Protocol

Preparation of Peptides from Yeast Cells for iTRAQ Analysis

Eric S. Simon

INTRODUCTION

A commercial reagent, known as the isobaric tag for relative and absolute quantification (iTRAQ), makes it possible to analyze multiple samples simultaneously. The ability of iTRAQ to compare relative protein abundances across as many as eight samples is a significant advantage over other stable isotope strategies, such as stable isotope labeling by amino acids in cell culture (SILAC) and isotope-coded affinity tag (ICAT). The iTRAQ protocol set compares the proteome of *Saccharomyces cerevisiae* under two different metabolic states (fermentation versus respiration) with the goal of determining the proteins involved in each of the activated pathways by measuring their relative abundances using iTRAQ. This protocol describes the preparation of yeast peptides in different metabolic states for iTRAQ analysis.

RELATED INFORMATION

Protocols are available for **Labeling Yeast Peptides with the iTRAQ Reagent** (Simon 2011a), **iTRAQ-Labeled Yeast Peptide Clean-Up Using a Reversed-Phase Column** (Simon 2011b), and **Isoelectric Focusing of iTRAQ-Labeled Yeast** (Simon 2011c). See also **Bradford Assay** (Harlow and Lane 2006).

The original iTRAQ reagent can compare up to four samples in parallel (Ross et al. 2004), whereas a more current version can handle multiplexing of up to eight samples (Choe et al. 2007). Figure 1 provides a diagram of the workflow that will be followed in the iTRAQ protocols. Briefly:

1. The peptides are labeled with different iTRAQ tags. With four iTRAQ tags (114, 115, 116, and 117) and only two samples, a double duplex experiment can be performed. Two aliquots of the peptides generated from the cells grown under fermentative conditions are labeled with tags 114 and 115. The peptides generated from the cells grown under respiration are labeled with tags 116 and 117.
2. After quenching the labeling reactions, the peptides are combined into one tube and cleaned up on a reversed-phase C18 cartridge to remove salts and by-products of the iTRAQ reaction.
3. The peptides are fractionated by isoelectric focusing (IEF).
4. The peptides are separated by reversed-phase chromatography, and fractions are incrementally mixed with matrix and spotted by an automated matrix-assisted laser desorption/ionization (MALDI) plate-spotting robot.
5. The MALDI plates are analyzed with a 4700 MALDI time-of-flight (TOF)/TOF mass spectrometer.
6. The data are then analyzed using ProteinPilot software.

MATERIALS

RECIPES: Please see the end of this article for recipes for reagents marked with <**R**>.

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

Adapted from *Proteomics: A Cold Spring Harbor Laboratory Course Manual* (ed. Link and LaBaer) CSHL Press, Cold Spring Harbor, NY, USA, 2009.
Cite as: Cold Spring Harb Protoc; 2011; doi:10.1101/pdb.prot5616

Reagents

Acetone, cold (chill at –20°C)
Bradford protein assay reagents
Dithiothreitol (200 mM) (DTT)
<R>DNase/RNase solution (used in Lysis solution for iTRAQ)
Iodoacetamide (200 mM)
<R>Lysis solution for iTRAQ
Triethylammonium bicarbonate (TEAB) (500 mM)
Trypsin (sequence grade; Promega)
Urea (7 M)/TEAB (500 mM)
Yeast cells (*S. cerevisiae* wild-type strain BJ1991)
<R>Yeast extract-peptone-dextrose growth medium (YEPD)
<R>Yeast extract-peptone-glycerol (YPG) medium for iTRAQ

Equipment

Cell culture plates
Centrifuge
H_2O bath or heat block set at 37°C
Micropipettor
Shaker
Vacuum evaporator (e.g., SpeedVac, Savant)

METHOD

Growing Yeast Cells

1. To grow cells:

   i. For fermentative growth, cultivate *S. cerevisiae* wild-type strain BJ1991 on rich YEPD growth medium. Inoculate a cell colony in 50 mL of liquid YEPD growth medium and grow to an optical density (OD) of 1 (~107 cells). Add 0.1–5 mL (volume depends on calculated doubling time and number of cells desired) of the starter culture to 1 L of liquid YEPD growth medium and grow to an OD of 1. Divide into 50-mL aliquots and pellet the cells by centrifugation (5000 rpm). This yields approximately 0.8–1 g of cells per 50 mL.

   ii. For growth under respiration conditions, grow a wild-type starter culture in rich YEPD growth medium to an OD of 1–2 (as described in Step 1i). Add 0.1–5 mL of the starter culture to 1 L of liquid YEPD growth medium and grow to an OD of 1 as described in Step 1i. Pellet the cells (50-mL aliquots) by centrifugation (5000 rpm). Transfer the cells to YPG medium for iTRAQ containing 3% (v/v) glycerol. Collect the cells by centrifugation from 50-mL aliquots (5000 rpm) after 16 h. This yields approximately 0.8–1 g of cells per 50 mL.

   The yeast cell pellets from Steps 1 and 2 can be stored frozen at –80°C.

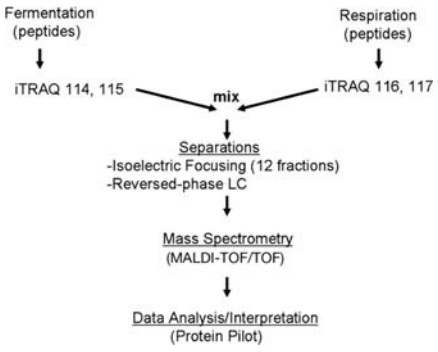

FIGURE 1. Workflow diagram for the yeast proteome experiment using the iTRAQ protocol.

Lysis of Yeast Cell Pellets

2. Allow the yeast cells to thaw, and then add 4 mL of lysis solution for iTRAQ to 0.9 g of yeast cell pellet. Shake the mixture gently for 30 min at room temperature.
3. Remove cell debris by centrifugation at 4°C.
4. Collect the soluble protein. Using the **Bradford method**, determine the amount of protein recovered, and store the sample at –80°C until needed.

Precipitation of Protein

5. Add cold acetone to the protein sample at a 5:1 ratio (acetone/sample, v/v). Incubate it for 2 h at –20°C.
6. Centrifuge the sample at 12,500*g* for 5 min at 4°C.
7. Discard the supernatant. Wash the pellet with cold acetone and centrifuge again.
8. Dry the protein pellet in a vacuum evaporator for 10 min to remove residual acetone.
9. Dissolve the pellet in 60 μL of 7 M urea/500 mM triethylammonium bicarbonate (TEAB).

   TEAB is used in place of ammonium bicarbonate because it has no primary amines, which would interfere with iTRAQ labeling.

Reduction, Alkylation, and Digestion of Proteins

10. Add 5 μL of 200 mM DTT to reduce the disulfide bonds. Incubate for 1 h at room temperature.
11. Add 20 μL of 200 mM iodoacetamide (carbamidomethylation). Incubate the reactions for 1 h in the dark at room temperature.
12. Quench the alkylation reaction by adding 20 μL of 200 mM DTT and incubating for 1 h at room temperature.
13. Dilute the protein samples with 500 mM TEAB, so that the urea concentration is <2 M.
14. Add trypsin to a final 1:25 ratio (trypsin/sample). Incubate the mixture overnight at 37°C.
15. Store the peptides at –20°C.

DISCUSSION

The field of proteomics, in its brief history, has progressed rapidly from merely identifying proteins within large, complex mixtures to additionally determining the relative abundance of proteins between two or more samples. This progression to quantitative measurements can be attributed to the advancements made in mass spectrometry (MS) and the adoption of stable isotope labeling strategies for distinguishing the relative abundance of like peptides between samples (Gygi et al. 1999; Ong and Mann 2005). Typically, a set of reagents consists of one tag that has a natural isotope distribution and a complementary tag consisting of the exact elemental composition and chemical structure, except that it is isotope enriched, typically with ^{13}C, although ^{2}H and ^{15}N can be used as well. The isotope-enriched tag, or heavy reagent, is chemically equivalent to the light reagent but has a higher mass. In a typical workflow, peptides from a control sample are labeled with the light reagent, and an experimental sample is labeled with the heavy reagent. The two labeled peptide samples are then mixed, fractionated, and analyzed by MS. A peptide present in both samples will have essentially the same physicochemical properties, including charge state, isoelectric point, and hydrophobicity, despite having tags of slightly different mass. As a peptide elutes, relative quantification is determined in the MS mode by integrating the peaks under the extracted ion chromatograms of each of the light and heavy labeled peptides across their chromatographic peak (Ong and Mann 2005). Figure 2 provides an example of this concept. Examples of stable isotope labeling strategies that yield MS-mode quantification in this way are SILAC (Ong et al. 2002) and ICAT (Gygi et al. 1999).

Despite the effectiveness of these strategies, no more than two or three samples can be compared in one experiment. This is due to the inherent increase in complexity of the spectra in the MS mode when multiple isotope-enriched tags are used. For example, if a control is tagged with a light reagent and the

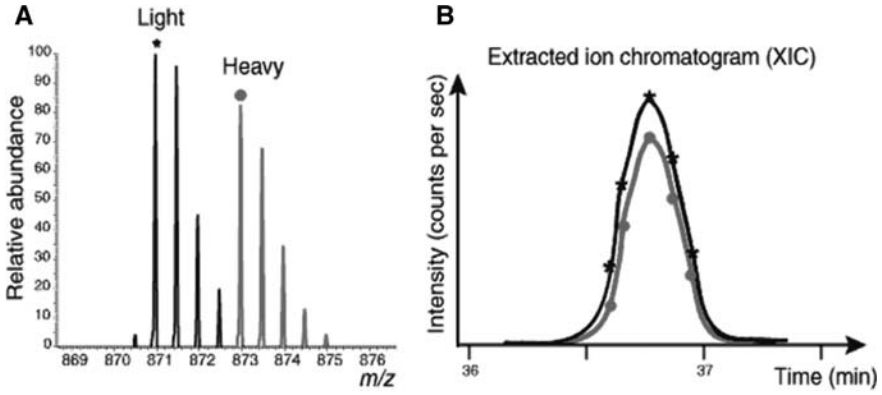

FIGURE 2. (*A*) Isotope clusters of a peptide labeled with light and heavy stable isotope reagents for relative quantification. The intensity of the monoisotopic peak of the light (*) and heavy (•) analog across the peptides elution profile is called the extracted ion chromatogram for each ion (XIC). (*B*) The area under each XIC correlates to the relative abundance of the peptide between the two samples. (Adapted from Ong and Mann 2005 and reprinted with permission from Nature Publishing Group © 2005.)

test sample is tagged with a corresponding, isotope-enriched heavy reagent, then the mass spectrometer registers two peaks representing that peptide as opposed to just one. As the number of samples increases, the number of higher mass reagents increases, thus increasing the number of peaks in the mass spectrum. This envelope of peaks representing the same peptide can overlap in the spectrum with envelopes of other peptides and can be extremely difficult to sort out.

The iTRAQ reagent allows the comparative analysis of multiple samples simultaneously. Figure 3 provides an illustration of the iTRAQ concept. The reagent itself is made up of three components. The reporter group, an *N*-methylpiperazine moiety, serves as the quantitative component, or reporter ion, observed in the low mass region of MS/MS spectra. Depending on the tag used, it will have a mass of

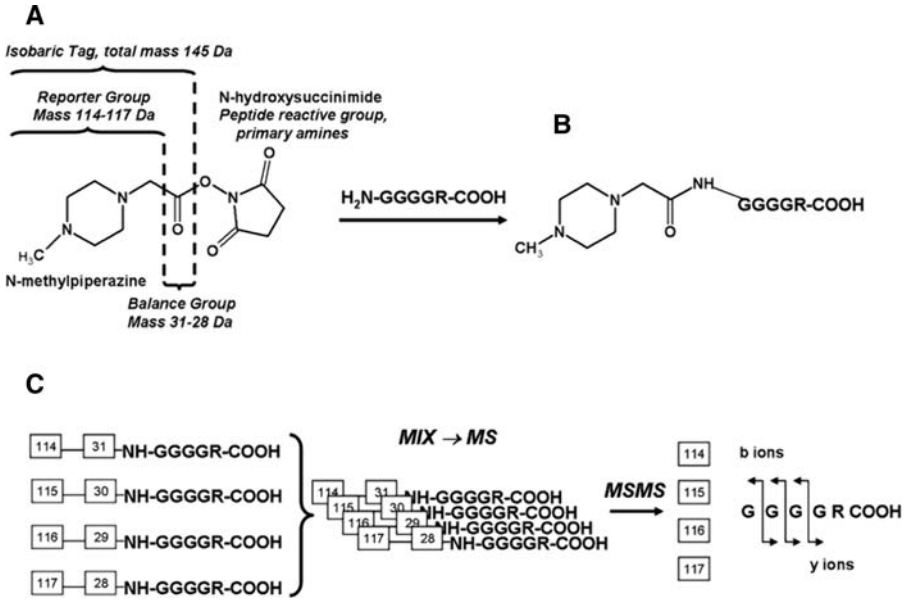

FIGURE 3. (*A*) The functional components of the iTRAQ reagent. (*B*) The *N*-hydroxysuccinimide component reacts with primary amines, resulting in modification of peptides on the amino-terminus and side-chain lysine residues. (*C*) After mixing peptides with different tags, they are indistinguishable in MS mode, but subsequent MS/MS spectra yield quantitative reporter ions at *m/z* 114–117 along with amide backbone fragments. (Modified from Ross et al. 2004 and reprinted with permission from American Society for Biochemistry and Molecular Biology, Inc.)

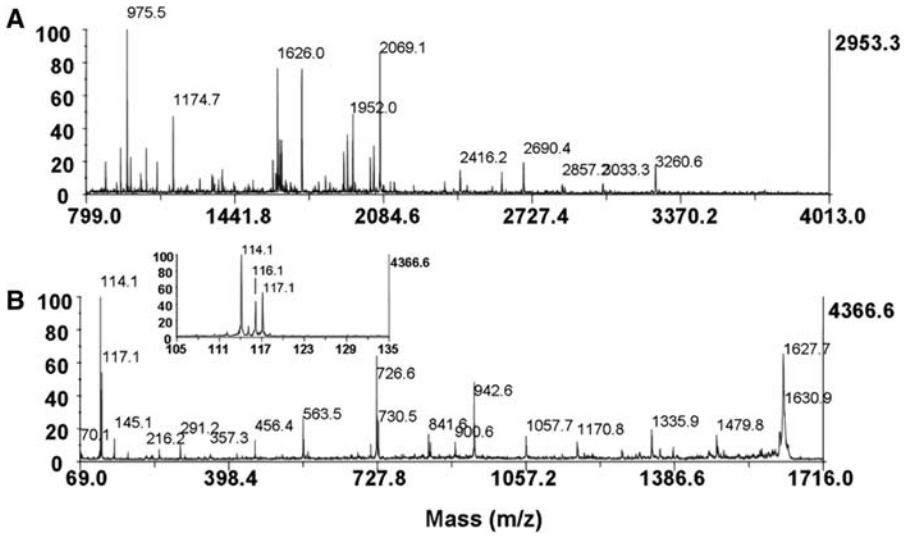

FIGURE 4. (*A*) A representative MS spectrum of iTRAQ-labeled peptides acquired on a MALDI TOF/TOF mass spectrometer. (*B*) A subsequent MS/MS spectrum of precursor ion *m/z* 1626.0 displaying reporter ion (*inset*) and backbone fragments.

114, 115, 116, or 117 Da. The next component of iTRAQ is the mass balancer, a carbonyl group, which is isotope enriched to have an offset mass of 31, 30, 29, or 28 Da, depending on the mass of the reporter group it is paired with. The reporter and mass balancer combine to yield a mass equivalence of 145 Da [(114 + 31), (115 + 30), (116 + 29), and (117 + 28)] across a set of four isobaric tags. The third component of iTRAQ is an amine-reactive group, *N*-hydroxysuccinimide, which derivatizes peptides at primary amines, thus targeting the amino termini and lysine side chains. Once the peptides have been derivatized, they are mixed together. A peptide present in all four samples will appear as a single peak in an MS survey scan. However, once fragmented, the peptide's MS/MS spectrum will contain the signature series of reporter peaks at 114–117 Da that are used for quantification. Also present in the MS/MS spectrum are a series of peaks representing amide backbone fragments, like b and y ions, that are used for peptide identification. Figure 4 displays two spectra acquired on a MALDI TOF/TOF mass spectrometer that illustrate these concepts. Figure 4A shows a precursor spectrum of iTRAQ-labeled peptides. Figure 4B shows an MS/MS spectrum of the precursor ion of *m/z* 1626.0. Each of the iTRAQ tags contains an isotope distribution such that the derivatized peptides are indistinguishable in MS spectra but yield signature ions in the low mass region of MS/MS spectra. The inset (Fig. 4B) shows the region of the MS/MS spectrum that contains the peaks corresponding to the iTRAQ reporter ions. The intensities of the reporter ions reflect the relative abundance of the peptide in each of the samples queried.

REFERENCES

Choe L, D'Ascenzo M, Relkin NR, Pappin D, Ross P, Williamson B, Guertin S, Pribil P, Lee KH. 2007. 8-Plex quantitation of changes in cerebrospinal fluid protein expression in subjects undergoing intravenous immunoglobulin treatment for Alzheimer's disease. *Proteomics* **7:** 3651–3660.

Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R. 1999. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat Biotechnol* **17:** 994–999.

Harlow E, Lane D. 2006. Bradford assay. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot4644.

Ong SE, Mann M. 2005. Mass spectrometry-based proteomics turns quantitative. *Nat Chem Biol* **1:** 252–262.

Ong SE, Blagey B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, Mann M. 2002. Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol Cell Proteomics* **1:** 376–386.

Ross PL, Huang YN, Marchese JN, Williamson B, Parker K, Hattan S, Khainovski N, Pillai S, Dey S, Daniels S, et al. 2004. Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. *Mol Cell Proteomics* **3:** 1154–1169.

Simon ES. 2011a. Labeling yeast peptides with the iTRAQ reagent. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot5617.

Simon ES. 2011b. iTRAQ-labeled yeast peptide clean-up using a reversed-phase column. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot5618.

Simon ES. 2011c. Isoelectric focusing of iTRAQ-labeled yeast. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot5619.

RECIPES

NOTE: Recipes for reagents marked with the <R> symbol not listed below can be found online at http://www.cshprotocols.org/recipes.

DNase/RNase solution (used in Lysis solution for iTRAQ)

DNase I	1 mg/mL
RNase	0.25 mg/mL
Tris-Cl	500 mM, pH 7.0
$MgCl_2$	50 mM

Lysis solution for iTRAQ

CelLytic (Sigma-Aldrich C4482)	12 mL
<R>DNase I/RNase solution	150 µL
Protease tablets, non-EDTA (Roche 10946900)	3
Tris-carboxyethyl phosphine (TCEP)	5 mM

Yeast extract-peptone-dextrose growth medium (YEPD)

Reagent	Quantity (for 1 L)	Final concentration (w/v)
Bacto peptone	20 g	2%
Yeast extract	10 g	1%
Dextrose	20 g	2%
H_2O	to 1 L	

Sterilize by autoclaving.

Yeast extract-peptone-glycerol (YPG) medium for iTRAQ

Yeast extract	1% (w/v)
Peptone	2% (w/v)
Glycerol	3% (w/v)

Preparation of Peptides from Yeast Cells for iTRAQ Analysis

Eric S. Simon

Cold Spring Harb Protoc; doi: 10.1101/pdb.prot5616