Trypsin In-gel Digestion Protocol

Destaining

- Excise protein spot/ band and cut into ~1mm cubes. Transfer to a clean microcentrifuge tube.
- 250 ul water, 15 min. (can store in water for longer)
- Remove water.
- A): for silver staining: 50 ul destaining buffer, 20-30 min.
    - Destaining buffer: 3 mL 100 mM $Na_2S_2O_3$
      3 mL 30 mM $K_3Fe(CN)_6$
- B): for coomassie blue and Sypro Ruby staining: Destain 3 times by adding 100 ul of 25 mM NH_4HCO_3 in 50% acetonitrile, incubate for 15-30 minutes with occasional vortexing, and discard the liquid with each wash.
- Remove destaining solution.
- 500 ul water, 30 min. (store overnight in water if blue color is not disappearing)
- Add enough 100% acetonitrile to fully cover gel piece, incubate for 20-30 minutes, and discard acetonitrile.
    - Gel pieces should be white/ opaque and shrunken
- Dry gel pieces using Speed Vac.

Reduction of Disulfide Bonds

- Add 100-200 ul of 10 mM dithiothreitol (DTT) in 100 mM NH_4HCO_3 to the dried gel piece and incubate for 1 hour at 56 °C.

Alkylation with Iodoacetamide

- Let the gel pieces cool to room temp and discard DTT soln.
- Add 200 ul of 55 mM (10 mg/ml) iodoacetamide in 100 mM NH_4HCO_3 until gel pieces are covered.
- Wrap tube in aluminum foil to protect sample from light and incubate 45 minutes in dark at room temperature.
- Remove iodoacetamide/ NH_4HCO_3 solution and discard.
- Add 50-100 ul of 100 mM NH_4HCO_3 and incubate for 10 minutes at room temperature. Remove and discard solution
- Dehydrate with about 100 ul of 100% acetonitrile. Remove and discard solution when gel pieces are white/ opaque.
- Re-swell in 100 ul of 100 mM NH_4HCO_3 for 10 minutes. Remove and discard solution
- Dehydrate with about 100 ul of 100% acetonitrile. Remove and discard solution when gel pieces are white/ opaque.
- Use the Speed Vac to evaporate remaining solvents.

- Add enough Trypsin solution (Trypsin: protein ratio of 1:20-1:100, w/w) to re-swell gel pieces completely at 4 °C for 30 minutes.
    - If after 30 minutes, gel pieces are uncovered, add more buffer to cover gel pieces.
    - Trypsin should be 6 ng/ul for silver stain and 12 ng/ul for coomassie blue.
    - **Digestion buffer:** 2.5 mL 100% acetonitrile, 45.5 mL H_2O, 2 mL 1M NH_4HCO_3,
- Digest overnight at 37 °C.
    - Digest no more than 18 hours

Peptide Extraction

- Transfer supernatant into new labeled vial
- Solution A 30-50 ul, 1 hour at room temperature
- Transfer the solution to the same vial as 1
- Solution B 30-50 ul, 1 hour at room temperature
- Transfer the solution to the same vial as 1
- Add enough acetonitrile to dehydrate
- Transfer the solution to the same vial as 1
- Dry the solution using Speed Vac. (can store in 4°C once dry)
- **Solution A:** 5% Formic Acid in H_2O
- **Solution B:** 5% Formic Acid in 50:50 H_2O: Acetonitrile

<u>At this point, ship samples to VGN</u>

Re-suspension

- Add 8-10 ul loading solution (2.5% MeCN, 2.5% Formic Acid) and run on MS