In-solution digest using filters and guanidine adapted from Wis´niewski 2009.

Required supplies

- eppendorph tubes - RNAase and DNAase free is best to avoid polymer contamination.
- mass spec vials (Thermo 11mm PP vial #C4011-13 and cap #C4011-55R)
- 25 mM Ammonium Bicarbonate (ABC) - rinse lab ware with HPLC grade isopropyl alcohol and dry before making buffers
- 8M urea in 100mM Tris/HCl pH 8.5- rinse lab ware with HPLC grade isopropyl alcohol and dry before making buffers
- Trypsin 1 ug/uL, Promega gold MS sequencing grade (Fisher Scientific or VWR - PRV5111)
- BCA or comparable Protein Assay
- 30kD centrifugal filters 500uL (go 30kD unless you are looking for a really small protein less than 5kD, available in chemistry stockroom or VWR 82031-352 for larger quantities)
- 200 mM Dithiothreitol (DTT, >99+% sigma # D-5545). Make stock 31 mg/mL in dH2O and store at -20 °C
- 200 mM Iodoacetamide (IAM, 97% sigma #I-670-9). 36.8 mg/mL in dH2O (throw away after use).
- 100 mM phenylmethanesulfonylfluoride (PMSF, FW=174.2) in 17.4 mg/mL in ethanol (throw away after use)

Procedure

1. Set temperature control bath to 60 °C to prepare for DTT step
2. Lyse tissue or cells using 4% SDS in 100 mM Tric/HCl pH 8.5 (homogenization will be different depending on what kind of sample you are working with. Please use the appropriate method for your sample)
3. Centrifuge lysate at 21000g 20 minutes (Pellet insoluble cell parts)
4. Decant soluble portion into new tubes, measure protein concentration with BCA or appropriate method
5. Add volume of sample you wish to reduce and alkylate for mass spectrometry to a new tube
    a. if you are analyzing sample directly, use ~50 ug of protein,
    b. if you are fractionating the peptides i.e. for high pH HPLC, use 400ug total protein,
6. Add sufficient 200 mM stock DTT to sample to obtain 5 mM final concentration (~1.2 uL 200 mM DTT for every 50 uL solution) mix and incubate at 55 °C 15 min (in sand bath)
7. Cool to room temperature for 5 min then add 200 mM IAM to 15 mM final concentration (~3.8 uL 200 mM IAM for every 50 uL of solution) and put in dark for 1 hour. (IAM is light sensitive do not store long term and do not store in light)
8. **Potential Stopping Point (can be stored in fridge overnight)**
9. Put reduced and alkylated sample onto filter
10. Spin filter at 14000g 10min (20-30 min for 10kD or 5kD filters), discard flow through
11. Add 8M urea (two times the volume of the sample to the sample, minimum 100ul) to the sample mix and then centrifuge at 14000g (Repeat this step at least one time). discard flow through
12. Add 25mM ABC (two times the volume of the sample to the sample, minimum 100ul) to the sample mix and then centrifuge at 14000g (Repeat this step at least one time), discard flow through
13. Empty **collection tube** then clean it with ddH2O thoroughly three times
14. Added MS trypsin to 1:50 (w/w) ratio to the protein solution on top of the filter.
15. Incubate with shaking at 37 °C overnight.
16. Quench digestion with 100 mM PMSF stock (final concentration ~1mM)
17. Centrifuge 14000g 30 min.

18. Add 100 uL 25 mM ABC to the top of the filter, centrifuge 14000g 30min
19. Collect filtrant (below the filter), put in mass spec vials.
20. Speedvac mass spec vial till ~1 or till dry.
21. Resuspend in 3% ACN 0.1% FA to a concentration (assuming 100% of original protein) of ~1ug/uL.