TECH TIP # 69

Thermo SCIENTIFIC

Strategies for removal of non-reacted TMT tag

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Introduction

The labeling of proteins with Tandem Mass Tag (TMT*) Reagents (e.g., Thermo Scientific CysTMT Reagents) requires an enrichment step for labeled peptides. The immobilized Anti-TMT antibody column can be used to accomplish this objective. However, successful enrichment requires removal of non-reacted tag. Several approaches for carrying out this procedure are detailed below. In addition to removing free tag, the use of SDS-PAGE and strong cation exchange chromatography (SCX) provide the additional advantage of fractionation to give higher levels of enrichment and therefore more protein identifications with relative quantitation.

The instructions supplied with the Cysteine-Reactive Tandem Mass Tag Reagents provide a protocol for gel-based removal of non-reacted tag and protein digestion. However, solution based workflows might be more suitable for some applications. Several approaches for solution-based tag removal are provided here.

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Protocol for the removal of non-reacted tag by acetone precipitation

A. Material Preparation

- Cold Acetone stored at -20°C.
- Acetonitrile (Part No. 51101)

B. Procedure

Note: Precipitation of proteins in 6 M Urea or Guanidine is not advisable. If the sample is in a denaturing buffer, dilute the sample to less than 1.5M or dialyse the sample against a non-denaturing buffer. Be careful to avoid precipitating the sample during this process. Precipitation can be avoided by gradually lowering the denaturing concentration of the dialysis buffer. For best results, perform all dialysis at room temperature.

1. After performing the labeling reaction, combine all of the samples into one tube.
2. Add at least a 5-fold excess (preferably 10-fold) of acetone that has been chilled to -20°C. For example, add 6 mL of the chilled acetone to the 0.6mL of TMT labeled peptides.
3. After addition of the acetone, place the tube at -20°C for at least four hours (overnight is preferable).
4. A white precipitate will appear.
5. After incubation of the sample at -20°C, centrifuge the samples at 8000 × *g* for 20 minutes.
6. Carefully remove the acetone solution and wash the pellet once with cold acetone. Wash the pellet once or twice with acetonitrile to remove unbound tag.
7. Allow the pellet to dry for no more than 5 minutes.
8. Resuspend the sample in either 50mM Tris (pH ~8.0) or 50mM ammonium bicarbonate (pH 7.8) at 1mg/mL.

9. Vortex the sample gently to homogenize the precipitate.
10. Add trypsin (1:25 enzyme/substrate) and let the sample incubate with shaking (400-800 rpm) for 2 to 16 hours at 37°C.
11. At this point, TMT labeled peptides can be isolated using the Immobilized Anti-TMT Resin (Part No. 90076).

Protocol for TMT Tag removal using Zeba* Spin Desalting Columns, 7K MWCO

A. Materials Needed

- Thermo Scientific Zeba Spin Desalting column, 2.0 or 5.0mL (Part No. 89889 or 89891)
- Lysis/Equilibration buffer (i.e. 6M Urea, 75mM Tris, 1mM EDTA) for exchange
- Variable speed centrifuge with a swinging bucket rotor.
- 15mL conical collection tubes

B. Procedure

Note: This protocol assumes the use of the 5.0mL columns.

1. After the labeling reaction, you will have 6 samples. The combined volume will be 0.6mL, assuming that all of the sample will be desalted. Scale down as needed if less sample will be desalted.
2. Remove column's bottom closure and remove cap.
3. Place the column into a 2.0mL collection tube. Centrifuge at 1000 × *g* for 2 minutes to remove the storage buffer.
4. Place a mark on the side of the column where the compacted resin is slanted upward. Place the column in the microcentrifuge with the mark facing outward in all subsequent centrifugation steps.
5. Add 2.5mL of buffer on top of the resin bed. Centrifuge at 1000 × *g* for 3 minutes.
6. Repeat steps 4 and 5 three additional times, discarding buffer from the collection tube.
7. Place the column in a new collection tube, and slowly apply sample to the center of the compact resin bed.
8. Centrifuge at 1000 × *g* for 4 to 5 minutes to collect the sample. Discard the column after use.
9. Important: Repeat the desalting procedure one additional time with a new column. This is required to ensure complete removal of the free tag.
10. Proceed to the *Protocol for preparation of peptides for LC-MS/MS*.

Protocol for TMT Tag removal using Pierce* Detergent Removal Columns

Note: Thermo Scientific Pierce Detergent Removal Columns were developed to remove detergents from protein and peptide solutions. However, they have been found to effectively remove non-reacted cysTMT tag.

A. Materials Needed

- Pierce Detergent Removal Column, 4.0mL (Part No. 87779)
- Lysis/Equilibration Buffer (i.e. 6M Urea, 75mM Tris, 1mM EDTA) for exchange
- Variable speed centrifuge with a swinging bucket rotor
- 15mL conical collection tubes

B. Procedure

Note: The following protocol assumes that 0.6mL of TMT labeled protein will be processed through the column.

11. Remove the bottom closure from column and loosen cap (do not remove the cap).
12. Place column into a 15mL collection tube.
13. Centrifuge the columns for 1 minute at 1000 × *g*.

Note: When using fixed-angle rotors, place a mark on the side of the column where the compacted resin is slanted upward. Place column in centrifuge with the mark facing outward in all subsequent centrifugation steps. Improper orientation will result in reduced detergent removal efficiency.

14. Add wash/equilibration buffer (free of detergents) and centrifuge the columns at the times listed in step 3. Discard the buffer. Repeat this step two additional times.
15. Place column in a new collection tube.
16. Slowly apply the sample volume to the top of the compact resin bed and incubate for 2 minutes at room temperature.
17. Centrifuge for 2 minutes to collect the detergent and TMT tag free sample. Discard the column after use.
18. Important: Repeat the whole procedure one additional time using a fresh column.
19. Proceed to the *Protocol for preparation of peptides for LC-MS/MS*.

Protocol for TMT Tag removal using the Slide-A-Lyzer* G2 Dialysis Cassettes

A. Materials Needed

- Thermo Scientific Slide-A-Lyzer G2 Dialysis Cassettes, 10K MWCO, 15mL (Part No. 87731)
- Dialysis Buffers (i.e., 0, 1, 2, 4 and 6M Urea (each containing 75mM Tris or 75mM ammonium bicarbonate, 1mM EDTA).

B. Procedure

1. Dilute the protein containing solution to approximately 0.5mg/mL. Assuming a protein concentration of about 4mg/mL, you will need to dilute 0.6 to 5mL.
2. Using the supplied instructions for the cassettes, add all of the labeled protein solution to the chamber.
3. Dialyse the solution against the 6M Urea buffer for 2 hours at room temperature.
4. Replace this buffer one additional time.
5. Replace with 4M Urea buffer and then gradually replace with buffers containing decreasing amounts of urea, as indicated in the Materials section, until the buffer does not contain urea.
6. Replace the final buffer solution 2 additional times.
7. Add trypsin (40µg; 1:40 enzyme/substrate) to the sample and digest for 4 hours at 37°C.
8. Proceed to the *Procedure for peptide desalting using a C18 solid-phase extraction cartridge*.

Protocol for the preparation of peptides for LC-MS/MS

A. Materials Needed

- Trypsin or other suitable protease
- Digestion buffer (50mM Tris or ammonium bicarbonate)

B. Procedure

Note: Do not reduce or alkylate the CysTMT reagent-labeled sample. Doing so will result in removal of the cysTMT Tag.

1. If the sample is in urea or guanidine•HCl, dilute 1:4 with 50mM Tris pH~8.0. Adjust the concentration to 1 to 5mg/mL.
2. Add trypsin or other protease at an enzyme-to-substrate ratio of between 1:25 and 1:150. Incubate 4 hours to overnight at 37°C.
3. If the sample is in urea or guanidine•HCl, proceed to the *Procedure for peptide desalting using a C18 solid-phase extraction cartridge*.
4. The samples, at this point, are suitable for enrichment using the Immobilized Anti-TMT Resin (Part No. 90076).

Protocol for performing on-line SCX fractionation

A. Materials Required

Column	2.1mm × 20cm or 4.6mm × 20cm; 5µm particles; 300Å pore size, polysulfoethyl A strong cation exchange column (PolyLC). The void volume for the 2.1mm × 20cm column is 0.4mL and for the 4.6mm × 20cm column, the void volume is 2.2mL.
Injector Loop	1 to 2mL sized loop (Upchurch Scientific)
Buffer A: Binding buffer	5mM KH_2P0_4/ 25% acetonitrile, pH 2.8 or 0.1% formic/30% acetonitrile
Buffer B: Elution buffer	5mM KH_2P0_4/ 25% acetonitrile, pH 2.8 + 350mM KCl or 500mM ammonium formate/ 30% acetonitrile

B. Procedure:

1. Desalt cysTMT-labeled peptides as described below in the *Procedure for peptide desalting using a C18 solid-phase extraction cartridge* or dilute the sample into an equal volume of the SCX buffer A. If necessary acidify the sample with 1M phosphoric acid until the pH is below 3.0. If the sample is above this pH, the peptides will not stick to the column.
2. Lyophilize the peptides.
3. Dilute the peptides such that they are 0.2 to 0.5mg/mL in the binding buffer. Use pH paper to confirm that the peptides are acidic and if necessary use a conductivity meter to confirm that the conductivity of the sample is less than 5mS.
4. Load the peptides onto a pre-equilibrated SCX column (listed above).
5. Run a gradient as follows using a flow rate of 0.2mL per minute (1mL injection onto a 2mL sample loop) for the 2.1mm × 20cm column and for the 4.6mm × 200cm column use a flow rate of 0.8mL per minute:

Time	% B (Gradient)
0 to 5 minutes	0
5 to 35 minutes	25
35 to 55 minutes	100
55 to 65 minutes	100

6. Use a longer 0% B wash if the baseline needs more time to return to zero.
7. Collect 0.4 to 1mL fractions.
8. Pool the fractions into 10 to 20 fractions. Keep the most intense peaks as one fraction and pool other fractions such that all of the fractions have approximately equal intensity absorbances. Only enrich fractions that show a UV absorbance at both 214nm and 280nm. (The cysTMT tag has a detectable absorbance at 280nm as well).
9. Process each individual fraction with 0.3mL of Immobilized Anti-TMT Resin (Part No. 90076) using the supplied instructions. Larger amounts of starting material will require more resin. Adjust the volume of resin based on sample requirements.

Procedure for peptide desalting using a C18 solid-phase extraction cartridge

A. Materials Required

Column	Solid Phase C18 Cartridge, Hypersil, Part No. 60108-390 (50mg) or 60108-302 (100mg) or equivalent
Wetting Solution	50% acetonitrile
Buffer I	0.1% TFA in water
Buffer II	0.5% acetic acid in water
Elution Buffer	70% acetonitrile/0.5% acetic acid in water
Sample Dilution Buffer	10% TFA

B. Procedure

1. Dilute up to 5.0mg of sample into a buffer containing 0.1 to 0.5% TFA. (The binding capacity of most columns is 5% of the bed volume. For example, 5mg of sample is the maximum amount that can be applied to a 100mg cartridge).
2. Equilibrate the cartridge with the wetting solution (5 column volumes).
3. Equilibrate the cartridge with the equilibration buffer (5 column volumes).
4. Slowly (no more than 1.0mL per minute) add the sample to the equilibrated cartridge.
5. Wash the cartridge with 10 column volumes of equilibration buffer I.
6. Wash the cartridge with 3 column volumes of equilibration buffer II
7. Slowly elute the sample with 3 to 5 column volumes of elution buffer.

   Note: elution can either be accomplished directly with the elution buffer or with stepwise elutions (fractions kept separate) of 10% acetonitrile/0.5% acetic acid (buffer B), 15% buffer B, 20% buffer B, 25% buffer B, and then the elution buffer.
8. Freeze the sample and then lyophilize.
9. The resulting peptide should appear as a white cotton-like solid.

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