**Following In-gel tryptic digestion kit by Pierce.**

**Material prep**

**Trypsin stock** - Promega Trypsin gold MS grace porcine 100 µg Cat#V528A, hydrated with trypsin storage solution or reconstitute in 50 mM acetic acid. Freeze in 1 µl aliquots at -80°C. Store in -80°C freezer in Rm 2-26.

**Digestion Buffer (W1)** – 10 mg of ammonium bicarbonate + 5 ml ultrapure water (final conc 2 mg/ml = 25 mM). Store at 4°C for up to 3 months but **check pH with pH paper before use to make sure it is at pH 8**. Trypsin digestion will not work in acidic solution.

**Destaining Soln. (W2)** – Mix 80 mg of ammonium bicarbonate to 20 ml water(4 mg/ml in water) and then add 20 ml of acetonitrile. Store at 4°C for up to 3 months (check pH, should also be ~pH 8).

**Protocol for Coomassie Blue stained bands/spots**

Cutting out bands and destaining

1. 2DGE spots excised and stored at 4°C until ready for trypsin digestion.
2. When ready for trypsin digestion, cut gel pieces up into smaller pieces and return to tubes. Add 400 µl of Destaining solution (W2) to gel pieces. Incubate with shaking at room temp for at least 30 min.
3. Remove and discard destaining soln.
4. Repeat steps 2 and 3.
5. Turn on and set 60°C water bath so that it is ready for the following steps.

**Reduction and Alkylation**

1. Prepare reducing buffer using the formula 5.5 µl TCEP + 50 µl W1.
2. Add 50 µl of reducing buffer to each sample and incubate at 60°C for 10 min. Use water bath to heat samples to 60°C.
3. Discard reducing buffer.
4. Prepare Akylation buffer just before use by dissolving 1 mg of iodoacetic acid (IAA) for every 50 µl of W1 solution. Keep in dark.
5. Add 50 µl Alkylation Buffer to each sample. Incubate sample in dark at room temp for one hour.
6. Discard Alkylation Buffer.
7. Add 200 Destaining Buffer (W2) to each sample. Incubate at room temp with shaking for 15 min.
8. Discard W2.
9. Repeat steps 7 and 8.

**Digestion**

1. Shrink gel pieces by adding 50 µl acetonitrile (W3). Incubate sample of 15 min at 24°C.
2. Carefully remove acetonitrile. Dry gel pieces by using speed vac for 2-5 min.
3. Obtain 2 µl trypsin stock (1 µg trypsin stock/µl) in 1.5 ml microfuge tube aliquot stored in -80°C.
4. Add 198 µl of digestion buffer (W1) to the 1 µl of trypsin stock. Mix. Keep on ice.
5. Add 75 µl trypsin soln (1 µg/µl W1), enough to cover gel pieces.
6. Incubate sample at 37°C over night.
7. Collect digestion solution (now containing the peptides) and place in 1.5 ml tube.
8. Add 50 - 100 µl elution buffer (0.1% formic acid, 50% acetonitrile, 49.9% water vol/vol) to cover gel pieces. Incubate with mixing for 15 min. Alternately, tubes can be incubated in a sonicating water bath for 5-10 min.
9. Remove fluid to same 1.5 ml tube from step 7. Speed vac tube to dryness.
10. Repeat steps 8 and 9.
11. Sample is ready for zip tip.

**Zip Tip (μ-C18) Purification for Mass spec samples**

Fresh solutions to prepare and have on hand

1) 0.1% Formic acid in ultra pure water

2) 0.1% Formic acid in 50% acetonitrile 49.9% ultra pure water

3) Acetonitrile

Procedure

1. Dry samples after trypsin digestions or other treatment using speed vac.
2. Dissolve sample in 12 μl 0.1% formic acid/water.
3. For each sample, wet a zip tip with 10 μl acetonitrile. Discard the acetonitrile.
4. Wet the zip tip with 10 μl 0.1 % formic acid/water. Discard the 0.1% formic acid/water solution.
5. Pipet sample up and down at least 10 times.
6. Wash zip tip with 10 μl 0.1 % formic acid/water. Discard the 0.1% formic acid/water solution.
7. Repeat step 6.
8. Elute the sample into a sample vial insert using 0.1% formic acid/50% acetonitrile/49.9% water.
9. Repeat step 8.
10. Dry liquid in vial inserts using speed vac.
11. Store vial inserts at -20°C in 1.5 ml microfuge tubes until ready to run on mass spec if running sample another day (stored 7/31/12).
12. Resuspend sample in vial insert with 6 μl of 0.1% formic acid containing a 0.7:2.8 acetonitrile:water ratio.
13. Vial inserts are ready for mass spec.