

TMT Isobaric Mass Tagging Kits and Reagents

2073.4

Number	Description
90063	TMTduplex™ Isobaric Mass Tagging Kit , contains sufficient reagents for 10 samples Contents: TMT⁰ Label Reagent , 5 × 0.8 mg TMT²-126 Label Reagent , 5 × 0.8 mg TMT²-127 Label Reagent , 5 × 0.8 mg Dissolution Buffer (1 M triethyl ammonium bicarbonate), 5 ml Denaturing Reagent (10% SDS), 1 ml Reducing Reagent (0.5 M TCEP), 1 ml Iodoacetamide , 12 × 9 mg Quenching Reagent (50% hydroxylamine), 1 ml Trypsin , 2 × 20 µg Trypsin Storage Solution , 250 µl Albumin, Bovine , 2.5 mg
90064	TMTsixplex™ Isobaric Mass Tagging Kit , contains sufficient reagents for 30 samples Contents: TMT⁰ Label Reagent , 5 × 0.8 mg TMT⁶-126 Label Reagent , 5 × 0.8 mg TMT⁶-127 Label Reagent , 5 × 0.8 mg TMT⁶-128 Label Reagent , 5 × 0.8 mg TMT⁶-129 Label Reagent , 5 × 0.8 mg TMT⁶-130 Label Reagent , 5 × 0.8 mg TMT⁶-131 Label Reagent , 5 × 0.8 mg Dissolution Buffer (1 M triethyl ammonium bicarbonate), 5 ml Denaturing Reagent (10% SDS), 1 ml Reducing Reagent (0.5 M TCEP), 1 ml Iodoacetamide , 12 × 9 mg Quenching Reagent (50% hydroxylamine), 1 ml Trypsin , 5 × 20 µg Trypsin Storage Solution , 250 µl Albumin, Bovine , 2.5 mg
90065	TMTduplex Label Reagent Set , contains sufficient reagents for 10 samples Contents: TMT²-126 Label Reagent , 5 × 0.8 mg TMT²-127 Label Reagent , 5 × 0.8 mg

90066 TMTsixplex Label Reagent Set, contains sufficient reagents for 30 samples

Contents:

TMT⁶-126 Label Reagent, 5 × 0.8 mg

TMT⁶-127 Label Reagent, 5 × 0.8 mg

TMT⁶-128 Label Reagent, 5 × 0.8 mg

TMT⁶-129 Label Reagent, 5 × 0.8 mg

TMT⁶-130 Label Reagent, 5 × 0.8 mg

TMT⁶-131 Label Reagent, 5 × 0.8 mg

90068 TMTsixplex Label Reagent Set, contains sufficient reagents for 72 samples

Contents:

TMT⁶-126 Label Reagent, 2 × 5 mg

TMT⁶-127 Label Reagent, 2 × 5 mg

TMT⁶-128 Label Reagent, 2 × 5 mg

TMT⁶-129 Label Reagent, 2 × 5 mg

TMT⁶-130 Label Reagent, 2 × 5 mg

TMT⁶-131 Label Reagent, 2 × 5 mg

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

Storage: Upon receipt store at -20°C. Product shipped with dry ice.

Note: This product is for research use only. Do not use for diagnostic procedures.

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Introduction

The Thermo Scientific TMT Isobaric Mass Tagging Kits and Reagents enable quantitative labeling of proteins extracted from cells and tissues. Each isobaric tagging reagent 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., 126-127 Da for TMT² and 126-131 Da for TMT⁶ 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. Each reagent in the duplex and sixplex set has the same nominal parent (precursor) mass; however, the masses differ in the balancing and reporter region. By balancing these two components, the intact molecule has the same mass. For example, for the duplex reagent a given peptide will be modified by 225 Da per amine residue. At the MS/MS level, the reporter ions will be either 126 or 127 Da (see the Additional Information Section). The mass balance is compensated by a neutral loss of the reporter ion.

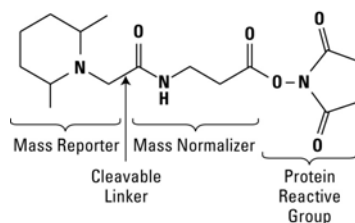


Figure 1. Chemical structure of the TMT Label Reagents.

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.

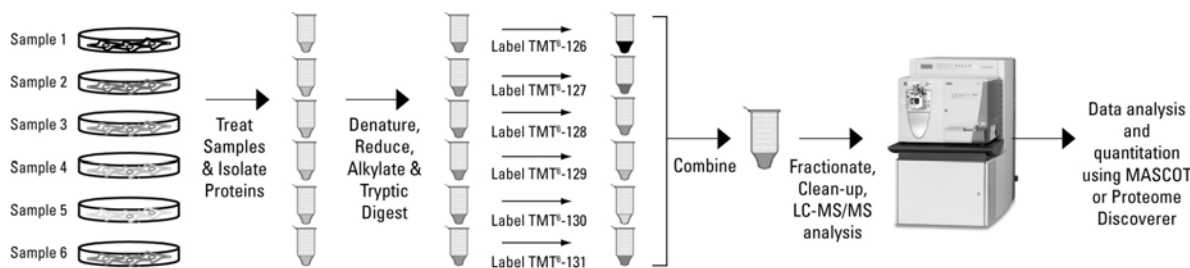


Figure 2. Schematic for using the Thermo Scientific TMTsixplex Isobaric Mass Tagging Reagents.

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 25-100 µg 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) or anhydrous dimethyl sulfoxide (DMSO), Sequanal grade (Product No. 20688)
- Glass syringe (100 µl)
- 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 8 M Urea (Product No. 29700)
- Protein assay such as Thermo Scientific Coomassie Plus (Bradford) Protein Assay (Product No. 23236), Pierce 660 nm Protein Assay (Product No. 22600) or Pierce BCA Protein Assay Kit (Product No. 22235)
- 75-300 μm capillary C_{18} 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, 0.5 ml (Product No. 66333)

Material Preparation

Albumin, Bovine (BSA)	Reconstitute BSA (2.5 mg) with 2.5 ml of ultrapure water. Divide solution into 100 μl aliquots and lyophilize to dryness.
200 mM TEAB (triethyl ammonium bicarbonate)	Add 100 μl of the Dissolution Buffer (1 M TEAB) to 400 μl of ultrapure water.
2% SDS	Add 50 μl of the Denaturing Reagent (10% SDS) to 200 μl of ultrapure water.
200 mM TCEP	Add 70 μl of the Reducing Reagent (0.5 M TCEP) to 70 μl of ultrapure water. Then add 35 μl of the Dissolution Buffer (1 M TEAB).
5% Hydroxylamine	Dilute the Quenching Reagent (50% hydroxylamine) 1:10 with 200 mM TEAB.

Preparing and Labeling Peptides with the TMT Isobaric Mass Tags

A. Preparing Whole Cell Protein Extracts

1. Culture cells to harvest at least 100 μg per condition. For best results, culture a minimum of 5×10^6 cells.
2. Lyse cells in either RIPA buffer, M-PER[®] Reagent or 8 M urea. Add protease and phosphatase inhibitors to the lysis reagent. Use 4 ml of lysis reagent for each milliliter of cells.
3. Perform a protein assay to determine the protein concentration. Use samples at ≥ 2 mg/ml. Less concentrated samples can be used; however it might be necessary to use larger volumes of reducing/alkylating reagents.
4. Place 100 μg per condition (two for the TMTduplex or six for the TMTsixplex Label Reagents) in a polypropylene microcentrifuge tube.
5. Add 45 μl of 200 mM TEAB to the sample and adjust to a final volume of 100 μl with ultrapure water.
Note: For labeling reactions > 100 μl use a large-volume centrifuge tube such as a 15 ml or 50 ml polypropylene conical tube. Optional: To solubilize complex protein mixtures, add 5 μl of 2% SDS before adjusting to final volume.
6. Add 5 μl of the 200 mM TCEP and incubate sample at 55°C for 1 hour.
7. Immediately before use, dissolve one tube of iodoacetamide (9 mg) with 132 μl of 200 mM TEAB to make 375 mM iodoacetamide. Protect solution from light.
8. Add 5 μl of the 375 mM iodoacetamide (with TEAB) to the sample and incubate for 30 minutes protected from light.
9. Add six volumes (~1 ml) of pre-chilled (-20°C) acetone. Allow the precipitation to proceed overnight.
10. Centrifuge the samples at $8,000 \times g$ 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 25-100 µg of purified or lyophilized protein per sample. If the protein is in solution, it must be free of amine-containing buffers. Use the BSA (100 µg) as a control sample for method optimization.

1. Suspend 100 µg acetone-precipitated (or lyophilized) protein pellets with 100 µl of 200 mM 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 20 µl 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., 2.5 µg of trypsin per 100 µg of protein).
3. Add 2.5 µl of trypsin (i.e., 2.5 µg) per 100 µg 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 0.8 mg vials, add 41 µl of anhydrous acetonitrile or DMSO to each tube. For the 5 mg vials, add 256 µl 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 DMSO are stable for one week when stored at -20°C and warmed to room temperature before opening.
2. Carefully add 41 µl of the TMT Label Reagent to each 25-100 µg sample. Alternatively, transfer the reduced and alkylated protein to the TMT reagent vial.
Note: A 100 µl 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 8 µl 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 7-9.
2. Immediately before use, equilibrate the TMT Label Reagents to room temperature. For the 0.8 mg vials, add 24 µl of anhydrous DMSO to each tube. For the 5 mg vials, add 150 µl 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 24 µl of the TMT Label Reagent to each 100 µg sample. Alternatively, transfer the reduced and alkylated protein to TMT reagent vial.
4. Incubate the reaction for 1 hour at room temperature.
5. Add 8 µl 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

Problem	Possible Cause	Solution
Poor labeling	An amine-based buffer was used	Use a non-amine-based buffer
	Incorrect buffer pH	Make sure the buffer pH is ~8.0
Protein precipitation	Lack of detergent present	Add detergent, such as 0.05% SDS to the preparation
	pH decreased	Make sure the pH is > 7.5
	Organic solvent too high	For protein labeling, dissolve TMT reagents in DMSO to minimize protein precipitation

Additional Information

A. Sample Cleanup 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 350 Da 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.
- 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.

<u>PolyLC Part #</u>	<u>Column</u>	<u>Particle</u>	<u>Pore</u>	<u>Load Range</u>	<u>Flow Range</u>
102SE0503	2.1 × 100	5 μm	300 Å	0.1-1.0 mg	0.14-0.2 ml/min
104SE0503	4.6 × 100	5 μm	300 Å	0.4-4.0 mg	0.7-1.0 ml/min

B. Data Acquisition Methods for Peptide Quantitation

Quantitation of peptides labeled with tandem mass tags 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 (Applied Biosystems), although optimal chromatography and fragmentation energy settings are slightly different.

Table 2. Instruments and MS/MS fragmentation options for peptide identification and quantitation with TMT reagents.

<u>Instrument</u>	<u>Fragmentation Method</u>	<u>Reference</u>
Thermo Scientific LTQ-Orbitrap Velos, LTQ-Orbitrap XL, or MALDI-Orbitrap XL	HCD, HCD/CID	Zhang, <i>et al.</i> (2008), Viner, <i>et al.</i> (2008), Strupat, <i>et al.</i> (2008)
Thermo Scientific LTQ-Orbitrap Discovery or LTQ ion trap	PQD, PQD/CID	Kiyonami, <i>et al.</i> (2008), Schwartz, <i>et al.</i> (2008), Bantscheff, <i>et al.</i> (2008)
Thermo Scientific LTQ-OrbitrapXL-ETD or LTQ-ETD	ETD	Schwartz, <i>et al.</i> (2008), Viner, <i>et al.</i> (2009)
Q-TOF	CID	Van Ulsen, <i>et al.</i> (2009)
TOF-TOF	CID	Dayon, <i>et al.</i> (2008)
Triple Quadrupole	CID	Byers, <i>et al.</i> (2009)

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 1.1, Thermo Scientific Bioworks 3.1.1, Matrix Science Mascot 2.1, 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 224 Da or 229 Da for every labeled lysine residue, respectively. Paired peaks with a 5 Da mass shift per labeled N-terminus and lysine residue are then quantified similarly to SILAC samples. This approach may also 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.

<u>Label Reagent</u>	<u>Modification Mass (monoisotopic)</u>	<u>Modification Mass (average)</u>	<u>CID Monoisotopic Reporter Mass</u>	<u>CID Average Reporter Mass</u>	<u>ETD Monoisotopic Reporter Mass*</u>	<u>ETD Average Reporter Mass*</u>
TMT ⁰ -126	224.152478	224.2994	126.127725	126.2193	114.127725	114.2086
TMT ² -126	225.155833	225.2921	126.127725	126.2193	114.127725	114.2086
TMT ² -127	225.155833	225.2921	127.131079	127.2120	114.127725	114.2086
TMT ⁶ -126	229.162932	229.2634	126.127725	126.2193	114.127725	114.2086
TMT ⁶ -127	229.162932	229.2634	127.131079	127.2120	114.127725	114.2086
TMT ⁶ -128	229.162932	229.2634	128.134433	128.2046	116.134433	116.1939
TMT ⁶ -129	229.162932	229.2634	129.137787	129.1973	116.134433	116.1939
TMT ⁶ -130	229.162932	229.2634	130.141141	130.1900	118.141141	118.1417
TMT ⁶ -131	229.162932	229.2634	131.138176	131.1834	119.138176	119.1727

*Because ETD cleaves TMT reagents at the N-C α bond, product ion spectra from the 126 and 127 or 128 and 129 labeling reagents generate structurally identical reporter peaks at m/z 114 and 116, respectively.

E. Information Available from the Web Site

- Tech Tip Protocol #49: Acetone Precipitation of Proteins
- Tech Tip Protocol #19: Remove Detergents from Protein Samples

Related Thermo Scientific Products

90008	Pierce Strong Cation Exchange Spin Column, Mini , 24 spin columns and 48 collection tubes
90009	Pierce Strong Cation Exchange Spin Column, Maxi , 8 spin columns and 16 collection tubes
89983	Pierce SILAC Protein Quantitation Kit – DMEM
89982	Pierce SILAC Protein Quantitation Kit – RPMI 1640
89870	PepClean C-18 Spin Columns , 25 columns
28904	Trifluoroacetic Acid, Sequanal Grade
23227	Pierce BCA Protein Assay
23208	Pre-Diluted Protein Assay Standards
89853	Phosphopeptide Isolation Kit
90003	Pierce Phosphoprotein Isolation Kit

89885 Pierce Concentrator, 20 ml/9K MWCO, 25 units
89893 Zeba Spin Desalting Columns, 10 ml, 5 columns

General References

- Bantscheff, M., *et al.* (2008). Robust and Sensitive iTRAQ Quantification on an LTQ Orbitrap Mass Spectrometer. *Mol Cell Proteomics* **7**:1702-13.
- Byers, H.L. (2009). Candidate verification of iron-regulated *Neisseria meningitidis* proteins using isotopic versions of tandem mass tags (TMT) and single reaction monitoring. *J Prot* doi:10.1016/j.jprot.2009.09.002. <http://dx.doi.org>
- Dayon, L., *et al.* (2008). Relative quantification of proteins in human cerebrospinal fluids by MS/MS using 6-plex isobaric tags. *Anal Chem* **80(8)**:2921-31.
- Kiyonami, R., *et al.* (2008). Identification and quantitation of iTRAQ labeled peptides on the LTQ using MS/MS and MS³. Application note # 353. www.thermo.com
- Ross, P.L., *et al.* (2004). Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. *Mol Cell Proteomics* **3(12)**:1154-69.
- Schwartz, J. *et al.* (2008). Relative quantitation of protein digests using tandem mass tags and pulsed-Q dissociation (PQD). Application note # 452. www.thermo.com
- Strupat K., *et al.* (2008). Accurate MS and MSⁿ Analysis with the Thermo Scientific MALDI LTQ Orbitrap. Application note # 30150. www.thermo.com
- Trinidad, J.C., *et al.* (2008). Quantitative analysis of synaptic phosphorylation and protein expression. *Mol Cell Proteomics* **7(4)**:684-96.
- Viner, R., *et al.* (2008). Quantitative profiling of DNA damage response proteins using iTRAQ labeling and the LTQ Orbitrap XL. Application note # 445. www.thermo.com
- Viner, R.I., *et al.* (2009). Quantification of post-translationally modified peptides of bovine α -crystallin using tandem mass tags and electron transfer dissociation. *J Proteomics* **72(5)**:874-85.
- Zhang, T., *et al.* (2008). Quantitation of iTRAQ labeled peptides using higher energy collisional dissociation on the LTQ Orbitrap. Application note # 421. www.thermo.com

TMT Reagents are protected by U.S. Patent #7,294,456 B2, European Patent EP1275004 B1 and patents pending.

TopTip™ is a trademark of PolyLC, Inc.

iTRAQ® is a registered trademark of Applied Biosystems.

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Current versions of product instructions are available at www.thermo.com/pierce. For a faxed copy, call 800-874-3723 or contact your local distributor.

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