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Trypsin-Catalyzed Oxygen-18 Labeling for Quantitative Proteomics

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Abstract

Stable isotope labeling based on relative peptide/protein abundance measurements is commonly applied for quantitative proteomics. Recently, trypsin-catalyzed oxygen-18 labeling has grown in popularity due to its simplicity, cost-effectiveness, and its ability to universally label peptides with high sample recovery. In ¹⁸O labeling, both C-terminal carboxyl group atoms of tryptic peptides can be enzymatically exchanged with ¹⁸O, thus providing the labeled peptide with a 4 Da mass shift from the ¹⁶O-labeled sample. Peptide ¹⁸O labeling is ideally suited for generating a labeled “universal” reference sample used for obtaining accurate and reproducible quantitative measurements across large number of samples in quantitative discovery proteomics.

Keywords

LC-MS; ¹⁸O labeling; quantitative proteomics; stable isotope labeling; enzymatic labelling

1. Introduction

Stable isotope labeling is commonly applied in shotgun proteomics for relative peptide/protein abundance quantitation. The incorporation of stable isotopes into peptides results in a fixed mass shift, yet does not affect the chemical properties of the peptides (e.g., peptide LC elution times or ionization efficiency), thus allowing relative abundances of labeled peptides from different samples to be accurately quantified during a single analysis using liquid chromatography coupled with mass spectrometry (LC-MS) (1, 2). In trypsin-catalyzed ¹⁸O labeling, a digested protein sample is placed into H₂¹⁸O where trypsin catalyzes the exchange of either one or both of the C-terminal carboxyl group ¹⁶O atoms of the peptide into ¹⁸O (see Fig. 3.1), thus providing the labeled peptide with a 2 or 4 Da mass shift, respectively, from the unlabeled (i.e., ¹⁶O-labeled) sample (see Fig. 3.2) (3). Similarly, other serine proteases such as Lys-C and Glu-C can also catalyze C-terminal carboxyl group oxygen exchange for ¹⁸O-labeling, although these enzymes target different amino acid C-termini than trypsin (4).

Trypsin-catalyzed ¹⁸O labeling is a popular approach for relative quantitation because it is simple, cost-effective, and flexible in its ability to specifically label all peptides that end with Arg or Lys residue on peptide C-terminal. Moreover, ¹⁶O/¹⁸O labeling has no lower limit on the amount of peptides that can be labeled and the procedure results in nearly 100% sample recovery (5), which is important for processing small amounts of samples. However, there

are two potential limitations of ^{18}O -labeling that need to be addressed in order to effectively apply such labeling for accurate quantitative measurements. The first limitation is the potentially incomplete labeling of different peptides, which has been recently addressed by de-coupling the enzymatic digestion and the ^{18}O -labeling steps. This strategy significantly improves the overall labeling efficiency where nearly all peptides can reach oxygen exchange equilibrium with optimized incubation time and enzyme concentration (5–8). The second limitation is the potential of oxygen “back-exchange” where ^{18}O is replaced with ^{16}O after labeling through a residual trypsin-catalyzed reaction when the sample is placed into a buffer containing ^{16}O water. Several approaches have been reported to minimize this back-exchange by inactivating trypsin by cysteine alkylation (8) or by using immobilized trypsin rather than solution-phase trypsin (9). More recently, we reported a simple procedure for effectively inactivating residue trypsin by boiling the labeled peptides for 10 min, followed by the addition of 5% (v/v) formic acid (5).

Stable ^{18}O -labeling is ideally suited for generating a labeled “universal” reference sample for large-scale quantitative discovery proteomics (10). As a pool across experimental samples, the “universal” reference ensures that all detected ^{16}O -labeled sample peptides will have their corresponding ^{18}O -labeled counterparts to serve as internal standards for quantitation. With an ^{18}O -labeled reference, it is unnecessary to label individual samples, thus eliminating additional sample handling and loss. The labeled reference that acts as comprehensive internal standards in every sample provides a means to obtain reproducible quantitative measurements for large-scale studies where LC-MS performance variation over time presents a significant challenge.

In this chapter, we present a detailed post-digestion trypsin-catalyzed ^{18}O -labeling protocol using solution-phase trypsin that provides effective labeling without the problem of oxygen back-exchange as well as a brief description of the quantitative data analysis strategy.

2. Materials

Unless otherwise noted, all reagents and equipment can be obtained from Sigma-Aldrich. Nanopure water is used for all experiments.

2.1. Protein Digestion

1. 50 mM ammonium bicarbonate (ABC) stock solution: dissolve 395 mg of ABC powder per 100 ml of water and adjust pH to 7.8 with HCl and/or NH_4OH . Store at 4°C for up to 6 months.
2. Urea in solid form.
3. 500 mM DL-dithiothreitol (DTT): prepare fresh DTT solution by dissolving 77 mg of DTT powder in 1 ml of water.
4. 400 mM iodoacetamide (IAA): prepare fresh IAA solution by dissolving 74 mg IAA powder in 1 ml of water. Note that iodoacetamide is light sensitive and should thus be kept in the dark!

5. 1 M calcium chloride: dissolve 147 mg of calcium chloride dihydrate powder in 1 ml of water.
6. 1 µg/µl trypsin: dissolve 20 µg of sequencing-grade porcine trypsin (Promega) in 20 µl of 50 mM ABC. Optional: incubate the re-dissolved trypsin at 37°C for 10 min before adding the trypsin to the sample.
7. 1.5 and 2.0 ml sterile, siliconized microcentrifuge tubes from Thermo Fisher Scientific, Inc.
8. 15 and/or 50 ml Falcon tubes (BD Biosciences).
9. Thermal mixer (Thermomixer R; Eppendorf) or oven that can be set to 37°C.
10. Bicinchoninic acid (BCA) protein assay kit (Thermo Scientific).
11. Absorbance reader (562 nm).

2.2. C18 Solid Phase Extraction (SPE) Cleanup Following Protein Digestion

1. 100% methanol.
2. 0.1% TFA (v/v) in water.
3. 5% (v/v) acetonitrile and 0.1% (v/v) TFA in water.
4. 80% (v/v) acetonitrile and 0.1% (v/v) TFA in water.
5. C18 SPE column of appropriate bed weight (i.e., a 1 ml column with a 100 mg bed weight can clean up to 5 mg of digested protein).
6. Vacuum manifold.
7. Speed-Vac[®] vacuum concentrator (Thermo Fisher Scientific, Inc.).

2.3. ¹⁸O-Labeling

1. 97% H₂¹⁸O (*see* Note 1).
2. 1 M ammonium bicarbonate (ABC) buffer in ¹⁸O water: dissolve 79.06 mg of ABC in ¹⁸O water. This buffer can be stored at 4°C for up to 6 months in a tube that is wrapped in a parafilm and placed into a larger container with desiccant.
3. 50 mM ammonium bicarbonate (ABC) buffer in ¹⁸O water: dilute the 1 M ABC buffer 20-fold with ¹⁸O water (storage conditions, *see* above).
4. 1 M calcium chloride (*see* step 5 of Section 2.1).
5. 1 µg/µl trypsin: dissolve 1 volume of 20 µg of sequencing-grade porcine trypsin (Promega, Madison, WI) in 20 µl of 50 mM ABC dissolved in ¹⁸O water.
6. 1 M formic acid in water.
7. Liquid nitrogen.

2.4. LC-MS Analysis

1. 65 cm long, 150 μ m I.D. fused silica reversed-phase capillary column packed with 3 μ m Jupiter C18-bonded particles.
2. Mobile phase A: 0.2% v/v acetic acid and 0.05% v/v trifluoroacetic acid in water.
3. Mobile phase B: 90% v/v acetonitrile and 0.1% v/v trifluoroacetic acid in water.

3. Methods

$^{16}\text{O}/^{18}\text{O}$ -labeling can be applied for both pair-wise comparative quantitation and relative quantitation across many biological samples by employing an ^{18}O -labeled “universal” reference sample (10). Sample processing typically starts with cell lysis, protein digestion, followed by post-digestion $^{16}\text{O}/^{18}\text{O}$ -labeling that utilizes solution-phase trypsin (*see* Fig. 3.3) that is later inactivated by boiling (5). Labeled samples with a mixture of $^{16}\text{O}/^{18}\text{O}$ -labeled peptides can be analyzed by LC-MS and LC-MS/MS with a high-resolution mass spectrometer. For pair-wise labeling, both biological samples will be processed using identical protocols with the exception that ^{16}O -labeling uses ^{16}O water while ^{18}O -labeling uses ^{18}O water. For applying the ^{18}O -labeled “universal” reference strategy, only one pooled sample needs to be labeled with ^{18}O , while all biological samples can be processed without an additional labeling step; however, residual trypsin from protein digestion in each unlabeled sample must be de-activated by boiling the peptide sample for 10 min prior to mixing it with the ^{18}O -labeled reference to prevent ^{18}O back-exchange. LC-MS data of the labeled samples are analyzed by a software algorithm that performs de-isotoping, feature and pair finding, and relative quantitative information based on $^{16}\text{O}/^{18}\text{O}$ -peptide pair abundance ratios (*see* Fig. 3.4).

3.1. Protein Digestion

1. Perform a BCA assay on the sample to determine protein concentration and total protein mass.
2. Dilute sample, if necessary, with 50 mM ABC so that the protein concentration does not exceed 10 mg/ml.
3. Add an appropriate amount of urea to obtain a final concentration of 8 M urea (i.e., 780 mg of urea per 1 ml of sample) to denature the proteins (*see* Note 2). Vortex briefly (i.e., ~ 1–3 s intervals) to mix thoroughly and spin down briefly to bring the sample and reagents to the bottom of the tube. Incubate at 37°C for 1 h (shaking at 450 rpm if possible), and following incubation, spin down briefly to remove condensation from the top of the lid.
4. To reduce the proteins, add DTT stock solution to the sample to obtain a final concentration of 10 mM DTT (i.e., 20 μ l of 500 mM DTT per 1 ml of sample). Vortex briefly to mix thoroughly and spin the sample briefly down. Incubate at 37°C for 1 h (shaking at 450 rpm if possible) and spin down briefly to remove all condensation from the top of the lid.

5. To alkylate the proteins (optional), add IAA stock solution to the sample to obtain a final concentration of 40 mM IAA (i.e., 111 μ l of 400 mM IAA per 1 ml of sample). Vortex, spin the sample down, and incubate for 37°C for 1 h in the dark. Following incubation, spin down briefly to remove condensation from the top of the lid.
6. For trypsin digestion, dilute the sample 10-fold with 50 mM ABC to reduce the salt concentration. Add calcium chloride to obtain a final concentration of 1 mM calcium chloride (i.e., 1 μ l of 1 M calcium chloride per 1 ml of sample). Add sequencing-grade porcine trypsin to the sample in a 1:50 trypsin:protein (w:w) ratio. Vortex briefly, spin down the sample briefly, and incubate at 37°C for 3–6 h, with shaking at 450 rpm if possible. After incubation, spin down briefly to remove condensation from the top of the lid (*see* Note 3).

3.2. C18 SPE Cleanup of the Protein Digest

1. Place the C18 SPE cartridge on the vacuum manifold, turn on the vacuum.
2. Condition the column by adding 3 ml of 100% methanol and letting the methanol flow no faster than one drop per second. Make sure that the column does not go dry before adding the next solution.
3. Equilibrate the column by adding 2 ml of 0.1% trifluoroacetic acid (TFA), letting the solution flow no faster than one drop per second.
4. Apply the sample, decreasing the flow to as slow as possible (i.e., approximately one drop every 3 s or even longer).
5. Wash the column by adding 4 ml of 5% (v/v) acetonitrile and 0.1% (v/v) TFA in water, letting the solution flow no faster than one drop each second.
6. Elute the sample slowly (i.e., one drop every 3 s or longer), collecting into a sterile and siliconized 1.5 ml microcentrifuge tube, with 1 ml of 80% (v/v) acetonitrile, 0.1% (v/v) TFA in water.
7. Discard the C18 SPE cartridge.
8. Concentrate sample in a Speed-Vac® vacuum concentrator down to ~50–100 μ l to remove most of the acetonitrile.
9. Perform a BCA assay to determine the peptide concentration (*see* Note 4).

3.3. Post-digestion ¹⁸O-Labeling (*see* Note 5)

1. Completely dry the peptide sample for ¹⁸O labeling in a Speed-Vac® vacuum concentrator (*see* Note 6).
2. Perform ¹⁸O labeling (*see* Note 7) by adding 100 μ l of 50 mM ABC buffer in ¹⁸O water to the dried peptide sample. Mix the sample by brief sonication (i.e., ~10 s) and vortex briefly (i.e., 1–3 s) (*see* Note 8) followed by a brief spin down to collect the sample at the bottom of the tube. Add 1.0 μ l of 1 M calcium chloride to the sample for a final concentration of 10 mM calcium chloride (*see*

Note 9). Add solution-phase porcine trypsin in a 1:50 trypsin:peptide (w:w) ratio to the sample. Wrap the tube lid in parafilm, vortex briefly, and spin down briefly to collect the sample at the bottom of the tube. Incubate at 37°C for 5 h (shaking at 450 rpm (*see* Note 10)).

3. Stop the ^{18}O -labeling reaction by placing the tubes into a floating tube rack into the boiling water (*see* Note 11) and boil the sample for 10 min (*see* Note 12). After boiling, immediately snap-freeze the sample in liquid nitrogen, and once the sample has thawed, immediately add 5 μl of formic acid (*see* Notes 13 and 14). Vortex briefly to mix and spin the sample down briefly (*see* Note 3).
4. To prepare samples for MS analysis, perform a BCA assay to determine the peptide concentration (*see* Note 15). (a) Verify the labeling efficiency of the ^{18}O -labeled sample to ensure accurate quantitation by analyzing the labeled sample by LC-MS before mixing the ^{18}O - and ^{16}O -labeled, or unlabeled, samples together (*see* Fig. 3.2b) (*see* Note 16). (b) Once high labeling efficiency of the ^{18}O -labeled sample has been verified, boil all of the unlabeled samples for 10 min. (c) Then, mix the ^{16}O - and ^{18}O -labeled samples in a 1:1 (w:w) ratio for LC-MS analysis.

3.4. Capillary LC-MS Analyses

In our laboratory, peptide samples are analyzed using a custom-built capillary LC system (11) coupled online to an LTQ-Orbitrap ion trap mass spectrometer equipped with an ESI interface:

1. Degas mobile phases using a vacuum degasser.
2. Inject 10 μl of peptide sample onto the reversed-phase capillary column and apply the following solvent gradient: first 100% of solvent A for 20 min, followed by increasing the solvent composition to 70% of solvent B over 100 min, using a stainless steel mixing chamber (*see* Note 17). This same gradient is applied for both LC-MS/MS and LC-FTICR analyses.
3. The mass spectrometers are operated under standard conditions, details of which are available in references (10, 11).

3.5. Quantitative LC-MS Data Analysis

In our laboratory, LC-MS data analysis and processing steps are automated using an in-house developed software package that includes the informatics tools Decon2LS and VIPER (12, 13), which can be downloaded at ncrr.pnl.gov. The details of data analysis are available in references (7, 10). The process is briefly described as follows:

1. The initial analysis of raw LC-MS data involves a mass transformation or de-isotoping step using Decon2LS, an analysis tool based on the THRASH algorithm, which generates a text file report for each LC-MS data set including both the monoisotopic masses and the corresponding intensities for all detected species for each mass spectrum.

2. Following Decon2LS analysis, each data set is processed by the feature matching tool (VIPER) for peptide identification and quantification. Data sets are displayed in a two-dimensional mass and normalized LC elution time (NET) format. The feature matching process includes the “distinct feature” (i.e., a peak with unique mass and elution time) finding that searches for $^{16}\text{O}/^{18}\text{O}$ feature pairs and computes abundance ratios for feature pairs. An intensity report for all detected features is created, which normalizes LC elution times via alignment to a database that allows the peptides to be identified.
3. Feature identification is performed by matching the accurately measured masses and normalized elution time (NET) values of each detected feature to a pre-established accurate mass and time (AMT) tag database from prior LC-MS/MS analyses.
4. For each identified peptide, the relative $^{16}\text{O}/^{18}\text{O}$ abundance ratio is reported if the feature is paired. The details for computing the $^{16}\text{O}/^{18}\text{O}$ abundance ratios using VIPER have been previously described (7).

4. Notes

1. The purity of ^{18}O water is directly related to the labeling efficiency because oxygen exchange is dependent upon the concentration of the available isotopes. In other words, the higher the purity of the ^{18}O water, the higher the ^{18}O -labeling efficiency.
2. Each milligram of urea adds an additional $\sim 0.8 \mu\text{l}$ to the sample volume.
3. At this point, the sample can be frozen.
4. Sample recovery after urea digestion and C18 SPE is $\sim 50\%$, by weight (i.e., final peptide weight versus starting protein amount).
5. The procedure can also be applied toward ^{16}O labeling using H_2^{16}O for comparative analyses. ^{16}O - and ^{18}O -labeled samples must be kept separate until the boiling step is done (step 4c of Section 3.3).
6. As much as 1 mg of digested protein in $100 \mu\text{l}$ of H_2^{18}O can be successfully labeled.
7. Peptide samples should be cleaned using SPE or a method that can effectively remove detergents and contaminants such as SDS that may interfere with labeling.
8. For small sample amounts, sample mixing should be performed via sonication rather than vortexing to improve sample recovery.
9. 1 M of calcium chloride is dissolved in deionized ^{16}O water, but could be made in ^{18}O water. The small amount of ^{16}O from the calcium chloride solution has a minimal effect on labeling efficiency when compared to the amount of ^{18}O in the final labeling mixture.

10. Labeling for longer than 5 h will not hurt the labeling efficiency but it does not appear to improve it either.
11. Microcentrifuge tube lids can pop open during boiling if the ratio of the sample volume to tube volume is high enough. For this protocol, a 100 μ l sample volume will not cause the lid of a 1.5 ml tube to pop open during the 10 min boil. However, 100 μ l sample volume in a 0.6 ml tube will usually cause the lid to pop open during boiling, which may result in sample loss and/or contamination. To minimize this from happening, position the tube in the floating rack so that the sample is covered with boiling water, but not pushed to the maximum depth.
12. The boiling step to inactivate the residue trypsin is the most critical step in this protocol; otherwise, back-exchange will be a significant issue.
13. 5% formic acid (v/v) added to the sample was observed to be beneficial for achieving high labeling efficiency.
14. It is essential that the formic acid is added as soon after thawing as possible to ensure good labeling efficiency. Rub the tube back and forth between the hands to accelerate the thawing. Once partially thawed, add the formic acid. Then, vortex in short bursts (i.e., 1–3 s) to mix the formic acid with the sample until the sample is completely thawed.
15. Sample recovery after labeling is ~100% by weight since the labeling protocol does not introduce sample loss.
16. ^{18}O -Labeling efficiency can be determined by visually evaluating a handful of chromatograms representing ^{18}O -labeled tryptically digested peptides. Although labeling may vary slightly from peptide to peptide, a well-labeled sample will have a narrow range of high labeling efficiency (i.e., minimal incorporation of ^{16}O) across all tryptically digested peptides and will be very similar to that shown in Fig. 3.2b.
17. The $^{16}\text{O}/^{18}\text{O}$ -labeling strategy is compatible with any type of mobile phase.

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Abbreviations:

ABC	ammonium bicarbonate
AMT	accurate mass and time
BCA	bicinchoninic acid
DTT	DL-dithiothreitol

ESI	electrospray ionization
FTICR	Fourier transform ion cyclotron resonance
IAA	iodoacetamide
I.D.	inner diameter
LC	liquid chromatography
MS	mass spectrometry
NET	normalized elution time
SPE	solid phase extraction

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Fig. 3.1. Mechanism of enzyme-catalyzed oxygen exchange on the C-terminal carboxyl groups of peptides. Serine proteases, like trypsin, bind to the C-terminal carboxyl group and catalyze the exchange of both oxygen atoms with ¹⁸O water.

Fig. 3.2.

Mass spectral patterns for $^{16}\text{O}/^{18}\text{O}$ -labeled peptides. (a) An ^{16}O -labeled, or unlabeled, peptide. (b) An ^{18}O -labeled peptide. Since the ^{18}O water is not 100% pure, a percentage of the peptide will have ^{16}O incorporated into one or two C-terminal carboxyl groups. A well-labeled peptide will have minimal ^{16}O incorporation. (c) ^{16}O - and ^{18}O -labeled samples mixed in a 1:1 ratio (w:w).

Fig. 3.3.
A workflow for post-digestion ^{18}O labeling.

Fig. 3.4.

An overview of LC-MS quantitative data analysis process. Each LC-MS data set was displayed in mass and normalized elution time (NET) dimensions for feature and pair finding and peptide identification. $^{16}\text{O}/^{18}\text{O}$ abundance ratio is calculated based on the light and heavy versions of peptide elution profiles for a given peptide.