## Reagents:

1. Ammonium Bicarbonate (NH4HCO3) pH 7.8 (if made fresh will already have this pH)
2. Acetonitrile (ACN) LC-MS grade
3. DTT (154.25 g/mol)
4. Iodoacetamide (IAA: 184.96 g/mol)
5. C18 columns/tips
6. Sequencing grade trypsin (Promega or Sigma)
7. Formic Acid LC-MS grade

## Protocol:

1. Run 1D SDS PAGE gel and stain with Sypro Ruby or Coomassie Blue.
2. Whilst the gel is running or staining make 3 vials containing: 1) 100 mM NH4HCO3 2) 50 mM NH4HCO3/50% ACN and 3) 100% ACN.
3. Cut protein bands of interest and/or gel lanes into equal portion sizes, and slice further until each gel pieces are approximately 2-5 mm in size
4. Buffer exchange gel pieces with 100 mM NH4HCO3, pH 7.8 and incubate for 10 minutes. Remove buffer. This step is to give the protein and gel pieces the correct pH for digestion.
5. Add 50 mM NH4HCO3 /50 % ACN to gel pieces and incubate for 20 minutes with intermittent vortexing. Remove buffer solution. This step is to slowly dehydrate the gel pieces.
6. Add 100 % ACN to gel pieces and vortex for 20 minutes. Remove ACN and SpeedVac dry (gel pieces will be a lot smaller and white, because the gel pieces have been completely dehydrated
7. Reduce samples with 25 mM DTT in 100 mM NH4HCO3 for 30 minutes and remove DTT. This step is to reduce the disulfide bonds on the protein (acts as a precautionary step even though proteins are reduced prior to 1D SDS PAGE)
8. Alkylate with 55 mM IAA in 100 mM NH4HCO3 in the dark for 45 minutes. Remove IAA. This step seals the free-radical sulfur groups on the cysteines from reforming by adding an amide group.
9. Follow step 3 – 5 again. Gel pieces will be white and smaller.
10. Make up trypsin by adding 20 µl trypsin resuspension solution (provided) to lyophilized trypsin. Aliquot 5 µl (1 µg/µl) and add 395 µl 100 mM NH4HCO3. This will give you a trypsin concentration of 12.5 ng/µl.
11. Add 20 µl trypsin to each gel fraction and incubate at 4 ºC for 10 minutes.  Add another 25 µl 100 mM NH4HCO3 and incubate at 37 ºC overnight. This step allows the dehydrated gel pieces to soak up the trypsin.
12. Recover the supernatants (if any) from the gel fractions into new tubes.
13. Extract the peptides from the gel pieces by adding 50% ACN/2% formic acid and incubate for 5 mins with intermittent vortexing. This step dehydrates the gel pieces allowing the peptides to diffuse, and the formic acid gives the peptides a positive charge.
14. Recover the supernatants and combine with the supernatants from step 12.
15. Repeat 13 and 14
16. SpeedVac dry the supernants to remove all the ACN until approximately 10-20 ul is leftover. SpeedVac setting must be on low and DO NOT DRY COMPLETELY as the peptides will stick to the walls of the tubes. Setting must be on low as heat will cause the peptides to aggregate and stick to the tube.
17. (Optional but recommended): Desalt peptides using C18 tips (PerfectPure C18 Tips) (Eppendorf, Germany) dry eluates using a vacuum centrifuge followed by resuspension in 0.1% (v/v) formic acid in preparation for nanoLC-MS/MS