INSTRUCTIONS

TMT Mass Tagging Kits and Reagents

90060-90068

2073.13

Number	Description
90060	**TMTduplex Isotopic Label Reagent Set,** sufficient reagents for 5 duplex isotopic experiments **Contents:** **TMT^{0} Label Reagent,** 5 × 0.8mg **TMT^{6}-127 Label Reagent,** 5 × 0.8mg
90061	**TMTsixplex Isobaric Label Reagent Set,** sufficient reagents for 1 sixplex isobaric experiment **Contents:** **TMT^{6}-126 Label Reagent,** 1 × 0.8mg **TMT^{6}-127 Label Reagent,** 1 × 0.8mg **TMT^{6}-128 Label Reagent,** 1 × 0.8mg **TMT^{6}-129 Label Reagent,** 1 × 0.8mg **TMT^{6}-130 Label Reagent,** 1 × 0.8mg **TMT^{6}-131 Label Reagent,** 1 × 0.8mg
90062	**TMTsixplex Isobaric Label Reagent Set,** sufficient reagents for 2 sixplex isobaric experiments **Contents:** **TMT^{6}-126 Label Reagent,** 2 × 0.8mg **TMT^{6}-127 Label Reagent,** 2 × 0.8mg **TMT^{6}-128 Label Reagent,** 2 × 0.8mg **TMT^{6}-129 Label Reagent,** 2 × 0.8mg **TMT^{6}-130 Label Reagent,** 2 × 0.8mg **TMT^{6}-131 Label Reagent,** 2 × 0.8mg
90063	**TMTduplex Isobaric Mass Tagging Kit,** sufficient reagents for 5 duplex isobaric experiments **Contents:** **TMT^{0} Label Reagent,** 5 × 0.8mg **TMT^{2}-126 Label Reagent,** 5 × 0.8mg **TMT^{2}-127 Label Reagent,** 5 × 0.8mg **Dissolution Buffer** (1 M triethyl ammonium bicarbonate), 5mL **Denaturing Reagent** (10% SDS), 1mL **Reducing Reagent** (0.5M TCEP), 1mL **Iodoacetamide,** 12 × 9mg **Quenching Reagent** (50% hydroxylamine), 1mL **Pierce™ Trypsin Protease, MS Grade,** 2 × 20µg **Trypsin Storage Solution,** 250µL **Albumin, Bovine,** 2.5mg

Pierce Biotechnology 3747 N. Meridian Road | PO Box 117 Rockford, IL 61105 USA | (815) 968-0747 (815) 968-7316 fax | www.thermoscientific.com/pierce

90064	**TMTsixplex Isobaric Mass Tagging Kit,** sufficient reagents for 5 sixplex isobaric experiments **Contents:** **TMT^{0} Label Reagent,** 5 × 0.8mg **TMT^{6}-126 Label Reagent,** 5 × 0.8mg **TMT^{6}-127 Label Reagent,** 5 × 0.8mg **TMT^{6}-128 Label Reagent,** 5 × 0.8mg **TMT^{6}-129 Label Reagent,** 5 × 0.8mg **TMT^{6}-130 Label Reagent,** 5 × 0.8mg **TMT^{6}-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 **Pierce Trypsin Protease, MS Grade,** 5 × 20µg **Trypsin Storage Solution,** 250µL **Albumin, Bovine,** 2.5mg
90065	**TMTduplex Isobaric Label Reagent Set,** sufficient reagents for 5 duplex isobaric experiments **Contents:** **TMT^{2}-126 Label Reagent,** 5 × 0.8mg **TMT^{2}-127 Label Reagent,** 5 × 0.8mg
90066	**TMTsixplex Label Reagent Set,** sufficient reagents for 5 sixplex isobaric experiments **Contents:** **TMT^{6}-126 Label Reagent**, 5 × 0.8mg **TMT^{6}-127 Label Reagent,** 5 × 0.8mg **TMT^{6}-128 Label Reagent,** 5 × 0.8mg **TMT^{6}-129 Label Reagent,** 5 × 0.8mg **TMT^{6}-130 Label Reagent,** 5 × 0.8mg **TMT^{6}-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:** **TMT^{6}-126 Label Reagent**, 2 × 5mg **TMT^{6}-127 Label Reagent,** 2 × 5mg **TMT^{6}-128 Label Reagent,** 2 × 5mg **TMT^{6}-129 Label Reagent,** 2 × 5mg **TMT^{6}-130 Label Reagent,** 2 × 5mg **TMT^{6}-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.

Contents

Introduction

The Thermo Scientific™ TMT™ Isobaric Mass Tagging Kits and Reagents enable multiplex relative quantitation by mass spectrometry (MS). Each mass-tagging reagent within a set has the same nominal mass (i.e., isobaric) and chemical structure composed of an amine-reactive NHS-ester group, a spacer arm and an MS/MS reporter (Figure 1). The reagent sets can be used to label two or six peptide samples prepared from cells or tissues. For each sample, a unique reporter in the low mass region of the MS/MS spectrum (i.e., 126-127Da for TMT^2 and 126-131Da for TMT^6 Isobaric Label Reagents) is used to measure relative protein expression levels during peptide fragmentation.

The TMTduplex™ Isotopic Label Reagent Set contains TMTzero™ and one of the TMTsixplex™ Reagents (TMT^6-127) to be used as "light" and "heavy" tags for MS-level peptide quantitation similar to duplex isotopic metabolic labeling (e.g., SILAC) or isotopic dimethylation labeling. These isotopic pairs can also be used in targeted quantitation strategies, including selective reaction monitoring (SRM, see the Additional Information Section). Advantages of the TMTduplex and TMTsixplex Isobaric Label Reagents include increased sample multiplexing for relative quantitation, increased sample throughput and fewer missing quantitative channels among samples.

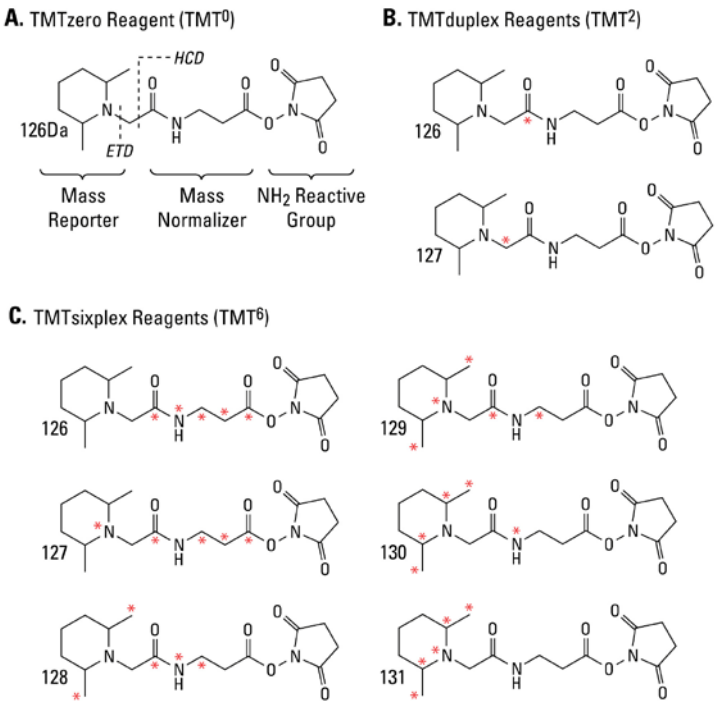

Figure 1. Chemical structure of the TMT Label Reagents. A. Functional regions of the reagent structure, including MS/MS fragmentation sites by higher energy collision dissociation (HCD) and electron transfer dissociation (ETD). **B.** TMTduplex Reagent structures and isotope positions (*); only HCD differentiates between these two reporters. **C.** TMTsixplex Reagent structures and isotope positions (*).

Procedure Summary

Protein extracts isolated from cells or tissues are reduced, alkylated and digested overnight. Samples are labeled with the TMT Reagents and then mixed before sample fractionation and clean-up. Labeled samples are analyzed by high resolution Orbitrap LC-MS/MS before data analysis to identify peptides and quantify reporter ion relative abundance (Figure 2).

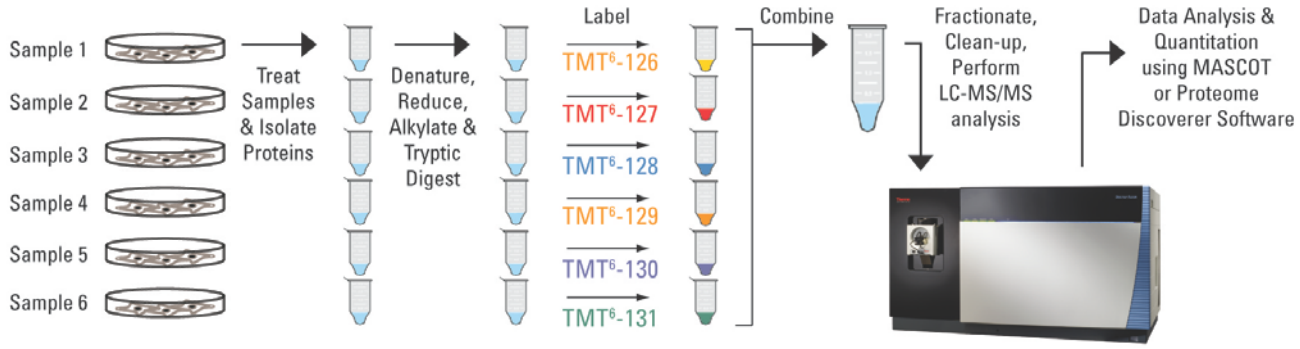

Figure 2. Schematic for using the Thermo Scientific TMTsixplex Isobaric Mass Tagging Reagents.

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.
- Anhydrous acetonitrile is the recommended solvent to dissolve reagents. Stock solutions are stable for one week when stored at -20°C. For long term storage of unused reagent, remove all solvent by drying and store with desiccant at -20°C. Anhydrous ethanol can be used as an alternative solvent to dissolve reagents but is not recommended for stock solution storage.
- 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.
- 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

- Microcentrifuge tubes
- Anhydrous acetonitrile (Thermo Scientific™ Acetonitrile HPLC grade, Product No. 51101)
- Water, LC-MS Grade (Product No. 51140)
- Chilled (-20°C) acetone
- Protein assay (e.g., Thermo Scientific™ BCA Protein Assay Kit, Product No. 22235)
- 75-300µm capillary C_{18} reversed-phase column
- High-resolution Orbitrap Mass Spectrometer, 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: C18 spin tips or columns (e.g., Thermo Scientific™ Pierce™ C18 Spin Columns, Product No. 89870 or Pierce™ C18 Tips, Product No. 87784)

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. The amounts listed below are sufficient for preparing and labeling 6 samples.

100mM TEAB (triethyl ammonium bicarbonate)	Add 500µL of the Dissolution Buffer (1M TEAB) to 4.5mL of ultrapure water.
Lysis Buffer	Add 200µL of the Denaturing Reagent (10% SDS) to 1.8mL of 100mM TEAB.
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	Add 50µL of the Quenching Reagent (50% hydroxylamine) to 450µL of 100mM TEAB.

Preparing and Labeling Peptides with the TMT Isobaric Mass Tags

Note: BSA can be used as a control sample for method optimization. Dissolve BSA to 1mg/mL using 100mM TEAB. Use 25-100µg of protein per labeling reaction. The Thermo Scientific™ Pierce™ Mass Spec Sample Prep Kit for Cultured Cells can also be used to prepare peptide digests for TMT reagent labeling.

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 2×10^6 cells.

   Note: Rinse cells 2-3 times with 1X PBS to remove cell culture media. Pellet cells using low-speed centrifugation (i.e., < 1000 × *g*) to prevent premature cell lysis.

2. Lyse the cells by adding five cell-pellet volumes of Lysis Buffer (i.e., 100µL of Lysis Buffer for a 20µL cell pellet).

   Note: Lysis buffers such as Thermo Scientific™ RIPA Lysis and Extraction Buffer (Product No. 89901) or 8M urea (Product No. 29700) in 50mM TEAB or HEPES buffer, pH 8 may be used as alternative denaturing cell lysis buffers. For urea-based lysis buffer, protein samples must be diluted to < 1M urea before digestion, and the final C18 desalting step (C.6) is not optional. Addition of protease and/or phosphatase inhibitors during lysis is optional and may interfere with MS analysis.

   Note: Depending on the Lysis Buffer used it may be necessary to reduce sample viscosity by shearing DNA using a microtip sonicator or addition of a nuclease (e.g., Thermo Scientific™ Pierce™ Universal Nuclease for Cell Lysis, Product No. 88700)

3. Centrifuge lysate at 16,000 × *g* for 10 minutes at 4°C.
4. Carefully separate the supernatant and transfer into a new tube.
5. Determine the protein concentration of the supernatant using established methods such as the BCA Protein Assay Kit (Product No. 23227).

   Note: Use samples at ≥ 2mg/mL. Less concentrated samples may be used; however, it might be necessary to use larger volumes of reducing/alkylating reagents.

6. Transfer 100µg per condition (two for the TMTduplex or six for the TMTsixplex Label Reagents) into a new microcentrifuge tube and adjust to a final volume of 100µL with 100mM TEAB.
7. Add 5µL of the 200mM TCEP and incubate sample at 55°C for 1 hour.
8. Immediately before use, dissolve one tube of iodoacetamide (9mg) with 132µL of 100mM TEAB to make 375mM iodoacetamide. Protect solution from light.
9. Add 5µL of the 375mM iodoacetamide to the sample and incubate for 30 minutes protected from light at room temperature.
10. Add six volumes (~600µL) of pre-chilled (-20°C) acetone and freeze at -20°C. Allow the precipitation to proceed for at least 4 hours up to overnight.
11. 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 2-3 minutes.

B. Protein Digestion

1. Resuspend 100µg of 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 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 are stable for one week when stored at -20°C. Anhydrous ethanol can be used as an alternative solvent to dissolve reagents but is not recommended for stock solution storage.

2. Carefully add 41µL of the TMT Label Reagent to each 25-100µg sample. Alternatively, transfer the peptide sample 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.

6. Optional: Clean-up samples with C18 spin tips (Product No. 87784) or columns (Product No. 89870) before LC-MS analysis. Peptide clean up is recommended before LC-MS analysis but is not required.

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
	Too much sample was used	Label 25-100µg per sample
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

Additional Information

A. Data Acquisition Methods

Quantitation of peptides labeled with Thermo Scientific™ 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. Higher energy collision dissociation (HCD) is recommended for TMT reporter ion fragmentation. Optimal HCD fragmentation energy is instrument-dependent and can be optimized using TMTzero Reagents. Electron transfer dissociation (ETD) may be used as an alternative fragmentation method for peptide identification and quantitation. 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). TMT Reagent reporter ions are not visible in ion traps following traditional CID fragmentation.

Table 1. Instruments and MS/MS fragmentation options for peptide identification and quantitation with Thermo Scientific TMT Reagents.

Instrument	Fragmentation Method	Reference(s)
Thermo Scientific Orbitrap™ Fusion™ Tribrid™ Mass Spectrometer	HCD/SPS-MS3	Viner, *et al.* (2013)
Thermo Scientific Orbitrap Elite™ Mass Spectrometer	HCD/MS3	McAllister, G.C., *et al*. (2012), Viner, *et al.* (2012)
Thermo Scientific Q Exactive™ Mass Spectrometer	HCD/MS2	Wühr, *et al.* (2012)
Thermo Scientific Orbitrap Velos Pro™, LTQ-Orbitrap™ XL, or MALDI-Orbitrap™ XL Mass Spectrometer	HCD/MS2	Ting, *et al.* (2011), Wenger, *et al* (2011), Schirle, *et al.* (2012), Lee, et al (2011), Xiong, *et al.* (2011), Strupat, *et al.* (2008)
Thermo Scientific™ Velos Pro™ ion trap	Trap HCD/MS2	Biringer, *et al.* (2011)
Thermo Scientific Orbitrap Elite ETD, Velos Pro ETD, LTQ-OrbitrapXL ETD	HCD/MS2 or ETD/MS2	Viner, *et al.* (2009)
Q-TOF	CID	Van Ulsen, *et al*. (2009)
TOF-TOF	CID	Dayon, *et al*. (2008)
Triple Quadrupole	CID/SRM	Stella, *et al* (2011), Byers, *et al.* (2009)

B. 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 and above, 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/MS3 or HCD/ETD), Proteome Discoverer may be necessary to merge spectra for identification and quantitation.

Table 2. Modification masses of the Thermo Scientific TMT Label Reagents.

Label Reagent	Modification Mass (monoisotopic)	Modification Mass (average)	HCD Monoisotopic Reporter Mass*	ETD Monoisotopic Reporter Mass**
TMT^0-126	224.152478	224.2994	126.127725	114.127725
TMT^2-126	225.155833	225.2921	126.127725	114.127725
TMT^2-127	225.155833	225.2921	127.131079	114.127725
TMT^6-126	229.162932	229.2634	126.127725	114.127725
TMT^6-127	229.162932	229.2634	127.124760	115.124760
TMT^6-128	229.162932	229.2634	128.134433	116.134433
TMT^6-129	229.162932	229.2634	129.131468	117.131468
TMT^6-130	229.162932	229.2634	130.141141	118.141141
TMT^6-131	229.162932	229.2634	131.138176	119.138176

***** HCD is a collisional fragmentation method that generates six unique reporter ions from 126 to 131Da.
******ETD is a non-ergodic fragmentation method that generates six unique reporter ions from 114 to 119Da.

C. 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

90110	**TMT10plex™ Isobaric Label Reagent Set,** 10 × 0.8mg
90113	**TMT10plex Isobaric Mass Tag Labeling Kit**
90406	**TMT10plex Isobaric Label Reagent Set,** 10 × 5mg
90114	**1M Triethylammonium bicarbonate (TEAB),** 50mL
90115	**50% Hydroxylamine,** 5mL
90100	**iodoTMTzero™ Label Reagent,** 5 × 0.2mg
90101	**iodoTMTsixplex™ Label Reagent Set,** 1 × 0.2mg
90103	**iodoTMTsixplex Isobaric Mass Tag Labeling Kit**
90076	**Immobilized Anti-TMT Antibody Resin**
90075	**Anti-TMT Antibody,** 0.1mL
90104	**TMT Elution Buffer,** 20mL
84840	**Pierce™ Mass Spec Sample Prep Kit for Cultured Cells**
23227	**BCA Protein Assay Kit**
90057	**Pierce Trypsin Protease, MS Grade**
90051	**Lys-C Protease, MS Grade**
88300	**Fe-NTA Phosphopeptide Enrichment Kit**
88301	**Pierce TiO_2 Phosphopeptide Enrichment and Clean-up Kit**
88321	**Pierce Peptide Retention Time Calibration Mixture,** 200µL
87784	**Pierce C18 Tips, 100µL bed,** 96 tips
89870	**Pierce C18 Spin Columns,** 25 columns
28904	**Trifluoroacetic Acid, Sequanal Grade**

General References

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