**INSTRUCTIONS**

**TMT® Mass Tagging Kits and Reagents**

<table>
<thead>
<tr>
<th>Number</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>90060</td>
<td><strong>TMTduplex™ Isotopic Label Reagent Set</strong>, sufficient reagents for 5 duplex isotopic experiments</td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
<td>TMT⁰ Label Reagent, 5 × 0.8mg</td>
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<tr>
<td></td>
<td>TMT⁶-127 Label Reagent, 5 × 0.8mg</td>
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<tr>
<td>90061</td>
<td><strong>TMTsixplex™ Isobaric Label Reagent Set</strong>, sufficient reagents for 1 sixplex isobaric experiment</td>
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<td></td>
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<td><strong>TMTduplex Isobaric Mass Tagging Kit</strong>, sufficient reagents for 5 duplex isobaric experiments</td>
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<td></td>
<td>Dissolution <strong>Buffer</strong> (1 M triethyl ammonium bicarbonate), 5mL</td>
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<td>Denaturing <strong>Reagent</strong> (10% SDS), 1mL</td>
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<td>Reducing <strong>Reagent</strong> (0.5M TCEP), 1mL</td>
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<td>Iodoacetamide, 12 × 9mg</td>
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<td>Quenching <strong>Reagent</strong> (50% hydroxylamine), 1mL</td>
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<td>Trypsin, 2 × 20µg</td>
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<td></td>
<td>Trypsin Storage Solution, 250µL</td>
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<tr>
<td></td>
<td>Albumin, Bovine, 2.5mg</td>
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90064 TMTsixplex™ Isobaric Mass Tagging Kit, sufficient reagents for 5 sixplex isobaric experiments
Contents:
TMT⁰ Label Reagent, 5 × 0.8mg
TMT⁶-126 Label Reagent, 5 × 0.8mg
TMT⁶-127 Label Reagent, 5 × 0.8mg
TMT⁶-128 Label Reagent, 5 × 0.8mg
TMT⁶-129 Label Reagent, 5 × 0.8mg
TMT⁶-130 Label Reagent, 5 × 0.8mg
TMT⁶-131 Label Reagent, 5 × 0.8mg
Dissolution Buffer (1M triethyl ammonium bicarbonate), 5mL
Denaturing Reagent (10% SDS), 1mL
Reducing Reagent (0.5 M TCEP), 1mL
Iodoacetamide, 12 × 9mg
Quenching Reagent (50% hydroxylamine), 1mL
Trypsin, 5 × 20μg
Trypsin Storage Solution, 250μL
Albumin, Bovine, 2.5mg

90065 TMTduplex Isobaric Label Reagent Set, sufficient reagents for 5 duplex isobaric experiments
Contents:
TMT²-126 Label Reagent, 5 × 0.8mg
TMT²-127 Label Reagent, 5 × 0.8mg

90066 TMTsixplex Label Reagent Set, sufficient reagents for 5 sixplex isobaric experiments
Contents:
TMT⁶-126 Label Reagent, 5 × 0.8mg
TMT⁶-127 Label Reagent, 5 × 0.8mg
TMT⁶-128 Label Reagent, 5 × 0.8mg
TMT⁶-129 Label Reagent, 5 × 0.8mg
TMT⁶-130 Label Reagent, 5 × 0.8mg
TMT⁶-131 Label Reagent, 5 × 0.8mg

90067 TMTzero™ Label Reagent, 5 × 0.8mg, sufficient reagents for 5 samples

90068 TMTsixplex Label Reagent Set, sufficient reagents for 12 sixplex isobaric experiments
Contents:
TMT⁶-126 Label Reagent, 2 × 5mg
TMT⁶-127 Label Reagent, 2 × 5mg
TMT⁶-128 Label Reagent, 2 × 5mg
TMT⁶-129 Label Reagent, 2 × 5mg
TMT⁶-130 Label Reagent, 2 × 5mg
TMT⁶-131 Label Reagent, 2 × 5mg

Storage: Upon receipt store at -20°C. Reagents are shipped with dry ice.
Note: These products are for research use only – do not use for diagnostic procedures.
## Introduction

The Thermo Scientific TMT Isobaric and Isotopic Mass Tagging Kits and Reagents enable quantitative labeling of proteins extracted from cells and tissues. Each isobaric tagging reagent within a set has the same nominal parent (precursor) mass and is composed of an amine-reactive NHS-ester group, a spacer arm and an MS/MS reporter (Figure 1). The reagents label peptides prepared from cell-based or tissue samples, either two samples for the duplex kit or six samples for the sixplex kit. For each sample, a unique reporter mass results in the MS/MS spectrum (i.e., 126-127Da for TMT² and 126-131Da for TMT⁶ Isobaric Label Reagents). These reporter ions are in the low mass region of the MS/MS spectrum and are used to report relative protein expression levels during peptide fragmentation.

The TMT Reagents share an identical structure, allowing TMTzero and TMTsixplex Reagents to be used also as isotopic “light” and “heavy” duplex tags. These tags are used to quantitate protein expression changes in cell-based or tissue samples that may not be amenable to metabolic isotopic labeling strategies (e.g., SILAC). These isotopic pairs can also be used in targeted quantitation strategies, including selective reaction monitoring (SRM, see the Additional Information Section). Peptides and proteins labeled with all TMT Reagents can be enriched with the anti-TMT Antibody Resin and TMT Enrichment Kit (see the Additional Information and Related Thermo Scientific Products Sections).

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![Figure 1. Chemical structure of the TMT Label Reagents. Panel A: Functional regions of the reagent structure. Panel B: Structures, isotope positions, MS/MS fragmentation sites and collision-induced reporter ions for each reagent.](image-url)
Procedure Summary

Protein extracts are isolated from cells grown in culture or from tissue samples. After removing amine-based buffers and thiol reagents, samples are reduced, alkylated and digested overnight. Samples are labeled with the TMT Reagents and then mixed at the duplex or the sixplex level. Strong-cation exchange (SCX) fractionation simplifies complex samples before LC-MS/MS analysis. Data analysis software is used to analyze the reporter ions in the low mass region (Figure 2).

Peptides are typically labeled with TMT Reagents because it allows quantitation of every peptide, but intact proteins can also be labeled. There are several advantages to labeling intact proteins. For example, combining labeled samples earlier in the sample process will reduce sample variability. Also, mixed samples enable single processing for fractionation and digestion. Fractionation methods include ion exchange chromatography, 1D-PAGE and phosphoprotein enrichment.

Figure 2. Schematic for using the Thermo Scientific TMTsixplex Isobaric Mass Tagging Reagents.

Important Product Information

- The TMT Reagents are moisture-sensitive. To avoid moisture condensation onto the product, vial must be equilibrated to room temperature before opening.
- The TMT Reagents are amine-reactive and modify lysine residues and the peptide N-termini. All amine-containing buffers and additives must be removed before digestion and labeling.
- All samples must be digested, labeled and then mixed equally before desalting, fractionation and LC-MS/MS. For optimal results, use 25-100µg of peptide for each labeling reaction.
- To avoid contamination of MS samples, always wear gloves when handling samples and gels. Use ultrapure MS-grade reagents. Perform sample preparation in a cleaned work area cleaned with 70% methanol (Fisher Product No. A454-1).
- The TMTzero Label Reagent can be used to optimize methods before multiplexed analysis of samples with the TMTduplex or TMTsixplex Reagent Set.

Additional Materials Required

- Anhydrous acetonitrile (Thermo Scientific Acetonitrile HPLC grade, Product No. 51101), 100 proof ethanol or, for protein labeling, anhydrous dimethyl sulfoxide (DMSO), Sequanal grade (Product No. 20688)
- Glass syringe (100µL)
- HPLC grade water (Fisher, Product No. W6-4)
- Chilled (-20°C) acetone
- Protease inhibitors (Thermo Scientific Halt Protease Inhibitor Single-Use Cocktail, EDTA-free, Product No. 78425)
- Phosphatase inhibitors (Thermo Scientific Halt Phosphatase Inhibitor Cocktail, Product No. 78420)
- Cell lysis reagent such as Thermo Scientific M-PER Mammalian Protein Extraction Reagent (Product No. 78501), RIPA Lysis and Extraction Buffer (Product No. 89901) or 8M Urea (Product No. 29700)
- Protein assay such as Thermo Scientific Coomassie Plus (Bradford) Protein Assay (Product No. 23236), Pierce 660nm Protein Assay (Product No. 22600) or Pierce BCA Protein Assay Kit (Product No. 22235)
- 75-300µm capillary C18 reversed-phase column
- Ion trap or time-of-flight (TOF) mass spectrometer with online or offline liquid chromatography (LC) system
- Data analysis software such as Thermo Scientific Proteome Discoverer or Mascot Software (Matrix Science, Ltd.)
• Optional: Thermo Scientific Zeba Spin Desalting Columns (Product No. 89882) or Slide-A-Lyzer Dialysis Cassettes, 3.5K MWCO, 0.5mL (Product No. 66333)

Material Preparation

Note: The 50% hydroxylamine and 10% SDS stock solutions provided with the kit may precipitate during storage. Warm both solutions to room temperature and vortex before use.

Albumin, Bovine (BSA) Reconstitute BSA (2.5mg) with 2.5mL of ultrapure water. Divide solution into 100µL aliquots and lyophilize to dryness.

100mM TEAB (triethyl ammonium bicarbonate) Add 50µL of the Dissolution Buffer (1M TEAB) to 450µL of ultrapure water.

2% SDS Add 50µL of the Denaturing Reagent (10% SDS) to 200µL of ultrapure water.

200mM TCEP Add 70µL of the Reducing Reagent (0.5M TCEP) to 70µL of ultrapure water. Then add 35µL of the Dissolution Buffer (1M TEAB).

5% Hydroxylamine Dilute the Quenching Reagent (50% hydroxylamine) 1:10 with 200mM TEAB.

Preparing and Labeling Peptides with the TMT Isobaric Mass Tags

A. Preparing Whole Cell Protein Extracts

1. Culture cells to harvest at least 100µg of protein per condition. For best results, culture a minimum of 5 × 10⁶ cells.

2. Lyse cells in either RIPA buffer, M-PER® Reagent or 8M urea. Add protease and phosphatase inhibitors to the lysis reagent. Use 4mL of lysis reagent for each milliliter of cells.

3. Perform a protein assay to determine the protein concentration. Use samples at ≥2mg/mL. Less concentrated samples can be used; however, it might be necessary to use larger volumes of reducing/alkylating reagents.

4. Place 100µg per condition (two for the TMTduplex or six for the TMTsixplex Label Reagents) in a polypropylene microcentrifuge tube.

5. Add 45µL of 100mM TEAB to the sample and adjust to a final volume of 100µL with ultrapure water. For labeling reactions > 100µL use a large-volume centrifuge tube such as a 15mL or 50mL polypropylene conical tube.

Optional: To solubilize complex protein mixtures, add 5µL of 2% SDS before adjusting to final volume.

6. Add 5µL of the 200mM TCEP and incubate sample at 55°C for 1 hour.

7. Immediately before use, dissolve one tube of iodoacetamide (9mg) with 132µL of 100mM TEAB to make 375mM iodoacetamide. Protect solution from light.

8. Add 5µL of the 375mM iodoacetamide (with TEAB) to the sample and incubate for 30 minutes protected from light.

9. Add six volumes (~1mL) of pre-chilled (-20°C) acetone. Allow the precipitation to proceed overnight.

10. Centrifuge the samples at 8000 × g for 10 minutes at 4°C. Carefully invert the tubes to decant the acetone without disturbing the white pellet. Allow the pellet to dry for 10 minutes.

B. Protein Digestion

Note: Use 25-100µg of purified or lyophilized protein per sample. If the protein is in solution, it must be free of amine-containing buffers. Use the BSA (100µg) as a control sample for method optimization.

1. Suspend 100µg acetone-precipitated (or lyophilized) protein pellets with 100µL of 100mM TEAB.

   Note: An acetone-precipitated pellet might not completely dissolve; however, after proteolysis at 37°C, all the protein (peptides) will be solubilized.

2. Immediately before use, add 20µL of the Trypsin Storage Solution to the bottom of the trypsin glass vial and incubate for 5 minutes. Store any remaining reagent in single-use volumes at -80°C (e.g., 2.5µg of trypsin per 100µg of protein).

3. Add 2.5µL of trypsin (i.e., 2.5µg) per 100µg of protein. Digest the sample overnight at 37°C.
C. Peptide Labeling

1. Immediately before use, equilibrate the TMT Label Reagents to room temperature. For the 0.8mg vials, add 41µL of anhydrous acetonitrile or ethanol to each tube. For the 5mg vials, add 256µL of solvent to each tube. Allow the reagent to dissolve for 5 minutes with occasional vortexing. Briefly centrifuge the tube to gather the solution.

   Note: Reagents dissolved in anhydrous acetonitrile or ethanol are stable for one week when stored at -20°C and warmed to room temperature before opening.

2. Carefully add 41µL of the TMT Label Reagent to each 25-100µg sample. Alternatively, transfer the reduced and alkylated protein to the TMT Reagent vial.

   Note: A 100µL glass syringe or positive displacement pipette may be necessary to accurately measure and dispense TMT Reagents in volatile acetonitrile solvent.

3. Incubate the reaction for 1 hour at room temperature.

4. Add 8µL of 5% hydroxylamine to the sample and incubate for 15 minutes to quench the reaction.

5. Combine samples at equal amounts and store at -80°C.

Preparing and Labeling Intact Proteins with the TMT Isobaric Mass Tags

Note: Protein labeling results in the modification of lysine residues and non-acetylated protein N-termini. Because trypsin does not recognize modified lysines, trypsin digestion cleaves only on the C-terminal side of arginine residues. The result is fewer, larger peptides and a less complex digest. Labeled proteins can be digested with other enzymes, including chymotrypsin and Glu-C.

1. Solubilize and quantify protein samples as described above in Section A: Preparing Whole Cell Protein Extracts, steps 1-8.

   Note: If primary amine containing lysis buffers were used to prepare whole cell protein extracts, samples must be exchanged using dialysis or desalting into a suitable non-primary amine, containing buffer (e.g., TEAB, PBS, HEPES or bicine) at pH 7 to 9.

2. Immediately before use, equilibrate the TMT Label Reagents to room temperature. For the 0.8mg vials, add 24µL of anhydrous DMSO to each tube. For the 5mg vials, add 150µL DMSO to each tube. Allow the reagent to dissolve for 5 minutes with occasional vortexing. Briefly centrifuge the tube to gather the solution.

   Note: Reagents dissolved in DMSO are stable for one week when stored at -20°C and warmed to room temperature before opening.

3. Carefully add 24µL of the TMT Label Reagent to each 100µg sample. Alternatively, transfer the reduced and alkylated protein to TMT Reagent vial.

4. Incubate the reaction for 1 hour at room temperature.

5. Add 8µL of 5% hydroxylamine to the sample and incubate for 15 minutes to quench the reaction.

6. Combine samples at equal amounts. Store samples at -80°C or fractionate to remove excess tag (e.g., acetone precipitation, desalting or SDS-PAGE) before enzymatic digestion.
Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible Cause</th>
<th>Solution</th>
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<tr>
<td>Poor labeling</td>
<td>An amine-based buffer was used</td>
<td>Use a non-amine-based buffer</td>
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<tr>
<td>Correct buffer pH</td>
<td></td>
<td>Make sure the buffer pH is ~8.0</td>
</tr>
<tr>
<td>Protein precipitation</td>
<td>Lack of detergent present</td>
<td>Add detergent, such as 0.05% SDS to the preparation</td>
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<tr>
<td></td>
<td>pH decreased</td>
<td>Make sure the pH is &gt; 7.5</td>
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<tr>
<td></td>
<td>Organic solvent too high</td>
<td>For protein labeling, dissolve TMT Reagents in DMSO to minimize protein precipitation</td>
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</table>

Additional Information

A. Sample Clean-up, Enrichment and Fractionation

Listed below are some options for peptide cleanup before MS analysis.

- If SDS and DMSO were avoided during the preparation, acetonitrile may be removed by vacuum centrifugation and samples analyzed directly by LC-MS/MS. Collect MS data above 350Da to avoid signal from unincorporated tag.
- SDS, solvent and unincorporated tags can be removed using TopTip™ Strong Cation-Exchange Tips (PolyLC, Product No. TT1000SEA-2003), according to the manufacturer’s instructions.
- Salt and unincorporated tags can be removed using Thermo Scientific Pierce C18 Spin Columns (Product No. 89870).
- Proteins labeled with TMT Reagents can be detected with the anti-TMT Antibody (Product No. 90075). Proteins and peptides labeled with TMT Reagents can be enriched with the Immobilized anti-TMT Antibody Resin and the TMT Enrichment Kit (Product No 90076 and 90077).
- For best results, use an HPLC system to perform strong cation exchange fractionation to remove SDS and to fractionate complex proteomic extracts. Perform the separation with a strong-cation exchange column (PolyLC, Inc., Table 1). See also www.polylc.com Proteomics v.2.htm and Trinidad, J.C. (2008).

Table 1. Strong-cation exchange column information.

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<tr>
<th>PolyLC Part #</th>
<th>Column</th>
<th>Particle</th>
<th>Pore</th>
<th>Load Range</th>
<th>Flow Range</th>
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<tr>
<td>102SE0503</td>
<td>2.1 × 100</td>
<td>5µm</td>
<td>300Å</td>
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<td>104SE0503</td>
<td>4.6 × 100</td>
<td>5µm</td>
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<td>0.4-4.0mg</td>
<td>0.7-1.0mL/min</td>
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B. Data Acquisition Methods for Peptide Quantitation

Quantitation of peptides labeled with Tandem Mass Tag® Reagents requires a mass spectrometer capable of MS/MS fragmentation, such as an ion trap, quadrupole time of flight, time of flight-time of flight (TOF-TOF) or triple quadrupole instrument. The choice of MS/MS fragmentation method(s) depends on the instrument capabilities such as collisionally induced dissociation (CID), pulsed-Q dissociation (PQD), higher energy collisional dissociation (HCD), or electron transfer dissociation (ETD), and the desire either to optimize one fragmentation method for both peptide identification and quantitation, or to use two methods that are each optimized for peptide identification or quantitation. For example, TMT Reagent reporter ions are not visible in ion traps following traditional CID fragmentation. Instead, quantify and identify peptides on an ion trap with PQD fragmentation or alternate PQD and CID methods optimized for identification and quantitation, respectively (Table 2). The TMT tags behave similarly to iTRAQ® Reagents (Life Technologies Corp.), although optimal chromatography and fragmentation energy settings are slightly different.
Table 2. Instruments and MS/MS fragmentation options for peptide identification and quantitation with Thermo Scientific TMT Reagents.

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Fragmentation Method</th>
<th>Reference</th>
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<tr>
<td>Thermo Scientific Velos Pro ion trap</td>
<td>Trap HCD</td>
<td>Biringer, et al. (2011)</td>
</tr>
<tr>
<td>Thermo Scientific LTQ-Orbitrap Discovery or LTQ ion trap</td>
<td>PQD, PQD/CID</td>
<td>Bantscheff, et al. (2008), Schwartz, et al. (2008),</td>
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<td>Thermo Scientific LTQ-OrbitrapXL-ETD or LTQ-ETD</td>
<td>ETD</td>
<td>Viner, et al. (2009)</td>
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<td>Q-TOF</td>
<td>CID</td>
<td>Van Ulsen, et al. (2009)</td>
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<tr>
<td>TOF-TOF</td>
<td>CID</td>
<td>Dayon, et al. (2008)</td>
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</table>

C. Data Analysis and Quantitation

The masses for peptide modification by the TMT zero, duplex, and sixplex reagents are present in the UNIMOD database (www.unimod.org) and are listed below. Several software packages directly support the modifications by TMT Reagents and the relative quantitation of reporter ions released from labeled peptides, including Thermo Scientific Proteome Discoverer (all versions) 1.1, Thermo Scientific Bioworks 3.1.1, Matrix Science Mascot 2.1 and above, and Proteome Software Scaffold Q+. For data acquired using a combination of fragmentation methods (i.e. HCD/CID or PQD/CID), Proteome Discoverer 1.1 or custom software might be necessary to merge spectra for identification and quantitation.

D. Mass Modification

All TMT Reagents share an identical chemical structure. Therefore, labeled samples behave identically during LC-MS or MALDI-MS analysis and can be quantified at either the MS/MS or MS level. For MS/MS quantitation, duplex or sixplex samples may be quantified with TMTduplex or TMTsixplex Reagent Sets. This strategy allows higher plexing and the ability to quantify specific, singly charged reporter ions without increasing sample complexity. For duplex MS quantitation, samples or internal standards labeled with TMTzero may be combined with samples labeled with a TMTsixplex Reagent, resulting in a modification of 224Da or 229Da for every labeled lysine residue, respectively. Paired peaks with a 5Da mass shift per labeled N-terminus and lysine residue are then quantified similarly to SILAC samples. This approach also may be used to quantitate specific parent and transition ions using selective reaction monitoring (SRM) strategies.
Table 3. Modification masses of the Thermo Scientific TMT Label Reagents.

<table>
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<tr>
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<td>224.152478</td>
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<td>225.2921</td>
<td>126.127725</td>
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<td>114.127725</td>
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<td>131.138176</td>
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* CID, HCD, and PQD are collisional fragmentation methods that generate reporter ions from 126 to 131Da.
**ETD is a non-ergodic fragmentation method that generates six unique reporter ions from 114 to 119Da.

E. Information Available from our Website

- Tech Tip Protocol #49: Acetone precipitation of proteins
- Tech Tip Protocol #19: Remove detergent from protein samples

Related Thermo Scientific Products

- 90076 Immobilized Anti-TMT Antibody Resin
- 90075 Anti-TMT Antibody, 0.1mL
- 88320 Pierce Peptide Retention Time Calibration Mixture, 50uL
- 88321 Pierce Peptide Retention Time Calibration Mixture, 200uL
- 89983 SILAC Protein Quantitation Kit – DMEM
- 89982 SILAC Protein Quantitation Kit – RPMI 1640
- 88439 SILAC Protein Quantitation Kit - DMEM:F12
- 87784 Pierce C18 Tips, 100µl bed, 96 tips
- 89870 Pierce C18 Spin Columns, 25 columns
- 28904 Trifluoroacetic Acid, Sequanal Grade
- 23227 Pierce BCA Protein Assay
- 23208 Pre-Diluted Protein Assay Standards
- 88300 Fe-NTA Phosphopeptide Enrichment Kit
- 88301 Pierce TiO2 Phosphopeptide Enrichment and Clean-up Kit
- 90003 Pierce Phosphoprotein Isolation Kit
- 88513 Pierce Concentrator, PES, 10K MWCO, 0.5mL
- 89893 Zeba Spin Desalting Columns, 10mL, 5 columns

General References


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TopTip is a trademark of PolyLC, Inc.

iTRAQ is a trademark of Life Technologies Corp.

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