In-Gel Trypsin Digest

When running and handling gels for down stream mass spectrometry all efforts should be made to reduce keratin contamination. Wear a white coat and gloves. Use fresh buffers (ideally use commercial buffers and pre-cast gels). Gel apparatus should be kept clean. Staining trays should be kept exclusively for this purpose and should be covered.
All solutions below should be freshly prepared in LC grade water from high purity reagents.
Note – always refer to the manufacturers notes when using trypsin. *The instructions given below are for Promega sequencing grade, other manufacturers instructions may vary*.

Solutions

- 200 mM NH_4HCO_3 – ammonium bicarbonate (mol wt 79.06), 15.81 mg/ml or 790 mg in 50 ml ddH_2O
- 100mM NH_4HCO_3 (Ambic) – dilute 200mM NH_4HCO_3 with an equal volume of ddH_2O
- 50mM NH_4HCO_3 (Ambic) – dilute 100mM NH_4HCO_3 with an equal volume of ddH_2O
- Ambic:ACN Add equal volumes of 100mM Ammonium bicarbonate and Acetonitrile (50:50 mixture)
- 10mM DTT (Dithiothreitol) in 50mM Ammonium bicarbonate
- 55mM IAA (Iodoacetamide) in 50mM Ammonium bicarbonate
- 25 mM NH_4HCO_3 prepared fresh from stock 200 mM NH_4HCO_3 (chilled).
- **Promega Trypsin** sequencing grade modified (V5111) prepared as follows:- resuspend a single vial of lyophilised trypsin (20 ug) in 100 ul of the accompanying resuspension buffer. To this add 900 ul of 25 mM NH_4HCO_3 (giving 20 ng/ul). *Keep on ice.*
- 70% Acetonitrile; 5% Formic Acid ; 25% LC-MS Water

Steps

A. Excise band
B. De-stain
C. Reduce and Alkylate
D. Digest
E. Extract

Procedure

A. Wash and Excise bands

- Excise the band of interest using a fresh scalpel on a clean surface (e.g. glass wiped down with ethanol). Avoid cutting excess gel by cutting as close as possible to the stained band. Cut into 1-2 mm pieces and place into a microfuge tube.
- Wash the excised pieces with approximately 100-200ul H_2O, leave for 5 minutes
- Remove and discard H_2O (with gel-loading tips if available)

B. De-stain (Coomassie)

1. Add 100-200ul Ambic:Acetronitrile, incubate for 5 minutes at 37°C on Thermomixer with shaking
2. Remove and discard supernatant
3. Repeat steps 1 and 2 until the gel pieces are colourless
4. Add 50ul Acetonitrile, incubate for 5 minutes at 37°C on Thermomixer with shaking until gel pieces shrink and turn white
5. Remove and discard Acetronitrile
6. Dry the gel pieces on a Speedivac for approximately 10 minutes

(Dried gel pieces can be stored at -20°C if necessary)

C. Reduce and Alkylate

1. Add 40ul of 10mM-DTT in 50mM-Ambic to the dried gel pieces and incubate for 45 mins at 56°C (the gel pieces should re-swell)
2. *Remove and discard* the supernatant and add 100ul Acetonitrile, incubate for 5 minutes at 37°C on Thermomixer with shaking until gel pieces shrink and turn white
3. *Remove and discard* Acetronitrile
4. Dry the gel pieces on a Speedivac if needed. (They should be white when dry).
5. Add 40ul of 55mM-Iodoacetamide in 50mM-ambic to cover gel pieces. Incubate for 30 mins at room temperature in the dark.
6. *Remove and discard* the supernatant.
7. Add 100ul of acetonitrile and incubate at room temperature for 5 minutes until gel piece turn white. *Remove acetonitrile*.
8. Dry down gel pieces on SpeedVac if needed.

D. Digestion of peptides

1. Rehydrate gel pieces in 40ul Trypsin solution (20ng/ul in 25mM NH_4HCO_3) for 45 minutes at 4°C (i.e. on ice).
2. After 15 minutes check if the digestion solution has been completely absorbed by the gel pieces. If so, add enough to cover the gel.
3. After 45 minutes remove and discard the supernatant.
4. Add sufficient 25mM-ambic to cover the gel pieces and incubate at 37°C overnight on Thermomixer (with gentle agitation)

E. Extraction of tryptic peptides

1. Remove and *retain* the supernatant, transferring to a fresh labeled 0.5ml tube.
2. Add 50ul of 70% Acetonitrile; 5% Formic Acid to extract the peptides from the gel pieces. Place closed tube in sonicating water bath for 5 minutes.
3. Remove and *retain* the supernatant, combining with supernatant from step 1.
4. Repeat steps 2 and 3 twice more, combining the supernatants each time.
5. Dry the combined extracts on a Speedvac

Tryptic peptides are re-suspended in 0.1% formic acid prior to running on LC-MS. Resuspended peptides can be read on the DeNovix spectrophotometer (against a blank of 0.1% Formic Acid) to determine the approximate concentration.