IN-GEL DIGESTION PROTOCOL FOR MS ANALYSIS

Materials:

- Stained polyacrylamide gel containing protein(s) of interest
- Methanol/50% (v/v) acetonitrile/0.1% (v/v) Trifluoroacetic acid (TFA)
- 0.1% (v/v) acetic acid in 50% (v/v) methanol
- 50 mM ammonium bicarbonate, pH 7.8
- LC-MS grade acetonitrile
- 10 mM dithiothreitol (DTT) in 50 mM ammonium bicarbonate
- 55 mM iodoacetamide in 50 mM ammonium bicarbonate
- 1% (v/v) formic acid
- Trypsin solution
- 1% (v/v) formic acid in 50% (v/v) acetonitrile

NOTE: To avoid/reduce contamination with human keratins, one MUST wear gloves prior handling the gel and preferably work in a dust free area.

- **Excise and washing of gel fragments**

1. Excise protein bands/spots of interest from the gel using a clean razor and mince it into pieces of 1 mm^3.
2. Place it into an eppendorf tube (washed previously with 200 µL of methanol/50% acetonitrile/0.1% TFA, **do not leave the lids off the eppendorf tubes for prolonged period**).
3. Add 300 µL of 50% methanol containing 0.1% acetic acid and incubate at 37ºC for 45 minutes. Repeat the wash step until no more stain is entering the de-staining solution.
4. Wash the gel pieces with 200 µL of 50 mM ammonium bicarbonate, pH 7.8 and discard the solution post washing. Resulting gel pieces should be clear.
5. Add 500 µL of LC-MS grade acetonitrile to the gel pieces and rotate/shake for 30 minutes. Repeat this step if necessary.

NOTE: The addition of acetonitrile causes quick dehydration (removal of water) of gel pieces. At this point the gel pieces/spot is sufficiently dehydrated causing it to shrink and become opaque-white color.

6. Remove acetonitrile by letting the gel pieces dry in speed vacuum for 10-15 minutes at room temperature.

- **Reduction and alkylation of cysteine residues**

7. Add 200 µL of 50 mM ammonium bicarbonate, pH 7.8 containing 10 mM DTT to cover the gel pieces, vortex, and centrifuge briefly. Incubate for 1 hour at 37ºC. **[Before carboamidomethylation any disulphide bridges that have reformed needs to be re-broken using DTT or DTE].**
8. Discard excess DTT solution and wash with 300 µL of 50 mM ammonium bicarbonate, pH 7.8. **[At this step all disulphide bridges are broken]**.
9. Add 300 µL of 100 mM ammonium bicarbonate containing 55 mM iodoacetamide. Incubate for 45 minutes at room temperature in the dark.
10. Discard excess solution and wash it thrice with 800 µL of 50 mM ammonium bicarbonate, pH 7.8. **[At this point all cysteine have now been carboamidomethylated permanently].**

NOTE: This procedure is not necessary for the analysis of protein from 2D-PA gels because the proteins have already been carboamidomethylated prior to SDS-PAGE]

- **Proteolysis**

For silver-stained gels: Add 60 µL of trypsin solution (12.5 ng/µL in 50 mM ammonium bicarbonate, pH 7.8). Incubate in a waterbath for 12 hours or overnight at 37ºC. **Add 1.6 mL of 50 mM ammonium bicarbonate to one aliquot.**

For coomassie blue stained gels: Add 60 µL of 25 ng of trypsin solution. Incubate in a waterbath for 12 hours or overnight at 37ºC. **Add 0.8 mL of 50 mM ammonium bicarbonate to one aliquot**.

11. After digestion, add 200 µL of 1% (v/v) formic acid, vortex and shake for 20 minutes. **[This wash will contain more hydrophilic peptides]**.
12. Remove 1% (v/v) formic acid wash and transfer wash solution to a new, washed eppendorf tubes.
13. To the gel pieces, add 300 µL of 50% (v/v) acetonitrile containing 1% formic acid. Vortex and shake for 20 minutes. **[This wash will contain most of the tryptic peptides]**.
14. Remove the 50% acetonitrile containing 1% formic acid wash (having tryptic peptides) and transfer to the eppendorf tubes containing the previous 1% formic acid wash solution.
15. Repeat step 13
16. Remove the final supernatant (50% acetonitrile containing 1% formic acid wash) and transfer it to the eppendorf tube containing previous washes (supernatant) thus pooling it at one place.
17. Add 200µL of 1% (v/v) formic acid to the pooled supernatant, vortex and freeze-dry.