Published in final edited form as:

Curr Protoc Protein Sci. ; 76: 23.4.1–23.4.9. doi:10.1002/0471140864.ps2304s76.

Quantitative Protein Analysis Using Enzymatic [^{18}O]Water Labeling

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Abstract

This unit describes the procedure for enzymatic [^{18}O]water labeling of peptides in order to quantitate relative protein levels for a comparative proteomic experiment.

Keywords

^{18}O -Labeling; Enzymatic Oxygen Labeling; Quantitative Proteomics; Immobilized Trypsin; Back-exchange

BASIC PROTOCOL

As the sequencing of many species' genomes is completed, the study of the protein complement to the genome, the proteome, has emerged as a dynamic field of research. A common approach to characterizing the various states in which a protein exists is comparative proteomics. In this method, the relative amounts of proteins present in two or more samples are compared. An enzyme-catalyzed method for ^{18}O labeling can be used to determine the relative amounts of the proteins present and is described in detail in this unit. Pools of proteins are enzymatically digested in parallel in [^{16}O] (i.e., standard deionized) and [^{18}O]water. In the latter pool, two atoms of ^{18}O are incorporated into the carboxyl terminus of each new peptide. Comparative proteomic studies are performed by mixing the pool of unlabeled peptide (generated in [^{16}O]water) and the pool of isotope-labeled peptide (from [^{18}O]water) and analyzing the peptide pairs by mass spectrometry. Relative quantification is derived from ratios of the isotope pairs. Tandem mass spectrometry experiments provide sequence information from the peptides, which is used to identify the proteins.

This protocol contains optional, but recommended, steps to prepare a protein sample for proteolytic digestion and ^{18}O labeling. This step will reduce and alkylate cysteine residues, as well as denature the protein. Alkylating the protein sample will allow more efficient digestion and avoid mixed disulfide bonds between digested peptides.

Figure 24.3.1 gives an outline of the procedure. Note that both pools (i.e., labeled and unlabeled) must first be digested by trypsin before comparative proteomic investigation.

Materials

Protein samples

50 mM Tris (tris(hydroxymethyl)amino methane)-HCl, pH 8.0 (*APPENDIX 2E*; prepare fresh)

Urea (Sigma)

100 mM Dithiothreitol (DTT, Sigma)

0.5 M iodoacetamide (Sigma)

10 mM Tris-HCl, pH 7.4 (*APPENDIX 2E*)

Modified trypsin, sequencing grade (Promega)

Immobilized trypsin on Poros materials (Applied Biosystems)

Benchtop rotator

BioSpin 6 Column (BioRad)

10% Trifluoroacetic acid (TFA, Pierce)

0.2% Formic acid (FA, Sigma)

Phenylmethanesulfonylfluoride (PMSF, Sigma)

Triethylammonium bicarbonate buffer, pH 8.0 (TEBA, Sigma)

Oasis HLB Material (Waters) packed in-house in TopTips (Glygen)

IsoPro 3.0 software (<http://members.aol.com/msmsoft>)

Additional reagents and equipment for mass spectrometry (Chapter 16)

- 1** Prepare the protein or proteins of interest in a freshly made solution of 50 mM Tris-HCl, pH 8, so that the concentration is ~1 µg/µL.
For the purpose of this protocol, the concentration is 100 µg protein in 100 µL buffer.
- 2** Add 0.024 g urea.
The urea concentration is now 4 M.
- 3** Reduce cysteine residues by adding 10 µL of 100 mM DTT and rotating 1 hr at room temperature.
The DTT concentration is ~10 mM.
- 4** Alkylate the reduced cysteine residues by adding 30 µL of 0.5 M iodoacetamide and rotate 1 hr in the dark at room temperature.

The iodoacetamide concentration is approximately ten times the DTT concentration.

- 5 Desalt the reduced and alkylated protein sample using a BioSpin 6 column. Recover the sample in 10 mM Tris-HCl, pH 7.4 and pool the recovered samples.

These columns are provided by the manufacturer in buffer solution. Directions are also provided about how to remove the buffer, as well as how to load, wash, and recover the protein sample. The manufacturer reports 90% to 95% recovery.

One spin column can desalt 75 μ L of the protein solution, so two columns used in parallel are necessary.

This procedure also removes excess iodoacetamide.

Perform initial protein digestion

- 6 Add 20 μ L of 50 mM Tris-HCl, pH 8, directly to one vial of 20 μ g sequencing-grade modified trypsin.

The final trypsin concentration is 1 μ g/ μ L.

- 7 Add 1 μ L (i.e., 1 μ g) dissolved trypsin per 50 to 100 μ g protein in the sample.

This is 0.5 to 1 μ L when 100 μ g protein has been dissolved in 100 μ L (step 1).

The remaining trypsin can be stored up to 6 months at -80°C in conveniently sized aliquots to avoid repetitive freeze-thaw cycles.

- 8 Check the pH to ensure that it is between 7 and 8.

The pH can be adjusted by adding additional Tris-HCl buffer.

- 9 Incubate overnight at 37°C to allow complete digestion.

- 10 Quench with 10% trifluoroacetic acid to lower the pH to < 3 .

- 11 Desalt the peptide pool using the HLB material. [Desalting protocol]

- 12 Dry the samples for 30 minutes in the speed vac, freeze dry and lyophilized until completely dried.

The following section outlines the steps for incorporating two atoms of ^{18}O into each peptide C-terminus (Fig. 23.4.1). Only one of the peptide pools in the comparative proteomic experiment is labeled with ^{18}O ; however, the procedure is applied to both pools in order to minimize differential sample loss before pooling of two samples for relative quantitation.

Enzymatic $^{18}\text{O}_2$ -labeling

- 13 Clean the immobilized trypsin by washing with 5 vol. of water. Centrifuge in a benchtop centrifuge 1 min at 8000 rpm and discard the supernatant. Repeat two more times. Freeze-dry and lyophilize.

Immobilized trypsin MUST be cleaned prior to use. Prepare an amount of immobilized trypsin equal to ~20% of the sample volume. This is ~20 μL if 100 μg protein was dissolved in 100 μL buffer (step 1).

- 14 Completely dry 500 μL of 50 mM Tris-HCl buffer (pH 8) with lyophilizer. Add 500 μL of H_2^{18}O to make the ^{18}O buffer.

If SpeedVac is used to dry Tris-HCl, a minimal 3 times of addition of anhydrous acetonitrile is needed to completely dry the buffer for reconstitution with H_2^{18}O .

- 15 Reconstitute desalted digested protein sample with 20 μL of acetonitrile.
- 16 Add 30 μL of ^{18}O buffer. Incubate for 10 minutes. Add 50 μL of ^{18}O buffer and incubate for 10 more minutes. For the unlabeled pool, ^{16}O Tris-HCl buffer was added.
- 17 Transfer all 100 μL to previously-washed immobilized trypsin.
- 18 Incubate at 37°C overnight.
- 19 Add 100 μL of anhydrous acetonitrile. Recover the peptides in the supernatant following a 2 min microcentrifugation at 8000 rpm.

Additional or less time may be necessary depending on the particular peptides in the sample.

- 20 **For** desalting with the HLB material (desalting protocol).

- 21 Reconstitute with 0.2% FA. Mix the labeled and unlabeled peptide pools and analyze by mass spectrometry (Fig 23.4.2; also see Chapter 16).

Samples can be analyzed by nanospray, electrospray, LC-MS, or MALDI. The method is compatible with most commercial mass spectrometers as long as they are able to resolve 4 Da.

The mixing ratio can be varied from 1:1 and taken into account in the calculations.

Interpretation and protein quantitation

- 22 Calculate values for the theoretical peak areas for the monoisotope peak of a peptide with known composition (M_0), the peak 2 Da higher (M_2), and the peak 4 Da higher (M_4) for each peptide using IsoPro 3.0 software or a similar software.

The elemental composition and charge state are entered into the software program and the program calculates the theoretical isotopic distribution. The distribution of ^{13}C isotopes in the unlabeled and labeled peptides is assumed to be identical.

- 23 Calculate the ratios of [$^{18}\text{O}/^{16}\text{O}$] peptides using the following equation.

$$\text{Ratio 1} = \frac{I_4 - \frac{M_4}{M_0} I_0 - \frac{M_2}{M_0} \left(I_2 - \frac{M_2}{M_0} I_0 \right) + \left(I_2 - \frac{M_2}{M_0} I_0 \right)}{I_0}$$

where I_0 , I_2 , and I_4 are the observed peak areas for the monoisotopic peak for the peptides without ^{18}O label, the peak 2 Da higher, and the peak 4 Da higher (double ^{18}O labeled peak), respectively.

This equation takes into account not only the ratio of labeled and unlabeled peak areas, but also contributions from incomplete labeling and corrects for the theoretical isotopic distribution of a peptide.

If the peptide sequence is not known, an estimate of the $^{18}\text{O}/^{16}\text{O}$ ratio can be made by taking the ratio of the peak areas of I_4 and I_0 .

Desalting protocol

(Optional; commercial reversed-phased desalting products and procedures can also be used)

1. Solution Preparation:

Solution A: 20% FA in deionized (DI) H_2O ;

Solution B: 70% ACN + 30% (0.2% FA in DI H_2O);

Solution C: (0.5% ACN + 0.1% TFA) in DI H_2O ;

Solution D: (0.5% ACN + 0.2% FA) in DI H_2O .

2. Add 20 μL of solution A to the sample for 5 mins. DO NOT shake or vortex, even if solids were observed. Vortex slight and spin, and then incubate the sample for another 5 mins. Add 1 mL of solution C and incubate for 2 mins. Vortex and spin.
3. Condition the HLB material packed in empty toptip with solutions B and C, consecutively ($3 \times 200 \mu\text{L}$ for each solution). Avoid air bubbles during pipetting and keep the HLB beads wet all the time.
4. Load the sample from Step 2 (150 μL each time) and collect the flow-through in a new clean tube slowly by pipetting out drop by drop.
5. Wash the beads with solutions C and D, consecutively ($3 \times 200 \mu\text{L}$ for each solution).
6. Pipet 20 μL of solution B in a clean tube. Pipette the solution up and down for 5 times to elute the bound peptides. Avoid air bubbles during the process and then pipet out completely at the last time. Then dilute the elution with 180 μL of solution. This sample is ready for immediate LC-MS analysis or Freeze-Dry.

COMMENTARY

Background Information

Enzyme-catalyzed ^{18}O labeling enables relative protein quantitation in high throughput proteomic strategies (Fenselau, et al., 2009). Historically, comparative proteomic studies

have been carried out using two-dimensional gel electrophoresis (*UNITS 10.4 & 22.1*). Currently, there is a push to find alternatives to two-dimensional gel electrophoresis because of its limited dynamic range, loss of hydrophobic proteins, and tedious procedures.

Enzyme-catalyzed ^{18}O labeling provides highly specific isotopic labeling of all but one of the peptide products from each protein. Labeling has been demonstrated to be complete and stable. The stability of the label makes the method compatible with multiple separation techniques that can add to the diversity of the experiment (e.g., affinity chromatography to isolate cysteine-containing peptides). The double ^{18}O labels can be introduced by various enzymes including trypsin, Lys-C, chymotrypsin, and Glu-C, as long as the same proteolytic enzyme is used to generate the peptides. The mechanism of incorporation is shown schematically in Figure 23.4.3. The enzyme covalently bonds the target residue and the intermediate then reacts with ^{18}O or ^{16}O water. Double ^{18}O labeling is achieved because the enzyme rebinds the C-terminal residue of the peptide product repetitively. In order to achieve complete double ^{18}O labeling, the sample must be digested in highly enriched (>95%) ^{18}O water.

Comparative proteomic studies are carried out by incubating one pool of proteins or peptides with an enzyme in ^{16}O water (unlabeled) and the other pool with the same enzyme in ^{18}O (double ^{18}O labeled). The two pools are then mixed and analyzed by mass spectrometry. The labeled and unlabeled peptides co-elute chromatographically and appear as isotopic doublets in the mass spectrometer (Fig. 23.4.2). Therefore, the relative abundance of each peptide can be directly quantitated based on peak areas. The double ^{18}O label is located at the peptide C-terminus. This label aids peptide sequencing in tandem mass spectrometry experiments because the y -ions, or C-termini-containing ions, carry the double label and can be readily identified in the mass spectrum. It then becomes trivial to walk down the sequence by following the y -ion doublets. The parent protein is then identified by querying the sequence information in a protein database (e.g., Mascot, Matrix Science).

Previous studies on ^{18}O proteolytic labeling performed digestion and labeling in one step (Yao et al., 2001; Reynolds et al., 2002). The current method, detailed in this unit, decouples the digestion and labeling steps (Yao et al., 2003). The decoupling adds to the versatility of the method because each step can be optimized individually. For example, if a high urea concentration is necessary to maintain a protein in solution, the sample could be digested by Lys-C and then the more soluble peptides could be labeled by trypsin. Most importantly, the two-step procedure eliminates the need to redissolve dried proteins in ^{18}O water before the label is incorporated. Use of immobilized enzyme for the oxygen incorporation makes possible the ready enzyme removal. The residual enzyme is accounted for literature observations in the back-exchange of the oxygen labels during storage and sample preparations (Fenselau, et al., 2009).

This method has also been applied to determine sites of N-linked glycosylation (Reynolds, et al., 2002). When N-linked sugars are enzymatically cleaved from a protein by glycopeptidase ^{18}O water, a single ^{18}O atom is incorporated. Following further digestion in ^{18}O water by trypsin, Glu-C, or other equivalent enzymes, the peptides that contained N-glycosylation sites have three ^{18}O labels and are readily recognized by mass spectrometry,

as shown in Figure 23.4.4. The site can usually be located by mass changes in the sequence ions from a tandem mass spectrometry experiment.

In contrast, acid-catalyzed oxygen labeling can incorporate higher number of ^{18}O labels into peptides, but side-reactions associated with highly acidic reaction conditions limits its application for complex proteomic samples (Jiang et al., 2010; Wang et al., 2011).

In summary, proteolytic ^{18}O labeling is a flexible, global, and robust strategy for comparative proteomics. Currently, this method is being employed in the authors' laboratory for various quantitative proteomic measurements in studying human diseases.

Critical Parameters

In this unit, proteolytic ^{18}O labeling is described using trypsin to catalyze isotope incorporation. (Other proteases are known to catalyze double ^{18}O incorporation, including Lys-C and Glu-C.) Since these enzymes are necessary to digest the protein and catalyze isotope incorporation into the peptide products, the sample conditions must be compatible with the enzyme—i.e., proper pH and salt concentrations should be used to keep the protease folded and functional. The volumes and concentrations described in this unit may be scaled up or down to suit the experiment.

Troubleshooting

If complete double ^{18}O incorporation is not observed, additional incubation time or addition of a second aliquot of enzyme may be necessary. Also, check the pH to ensure that the enzyme is functional.

Anticipated Results

This protocol should produce a set of proteolytic peptides, all of which, other than the peptide containing the original C-terminus, are labeled >95% with two ^{18}O atoms. The $^{18}\text{O}_2$ labeled peptides can then be mixed with the unlabeled pool and relative quantitative information derived by mass spectrometry. The procedure has been successfully applied to study adenovirus strains using MALDI-TOF mass spectrometry (Yao et al., 2001). The method has also been used to quantitate changes in phosphorylation (Bonenfant et al., 2003). Figure 23.4.5 shows an electrospray mass spectrum of the human protein cofilin isolated from a human breast cancer cell line. The $^{18}\text{O}_2$ -labeled peptide was isolated from drug-susceptible cells, while the unlabeled peptide counterpart was isolated from doxorubicin-resistant cells. The peptides were observed in a 1:1 ratio, so no change in regulation was observed.

Time Considerations

The labeling step has been evaluated using trypsin with a mixture of peptides terminating in lysine or arginine (Yao et al., 2003). The time required for 95% incorporation of two ^{18}O atoms varied from 5 to 90 min, although over-night incubation can be used as a default setting for labeling complex proteome digests.

Stability of ^{18}O Labels

If solution-phase trypsin in the first steps of digestion was not inactivated or removed, back-exchange of ^{18}O may occur if the immediate sample analysis is not performed. The stability of oxygen labels was investigated by storing the $^{18}\text{O}_2$ -labeled peptide solutions at 4°C under four conditions for 7 days: (1) 0.2% FA, (2) 0.2% FA with $2\ \mu\text{M}$ PMSF, (3) 20 mM TEAB, and (4) 20 mM TEAB with $5\ \mu\text{M}$ PMSF. No measureable increases in the signals for peptides with the single and zero ^{18}O labels were observed for all conditions. Effect of PMSF is not evident, which is attributed to efficient inactivation or removal of solution-phase trypsin.

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Figure 23.4.1.

Schematic representation of the enzyme-catalyzed ^{18}O -labeling strategy for comparative proteomics with digestion and labeling steps decoupled.

Figure 23.4.2.

Electrospray mass spectrum of a pair of doubly charged peptides, unlabeled (I_0) and labeled (I_4), and the theoretical molecular ion profile of the unlabeled peptide. **(A)** Observed isotopic distribution of ^{18}O and ^{16}O mixed samples. **(B)** Theoretical isotopic distribution without ^{18}O labeling.

Figure 23.4.3.

Schematic representation of the mechanism of double ^{18}O incorporation with digestion and labeling in a single step.

Figure 23.4.4.

Electrospray mass spectrum of two peptides, which were deglycosylated and digested in [^{18}O]water in parallel with the [^{16}O] counterparts. A triply charged peptide that did not contain a glycosylation site, and a singly charged peptide that had been glycosylated are detected with the respective 4 and 6 Da mass increments. (Reynolds et al., 2002) Reprinted with permission.

Figure 23.4.5.

Electrospray mass spectrum of doubly charged N-terminally acetylated peptide ASGVAVSDGVIK from human cofilin isolated from MCF-7 human breast cancer cell lines. The unlabeled peptide was isolated from drug-susceptible cells, while the double ^{18}O labeled peptide was isolated from doxorubicin-resistant cells. The ratio of cofilin in the two cell lines was calculated as resistant/susceptible: 1.2 ± 0.2 .