In-Gel Tryptic Digestion Protocols

Using Promega Modified Sequencing Grade Trypsin (Porcine)
3 hour - overnight digestion

Wear gloves and a clean lab coat to avoid keratin contamination.
Prepare all solutions containing ammonium bicarbonate fresh daily.

Day 1

1. Excise protein spot/band on a clean glass surface using a scalpel. Cut the gel as close to the band as possible to reduce the volume of gel to be processed. Cut into 4-5 pieces and place into a 500 µL microcentrifuge tube. Push gel pieces down with a pipet tip.

2. If storing the gel, add 40 µL of 1% acetic acid in Milli-Q water. Store at 4°C until ready for digestion.

3. Prepare the following solutions:

   50mM Ammonium bicarbonate
   0.0396 g in 10 mL of Milli-Q water
   pH should be ~8

   10 mM DTT in 50 mM ammonium bicarbonate
   0.0015 g dithiothreitol, electrophoresis grade in 1 mL of 50 mM ammonium bicarbonate

   100 mM iodoacetamide in 50 mM ammonium bicarbonate
   0.0185 g iodoacetamide in 1 mL of 50 mM ammonium bicarbonate

   50% Acetonitrile/25 mM ammonium bicarbonate
   1 mL of acetonitrile
   1 mL of 50 mM ammonium bicarbonate

4. Remove the acetic acid from the tube.

5. **Destaining**

   Coomassie stained Bands. This can also be used to wash Sypro-Ruby stained gels

   - Wash the gel with 50 µL of 50 mM ammonium bicarbonate. Vortex mix and let stand for 5 minutes. Discard liquid into waste.
   - Shrink the gel with 50 µL of 50% acetonitrile/25 mM ammonium bicarbonate. Vortex mix and let stand for 10 minutes. Remove and discard the supernatant into waste.

   Silver-stained bands

   - The silver-stained band/spot is incubated with a fresh mixture (v 1: 1) of 30 mM potassium ferricyanide (98 mg/10ml H_2O) and 100mM sodium thiosulfate (248 mg/10ml H_2O) for 15 minutes. Remove and discard the supernatant into waste.
   - Wash with 50 µL deionized water. . Vortex mix and let stand for 5 minutes. Discard liquid into waste.
   - Wash the gel with 50 µL of 50 mM ammonium bicarbonate. Vortex mix and let stand for 5 minutes. Discard liquid into waste.
   - Shrink the gel with 50 µL of 50% acetonitrile/25 mM ammonium bicarbonate. Vortex mix and let stand for 10 minutes. Remove and discard the supernatant into waste.

6. **Reduction**

   - Reduce with 30 µL of 10 mM DTT for 30 minutes at 56°C. Remove and discard the supernatant liquid into waste.

7. **Alkylation**

- Alkylate with 30 µL of 100 mM iodoacetamide for 15 minutes in the dark at room temperature. Remove and discard the supernatant liquid into waste.

8. Shrink with 50 µL of 50% acetonitrile/25 mM ammonium bicarbonate. Vortex mix and let stand for 15 minutes. Remove and discard the supernatant into waste.

9. **Digestion**

   Prepare the trypsin solution:
   Trypsin Stock (0.2 µg/µL): Add 100 µL of Promega resuspension buffer to 20 µg of lyophilized trypsin (porcine, modified sequencing grade). Store at -20ºC.

   13 ng/µL trypsin in 50 mM ammonium bicarbonate
   10 µL of trypsin stock
   140 µL of 50 mM ammonium bicarbonate

   - Cover the gel pieces with 13 ng/ µL trypsin to the gel pieces. Incubate for ***at least*** 3 hours at 37˚C.

10. **Extraction of Peptides**

    Prepare the following solutions:
    25 mM ammonium bicarbonate
    1 mL of 50mM ammonium bicarbonate
    1 mL of Milli-Q water

    5% formic acid in water
    4.75 mL of Milli-Q water
    250 µL of formic acid

    a) Remove the tubes from the incubation. From this point on the supernatant will be collected into a new 500 µL microcentrifuge tube.
    b) Transfer the liquid to a new tube.
    c) Add 20 µL of 25 mM ammonium bicarbonate. Vortex mix and let stand for 10 minutes. Add to the supernatant collected in step b).
    d) Add 20 µL of 5% formic acid. Vortex mix and let stand for 10 minutes. Add to the supernatant collected in step b).
    e) Add 20 µL of 100% acetonitrile. Vortex mix and let stand for 10 minutes. Add to the supernatant collected in step b).
    f) Add 20 µL of 5% formic acid. Vortex mix and let stand for 10 minutes. Add to the supernatant collected in step b).
    g) Add 20 µL of 100% acetonitrile. Vortex mix and let stand for 10 minutes. Add to the supernatant collected in step b).

11. Store the extracted peptides at -20˚C until ready to evaporate down.

12. **Preparation of Analysis**
    Evaporate down to dryness and reconstitute in 5 µL of 0.1% formic acid in water or 0.1% TFA in water. Samples are ready for LC MS/MS analysis with no further clean-up needed. Samples will need to undergo C18 Ziptip cleanup before ESI or MALDI MS analysis.