APEX TMT labeling quantitative mass spec protocol

Introduction

Sample prep for Tandem mass spec labeling

Materials

- Biotin-phenol
- H_2O_2
- 2X Quenching solution
  - Trolox 10 mM
  - Sodium Ascorbate 20 mM
  - Sodium Azide 20 mM
  - D-PBS
- 1X Quenching solution
  - Trolox 5 mM
  - Sodium Ascorbate 10 mM
  - Sodium Azide 10 mM
  - D-PBS
- T1 streptavidin beads (or Pierce streptavidin beads)
  - 8:5 bead vol to lysate vol for T1 beads
  - *Note: Pierce beads have higher binding capacity and I recommend over T1.*
- lysis buffer
  - 20 mM Tris, pH 7.4
  - 150 mM NaCl
  - 5 mM $MgCl_2$
  - 1% Triton X-100
  - 0.1 % SDS
  - EDTA-free complete protease inhibitor cocktail
  - Trolox 5 mM
  - Sodium Azide 10 mM
  - Sodium Ascorbate 10 mM
  - dH_2O
- KCl wash buffer
  - 1 M KCl in dH_2O

- Urea wash buffer
  - 2M Urea
  - 10 mM Tris, pH 8.0
- TEAB (triethyl ammonium bicarbonate)
- TCEP 200 mM
- Iodoacetamide 375 mM
- Mass spec grade Trypsin (pierce)
- Hydroxylamine
- TMTsixplex™ Isobaric Label Reagent Set, 1 x 0.8 mg

Procedure

in vivo APEX labeling

1. Plate cells in 15 cm dish with appropriate TET for overexpression of fusion protein
2. In day of experiment, make Biotin-phenol (500 uM final) in warm DMEM media and change 15 cm dish media by adding this solution.
3. Incubate at 37 C for 30 min.
4. Add H_2O_2 to dish for a final concentration of 1 mM H_2O_2 for 1 min
5. Agitate the plate gently for 1 min. *if samples underwent stress prior to H_2O_2 induction, becareful not to detach cells as agitation may disrupt them*
6. Quench the reaction by addition of 2X quenching solution (10 mM Trolox and 20 mM sodium ascorbate in DPBS) for a final concentration of 5 mM Trolox and 10 mM sodium ascorbate.
7. Perform one 1X quenching solution wash
8. Lysing cells: Approach 1

   .detach cells with 14 mL 1X wash solution and pippeter pressure
   .collected in 14 mL falcon tube & spin down at 200g for 10 min
   .remove supernatant and add 800 uL of lysis buffer
9. Lysing cells: Approach 2

   .add 800 uL of lysis buffer to 15 cm dish and scrape cells with cell scraper. In this approach expect final volume to exceed 800 uL as some remaining wash solution will remain on plate.

Streptavidin pulldown

Note: Use 1 mL volumes at 4 C for washes.

10. Equilibrate streptavidin beads with lysis buffer (2-3 washes)

11. PAUSE Add lysate and incubate at RT for 1 h or 4C overnight.

12. Wash beads twice with lysis buffer (with detergent)

13. Wash beads once with 1M KCl

14. Wash beads once with 2M urea in tris, pH 8

15. Wash beads twice with lysis buffer (no detergent)

Protein elution

16. elute protein from streptavidin beads by adding 8M urea in tris, pH 8 and boiling

17. The use of HiPPR™ Detergent Removal Spin Column Kit can be performed, however, I have not had detergent problems with the current wash solution and protein precipitations I've done. Also, expect sample loss if using HiPPR columns.

    CRITICAL Note: the volume capacity is 25-200 uL, however, step 18. requires we adjust our sample to 100 uL with TEAB.
    Note: This step is optional. The acetone wash/percipitation step was sufficient in removing my detergent (1% triton, 0.1% SDS).

Preparing pulldown for TMT labeling

18. transfer 150-200 µg per condition into a new microcentrifuge tube and adjust to a final volume of 100µL with 100mM TEAB.

19. Add 5µL of 200mM TCEP and incubate sample at 55°C for 1 hour.

20. Immediately before use, iodoacetamide with 100mM TEAB to make 375mM iodoacetamide. Protect solution from light.

21. Add 5µL of the 375mM iodoacetamide to the sample and incubate for 30 minutes protected from light at room temperature.

22. Add six volumes (or more) (~600µL) of pre-chilled (-20°C) acetone and freeze at -20°C.

23. PAUSE Allow the precipitation to proceed for at least 4 hours up to overnight.

    Note: Methanol/chloroform is the recommended solvent for precipitation of proteins derived from tissue extracts.

24. Centrifuge the samples at 8000× 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

Protein digestion

25. Resuspend 100µg of acetone-precipitated (or lyophilized) protein pellets with 100µL of 50mM TEAB.

    CRITICAL 50 mM TEAB is recommended because the peptide quantitation kit has a TEAB concentration upper limit

    Note: An acetone-precipitated pellet might not completely dissolve; however, after proteolysis at 37°C, all the protein (peptides) will be solubilized.

26. 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).

27. Add 2.5µL of trypsin (i.e., 2.5µg) per 100µg of protein.

28. PAUSE Digest the sample overnight at 37°C.

Peptide Labeling

29. 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.

30. Carefully add 41µL of the TMT Label Reagent to each 100µL sample (25-100µg protein digest). Alternatively, transfer the reduced and alkylated protein digest to the TMT Reagent vial.

    Note: Labeling more than 100µg of protein digest per reaction requires additional TMT Label Reagent.

31. Incubate the reaction for 1 hour at room temperature.

32. Add 8µL of 5% hydroxylamine to the sample and incubate for 15 minutes to quench the reaction.

33. Combine samples at equal amounts in new microcentrifuge tube and store at -80°C.

34. PAUSE Speed vac the pooled sample dry.

    Note: The Brün Lab speed vac takes 4-6 hr on medium heat to dry ~850 uL.