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ProductInformation

^{18}O Proteome Profiler™ Kit

Product Code **P3623**
Storage Temperature 2–8 °C

TECHNICAL BULLETIN

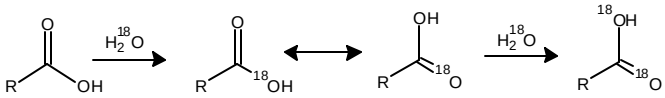
Product Description

The ^{18}O Proteome Profiler™ Kit provides all reagents necessary for performing relative quantitation of protein samples using Mass Spectrometric (MS) analysis. The basis of the kit is the enzymatic incorporation of a stable isotope label (^{18}O) into virtually all peptides of a tryptic digest.[1-8] Because oxygen atoms do not rapidly exchange in the absence of the protease, this method is highly specific for stable isotope incorporation into the carboxyl terminus of each peptide. Also, this method is generally considered to be a global labeling procedure, since the only peptide without a stable isotope label is the one containing the C-terminus of the original protein, unless the protein C-terminal amino acid is an arginine or lysine.

The enzymatic labeling method starts with two samples, which are being compared to determine relative levels of specific proteins. The samples are prepared in parallel through the tryptic digestion step. At this stage, the peptide samples are dried and then reconstituted in either natural water ($H_2{}^{16}O$) or stable isotope labeled water ($H_2{}^{18}O$). In the presence of trypsin, the oxygen isotope will be incorporated into the C-terminus of the digest peptides. The samples can then be mixed and analyzed by MS. Alternatively, the stable isotope label can be integrated into the peptides during the tryptic digestion process. The latter method, however, is less efficient at incorporating the ^{18}O isotope and overall has more limitations.[3,7,9,10]

When used in an enzymatic labeling procedure, trypsin is capable of incorporating two ^{18}O atoms per peptide (see Figure 1). This is due to the nature of the extended interaction between the peptide and protease, allowing for equilibration of both carboxyl oxygen atoms at the C- terminus.[4,11]

Figure 1.
Trypsin Mediated Incorporation of ^{18}O into Both Carboxyl Oxygen Atoms of the C-terminal Amino Acid



When comparing sets of samples, either labeled with ^{18}O or unlabeled, the mass difference achieved is primarily 4 Da. In some cases, depending on the size and sequence of the peptide, the equilibration of the second ^{18}O atom is slow and it may not be completely incorporated in the timeframe allotted.[4,10,11] In this situation, only a 2 Da mass increase is observed. In general, peptides with a C-terminal arginine residue will incorporate the ^{18}O label faster than those with a C-terminal lysine residue. It is, therefore, important to allow sufficient labeling time to fully incorporate the double label, thus simplifying data analysis and quantitation.

Components

Sufficient reagents are supplied for 12 protein quantitation experiments, each derived from two 50 µg protein samples.

- Water-^{18}O, 99 atom % ^{18}O (Isotopic Purity) 550 µl
  Normalized with respect to hydrogen
  Product Code Q34608
- Trypsin, Proteomics Grade 2 X 20 µg
  Product Code T6567
- Trypsin Singles™, Proteomics Grade, Enzyme 24 X 1 µg
  Product Code T7200
- Trypsin Solubilization Reagent 1 ml
  Cap Color: Clear
  Product Code T2073
- Enzyme Reaction Buffer 2 X 25 ml
  (50 mM Tris HCl with 50 mM $CaCl_2$)
  Product Code B9185

- Microspin Columns and Collection Tubes 24 each
  Product Code M1819
- Acetonitrile, 1 ml
  Use Tested for MALDI-TOF-MS
  Cap Color: Red
  Product Code A8596
- 1.0% Trifluoroacetic Acid (TFA) in Water, 1 ml
  Use Tested for MALDI-TOF-MS
  Cap Color: Blue
  Product Code T3693
- Clear-View Snap-Cap Microtubes, 0.6 ml 50 each
  (siliconized microcentrifuge tubes)
  Product Code T4691
- 1 ml Polypropylene Tuberculin Syringes 12 each
  Product Code Z192090
- B-D Precisionglide® Syringe Needles 12 each
  23 Gauge
  Product Code Z192457

Reagents Recommended for Sample Preparation but Not Provided
(Product Codes have been given where appropriate)

- 8 M Guanidine, buffered at pH 8.5 (Product Code G7294)
- 8 M Urea (Product Code U4883)
- Protein Extraction Reagent Type 4 (Product Code C0356)
- Bis-Tris-Propane (Product Code B9410)
- Trizma® base (Product Code T6791)
- ProteoPrep® Reduction and Alkylation Kit (Product Code PROTRA)
- 1.0 M DTT Solution (Product Code 646563)
- 0.5 M TCEP Solution (Product Code 646547)
- Iodoacetamide (Product Code A3221)
- 200 mM Tributylphosphine (TBP) Solution (Product Code T7567)
- Myoglobin (Product Code M0630)

Precautions and Disclaimer
This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions
All components, except as noted, are ready to use.

Trypsin Solution – Reconstitute a 20 µg package of Trypsin, Proteomics Grade (Product Code T6567) with 40 µl of Trypsin Solubilization Reagent (clear cap, Product Code T2073) to obtain a final concentration of 0.5 µg/µl. This solution should be frozen in aliquots for multiple uses (see Storage/Stability).

Microspin Columns (Product Code M1819)
Note: The columns provided are for use with 20 µl to 75 µl sample sizes only. The molecular weight cutoff (MWCO) of the resin is ~6 kDa.

1. Prepare the column for use by first removing it from the cooler and allowing it to warm to room temperature. This temperature equilibration should take ~15 minutes.
2. To remove air pockets that may have formed during shipping, shake the column so the resin is forced to the bottom of the column next to the frit.
3. Break off the bottom piece of the column and discard.
4. Place the column in a collection tube and remove the cap from the column.
5. Centrifuge the column at 1,000 x *g* for 2 minutes to remove the storage buffer. Discard the buffer in the collection tube.
6. To equilibrate the resin, add 0.5 ml of the Enzyme Reaction Buffer to the top of the resin bed.
7. Centrifuge the column at 1,000 x *g* for 2 minutes and discard the buffer in the collection tube.
8. Repeat steps 6 and 7 two additional times.
9. The column is now ready for use in the Sample Clean Up Procedure.

Storage/Stability
The kit should be stored at 2–8 °C. All components are stable for at least one year when stored properly.

DO NOT PLACE THE KIT IN THE FREEZER. The Microspin Columns will be irreversibly damaged if stored below 0 °C.

The prepared Trypsin Solution can be stored at 2–8 °C for 2 weeks or at –20 °C for up to 4 weeks. The Trypsin Solution is stable for at least 3 freeze-thaw cycles.

Procedure
This procedure is designed to compare two different protein samples (~50 µg each). The two samples should be processed in parallel, using the same reagents and with the same timing. This will minimize variability in results due to differential sample handling.

It is recommended that a protein of known concentration be analyzed along with the samples being tested. This internal control will aid in optimizing the procedure to fit individual needs. One protein suitable for use as an internal control is myoglobin (Product Code M0630).

Preparation of samples for use with this kit should include protein denaturation followed by reduction and alkylation. Following initial preparation, the samples must be cleaned up using an appropriate method. The spin columns provided with this kit are ideal for this purpose. Other acceptable methods are dialysis or gel electrophoresis. Once all detergents, chaotropes, reduction and alkylation reagents are removed, the samples are digested with trypsin. Following proteolysis the tryptic peptides are enzymatically labeled with $H_2{}^{18}O$ and then analyzed via an appropriate method such as MALDI-TOF MS.

I. Sample Preparation

To allow for the most efficient tryptic digestion it is recommended to denature protein samples prior to using this kit. Suggested reagents for denaturation include: 8 M guanidine HCl, buffered at pH 8.5 (Product Code G7294), 8 M Urea (Product Code U4883), or Protein Extraction Reagent Type 4 (Product Code C0356). Lower concentrations of chaotropes (3–8 M) may be utilized along with other detergents. A solution buffered at ~pH 8.5 is optimal at this step, especially if the protein is to be reduced and alkylated. Suitable buffers for this pH include Bis-Tris-Propane (Product Code B9410) or Trizma base (Product Code T6791).

After denaturation, it is recommended that the samples be reduced and alkylated prior to labeling. This will ensure that the tryptic digestions are as efficient as possible. Suggested reagents for this include: the ProteoPrep Reduction and Alkylation Kit (Product Code PROTRA), or either 1.0 M DTT Solution (Product Code 646563) or 0.5 M TCEP Solution (Product Code 646547) used with Iodoacetamide (Product Code A3221).

1. Prepare the two protein samples for relative quantitation in parallel.
2. Denature proteins by mixing each sample with an equal volume of 8 M guanidine HCl, buffered at pH 8.5 with 50 mM Bicine (Product Code G7294). The total protein concentration should ideally be ~1 mg/ml.
3. Reduce protein disulfides by adding a 200 mM stock solution of Tributylphosphine (TBP; Product Code T7567) to obtain a final concentration of 5 mM.
4. Incubate each sample for 30 minutes at room temperature.
5. Alkylate protein sulfhydryl moieties by adding a 0.5 M stock solution of Iodoacetamide (Product Code A3221) to obtain a final concentration of 15 mM.
6. Incubate the samples for 2 hours at 37 °C.
7. Protein samples may now be directly labeled using the kit procedure, or alternatively, fractionated using commonly used proteomics techniques, including gel electrophoresis, affinity chromatography, or HPLC.

II. Sample Clean Up

In order to obtain optimal kit performance, samples must have all denaturants, chaotropes, detergents, and reduction/alkylation reagents removed prior to the labeling step. **Failure to remove these interfering substances will cause incomplete proteolysis, inefficient labeling, and low quality data.** This step is optional if the protein sample(s) were fractionated by gel electrophoresis.

The Microspin Columns provided in the kit are ideal for the clean up of samples containing proteins >6 kDa. Do not use the provided spin columns for proteins with molecular weights (MW) <6 kDa. The clean up of proteins with molecular weights (MW) <6 kDa may be accomplished by dialysis with a MWCO lower than the MW of the protein of interest, hydrophobic chromatography (reverse phase HPLC or C18 clean up devices), or gel electrophoresis. Refer to the manufacturer's recommended protocol for each of these methods.

1. Prepare two Microspin Columns for use (see Preparation Instructions). Label these columns as appropriate ("test" and "control")
2. Carefully apply 50–75 µl of the appropriate protein sample, containing approximately 50 µg of protein, to the top of the resin bed. Ensure the column is seated in a clean collection tube with all wash buffer removed. Label the collection tubes similarly to the spin columns.
3. Centrifuge the column at 1,000 x *g* for 4 minutes to spin the sample through the resin.
4. Recover each sample from the collection tube and transfer to one of the Clear-View Snap-Cap Microtubes (Product Code T4691).
   Note: Depending on the protein being assayed, some sample loss may occur.

III. Two Step Procedure for Trypsin Digestion and Subsequent ^{18}O Labeling (Recommended)

1. Prepare the Trypsin Solution (see Preparation Instructions). If the Trypsin Solution was previously aliquoted and frozen, remove an aliquot from the freezer and allow it to thaw completely before use.
2. Add 2 µl of the Trypsin Solution to each protein sample. This is results in ~1:50 (w/w) ratio of trypsin to protein.

3. Add Acetonitrile (red cap, Product Code A8596) to the sample at 9% of the total volume (~4 µl).
4. Incubate overnight (12–18 hours) at 37 °C.
5. Dry both protein samples completely in a vacuum centrifuge. Samples should be visually checked to verify complete removal of water. Drying will take 30 minutes to 2 hours depending on the type of vacuum centrifuge used and the total volume of the sample.
6. Add 10 µl of Acetonitrile (red cap) to each dried peptide sample.
7. Obtain ultrapure water (HPLC Grade) for the $H_2{}^{16}O$ sample.
8. Invert the serum vial containing the $H_2{}^{18}O$ and use the provided syringe/needle to remove the required amount of $H_2{}^{18}O$. Approximately 40 µl of water per sample is required; however, more may be removed and transferred to a clean, dry microcentrifuge tube. Seal the microcentrifuge tube tightly with wrap until required for use. Handle in a dry room if available.
9. Using a calibrated pipette, add 40 µl of the appropriate type of water to one Trypsin Singles (Product Code T7200) tube. Pipette up and down repeatedly to ensure that the trypsin is completely dissolved.
10. Transfer the dissolved contents of the Trypsin Singles tube to the appropriate sample from step 6. For example, add trypsin in $H_2{}^{16}O$ to the test sample and trypsin in $H_2{}^{18}O$ to the control sample. Note: Adding 1 µl of 1.0% (TFA) in Water (blue cap, Product Code T3693) to each sample may improve the mass spectrometric signal intensity of the peptides.[9]
11. Vortex the tubes to completely resuspend the peptides.
12. Incubate at 37 °C for 12–24 hours. Longer incubation times ensure complete labeling of all peptides. See Product Description for further details and the theoretical aspects.
13. Stop the labeling process by adding 5 µl of 1.0% TFA in Water (blue cap) to each sample. Mix well. If samples will not be analyzed immediately, dry both samples separately in a vacuum centrifuge to complete dryness. Samples may be stored in the freezer for future analysis. Ensure that the tube is well sealed to protect from moisture contamination.
14. Mass Spectrometric analysis:
    a. If the samples are to be analyzed immediately, mix the two samples in a 1:1 (v/v) ratio prior to analysis.
    b. If the samples were dried, reconstitute samples with 50 µl of a 0.1% TFA solution. This solution may be prepared by diluting the supplied 1.0% TFA in Water (blue cap) solution 10-fold with ultrapure $H_2{}^{16}O$ water. Mix the two samples in a 1:1 (v/v) ratio. Smaller volumes may be used for reconstitution if sample concentration is a concern.
15. Samples, once mixed, are stable for approximately 1 hour. Back exchange of the ^{18}O labels is very slow in the presence of 0.1% TFA,[12] but will occur if samples are allowed to stand at room temperature for extended periods.

IV. Single Step Procedure for Trypsin Digestion and ^{18}O Labeling (Alternative)

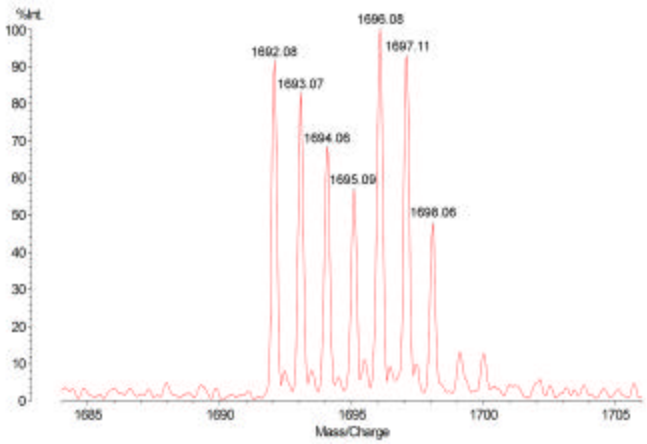
1. After following the procedures for Sample Preparation (Section I) and Sample Clean Up (Section II), dry both protein samples completely in a vacuum centrifuge. Samples should be visually checked to verify complete removal of water. Drying will take 30 minutes to 2 hours depending on the type of vacuum centrifuge used and the total volume of the sample.
2. Obtain ultrapure water (HPLC Grade) for the $H_2{}^{16}O$ sample.
3. Invert the serum vial containing the $H_2{}^{18}O$ and use the provided syringe/needle to remove the required amount of $H_2{}^{18}O$. Approximately 40 µl of water per sample is required; however, more may be removed and transferred to a clean, dry microcentrifuge tube. Seal the microcentrifuge tube tightly with wrap until required for use. Handle in a dry room if available.
4. Using a calibrated pipette, add 40 µl of the appropriate type of water to one Trypsin Singles (Product Code T7200) tube. Pipette up and down repeatedly to ensure that the trypsin is completely dissolved.
5. Transfer the dissolved contents of the Trypsin Singles tube to the appropriate sample from step 1. For example, add trypsin in $H_2{}^{16}O$ to the test sample and trypsin in $H_2{}^{18}O$ to the control sample.
6. Add 10 µl of Acetonitrile (red cap) to each sample.
7. Vortex the tubes to completely resuspend the peptides.
8. Incubate at 37 °C for 12–24 hours.
9. To complete the procedure, follow steps 13–15 of the two step method (Section III).

Results

Mass spectrum of labeled and unlabeled peptide from a typtic digest of aldolase is shown in Figure 2.

Figure 2.

MALDI-TOF Mass Spectrum of the Peptide YSHEEIAMATVTALR Derived from Aldolase



This mass spectrum represents expected results obtained when two protein solutions of equal concentration are analyzed using this kit. The base peak at m/z 1692.08 (mass + 0 Da) has approximately the same intensity (I_0) or integrated peak area as the peak at m/z 1696.08 (mass + 4 Da, I_4). The peaks at 1693.07, 1694.06, 1695.09, 1697.11, and 1698.06 represent the normal isotopic distribution obtained by the mass spectrometric analysis of a peptide.

Calculations

This kit is fully qualified for analysis by MALDI-TOF MS. If LC-MS is employed using relatively low resolution ion trap instruments, the data should be analyzed by interpreting only the monovalent and divalent ions.

Equation 1 - Data analysis may be accomplished using the following equation:[1]

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

I_0, I_2, and I_4 represent the observed intensities of the peaks 0, 2 or 4 Da from the base peak (the monoisotopic ^{16}O peak). Similarly, M_0, M_2, and M_4 represent the theoretical intensities of each native or unlabeled (^{16}O) isotope peak. The "M values" can be obtained from appropriate software,[13] following identity determination of the peptide, or alternatively may be calculated using equations 3 and 4. The above equation allows for more accurate quantitation ratios in instances where only one ^{18}O atom incorporates into a peptide and also takes into account the natural isotopic distribution for the peptides of interest.

Sample Calculation - The data for Equation 1 is derived from the mass spectrum shown in Figure 2. The theoretical intensities of each peak (M_x) were obtained by analyzing the peptide sequence with GPMAW software.[13] The observed intensities were obtained via a quantitative analysis of the MALDI-TOF MS spectrum.

The theoretical and observed intensities were as follows:

$M_0 = 100.0$	$M_2 = 51.2$	$M_4 = 7.0$
$I_0 = 44.4$	$I_2 = 33.1$	$I_4 = 48.5$

$$1.14 = \frac{48.5 - \frac{(7.0)\ 44.4}{100} - \frac{51.2}{100}\left(33.1 - \frac{(51.2)\ 44.4}{100}\right) + \left(33.1 - \frac{(51.2)\ 44.4}{100}\right)}{44.4}$$

The calculated ratio of 1.14 compares very favorably to the expected (theoretical) ratio of 1.0.

Equation 2 - A simpler, but less accurate, equation that may be used is:

Ratio = I_4 / I_0

Sample Calculation - I_0 and I_4 represent the intensities of the peaks 0 or 4 Da from the base peak (the monoisotopic ^{16}O peak). This equation is much simpler to use, but does not account for incomplete labeling or isotopic distribution differences due to variable peptide sequences.

The observed intensities were as follows:
$I_0 = 44.4$ $I_4 = 48.5$

$$1.09 = \frac{48.5}{44.4}$$

Equation 3 – Calculation for determining M_2/M_0 values.[2]
Note: M_r denotes the peptide monoisotopic mass.

$$\frac{M_2}{M_0} = 3 \times 10^{-7} M_r^{1.9241}$$

Equation 4 – Calculation for determining M_4/M_0 values.[2]
Note: M_r denotes the peptide monoisotopic mass.

$$\frac{M_4}{M_0} = 2 \times 10^{-12} M_r^{3.2684}$$

Equations 3 and 4 should be used for peptides with m/z ≥1500, since the contribution to the M_4 term by naturally occurring isotopes is less than 5%.

To obtain relative quantitative proteomics data of high veracity and reproducibility it is recommended to perform the entire procedure with as many replicates (start to finish) as is practical.

References

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