## Protein extraction, digestion, and TMT labeling

**Lysis buffer**:

RIPA lysis buffer kit, SC-24948. 10 mL
NP-40, 100 μL
Protease inhibitor cocktail, 1 tablet
100 mM PMSF, 100μL

## Cell lysis procedure

1. ~$10^7$ cells are transferred from cell culture dishes or flask to 1.5 or 2.0 mL Eppendorf tubes after washed with cold PBS x 2. Then, 200 μL of lysis buffer solution is added to the cell pellets (Note: The volume of lysis buffer used varies depending on the amount of cells).
2. The cells are homogenized by sonication on ice by 30 strokes x 2.
3. The homogenized cells are incubated on ice by 1~2 hours.
4. After centrifugation at 14,000g for 10 min, the supernatant is pipetted to a clean 1.5 mL Eppendorf tube.
5. Another 200 μL of lysis buffer solution is added to the cell pellets and steps3 is repeated.
6. After centrifugation at 14,000g for 10 min, the supernatant is combined to the first supernatant.
7. Protein concentration is measured by BCA.

## Protein digestion

1. 300 μg of protein sample is diluted in 1mg/mL concentration by 50 mM Tris-HCl (pH 8.6) and 0.1% SDS buffer.
2. Proteins are reduced by incubation in 10 mM DTT at 50ºC for 30 min followed by carboxymethylation with 25 mM iodoacetamide in the dark for 2 h.
3. Proteins are precipitated using cold acetone (85% or six volumes of sample volume) at - 20ºC overnight. The protein pellet is generated by centrifugation at 15000 rpm for 10 min and the supernatant is removed using glass Pasteur pipette.
4. The protein pellet is dissolved in 500 μl of 80 mM triethylammonium bicarbonate buffer. The protein solution should be clear after vortex or sonication. If not, add 1 or 2 μg trypsin to digest for 4 hours. The protein concentration in each sample is measured again by BCA.
5. Divide the protein solution in 60~100 aliquots.
6. One aliquot of proteins (60μg) is digested using trypsin at a protein/enzyme ratio of 30:1 (by mass) at 37ºC for overnights. Trypsin is added by two time-intervals, one (1 μg) at the start and the other (1 μg) after digestion for 4 hours.

## TMT labeling

1. Immediately before use, equilibrate the TMT Label Reagents to room temperature. For the 0.8 mg vials, add 40 µl of anhydrous acetonitrile 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 or DMSO are stable for one week when stored at -20°C and warmed to room temperature before opening.
2. Carefully add ~ 40 µl of the TMT Label Reagent to each 25-100 µg sample. Each sample is labeled with one specific TMT reagent (TMT-126 - TMT-131). Normally, duplicated samples are prepared and labeled with two different TMT reagents to have two analytical repeats. Alternatively, transfer the previously reduced and alkylated protein digests to the TMT Reagent vial. **Note:** A 100 µl 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 2 hour at room temperature.
4. Add 15 µl of 5% hydroxylamine to the sample and incubate for 15 minutes to quench the reaction.
5. Combine all of the TMT-labeled samples.
6. Desalt the combined sample using a C18/hypercarbon desalting column.
7. Run LC-MS/MS for the combined samples.

*Alternatively with more cautions:*

5. Desalt each sample using a C18/hypercarbon desalting column.
6. Run LC-MS/MS to determine the signal of β-actin for each sample.
7. Combine samples at equal amounts based on β-actin signals.
8. Run LC-MS/MS for the combined samples.

**Note:** For label-free proteomics, the TMT labeling step is skipped and the triethylammonium bicarbonate buffer is replaced by ammonium bicarbonate.