INSTRUCTIONS

aminoxyTMT Mass Tag Labeling Reagents

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Rev B.0

90400 90401 90402

Number	Description
90400	**aminoxyTMTzero Label Reagent,** sufficient reagent to label six samples **Contents:** **aminoxyTMTzero Label Reagent**, 6 × 0.2mg
90401	**aminoxyTMTsixplex Label Reagent Set,** sufficient reagents for one sixplex isobaric experiment **Contents:** **aminoxyTMT6-126 Label Reagent,** 1 × 0.2mg **aminoxyTMT6-127 Label Reagent,** 1 × 0.2mg **aminoxyTMT6-128 Label Reagent,** 1 × 0.2mg **aminoxyTMT6-129 Label Reagent,** 1 × 0.2mg **aminoxyTMT6-130 Label Reagent,** 1 × 0.2mg **aminoxyTMT6-131 Label Reagent,** 1 × 0.2mg
90402	**aminoxyTMTsixplex Label Reagent Set,** sufficient reagents for five sixplex isobaric experiments **Contents:** **aminoxyTMT6-126 Label Reagent,** 5 × 0.2mg **aminoxyTMT6-127 Label Reagent,** 5 × 0.2mg **aminoxyTMT6-128 Label Reagent,** 5 × 0.2mg **aminoxyTMT6-129 Label Reagent,** 5 × 0.2mg **aminoxyTMT6-130 Label Reagent,** 5 × 0.2mg **aminoxyTMT6-131 Label Reagent,** 5 × 0.2mg

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

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

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Introduction

The Thermo Scientific™ aminoxyTMT™ Mass Tag Labeling Reagents enable multiplexed relative quantitation of carbonyl-containing compounds by mass spectrometry (MS). Each mass-tagging reagent within a set has the same nominal mass (i.e., isobaric) and chemical structure composed of a carbonyl-reactive aminoxy group, a spacer arm and a mass reporter (Figure 1). The chemistry of the aminoxy group is more advantageous than a hydrazide group because of its greater reactivity with carbonyls and better stability of the oxime bond of the labeled product. For each sample, a unique reporter mass (126-131Da) in the low mass region generated by MS/MS fragmentation is used to measure the relative abundance of labeled molecules. The aminoxyTMT Reagents may be used to quantitate a broad range of biologically important molecules including carbohydrates, steroids and oxidized proteins.

The Thermo Scientific™ aminoxyTMTzero™ Label Reagent contains no heavy isotopes and can be used to evaluate the reagent in different workflows. The Thermo Scientific™ aminoxyTMTsixplex™ Label Reagents share an identical structure with aminoxyTMTzero Reagent, but contain different numbers and combinations of ^{13}C and ^{15}N isotopes in the mass reporter. Advantages of using the aminoxyTMTsixplex Label Reagents include sample multiplexing for relative quantitation, increased sample throughput and fewer missing quantitative values among replicates. For glycobiology MS applications, these reagents enable profiling levels of glycan isoforms and discovery of glycan biomarkers. Using the aminoxyTMT Reagents for glycan analysis improves ionization of labeled glycans compared to unlabeled glycans for increased sensitivity and better retention of labeled glycans by reverse phase liquid chromatography (LC).

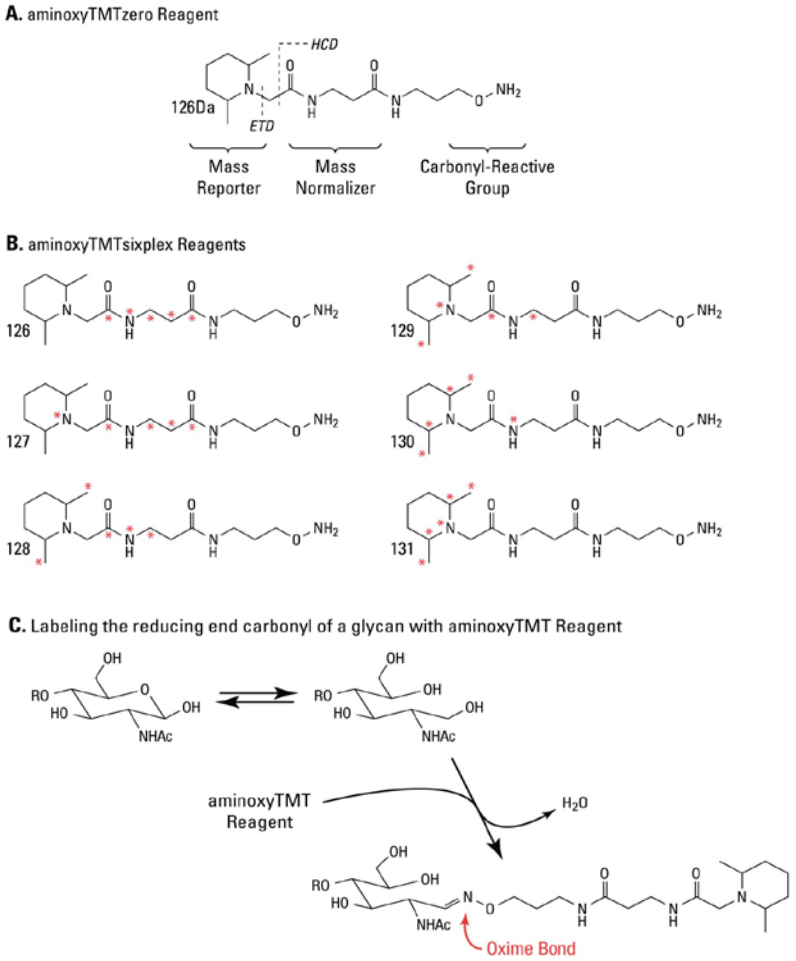

Figure 1. Chemical structures of the Thermo Scientific aminoxyTMTsixplex Label Reagents. A. Functional regions of the reagent structure including MS/MS fragmentation sites by higher energy collision dissociation (HCD) and electron transfer dissociation (ETD). **B.** aminoxyTMTsixplex Reagent structures and isotope positions (*). **C.** Reaction scheme for labeling of reducing-end sugars with aminoxyTMT Reagent.

Procedure Summary

Intact proteins or proteolytic digests of proteins extracted from cells, tissue or fluids are treated with PNGase F/A glycosidases to release *N*-linked glycans from the peptides. Glycans are separated from the protein or peptide material using a hydrophobic clean-up column (C18 or polymer-based resin) and labeled with the aminoxyTMT Reagents at the reducing end of the glycan. After a quenching step with acetone, individually labeled samples are combined and cleaned up using a HILIC column to remove residual label reagent. Labeled samples are analyzed using a mass spectrometer to identify glycoforms in the sample and quantify reporter ion relative abundance at MS^2-level (Figure 2).

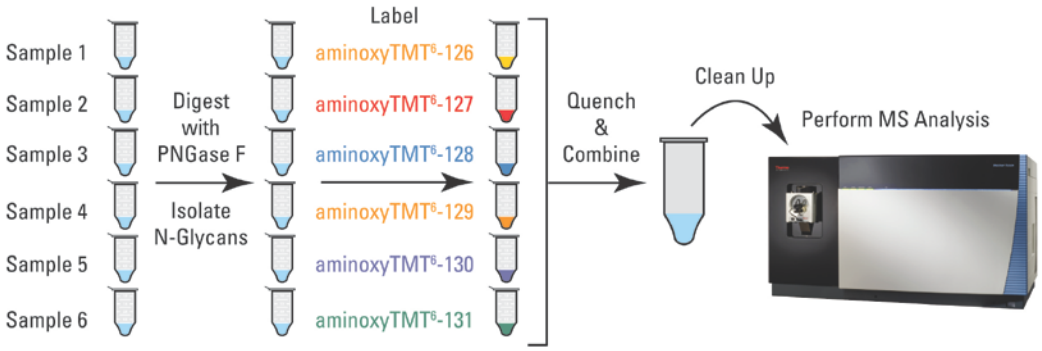

Figure 2. Procedure schematic for using the Thermo Scientific aminoxyTMTsixplex Label Reagents.

Important Product Information

- The aminoxyTMT Reagents are carbonyl-reactive and will rapidly react with ketones and aldehydes. The use of carbonyl-containing compounds and additives during sample preparation must be avoided. **Ensure samples do not contain acetone or traces of acetone!**
- To avoid contamination of MS samples, always wear gloves when handling samples. Use ultrapure MS-grade reagents. Perform sample preparation in a clean work area.
- The upstream processing reagents for preparation of the sample and digestion will vary based on application and are not included with the labeling reagents.

Additional Materials Required

- PNGase F glycosidase or PNGase A glycosidase (e.g., Prozyme Product No. GKE-5003, GKE-5011A)
- Waters Oasis™ HLB 3cc Vac Cartridge or equivalent (e.g., Waters Product No. WAT094226)
- 2.0mL microcentrifuge tubes
- Acetonitrile, LC-MS Grade (e.g., Thermo Scientific Product No. 51101)
- Water, LC-MS Grade (e.g., Thermo Scientific Product No. 51140)
- Glacial acetic acid (e.g., Fisher Scientific Catalog No. A35-500)
- Methanol (e.g., Fisher Scientific Catalog No. A456-500)
- Acetone (e.g., Fisher Scientific Catalog No. A949SK-4)
- Triethylammonium bicarbonate (TEAB), 1M (e.g., Thermo Scientific Product No. 90114)
- Vacuum centrifuge (e.g., Thermo Scientific™ Speed-Vac™ Vacuum Concentrator)
- Mass spectrometer with HCD or Q-TOF CID fragmentation capability

Material Preparation

20mM triethylammonium bicarbonate	Prepare 10mL of solution by adding 200µL of 1M triethylammonium bicarbonate, pH 8.5 to 9.8mL of water.
95% acetonitrile	Prepare 100mL of solution by adding 5mL of water to 95mL of acetonitrile.
50% acetonitrile	Prepare 100mL of solution by adding 50mL of acetonitrile to 50mL of water.
0.1% acetic acid	Prepare 100mL of solution by adding 100µL of glacial acetic acid to 100mL of water.
95% methanol, 0.1% acetic acid	Prepare 10mL of solution by adding 0.5mL of water and 10µL of glacial acetic acid to 9.5mL of methanol.
95% methanol	Prepare 10mL of solution by adding 0.5mL of water to 9.5mL of methanol.
10% acetone	Prepare 10mL of solution by adding 1mL of acetone to 9mL of water.

Preparing and Labeling *N*-glycans with the aminoxyTMT Isobaric Mass Tags

Important: Deglycosylation of *N*-linked glycans using PNGase F glycosidase (or PNGase A glycosidase for plant-derived samples) may be performed at the protein or peptide level. Typically, it is recommended that protein samples be reduced, alkylated and digested with trypsin prior to treatment with glycosidase to improve yields of released glycans. If deglycosylation is performed at the peptide level, we recommend removing reducing and alkylating reagents by solid-phase extraction prior to trypsin digestion. If protein samples were purified using acetone precipitation, ensure complete removal of acetone from the sample by drying in a vacuum centrifuge. If starting with carbohydrates with free reducing-ends, proceed directly to Step B.

A. Release and Purification of *N*-glycans

1. Dissolve 100-1000µg of digested or intact protein sample in 100µL of 20mM TEAB solution.

   Note: Total protein glycosylation varies with sample type. Each aminoxyTMT Reagent vial contains enough reagent to label up to 100nmol of released *N*-linked sugars.

2. Add recommended amount of glycosidase (typically 1-5µL) to the sample solution and incubate at 37°C with continuous mixing for 8-18 hours, depending on complexity of the original protein sample.

3. Condition Oasis HLB column by washing it with 3mL of 95% acetonitrile and then 3mL of 0.1% acetic acid solution. Allow the wash solutions to pass through the column completely for all steps.

   Note: Failure to allow wash solutions to pass through the column completely before addition of the next solution may result in poor recovery/purity of glycan.

4. Add 100µL of 0.1% acetic acid solution to the sample.

5. Add acidified sample to the column and collect the flow-through.

6. Wash the column again with 2mL of 0.1% acetic acid and combine the wash with the original flow-through. This fraction contains released *N*-glycans.

   Note: Some small hydrophilic peptides may be eluted with the glycans; however, most peptide material should remain bound to the column. If desired, peptides may be eluted by adding 2mL of 50% acetonitrile solution to the column.

7. Evaporate the liquid in the glycan-containing fraction using a vacuum centrifuge until the contents are completely dry.

   Note: Dry glycan samples can be stored at -20°C until used. Storage of glycans in low pH solutions is not recommended due to loss of acid-labile sialic acid residues.

B. Labeling of *N*-glycans with aminoxyTMT Reagent

1. Dissolve aminoxyTMT Reagent in 200µL of 95% methanol, 0.1% acetic acid solution and transfer into a sample tube containing released *N*-glycans.

2. Mix the sample by continuous shaking for 10 minutes at room temperature to dissolve and label the sample. Evaporate the liquid in a vacuum centrifuge until the sample is completely dry.

3. Add 200µL of 95% methanol to the sample to dissolve and mix again for 10 minutes. Evaporate the liquid in a vacuum centrifuge again until the sample is completely dry. All sample *N*-glycans are now labeled.
4. Add 100µL of 10% acetone solution to each labeled sample and incubate at room temperature for 10 minutes with continuous mixing by shaking or vortexing to quench excess reagent.

   Note: Excess aminoxyTMT Reagent must be quenched with acetone to avoid label exchange when performing a multiplex experiment.
5. Combine all multiplex samples into a single test tube and remove the liquid by evaporation in a vacuum centrifuge. Sample may be stored at -20°C until clean-up in Step C.

C. Labeled Glycan Clean-up for MS Analysis

1. Condition a new Oasis HLB column by sequentially washing it with 3mL of 95% acetonitrile, 1mL of 50% acetonitrile, and finally 3mL of 95% acetonitrile again.
2. Dissolve the labeled glycan sample in 200µL of 50% acetonitrile.
3. Fill the column with 3mL of 95% acetonitrile solution and immediately add 200µL of the sample to the solution in the column. Allow the contents to pass through the column completely. Discard the flow-through.
4. Add another 3mL of 95% acetonitrile solution to the column and allow the solution to pass through. Repeat one more time to ensure full removal of the quenched reagent. Discard the flow-through.
5. Elute labeled glycans by washing the column with 2mL of 50% acetonitrile solution, collecting the eluent into a clean sample tube.
6. Evaporate the liquid using a vacuum centrifuge and store the sample at - 20°C until ready for analysis.

D. MS Analysis

1. For direct infusion experiments using an ESI source, dissolve the sample in 20µM NaOH in 50% acetonitrile/water. Infuse at 5-10µL/min and analyze in the positive ion mode.
2. For LC/MS experiments, dissolve the sample in appropriate loading solvent and perform LC using either a hydrophilic interaction (HILIC) column (e.g., Thermo Scientific™ Accucore™ HILIC columns) or porous graphitized carbon (PGC) column.
3. Selected precursors are fragmented by HCD using optimized collision energy. Relative peak intensities of the reporter ions provide relative abundance of each glycoform in a set of samples.

Troubleshooting

Problem	Possible Cause	Solution
Poor labeling	Carbonyl-containing contaminant present in the sample	Modify clean-up procedure to remove carbonyl species
		Ensure sample does not contain ketones or aldehydes (e.g., acetone, formaldehyde)
		Avoid contact with acetone until the quenching step
	Too much sample was used	Do not label more than 100nmol of reducing-end sugars
	Sample stored in acidic solution (e.g., 0.1% acetic acid)	Storage of labeled samples in acidic solutions may lead to decomposition of the labeled products. Store samples dry at -20°C
Exchange of tags across multiple samples	Tags were not quenched with acetone	Quench unreacted tags in each sample separately with acetone before the samples are combined
	Excess quenched tag was not adequately removed	Remove quenched, unreacted reagent from the reaction mixture

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Poor glycan yield	Incomplete deglycosylation	Use higher quantities of PNGase F and incubate for longer time periods to improve the release of *N*-glycans
		Perform deglycosylation at peptide level to improve glycan yield
		Plant-derived samples may require deglycosylation with PNGase A glycosidase
	Loss of glycan during clean-up step	Ensure the use of appropriate solutions during column equilibration and elution
		Allow wash solutions to completely pass through the column before addition of the next solution

Additional Information

A. Data Acquisition Methods

Quantitation of carbohydrates labeled with aminoxyTMT Reagents requires a mass spectrometer capable of MS/MS fragmentation. Higher energy collision dissociation (HCD) is recommended for aminoxyTMTsixplex reporter ion generation. Optimal HCD fragmentation energy is instrument-dependent and can be optimized using aminoxyTMTzero Reagents. Electron transfer dissociation (ETD) may be used as an alternative fragmentation method for glycan identification and quantitation. An HCD-enabled Thermo Scientific™ Velos Pro™ instrument is also capable of producing accurate quantitation data; however, the use of high-resolution Thermo Scientific™ Orbitrap™ instruments is recommended to resolve mass tag reporter ions from interfering ions for improved accuracy. For example, an *N*-acetyl hexose fragment (126.0549Da) may interfere with reporter ion quantitation on lower resolution instruments.

B. Mass Modification

All TMT Reagents share an identical chemical structure but have a unique distribution of heavy stable isotopes resulting in distinct reporter ions (Table 1). Therefore, labeled samples behave identically during LC-MS analysis and can be quantified at either the MS/MS or MS level. 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 aminoxyTMTzero Reagent may be combined with samples labeled with an aminoxyTMTsixplex Reagent, resulting in a modification of 296Da or 301Da, respectively. This approach also may be used to quantitate specific parent and transition ions using selective reaction monitoring (SRM) strategies.

Table 1. Modification masses of the Thermo Scientific aminoxyTMT Label Reagents.

Label Reagent	Modification Mass (monoisotopic)	Modification Mass (average)	HCD Monoisotopic Reporter Mass*	ETD Monoisotopic Reporter Mass**
aminoxyTMT^{0}-126	296.2212	296.4084	126.1277	114.1277
aminoxyTMT^{6}-126	301.2317	301.3724	126.1277	114.1277
aminoxyTMT^{6}-127	301.2317	301.3724	127.1248	115.1248
aminoxyTMT^{6}-128	301.2317	301.3724	128.1344	116.1344
aminoxyTMT^{6}-129	301.2317	301.3724	129.1315	117.1315
aminoxyTMT^{6}-130	301.2317	301.3724	130.1411	118.1411
aminoxyTMT^{6}-131	301.2317	301.3724	131.1382	119.1382

***** 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.

Related Thermo Scientific Products

90067	**TMTzero Label Reagent,** 5 × 0.8mg
90061	**TMTsixplex Isobaric Label Reagent Set,** 1 × 0.8mg
90064	**TMTsixplex Isobaric Mass Tagging Kit**
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	**Pierce BCA Protein Assay Kit**
90057	**Pierce Trypsin Protease, MS Grade**
90051	**Lys-C Protease, MS Grade**
28904	**Trifluoroacetic Acid, Sequanal Grade**
51140	**Water, LC-MS Grade**
51101	**Acetonitrile (ACN), LC-MS Grade**

General References

Atwood III, J.A., *et al.* (2008). Quantitation by Isobaric Labeling: Applications to Glycomics. *J Proteome Res* **7(1)**:367-374.

Prien, J.M., *et al.* (2010). Mass spectrometric-based stable isotopic 2-aminobenzoic acid glycan mapping for rapid glycan screening of biotherapeutics. *Anal Chem.* **82(4):**1498-508.

Snovida, S., *et al.*, Applications of Aldehyde-Reactive Thermo Scientific Tandem Mass Tag (TMT) Reagents for Mass Spectrometry-based Quantitative Glycomics. ASMS 2013 conference proceedings.

Limited Use Label License: aminoxyTMT™ Labeling Kits and Reagents

(Thermo Scientific Product Nos. 90400, 90401 and 90402).

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