## 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. Buffer exchange proteins (~20µg is usually enough) with 100 mM NH4HCO3, pH 7.8 using a Microcon YM-10 Centrifugal Filter unit (Millipore, MA, USA).
2. Spin at 12,000g for 15 mins, flip the filter upside down into a new tube and spin for 1,000g for 3 mins.
3. Reduce samples with 25 mM DTT (~50µl) for 30 minutes at 37ºC
4. Alkylate with 55 mM IAA (~100µl) in the dark for 45 minutes.
5. Repeat step 1-2. Buffer exchange again with 100 mM NH4HCO3, pH 7.8. and concentrate to approximately 100 µl. Spin at 12,000g for 15 mins, flip the filter upside down into a new tube and spin for 1,000g for 3 mins
6. Digest proteins with trypsin in trypsin:protein (1:50) with sequencing-grade trypsin (Promega, Madison, WI) for 18 hours at 37 °C
7. Desalt peptides using C18 tips (PerfectPure C18 Tips/Varian) (Eppendorf, Germany). See below.
8. To desalt
   * - activate the tip (load from the top) with 100 µl 90% ACN/0.1 formic acid.
   * - equilibrate with 100 µl 5% ACN/0.1% formic acid
   * - load your sample (usually <200 µl)
   * - wash with 100 µl  5% ACN/0.1% formic acid
   * - elute with  100 µl 90% ACN/0.1% formic acid into a 0.5 ml eppedorf tube
9. Dry eluates using a vacuum centrifuge followed by resuspension in 0.1% (v/v) formic acid in preparation for nanoLC-MS/MS
   * SpeedVac dry the contents until only 10µl is left remaining, if there is less than 10µl top it up with 0.1% formic acid. NOTE: DO NOT DRY COMPLETELY!!!