevated flow rates, e.g., 10 µL/min) to remove the excess of iTRAQ reagent.
4. Separation of labeled peptides is achieved by standard "peptide mapping" RP C18 columns, most frequently used with inner diameter of 75 µm working at a flow rate between 180 and 300 nL/min.
5. For MS/MS, the routine settings in the mass spectrometer MS can be used. However, sometimes it is recommended to enhance the detection of fragment ions in the lower m/z range in the mass spectrometer (e.g., in the qQ-ToF Waters Ultima by setting the appropriate radio-frequency values).
6. Before submission of the data for database search, the raw data-processing parameter (to generate, e.g., centroid data) should be adjusted, in particular when the lower m/z mass range of a spectrum is being monitored.
7. To analyze the iTRAQ data, several software tools are available, both commercially (Mascot 2.2 (Matrix Science), Protein pilot (ABI)) and freely (iTracker *[43]*, Multi-Q *[44]*,

etc.). However, not all available software (in particular, the freely available software on the internet) can handle the processed instrument-specific data formats without any difficulties.

Our laboratory uses Mascot 2.2 to quantify the data. At searches with iTRAQ™-labeled peptides, modifications at the N-term, at lysine side chains and at tyrosine side chains (side reaction of the iTRAQ reagent) are taken into account. The labeling efficiency is checked by searching the fragment spectra with iTRAQ as a variable (i.e., optional) modification. The labeling efficiency must be ≥90%. All parameters for data-processing and -searching should be tested with known standards (see elsewhere in this chapter).

3.5. Conclusions

iTRAQ labeling from in-gel digested samples is a reliable approach to compare the relative protein quantities between different samples, in particular when working with samples of moderate protein complexity. Since the labeling step does not require any further desalting and/or chromatography steps, it can be implemented on a routine basis within the well-established in-gel digestion procedure of proteins. It offers the opportunity to compare the relative protein amount in a multitude of purified protein (–RNA) complexes that were isolated under different conditions or from different functional stages of a cell.

4. Notes

1. For all buffers and solutions p.a. grade water and solvents were used.
2. All NuPAGE® electrophoresis system components were used according to manufacturer's protocols.
3. All buffers for in-gel digestion, extraction of tryptic peptides, and iTRAQ-labeling were prepared fresh prior to use.
4. For LC-coupled MS/MS the routine solvents and buffers as well as the routine settings in the MS can be used.

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