

## Sample Preparation Approaches for iTRAQ Labeling and Quantitative Proteomic Analyses in Systems Biology

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### Abstract

Among a variety of global quantification strategies utilized in mass spectrometry (MS)-based proteomics, isobaric tags for relative and absolute quantitation (iTRAQ) are an attractive option for examining the relative amounts of proteins in different samples. The inherent complexity of mammalian proteomes and the diversity of protein physicochemical properties mean that complete proteome coverage is still unlikely from a single analytical method. Numerous options exist for reducing protein sample complexity and resolving digested peptides prior to MS analysis. Indeed, the reliability and efficiency of protein identification and quantitation from an iTRAQ workflow strongly depend on sample preparation upstream of MS. Here we describe our methods for: (1) total protein extraction from immortalized cells; (2) subcellular fractionation of murine tissue; (3) protein sample desalting, digestion, and iTRAQ labeling; (4) peptide separation by strong cation-exchange high-performance liquid chromatography; and (5) peptide separation by isoelectric focusing.

**Key words** Proteomics, Mass spectrometry, iTRAQ, Subcellular fractionation, High-performance liquid chromatography, Isoelectric focusing

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## 1 Introduction

Quantitative analysis of protein expression, function, and subcellular localization is fundamental to network biology. Mass spectrometry (MS)-based quantitative proteomic approaches have evolved rapidly in the last 15 years and are generating datasets essential for systems biology and the modeling of biological networks [1]. Discovery applications in MS-based proteomics have largely employed untargeted strategies where proteins in one or more samples (diseased or treated) are quantified relative to the amount of proteins in a separate sample (normal or control). Quantification of the peptides/proteins can either be done by comparative analysis of spectral features in a “label-free” workflow or alternatively be accomplished through isotopic labeling or the incorporation of a differential mass tag. Isobaric tags for relative and absolute

quantitation (iTRAQ) are widely used amine-specific, stable-isotope reagents which can be used to label the N terminus and side chain of lysines of peptides generated by tryptic digestion of extracted proteins. The reagents were designed for “multiplexing,” and four or eight separate iTRAQ labels are available permitting the simultaneous analysis of multiple samples. The labels consist of a low-mass reporter group, a balance group, and an amine-reactive group; they have isobaric masses in MS mode (145 and 305 Da for 4-plex or 8-plex reagents), but upon fragmentation release low-mass reporter ions ( $m/z$  values of 114.1, 115.1, 116.1, 117.1 for 4-plex, plus 113.1, 118.1, 119.1, 121.1 for 8-plex) allowing quantification at the MS/MS level. Unlike metabolic incorporation of stable isotopes, a key advantage to iTRAQ labels is that samples from any source, including patient material, can be chemically labeled. This fact, in combination with the ability to simultaneously analyze multiple samples, has likely contributed to the widespread use of these reagents [2, 3]. It should be noted that quantitation by iTRAQ is not perfect; contamination during precursor ion selection, specific to MS/MS quantitation, results in compression of the iTRAQ ratio and underestimation of relative protein abundance estimates; and variance is higher for low-intensity signals [4, 5]. Data processing and instrument-specific approaches aimed at addressing issues related to the precision and accuracy of iTRAQ quantitation continue to evolve and have been reviewed elsewhere [2, 3].

Technological advances in high-resolution MS instrumentation means that almost complete coverage of unicellular organisms such as yeast is now possible [6] and comprehensive analysis of mammalian proteomes is envisaged as feasible in the near future [7]. However, the required technology is not widely available and currently most researchers will find that proteome coverage is dependent on reducing sample complexity and their choice of multiple sample preparation steps including protein separation, digestion, and peptide fractionation steps. Proteomic workflows can be complex and the role of error propagation through multiple handling steps should not be underestimated [8]. Critical to the success of an iTRAQ experiment is the use of equal sample amounts, reproducible protein digestion, and efficient peptide labeling. Protein samples may be pre-fractionated either by subcellular fractionation or based on size by polyacrylamide gel electrophoresis. Proteins are most typically digested using trypsin, although other proteases can be used [9], and digested peptides are separated by either high-performance liquid chromatography (HPLC) or isoelectric focusing.

We have used iTRAQ reagents to examine differential protein expression in fatty acid-treated hepatocarcinoma cells and liver tissue mice fed a high-fat diet. Here we describe our methods for: (1) total protein extraction from immortalized cells; (2) subcellular

fractionation of murine liver tissue; (3) protein sample desalting, digestion, and iTRAQ labeling; (4) peptide separation by strong cation-exchange (SCX) HPLC; and (5) peptide separation by isoelectric focusing.

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## 2 Materials

All reagents should be of analytical grade and solvents either HPLC or LC-MS grade. All solutions should be prepared with distilled, deionized water (ddH<sub>2</sub>O), typically 18.2 MΩ·cm at 25 °C, except for LC-MS/MS buffers which require LC-MS-grade H<sub>2</sub>O.

### 2.1 Protein Extraction from Cells

1. Phosphate-buffered saline (PBS; 1×): 137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>.
2. Radioimmunoprecipitation assay buffer (RIPA): 150 mM NaCl, 1.0 % IGEPAL® CA-630, 0.5 % sodium deoxycholate, 0.1 % SDS, and 50 mM Tris, pH 8.0.
3. Protease inhibitor cocktail-EDTA free (PI): Mixture of several protease inhibitors inhibiting serine, cysteine, but not metalloproteases.
4. Low-speed swinging bucket centrifuge.
5. Refrigerated table top microcentrifuge.
6. QIAshredder columns (QIAGEN, UK).
7. BCA assay.

### 2.2 Protein Extraction from Liver Tissue

1. HEPES, EDTA, mannitol (HEM) buffer: 20 mM HEPES, 1 mM EDTA, 300 mM mannitol.
2. Protease inhibitor cocktail EDTA free (PI): Mixture of several protease inhibitors inhibiting serine, cysteine, but not metalloproteases.
3. 10 ml glass Dounce homogenizer with pestle attached to electric drill.
4. Refrigerated swinging bucket centrifuge.
5. Ultracentrifuge.
6. Appropriate polyallomer tubes for ultracentrifugation.
7. Amicon Ultra-15 Centrifugation Filter Device (NMWL of 3 kDa; Millipore, USA).
8. BCA assay (Pierce Scientific, UK).

### 2.3 Protein Desalting, Digestion, and iTRAQ Labeling

1. 2 ml ZEBRA columns (Pierce Scientific, UK; *see* **Note 6**).
2. RIPA buffer and PI as before.
3. Vacuum centrifuge (Eppendorf, UK).
4. Low-bind microcentrifuge tubes (*see* **Note 7**).

5. Bovine serum albumin (BSA) as control to monitor digestion and future LC separation efficiency.
6. Triethylammonium bicarbonate (TEAB) buffer, 1 M pH 8.5 (Sigma Aldrich, UK).
7. iTRAQ Reagents Multiplex Kit (ABSciex, UK).
8. Shaking dry heat block (Eppendorf, UK).
9. Mass spectrometry-grade trypsin gold (Promega, UK).
10. Sonication water bath.

#### **2.4 Peptide Separation by Strong Cation-Exchange HPLC**

1. HPLC instrument (Hewlett Packard 1100 series) with autosampler (Agilent Technologies 1200).
2. Polysulfoethyl A chromatography column (100×94 2.1 mm—300 Å; Hichrom Ltd, UK).
3. Guard cartridge (Hichrom Ltd, UK; *see Note 9*).
4. Buffer A: 10 mM KH<sub>2</sub>PO<sub>4</sub> (pH 2.75), 25 % acetonitrile (*see Note 10*).
5. Buffer B: 10 mM KH<sub>2</sub>PO<sub>4</sub>, 1 M KCl (pH 2.75), 25 % acetonitrile (*see Note 10*).
6. Vacuum centrifuge (Eppendorf, UK).

#### **2.5 Peptide Separation by Isoelectric Focusing**

1. 3100 OFFGEL Fractionator (Agilent Technologies, UK).
2. OFFGEL High Resolution Kit, pH 3–10, 12 samples, 24 fractions (Agilent Technologies, UK).
3. Vacuum centrifuge (Eppendorf, UK).

#### **2.6 Sample Cleanup Prior to Mass Spectrometry**

1. 0.1 % (v/v) trifluoroacetic acid (TFA; Sigma Aldrich, UK) in HPLC-grade H<sub>2</sub>O.
2. ZipTips C18 with 0.6 µl bed of chromatography media (Millipore, USA).
3. “Wet solution”: 25 % acetonitrile in HPLC-grade H<sub>2</sub>O.
4. “Equilibration and wash solution”: 0.1 % TFA in HPLC-grade H<sub>2</sub>O.
5. “Elution solution”: 50 % acetonitrile/0.1 % TFA in HPLC-grade H<sub>2</sub>O.

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### **3 Methods**

#### **3.1 Total Protein Extraction from Hepatocellular Carcinoma Cell Line**

1. Add protease inhibitor cocktail to RIPA buffer to a final concentration of 1.5×; keep on ice. Bring a microcentrifuge for the 10,000×*g* spin to 4 °C.
2. Following your preferred cell treatment, detach cells from flask surface using trypsin and centrifuge cell suspension for 5 min at 200×*g* RT.

3. Wash cell pellets with 1× PBS and centrifuge again for 5 min at  $1200\times g$ .
4. Resuspend cell pellet in 350  $\mu$ l RIPA buffer containing PI and leave on ice for 10 min.
5. Vortex lysed cells, then place on QIAshredder column, and centrifuge for 2 min at  $10,000\times g$ .
6. Measure protein concentration of resulting eluate using BCA assay.

### **3.2 Membrane and Cytosol Protein Extraction from Mouse Liver Tissue**

1. Bring both an ultracentrifuge and swinging bucket centrifuge to 4 °C.
2. Add protease inhibitor cocktail to of ice-cold HEM buffer (*see Note 1*) to a final concentration of 1.5×; keep on ice.
3. From frozen liver lobes (*see Note 2*) excise approximately 50 mg of liver with a razor blade on dry ice. Place into Dounce homogenizer with 6 ml of ice-cold HEM buffer and homogenize using electric drill (*see Note 3*).
4. Transfer sample to sterile 15 ml conical tube and keep on ice while repeating for all biological replicates. Rinse pestle and container in between each sample with ddH<sub>2</sub>O.
5. Centrifuge samples in swinging bucket centrifuge at  $2000\times g$  for 10 min at 4 °C.
6. Transfer supernatant (containing membrane and cytosol proteins) to polyallomer ultracentrifuge tubes on ice; add ~4 ml of HEM buffer with PI and balance tubes within 1/10th of a gram of each other. Discard pellet (*see Note 4*).
7. Centrifuge at  $100,000\times g$  for 30 min at 4 °C.
8. Transfer the supernatant, representing the cytosolic fraction, into Amicon Ultra-15 Centrifugation Filter Device and centrifuge for 1 h at  $4000\times g$  at 4 °C to collect concentrated cytosolic protein (>3 kDa).
9. Re-homogenize (*see Note 5*) the membrane-enriched pellet in 0.5–1.0 ml of fresh HEM buffer with PI.
10. Determine protein concentration using BCA assay before aliquoting samples and storing at –80 °C.

### **3.3 Protein Desalting/Buffer Exchange, Digestion, and iTRAQ Labeling**

1. Remove storage solution from 2 ml ZEBA columns (*see Note 6*) by centrifuging at  $2000\times g$  for 2 min. For tissue samples use RIPA for buffer exchange; for cells use ddH<sub>2</sub>O for desalting. Add 1 ml RIPA buffer with PI or ddH<sub>2</sub>O to column resin and centrifuge for 2 min at  $2000\times g$ ; repeat three times. Then add samples to column resin and centrifuge for 2 min at  $2000\times g$ . Process BSA control alongside protein samples up until iTRAQ labeling (**step 9**).

2. Collect the resulting desalted protein eluate and quantify protein concentration by BCA assay.
3. Aliquot 100  $\mu\text{g}$  of protein into low-bind microcentrifuge tubes (*see Note 7*) from each treatment and dry in vacuum centrifuge at 45 °C; dried sample can be stored at -80 °C prior to digestion.
4. If stored at -80 °C bring dried protein samples to RT, and then add 40  $\mu\text{l}$  of 1 M, pH 8.5, TEAB buffer (*see Note 8*) and 2  $\mu\text{l}$  of 2 % sodium dodecyl sulfate (SDS; denaturing agent from iTRAQ kit). Vortex thoroughly to solubilize, and then pulse spin to bring the sample to the bottom of the tube.
5. Add 4  $\mu\text{l}$  of *tris*(2-carboxyethyl)phosphine (TCEP; reducing agent from iTRAQ kit); vortex thoroughly, then pulse spin, and incubate for 1 h at 60 °C.
6. Add 1  $\mu\text{l}$  of methyl methanethiosulfonate (MMTS; cysteine blocking reagent from iTRAQ kit); vortex thoroughly, then pulse spin, and incubate for 10 min at RT.
7. Add 10  $\mu\text{l}$  of 1  $\mu\text{g}/\mu\text{l}$  trypsin solution (reconstituted in 50 mM acetic acid) vortex thoroughly, then pulse spin, and incubate, shaking, at 37 °C for 12–16 h.
8. Post-digestion, dry down samples by vacuum centrifugation at 45 °C and prepare 100  $\mu\text{l}/\text{sample}$  7:3 (v/v) ethanol (100 %): TEAB (0.5 M) solution.
9. Resuspend peptide digests in 80  $\mu\text{l}$  of ethanol:TEAB by vortexing and then sonicate for 5 min at RT in sonicating water bath. Repeat to ensure complete solubilization.
10. Bring required iTRAQ reagent vials (114, 115, 116, 117) to RT; pulse spin, add each peptide digest to the appropriate iTRAQ reagent vial, and vortex thoroughly.
11. Rinse each sample tube with the additional 20  $\mu\text{l}$  of ethanol:TEAB solution and transfer to the correct iTRAQ reagent vials. Vortex, pulse spin, and then incubate for 1 h at RT.
12. Combine iTRAQ-labeled samples into single tube; rinse empty tubes with one aliquot of 100  $\mu\text{l}$  of 50 % acetonitrile and add to combined tube.
13. Dry down pooled sample via vacuum centrifugation at 45 °C.

### **3.4 Peptide Separation by Strong Cation-Exchange HPLC**

1. The dried, digested, and iTRAQ-labeled sample (one tube) was resuspended in 100  $\mu\text{l}$  of buffer A.
2. Set up guard cartridge (*see Note 9*) before HPLC column.
3. Condition the HPLC column by introducing buffer A (*see Note 10*) for 30 min at a flow rate of 1.5 ml/min.
4. Use digested BSA sample to check both the efficiency of the tryptic digestion and the separation efficiency of the column using the stepped gradient (Table 1).

**Table 1**

**The stepped gradient that was followed during the SCX approach. The pressure was set to 400 bar**

| Time (min) | % Buffer B | Flow rate (ml/min) |
|------------|------------|--------------------|
| 0.00       | 0.00       | 1.00               |
| 0.01       | 0.00       | 0.80               |
| 10.00      | 0.00       | 0.80               |
| 10.01      | 0.10       | 0.80               |
| 10.02      | 6.00       | 0.80               |
| 12.02      | 6.00       | 0.80               |
| 12.03      | 9.00       | 0.80               |
| 14.03      | 9.00       | 0.80               |
| 14.04      | 11.00      | 0.80               |
| 16.04      | 11.00      | 0.80               |
| 16.05      | 13.00      | 0.80               |
| 18.05      | 13.00      | 0.80               |
| 18.06      | 15.00      | 0.80               |
| 20.06      | 15.00      | 0.80               |
| 20.07      | 17.00      | 0.80               |
| 22.07      | 17.00      | 0.80               |
| 22.08      | 19.00      | 0.80               |
| 24.08      | 19.00      | 0.80               |
| 24.09      | 23.00      | 0.80               |
| 26.09      | 23.00      | 0.80               |
| 26.10      | 27.00      | 0.80               |
| 28.10      | 27.00      | 0.80               |
| 28.11      | 33.00      | 0.80               |
| 30.12      | 33.00      | 0.80               |
| 30.13      | 40.00      | 0.80               |
| 32.13      | 40.00      | 0.80               |
| 33.14      | 50.00      | 0.80               |
| 38.14      | 50.00      | 0.80               |
| 39.15      | 100.00     | 0.80               |
| 42.16      | 100.00     | 0.80               |

(continued)

**Table 1**  
(continued)

| Time (min) | % Buffer B | Flow rate (ml/min) |
|------------|------------|--------------------|
| 42.17      | 6.00       | 0.80               |
| 43.17      | 6.00       | 0.80               |
| 43.18      | 0.00       | 0.50               |
| 50.01      | 0.00       | 0.10               |

- Inject the entire volume of sample with a 100  $\mu$ l injection volume and collect fractions in 2-min time slices; use LC results to assess how many fractions contain sample (*see Note 11*).
- Dry down collected fractions by vacuum centrifugation at 45 °C.

### 3.5 Peptide Separation by Isoelectric Focusing

- Make up “peptide OFFGEL stock solution (1.25 $\times$ )” and “peptide-immobilized pH gradient (IPG) strip rehydration solution” with the appropriate ampholytes depending on which IPG strips you are using according to kit instructions (*see Note 12*). Assemble the IPG strips, frames, and electrodes according to the manufacturer’s protocol.
- Resuspend by vortexing the dried peptide samples in 3.6 ml 1 $\times$  OFFGEL stock solution (e.g., 2.88 ml 1.25 $\times$  OFFGEL stock solution + 0.72 ml dH<sub>2</sub>O). Load 150  $\mu$ l of this sample in each of the 24 wells.
- Samples should be focused using a maximum current of 50  $\mu$ A and the focusing is stopped after the total voltage reaches 50 kVh (60 h). During the focusing, pads on the electrodes should be exchanged to prevent evaporation (*see Note 13*).
- After focusing, 50–150  $\mu$ l of peptide fractions should be recovered for each well and transferred to individual microfuge tubes. To recover as much as possible of the focused peptides, 150  $\mu$ l of HPLC-grade methanol should be added to each well, incubated for 15 min without voltage, and then added to the appropriate tube.
- Dry down collected fractions by vacuum centrifugation at 45 °C.

### 3.6 Sample Cleanup Prior to Mass Spectrometry

- Resuspend peptide samples (fractionated by either SCX or IEF) in 120  $\mu$ l 0.1 % TFA.
- Equilibrate the ZipTip by first aspirating 10  $\mu$ l of the “wet solution” and discarding to waste, repeat once; then aspirate 10  $\mu$ l of the “equilibration and wash solution” and discarding to waste, repeating three times.
- Bind peptides to ZipTip by cycling (aspirate-dispense-aspirate-dispense) ten times with your sample.

4. Wash sample by aspirating 10  $\mu$ l of the “equilibration and wash solution” and discarding to waste; repeat three times.
5. Elute sample by aspirating 10  $\mu$ l of the “elution solution” and collecting in microfuge tubes; repeat six times until 70  $\mu$ l is collected in all.
6. Dry down collected cleaned up fractions by vacuum centrifugation at 45 °C. Resuspend in 10  $\mu$ l formic acid just prior to nLC-ESI-MS/MS analysis.

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## 4 Notes

1. An alternative buffer for tissue lysis may be applied as deemed appropriate.
2. We recommend at the time of study termination excising the same liver lobe from all animals, immediate snap freezing in liquid nitrogen, and then keeping on dry ice until transfer to -80 °C.
3. In our case we used the lowest speed setting on a very powerful bench-top electric drill and used ten, slow, up-and-down strokes for each sample. This may vary depending on your setup; just keep consistent for all samples.
4. Pellet contains nuclear proteins and cellular debris; you may wish to use differential detergent fractionation here to isolate nuclear proteins as well.
5. We use pestle manually here in addition to pipetting.
6. Methanol-chloroform and other precipitation methods may be used, but we have experienced better recovery and reproducibility using Zeba columns for buffer exchange and sample concentration.
7. Drying down by vacuum centrifuge may result in sample adhering on the walls of the tube. To minimize any protein loss and to make resuspension easier, low-bind tubes should be used in all drying, by vacuum centrifuge, steps.
8. The TEAB buffer that is included in the iTRAQ kit is 0.5 M. We have had better recovery of the sample from the dry phase using 1 M TEAB for resuspension of the dried sample before the iTRAQ labeling process begins. For the next step and whenever required, the TEAB buffer provided by the iTRAQ kit should be used.
9. Although not necessary, it is advised to use a guard cartridge attached in front of the HPLC column. In case of blockage due to sample impurities the column remains unaffected and the guard cartridge is cheaper to replace.

10. For strong cation exchange, the pH of the buffers should be tightly controlled below or equal to 3. Whenever buffers need to be replaced, the pH levels should be exactly the same as previously.
11. In our case, separating peptide digests of total protein from HuH7 cells, we had sample in first 17 fractions.
12. The OFFGEL stock solution contains glycerol; this can make later sample resuspension difficult and interfere with the subsequent performance of the mass spectrometer by blocking the nLC lines. It has been suggested that the glycerol can be omitted or reduced but we have not tested this.
13. The total run for the OFFGEL fractionation is around 60 h. In order to prevent water evaporation from the pads that will lead to insufficient peptide separation, upper electrode pads should be changed every 15 h with fresh pads wetted in ddH<sub>2</sub>O.

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