INSTRUCTIONS

Iodoacetyl Tandem Mass Tag™ (iodoTMT™) Reagents

Pub. No. MAN0011795
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Rev B.0

90100 90101 90102 90103

Number	Description
90100	**iodoTMTzero™ Label Reagent Set,** 5 × 0.2mg, sufficient reagents for five samples
90101	**iodoTMTsixplex™ Isobaric Label Reagent Set,** sufficient reagents for one sixplex experiment **Contents:** **iodoTMT^6-126 Label Reagent,** 1 × 0.2mg **iodoTMT^6-127 Label Reagent,** 1 × 0.2mg **iodoTMT^6-128 Label Reagent,** 1 × 0.2mg **iodoTMT^6-129 Label Reagent,** 1 × 0.2mg **iodoTMT^6-130 Label Reagent,** 1 × 0.2mg **iodoTMT^6-131 Label Reagent,** 1 × 0.2mg
90102	**iodoTMTsixplex Isobaric Label Reagent Set,** sufficient reagents for five sixplex experiments **Contents:** **iodoTMT^6-126 Label Reagent,** 5 × 0.2mg **iodoTMT^6-127 Label Reagent,** 5 × 0.2mg **iodoTMT^6-128 Label Reagent,** 5 × 0.2mg **iodoTMT^6-129 Label Reagent,** 5 × 0.2mg **iodoTMT^6-130 Label Reagent,** 5 × 0.2mg **iodoTMT^6-131 Label Reagent,** 5 × 0.2mg
90103	**iodoTMTsixplex Isobaric Mass Tag Labeling Kit,** sufficient reagents for five sixplex isobaric experiments **Contents:** **iodoTMT^6-126 Label Reagent,** 5 × 0.2mg **iodoTMT^6-127 Label Reagent,** 5 × 0.2mg **iodoTMT^6-128 Label Reagent,** 5 × 0.2mg **iodoTMT^6-129 Label Reagent,** 5 × 0.2mg **iodoTMT^6-130 Label Reagent,** 5 × 0.2mg **iodoTMT^6-131 Label Reagent,** 5 × 0.2mg **HES Buffer** (50mM HEPES pH 8.0, 1mM EDTA, 0.1% SDS), 15mL **Bond-Breaker™ TCEP Solution,** 0.5mL **Dithiothreitol (DTT), No-Weigh™ Format,** 8 × 7.7mg **Pierce Trypsin Protease, MS Grade,** 5 × 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 | thermofisher.com

Storage: Upon receipt store all products at -20°C. Products are shipped with an ice pack.

Note: Products are for research use only. Do not use for diagnostic procedures.

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Introduction

The Thermo Scientific™ Iodoacetyl Tandem Mass Tag™ (iodoTMT™) Reagents enable selective labeling and relative duplex to sixplex mass spectrometry (MS) quantitation of cysteine-containing peptides derived from complex biological samples. The iodoTMT Reagents irreversibly label free sulfhydryl groups on cysteine residues, in contrast to previous reversible cysteine-reactive TMT (cysTMT™) Reagents.[1] To selectively analyze the cysteine-labeled peptides, the sample is enriched using the Thermo Scientific™ Immobilized Anti-TMT Antibody Resin and TMT Elution Buffer. This approach of selective thiol labeling and affinity enrichment is similar to isotope-coded affinity tags (ICAT™ Method)[2] but allows for affinity enrichment with a non-biological tag and higher multiplex quantitation.

Each isobaric iodoTMTsixplex Reagent within a set has the same nominal parent mass and is composed of a sulfhydryl-reactive iodoacetyl group, an MS-neutral spacer arm and an MS/MS reporter (Figure 1). The reagents label proteins prepared from up to six biological samples or treatments, which are combined into one sample for the quantitative analysis of relative protein expression or modification. During the MS/MS stage of acquisition to derive fragment ions and sequence information, a unique reporter ion mass is also generated (e.g., 126-131Da for the iodoTMT^6 Isobaric Label Reagents). These reporter ions are in the low mass region of the MS/MS spectrum, providing information on relative protein expression levels for up to six different sample conditions. The iodoTMTzero and iodoTMTsixplex Reagents can also be used as isotopic “light” and “heavy” duplex tags for MS-level quantitation. These tags enable quantitation of protein expression changes in cell-based and tissue samples that may not be amenable to metabolic isotopic labeling strategies (e.g., SILAC).

The iodoTMT Reagents, Immobilized Anti-TMT Resin and TMT Elution Buffer are effective for reducing sample complexity, improving dynamic range and studying cysteine modifications such as S-nitrosylation, oxidation and disulfide bonds. For detection of S-nitrosylated cysteines, a modified S-nitrosylation switch assay can be used for S-nitrosylation site mapping and quantititation.[3] In this method, unmodified cysteines are blocked using a sulfhydryl-reactive compound (e.g., MMTS). S-nitrosylated cysteines are then selectively reduced with ascorbate for specific labeling with iodoTMT Reagents.

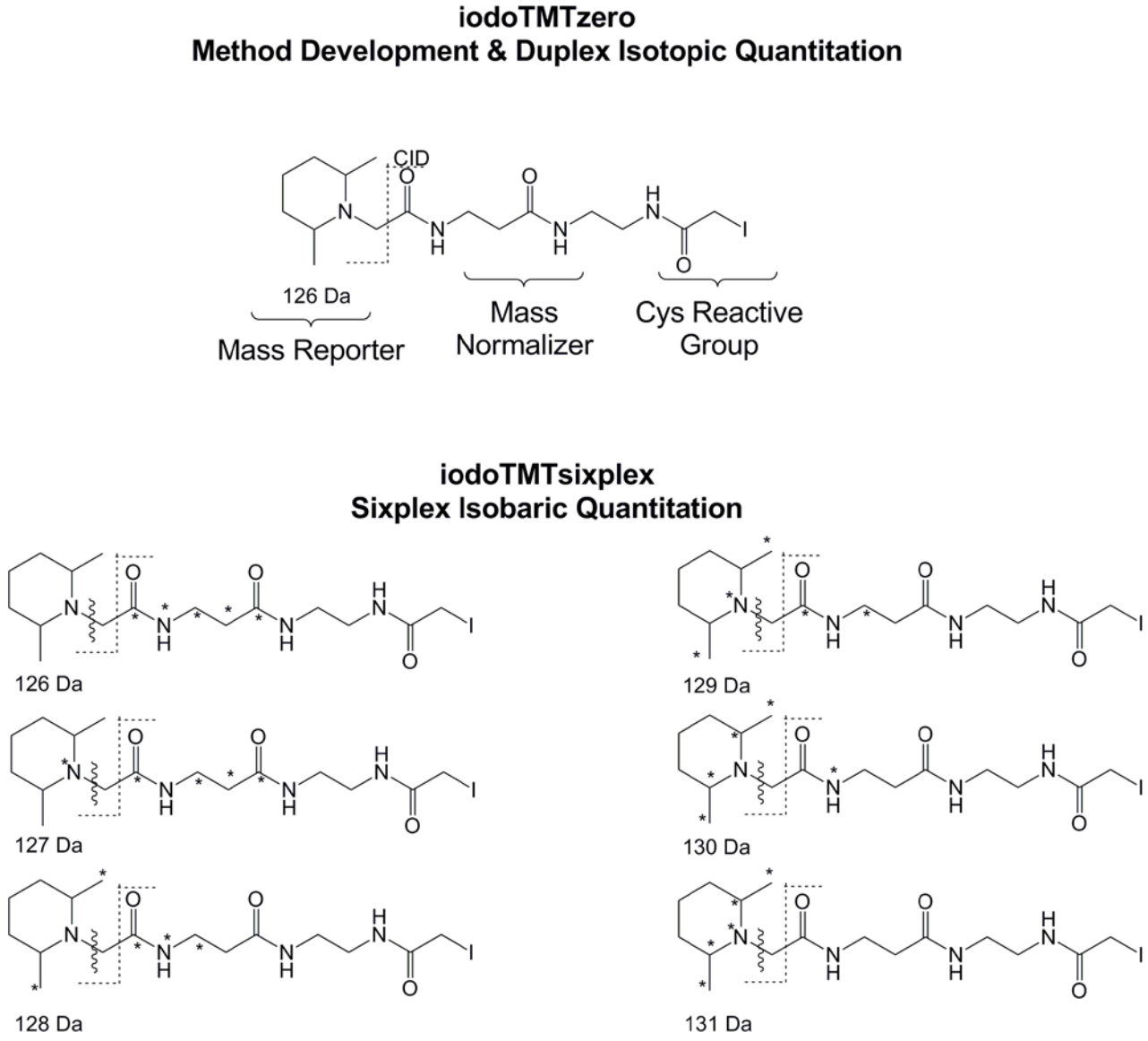

Figure 1. Chemical structure of the Thermo Scientific iodoTMT Label Reagents. Functional regions of the reagent structure with the isotope positions, MS/MS fragmentation sites and collision-induced reporter ions for each reagent. Molecular weight of the intact iodoTMTzero Reagent is 452.33Da. Molecular weight of the intact iodoTMTsixplex Reagent is 457.33Da.

Procedure Summary

To label and prepare samples for analysis (Figure 2), protein extracts are isolated from cultured cells or tissues. After proteins are denatured and reduced, each sample is individually labeled. Excess tag is removed by acetone precipitation, SDS-PAGE or Thermo Scientific™ Zeba™ Spin Desalting Columns. For LC-MS/MS analysis, proteins are digested with a site-specific endoproteinase. After digestion, labeled peptides are enriched using the Immobilized Anti-TMT Antibody Resin and TMT Elution Buffer. Data acquisition is performed on a Thermo Scientific™ LTQ Orbitrap™ or Orbitrap Velos Mass Spectrometer, and data analysis software is used for protein identification and relative quantitation of the six samples via reporter ions.

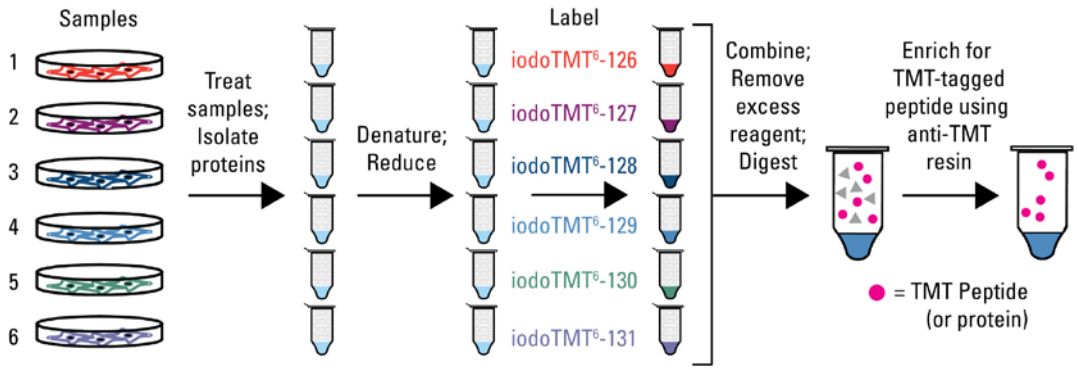

Figure 2. Workflow for the Thermo Scientific iodoTMT Reagents.

Important Product Information

- iodoTMT Reagents are light- and free radical-sensitive. Minimize exposure to light during labeling reactions by wrapping reaction tubes in aluminum foil or placing reactions in a space protected from light. Avoid water and buffers containing metal ions.
- The iodoacetyl group of the iodoTMT Reagent reacts specifically with free (reduced) sulfhydryls at pH 8-8.5. To prevent quenching of the labeling reagent, reducing agent concentration in reaction buffers should be 2- to 5-fold less than final iodoTMT Reagent concentration.
- To prevent reformation of disulfide bonds after reduction, denaturants (e.g., urea, guanidine or SDS) and EDTA (1-5mM) may be added to lysis buffers.
- For optimal results, use 100-200µg of protein per 100µL reaction. The concentration of the iodoTMT Reagent during labeling should be $\geq$ 4mM for complete labeling of protein cysteines. For best results, use a 10-fold excess of reagent over free thiol concentration. Ellman's Reagent (see Related Thermo Scientific Products) can be used to determine the sample's free thiol concentration.
- For S-nitrosylation switch assays, use 1-2mg of protein per 1mL reaction. The concentration of the iodoTMT Reagent during labeling should be 0.4-1mM for selective labeling of S-nitrosocysteines in a denaturing buffer (e.g., HENS).
- Removal of non-reacted iodoTMT Label Reagent is required for successful enrichment of labeled peptides (or proteins) from complex samples using Anti-TMT Resin (Product No. 90076). Strategies for removal of the label reagent are found in Tech Tip #69 on our website.
- Trypsin is used for protein digestion and is effective in most circumstances; however, proteases such as Lys-C (Product No. 90051), Glu-C (Product No. 90054), chymotrypsin (Product No. 90056) or Asp-N (Product No. 90053) may be used to increase protein sequence coverage and identification.
- To become familiar with the protocol, use the iodoTMTzero Label Reagent Set with a test sample [e.g., 100µg of bovine serum albumin (BSA)]. Protein labeling can be confirmed by Western blot using the Anti-TMT Antibody (Product No. 90075) or LC-MS/MS of tryptic peptide digests.

Additional Materials Required

Materials for iodoTMT Reagent Labeling and Analysis

- LC/MS grade methanol (Fisher Scientific, Product No. A456-1)
- LC/MS grade acetonitrile (Product No. 51101)
- LC/MS grade water (Product No. 51140)
- Trifluoroacetic acid (TFA) (Product No. 28904)
- Protein assay such as Thermo Scientific™ BCA Protein Assay (Product No. 23225) or Pierce™ 660nm Protein Assay (Product No. 22600)
- Acetone, chilled (-20°C)
- Tris Buffered Saline (TBS) (Product No. 28358)
- Immobilized Anti-TMT Resin (Product No. 90076)
- TMT Elution Buffer (Product No. 90104)
- 75-300µm capillary C_{18} reversed-phase column (10-25cm long) (Thermo Scientific™ Hypersil™ Product No. 25005-150065)
- 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™ (Matrix Science, Ltd.)
- Optional: Thermo Scientific™ Pierce™ Spin Columns – Screw Cap, 2mL (Product No. 69705)
- Optional: Thermo Scientific™ Pierce™ C18 Clean-up Columns or Tips (Product No. 89870, 87783 or equivalent)

Additional Materials for S-Nitrosylated Protein Labeling

- HENS Buffer (Product No. 90106)
- MMTS (Product No. 23011)
- Sodium ascorbate
- Dimethylformamide (DMF), Sequencing grade (Product No. 20673)
- Iodoacetamide (Product No. 90034)
- 50mg C18 SPE column (Thermo Scientific™ HyperSep™ C18 SPE Column, Product No. 60108-390 or Waters™ Sep-Pak™ tC18 SPE Column, Product No. WAT054960)
- Optional: S-nitrosoglutathione

Total Protein Cysteine Labeling Using iodoTMT Reagents

Note: Use 100-200µg of protein per sample depending on the number of free cysteine thiols available. Use BSA (100µg) as a control sample for method optimization.

A. Protein Extraction, Reduction and Cysteine Residue Labeling

1. Culture cells to harvest at least 100µg of protein per condition. For best results, culture at least 5×10^6 cells.
2. Lyse cell pellet with 4 cell-pellet volumes of HES Buffer (i.e., use 4mL of HES Buffer per milliliter of cells).

   Note: Sonicate lysates to reduce viscosity.
3. Centrifuge sample at 10,000 × *g* for 10 minutes.
4. Perform a protein assay (e.g., Pierce 660nm or BCA Protein Assay) to determine the protein concentration.
5. Prepare protein at 1-2mg/mL in at least 100µL of HES Buffer. Use 100µg per condition. Make sure the protein concentration is exactly the same for each of the six treatment conditions.

6. To each 100µL sample, add 1µL of 0.5M TCEP (5mM final concentration) and incubate for 1 hour at 50°C.
7. Optional**:** Use Ellman's Reagent (Product No. 22582) to estimate the concentration of free cysteine thiols.

   Note: Use Zeba Spin Desalting Columns to remove TCEP before using Ellman's Reagent.
8. While samples are reducing, add 10µL of LC/MS-grade methanol to the iodoTMT Reagent tubes to solubilize the reagent. Centrifuge the solubilized reagent at 1500 × *g* for 1 minute.
9. Add sample to the dissolved iodoTMT Reagent. Use a 10-fold excess of iodoTMT Reagent over free thiols.
10. Allow the reaction to proceed for 1 hour at 37°C protected from light.
11. Dissolve 7.7mg of DTT with 100µL of LC/MS-grade ultrapure water to make a 0.5M stock solution.
12. Quench the reaction by adding 4µL of 0.5M DTT (20mM final concentration) and incubating sample for 15 minutes at 37°C protected from light.
13. If labeling using iodoTMTsixplex Reagents, combine equal amounts of each labeled sample in a separate tube.
14. Add six volumes (e.g., 2.4mL for 600µg of combined sample) of pre-chilled (-20°C) acetone and freeze at -20°C. Allow the precipitation to proceed for at least 4 hours up to overnight.

    Note: Removal of non-reacted tag is required before iodoTMT Reagent labeled-peptide enrichment. In addition to acetone precipitation, non-reacted tag can be removed using desalting or SDS-PAGE. Refer to Tech Tip #69 from our website for more protocols.
15. Centrifuge the samples at 10,000 × *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. Labeled-Protein Digestion and Clean-Up

1. Dissolve the 100µg acetone-precipitated (or lyophilized) protein pellets with 100µL of 50mM ammonium bicarbonate buffer, pH 8.

   Note: An acetone-precipitated pellet may not completely dissolve; however, after proteolysis at 37°C, all of the protein (peptides) will be solubilized.
2. Immediately before use, add 20µL of Trypsin Storage Solution (i.e., 50mM acetic acid) 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 to sample (i.e., 1/50:trypsin/protein, w/w). Digest the sample 4 hours to overnight at 37°C.
4. After digestion, acidify samples by adding 4µL of 10% TFA.
5. Optional: Clean up peptides using C18 spin columns or tips.
6. Freeze peptides and lyophilize using a vacuum concentrator.
7. Proceed to the Enrichment of iodoTMT Reagent-labeled Peptides Section.

S-Nitrosylated Protein Labeling Using iodoTMT Reagents

Note: Use 1-2mg protein per sample as the number of nitroso-cysteine thiols are significantly lower in abundance compared to total cysteine free thiols. Use S-nitrosoglutathione to generate a control sample for method optimization

Material Preparation

1M MMTS	Dilute 20µL of MMTS with 180µL of DMF in fume hood.
1M Sodium ascorbate	Dissolve 200mg of sodium ascorbate with 1mL of water.
0.5M Iodoacetamide	Dissolve 18.6mg with 200µL of LC/MS-grade water.
0.5M DTT	Dissolve 7.7mg DTT with 100µL of LC/MS-grade ultrapure water.

A. Protein Extraction, Reduction and Cysteine Residue Labeling

1. Culture cells to harvest at least 1mg of protein per condition. For best results, culture at least 5×10^7 cells.
2. Lyse cells with 4 cell-pellet volumes of HENS Buffer (i.e., use 4mL of HENS Buffer per milliliter of cells).

   Note: Sonicate lysates to reduce viscosity.
3. Centrifuge sample at 10,000 × *g* for 10 minutes.
4. Perform a protein assay (e.g., BCA Protein Assay) to determine the protein concentration.
5. Prepare protein at 1-2mg/mL in at least 1mL of HENS Buffer. Use 1mg per condition. Make sure the protein concentration is exactly the same for each of the six treatment conditions.
6. Optional: Incubate sample with 200µM S-nitrosoglutathione for 30 minutes at room temperature to generate a control sample. Remove unreacted S-nitrosoglutathione using Zeba Spin Desalting Columns preequilibrated in HENS Buffer.
7. To each 1mL sample, add 20µL of 1M MMTS (20mM final concentration), vortex vigorously for 1 minute to mix and incubate for 30 minutes at room temperature to block free cysteine thiols.
8. Precipitate protein by adding 6mL of pre-chilled (-20°C) acetone to each sample and freeze at -20°C to remove MMTS. Allow the precipitation to proceed for at least 1 hour.
9. Centrifuge the samples at 10,000 × 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.
10. Resuspend precipitated samples in 1mL of HENS Buffer.

    Note: Removal of excess MMTS is required before iodoTMT Reagent labeling. In addition to acetone precipitation, MMTS can be removed using Zeba Spin Desalting Columns pre-equilibrated in HENS Buffer.
11. While samples are precipitating, add 10µL of LC/MS grade methanol to the iodoTMT Reagent tubes to solubilize the reagent. Centrifuge the solubilized reagent at 1500 × *g* for 1 minute.
12. Add the dissolved iodoTMT Reagent to each sample and briefly vortex to mix.
13. Add 20µL of 1M sodium acscorbate to each sample and briefly vortex to mix.

    Note: For negative control reactions, add 20µL of ultrapure water instead of sodium ascorbate.
14. Allow the reaction to proceed for 1 hour at 37°C, protected from light.
15. Quench the reaction by adding 40µL of 0.5M DTT (20mM final concentration), briefly vortexing and incubating sample for 15 minutes at 37°C protected from light.
16. Combine equal amounts of each sample labeled using iodoTMTsixplex Reagents in a separate tube.
17. Add six volumes (~24mL) of pre-chilled (-20°C) acetone and freeze at -20°C. Allow the precipitation to proceed for at least 1 hour.

18. Centrifuge the samples at 10,000 × *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.
19. Dissolve 6mg acetone-precipitated protein with 6mL of HENS Buffer.
20. Add 200µL of 0.5M iodoacetamide to sample and incubate at 37°C for 1 hour protected from light.
21. Add six volumes (~24mL) of pre-chilled (-20°C) acetone and freeze at -20°C. Allow the precipitation to proceed for at least 1 hour.
22. Centrifuge the samples at 10,000 × *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. Labeled-Protein Digestion and Clean-up

1. Dissolve 6mg of acetone-precipitated protein pellets with 6mL of 50mM ammonium bicarbonate buffer, pH 8.

   Note: An acetone-precipitated pellet may not completely dissolve; however, after proteolysis at 37°C, all the protein (peptides) will be solubilized.
2. Immediately before use, add 120µL of Trypsin Storage Solution (i.e., 50mM acetic acid) to 120µg of trypsin (i.e., 6 × 20µg glass vials) and incubate for 5 minutes.
3. Add 120µL of trypsin to sample (i.e., 25µg per 1mg of protein). Digest the sample 4 hours to overnight at 37°C.
4. After digestion, acidify samples by adding 250µL of 10% TFA.
5. Clean up peptides using 50mg C18 SPE columns.
6. Freeze peptides and lyophilize using a vacuum concentrator.
7. Proceed to the Enrichment of iodoTMT Reagent-labeled Peptides Section.

Enrichment of iodoTMT Reagent-Labeled Peptides

Note: Use at least 100µL of settled resin (200µL of 50% slurry) for every 100µg of total cysteine-labeled peptide digest. For S-nitrosylated protein-labeled samples use 100µL of settled resin (200µL of 50% slurry) for every 1mg of sample. Pierce Spin Columns – Screw Cap (Product No. 69705) may be used to facilitate resin washes.

1. Wash the Anti-TMT Resin three times with one column volume of 1X TBS.
2. Resuspend lyophilized peptides with 100µL of 1X TBS. Save a small portion of this unfractionated sample for direct analysis of the non-enriched samples.
3. Add peptides to the Anti-TMT Resin and incubate for 2 hours at room temperature or overnight with end-over-end mixing at 4°C.
4. Remove the supernatant and wash the resin five times (5 minutes/wash) with one column volume of TBS.

   Note: Addition of 2M urea or MS-compatible detergents (0.05-0.2%) to TBS wash buffers can be used to decrease nonspecific peptide binding.
5. Wash the resin three times with one column volume of 1X TBS.
6. Wash the resin three times with one column volume of water.
7. Elute the sample with four column volumes of TMT Elution Buffer.
8. Pool the eluate, freeze peptides and lyophilize using a vacuum concentrator.
9. Resuspend the samples in 25µL of 5% acetonitrile/0.1% formic acid and inject 1-5µL directly onto an LC-MS/MS system (e.g., Orbitrap Velos Mass Spectrometer).

MS Analysis of iodoTMT Reagent-Labeled Peptides

A. Recommended Parameters for Data Acquisition

Quantitation of labeled peptides requires a mass spectrometer capable of MS/MS fragmentation (e.g., ion trap, quadrupole time of flight (Q-TOF), 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 collision induced dissociation (CID), higher energy collisional dissociation (HCD) or electron transfer dissociation (ETD) and the desire 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, iodoTMT Reagent reporter ions are not visible in ion traps following traditional CID fragmentation. Instead, quantify and identify peptides on a hybrid-Orbitrap mass spectrometer with HCD fragmentation or alternate HCD and CID methods optimized for identification and quantitation, respectively (Table 1). The TMT Tags behave similarly to iTRAQ™ Reagents, although optimal chromatography and fragmentation energy settings are slightly different. See Tech Tip #70 from our website for data acquisition methods.

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

Instrument	Fragmentation Method	Reference
Thermo Scientific Q Exactive™	HCD	Schirle, *et al*. (2012), Bomgarden, *et al*. (2011), Zhang, *et al*. (2008), Viner, *et al.* (2008)
Thermo Scientific™ Orbitrap Velos, Velos Pro, LTQ Orbirap XL™ or Elite™	HCD, HCD/CID	
Thermo Scientific Orbitrap Velos or Elite	MS^3, PTR/HCD	Ting, *et al*. (2011), Wenger, *et al*. (2011)
Thermo Scientific Velos Pro	TrapHCD	Biringer, *et al*. (2011)
Thermo Scientific LTQ Orbitrap XL-ETD	ETD	Viner, *et al.* (2009)
Q-TOF	CAD	Van Ulsen, *et al*. (2009)
TOF-TOF	CAD	Dayon, *et al*. (2008)
Triple Quadrupole	CID	Stella, *et al*. (2012), Byers, *et al.* (2009)

B. Data Analysis and Quantitation

Masses for peptide modification by the zero and sixplex iodoTMT Reagents are present in the UNIMOD database (*www.unimod.org*) and listed below (Table 2). 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™ 1.3, 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), Proteome Discoverer 1.3 or custom software may be necessary to merge spectra for identification and quantitation.

C. Mass Modification

All TMT Reagents share identical chemical structure. Therefore, labeled samples behave identically during LC-MS or MALDI-MS analysis and can be quantified at the MS/MS or MS level. For MS/MS, quantitate duplex or sixplex samples with iodoTMTsixplex Reagents. This strategy allows higher mutiplexing and the ability to quantitate specific, singly charged reporter ions without increasing sample complexity. For duplex MS quantitation, combine samples or internal standards labeled with iodoTMTzero Reagent with samples labeled with an iodoTMTsixplex Reagent; this results in a modification of 324.2Da or 329.2Da for every labeled cysteine residue, respectively. Paired peaks with a 5Da mass shift per labeled cysteine residue are then quantified similarly to SILAC samples. This approach is also effective for quantitating specific parent and transition ions using selective reaction monitoring (SRM) strategies.

Table 2. Instruments and MS/MS fragmentation options for peptide identification and quantitation with Thermo Scientific TMT Reagents.

Label Reagent	Cysteine Modification Mass (monoisotopic)	Cysteine Modification Mass (average)	CID Monoisotopic Reporter Mass*	ETD Monoisotopic Reporter Mass**
iodoTMT^{0}-126	324.216141	324.4185	126.127725	114.127725
iodoTMT^{6}-126	329.226595	329.3825	126.127725	114.127725
iodoTMT^{6}-127	329.226595	329.3825	127.124760	115.124760
iodoTMT^{6}-128	329.226595	329.3825	128.134433	116.134433
iodoTMT^{6}-129	329.226595	329.3825	129.131468	117.131468
iodoTMT^{6}-130	329.226595	329.3825	130.141141	118.141141
iodoTMT^{6}-131	329.226595	329.3825	131.138176	119.138176

* 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.

Troubleshooting

Problem	Possible Cause	Solution
Protein precipitated during reduction	Protein was not denatured	Denature protein with 6M urea, 8M guanidine or 0.1% SDS detergent
	Protein concentration was too high	Reduce protein concentration
	Organic solvent amount was too high	Decrease amount of methanol used to solubilize tag
Poor labeling of cysteine residues	A denaturing buffer was not used	Use a denaturing buffer with 6M urea, 8M guanidine or 0.1% SDS detergent and add up to 5mM EDTA to keep proteins unfolded and less prone to oxidation
	iodoTMT Reagent concentration was too low	Increase iodoTMT Reagent concentration to > 5mM
	Reducing agent concentration was too high	Reduce amount of TCEP or DTT used for reduction
	Incorrect buffer pH	Use appropriate buffer to maintain pH 8-8.5
	Reagent exposed to light	Protect reagent from light during labeling reactions
Poor labeling of S-nitrosylated proteins	Free sulfhydryls were not completely blocked	Vigorously mix MMTS reagent with sample during blocking reactions
		Increase MMTS concentration, incubation time and/or temperature
	S-nitroso group is labile	Protect samples from light during labeling reactions
		Avoid reducing agents during sample prep
		Decrease total sample handling time by using Zeba Spin Desalting Columns instead of acetone precipitation
	Sodium ascorbate not used	Add sodium ascorbate to selectively reduce S-nitrosylated cysteines

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Poor enrichment of cysteine-labeled peptides by antibody resin	Nonspecific, unlabeled peptides bound antibody resin	Increase incubation time during wash steps
		Add urea or MS-cleavable detergent to wash buffers
	Nonspecific labeling of non-cysteine peptides	Quench non-reacted tag with excess DTT
		Maintain pH 8-8.5 to minimize labeling of non-cysteine amino acids
	Excess non-reacted tag was present	Perform labeling using a lower excess of reagent
		Desalt labeled samples using Zeba Spin Desalting Columns before enrichment
	Sample amount was insufficient	Increase sample load for Anti-TMT enrichment
		Load more sample onto the C18 column
Poor reporter ion quantitation	The MS/MS resolution in the reporter ion region was too low	Always use HCD-based MS/MS fragmentation at a resolution ≥ 7500
	Suboptimal reagent fragmentation	Optimize HCD-based MS/MS fragmentation collision energy

Additional Information

Information Available from our Website

- Tech Tip #69: Strategies for removal of non-reacted TMT tag
- Tech Tip #70: TMT data acquisition on the LTQ Orbitrap XL Mass Spectrometer
- Tech Tip #49: Acetone precipitation of proteins
- Tech Tip #19: Remove detergent from protein samples

Related Thermo Scientific Products

90076 **Immobilized Anti-TMT Resin,** 6mL
90104 **TMT Elution Buffer,** 20mL
90105 **Pierce S-Nitrosylation Western Blot Kit**
90106 **HENS Buffer,** 100mL
90075 **Anti-TMT Antibody,** 100µL
22582 **Ellman's Reagent,** 5g
23011 **MMTS,** 200mg
90034 **Iodoacetamide,** 24 × 9.3mg
90064 **TMTsixplex™ Mass Tagging Kit,** 5 reactions
90067 **TMTzero™ Label Reagent Set,** 5 reactions
77720 **Bond-Breaker™ TCEP Solution, Neutral pH,** 5mL
20291 **DTT (Dithiothreitol), No-Weigh Format,** 48 tubes
69705 **Pierce Spin Columns – Screw Cap,** 25/pkg
89890 **Zeba Spin Desalting Columns, 7K MWCO, 2mL,** 5/pkg
88305 **HiPPR™ Detergent Removal Spin Column Kit**
90057 **Pierce Platinum Trypsin Endoproteinase,** 1 × 20µg
90054 **Glu-C Endoproteinase,** 5 × 10µg
90051 **Lys-C Endoproteinase,** 1 × 20µg
89870 **Pierce C18 Spin Columns,** 25 columns
28904 **Trifluoroacetic Acid, Sequanal Grade**

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1. Murray C.I. (2012). Identification and quantification of S-nitrosylation by cysteine reactive tandem mass tag switch assay. *Mol Cell Proteomics* **11(2):**M111.013441.
2. Gygi, S.P., *et al*. (1999). Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat Biotech* **17:**994-9.
3. Jaffrey, S.R., *et al*. (2001). The biotin switch method for the detection of S-nitrosylated proteins. *Sci STKE* **86:**p11.

General References

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