

## Procedure for In Solution Digestion

### Materials

**Ammonium Bicarbonate:** 50 mM ammonium bicarbonate.

**6M Urea:** Dilute 1.0 g of urea in 2.5 ml of 50 mM ammonium bicarbonate solution.

**Reducing reagent:** Dissolve 30 mg of DTT in 1.0 ml of 50 mM ammonium bicarbonate solution to make 200 mM DTT

**Alkylating reagent:** Dissolve 36 mg (0.036 g) of iodoacetamide in 1 ml of 50 mM ammonium bicarbonate solution to make 200 mM iodoacetamide.

**Trypsin solution:** Trypsin (unmodified sequence grade from Roche) is made up by dissolving 20 µg of trypsin in 200 µL of 50 mM ammonium bicarbonate buffer. This 0.10 µg/µL solution can be used in a 1:20 ratio of enzyme: substrate by weight. This is an approximate value for the trypsin catalyst.

### Digestion Procedure

This procedure is based on 1 mg of total protein. The reagent volume should be adjusted according to the actual amount of protein.

1. Reconstitute protein sample in approximately 100 µl of 6.0 M urea, 50 mM ammonium bicarbonate buffer in a 1.5 ml plastic centrifuge tube.
2. Add 5 µl of Reducing Reagent and mix the sample by gentle vortex.
3. Reduce the mixture for 1 hour at room temperature or at 37 °C.
4. Add 20 µl of Alkylating Reagent and alkylate (wrap in aluminum foil to cover up the sample).
5. Add 20 µl of Reducing Reagent to consume any unreacted alkylating agent (so the trypsin is not alkylated). Mix the sample by gentle vortex and allow reaction to stand at room temperature for 1 h.
6. Add 900 µl of 50 mM ammonium bicarbonate solution to dilute the urea before digesting with trypsin.
7. Add trypsin in appropriate ratio (1:20) to approximate amount of protein by weight. Digest overnight at 37°C.
8. Stop the reaction and adjust the pH of the solution to 6 by adding concentrated acetic acid as needed.

### Peptide Mass Fingerprint by MALDI-TOF MS

1. If there is sufficient amount of protein, the sample can be submitted directly for MALDI analysis (consult with personnel at mass spec lab).

2. If there is not enough sample, preconcentrate by drying the sample down in a Speedvac to a smaller volume. However, this will also increase the urea concentration and make it difficult to see the ions directly by MALDI. You may have urea crystals crashing out at the bottom of the tube. You can stop the drying down process when there is still some (~50  $\mu$ L of liquid) left in the centrifuge tube. This sample can be taken (containing the peptides) and zip tipped to get rid of the extra urea for MALDI.

### **Purification of tryptic fragments by C18 zip tip**

Prior to zip tip acidify the above concentrated peptide sample by addition of glacial acetic acid to final concentration of ~1%.

1. Prepare a C18 zip tip by equilibrating it with 65% acetonitrile/1% acetic acid in about three cycles, and then 2% acetonitrile/1% acetic acid in about three cycles.
2. Allow sample to bind to zip tip by repeatedly drawing and dispensing sample through zip tip (slowly, about 3-5 times).
3. Wash zip tip with 2% acetonitrile/1% acetic acid slowly about 3 times.
4. Elute sample slowly from zip tip with 5  $\mu$ l of 65% acetonitrile/1% acetic acid to a clean 0.5 ml microcentrifuge tube.
5. The eluate is now ready for MALDI-MS analysis.

Information about zip tip suppliers can be found at following web sites:

<http://www.millipore.com/catalogue.nsf/docs/C5737>

### **Peptide Sequencing by LC/MS/MS**

The concentrated tryptic peptides after Speedvac (~50  $\mu$ l) can be directly analyzed by LC/MS/MS for peptide sequencing. Most of the urea will probably crash out at the bottom of the tube