In-Solution Digestion Protocol

Chemical Reagents:

- **Trypsin**: Promega sequencing grade modified trypsin (V511A), 20 ug lyophilized powder, can be stored in solution for several weeks at -20°C.
- **Endoproteinase ASP-N**: SIGMA (P 3303).
- **Glutamic Acid-C:** Princeton Separations Sequencing grade modified Glutamic Acid-C (EN-140).
- **ddH2O**: MilliQ water or HPLC water.

Solutions:

- **200 mM Dithiothreitol (DTT)**

  30.86 mg of DTT + 1000 µL of 100mM NH_4HCO_3

- **50 mM NH_4HCO_3:**

  50 µL of 1 M NH_4HCO_3 in 950 µL of ddH_2O

- **1M iodoacetamide in 100 mM NH_4HCO_3:**

  Add 37 mg of iodoacetamide to 200 µL of 100mM NH_4HCO_3

- **Buffer A**: 50% Acetonitrile (ACN) and 0.1% formic acid in ddH_2O

  25 mL of ACN + 50 µL of formic acid + 25 mL ddH_2O

- **Buffer B**: 0.1% formic acid in ddH_2O

1. ***Sample Preparation***

Lower sample volumes/higher protein concentrations work best here. Bring samples up to 100 ul in 50mM NH_4HCO_3. It is very important to adjust the pH to the appropriate pH for certain enzyme (see table 2 in in-gel digestion). It is highly recommended that the samples be free of any detergents before digestion (most detergents can be removed by protein precipitation and/or ion exchange chromatography – see separate protocols).

2. ***Disulfide Reduction***

Reduce the sample by adding 5 ul of DTT stock to the 100 ul sample and boil it for ten minutes **if there is no urea in it**, otherwise skip boiling and vortex the sample, spin it down with a quick burst in the centrifuge and let the sample reduce at room temp for 45 min-1hr.

3. ***Sulfhydryl Alkylation***

Alkylate the sample by adding 4 ul of the iodoacetamide stock to the sample and vortex followed by a quick spin to get the sample to the bottom of the tube. Alkylate for 45 min-1hr at room temp.

4. ***Stopping Alkylation***

Neutralize the remaining iodoacetamide by adding 20 ul of your DTT stock, vortex, spin, and incubate at room temp for 45 min-1hr.

5. ***Trypsin Digest***

1. The ratio of trypsin to sample should between 1:50 to1:20 – 1 mg of trypsin for every 50 to 20 mg of protein.

   In order for the trypsin to work, the concentration of detergents (ie. SDS) and other chaotropes (ie. Urea) need to be at a compatible final concentration. When you add the correct amount of trypsin make sure you add it in a volume that will dilute your sample to the desired concentration of the limiting interfering agent. See the table at the end of this section for other reagent compatibility for trypsin digestion. *****

2. Gently vortex and spin the sample. To allow complete digestion place the rack in the 37°C incubator overnight, or for at least 18 hrs. Gentle or periodic mixing is optional.

6. Sample Clean-up

If detergents are present in the digest, they must be removed first. For non-ionic detergents (ie. Tween, NP-40, CHAPS, Triton-X), or anionic (ie. SDS), strong cation exchange (SCX) chromatography is the recommended removal method.

Sample De-Salting and Concentration by SPE C18 Reverse Phase

This allows for the concentration of the digest and removal of hydrophilic buffer components prior to MS analysis.

1. It is recommended that an SPE cartridge with ~100 mg of resin and a volume of at least 1 ml is used. (ie. JT Baker 10-SPE 1mL C_{18} extraction columns #7020-1…or equivalent).

2. To activate the C_{18} column in order to bind your peptides wash the column with 3 X1mL of Buffer A, then 3 X 1 mL Buffer B, sending the flow through to the waste beaker.

3. Acidify your sample to 0.2% formic acid and pass it over the SPE cartridge twice. The peptide-depleted sample that contains the unbound material should be left in the sample tube – just in case.
4. Wash unbound components off the column with 3 X 1mL of Buffer B.
5. Elute the peptides off of the column with 400 ul of Buffer A into a 0.5 ml tube.
6. Reduce the sample volume and remove the acetonitrile by vacuum concentration.

Table: List of common reagents used for protein extraction and the limitations of their FINAL concentrations for maintaining the enzyme activity of trypsin.

	Maximum allowable concentration
Chaotropes:	Less than 1 M total
Urea	
Thiourea	
Detergents:	
SDS	0.05 %
Triton X-100	1 %
CHAPS	1 %
NP-40	1 %
Tween 20	1 %
Octyl glucopyranoside	1 %
Salts/Buffers/pH:	Less than 250 mM total pH ~ 8-9
Reducing Agents:	
DTT	20 mM
TCEP	5 mM
TBP	5 mM
Protease Inhibitors:	Digestion should be free of ALL serine protease inhibitors
Solvents:	
Acetonitrile	40 % (v/v)