In-Gel Trypsin Digest

(Adapted from EMBL Method)

- Wear gloves to minimize contamination from keratins. Do not lean, talk or breath over your gel when cutting bands. Do not have anyone walk by you when cutting out bands. Keratin is everywhere, so please take these precautions to minimize any exogenous keratin.
- Low retention or siliconized tubes are highly recommended for proteolytic digestions.
- A typical gel band is ~2x10 mm (1mm thick). The volumes in this protocol correspond to gel volumes of this size. Please scale accordingly if your gel band/region is larger. If the gel band is smaller, the stated volumes are suitable.

1. Excision of protein bands from polyacrylamide gels.

a) Excise the band of interest using a sterile scalpel, razor blade or sharpened microspatula. Cut as close to the protein band as possible to reduce the amount of "background" gel. Cut into ~2 x 2 mm cubes and place in 1.5 mL microfuge tube. If cutting multiple bands, wash cutting utensil with 1:1 water/ethanol in between each gel band.

b) If silver stain was used, destain bands with the protocol below; otherwise continue to step 2:

Silver Destaining

i. Excise the spot/band and place in very clean water (such as Milli-Q or nanopure water) in a siliconized microcentrifuge tube. Discard water.

ii. De-stain (remove silver stain) gel piece with 1:1 solution of 30 mM potassium ferricyanide and 100 mM sodium thiosulphate made FRESHLY before use. Use 300 – 500 uL, incubate for 8 minutes, and discard.

iii. Add 100 µL of the destaining solution if necessary, incubate for 1 minute and discard.

iv. Wash 4 x with 1 mL of Mill-Q water, 8 minutes per wash

2. Washing of gel pieces

a) Transfer 75 µL 1:1 100mM ammonium bicarbonate:acetonitrile to gel pieces, vortex briefly and incubate 15 min at room temperature. Remove solution, discard to waste and repeat this step for a total of two washes. If the band is heavily Coomassie stained, an extra wash is recommended.

b) Remove previous wash and add 75 µL of 100% acetonitrile. After pieces shrink and turn white and semi-opaque, remove acetonitrile (~30 sec – 1 min).

c) Remove acetonitrile and proceed to step 3.

3. Reduction & Alkylation - **note that DTT and iodoacetamide solutions should be made fresh for each digest*.

a) Rehydrate gel pieces with 75 µL 10 mM DTT in 50 mM ammonium bicarbonate. Incubate 1 hr at 56 °C in water bath. After incubation, briefly spin down tubes then remove DTT solution.

b) Add 75 µL of 55 mM iodoacetamide in 50 mM NH_4HCO_3 and incubate 30 min at room temperature in the dark (iodoacetamide is light sensitive). Remove iodoacetamide solution.

c) Wash gel plugs with 75 µL 1:1 acetonitrile:100 mM ammonium bicarbonate as in step 2. Repeat for a total of 2 washes. All the Coomassie should be removed at this time. If residual Coomassie remains, repeat wash with the acetonitrile:100 ammonium bicarbonate solution.

d) Remove previous wash and add 75 µL of 100% acetonitrile. After pieces shrink and turn opaque white, remove acetonitrile (~30 sec-1 min).

4. In-Gel Digest

a) Gel pieces should have the opaque white look from the last wash with 100% acetronitrile.

b) Rehydrate gel pieces in digestion buffer at 4°C (50 mM NH_4HCO_3, 5 mM $CaCl_2$, 5 ng/µL trypsin). Add a sufficient volume of buffer to cover gel pieces, ~20 µL, inspect visually. Add more if necessary.

c) Set on ice for 15 min. Remove supernatant and replace with 70 µL 50 mM NH_4HCO_3, 5 mM $CaCl_2$. Incubate at 37 °C overnight in a warm-air incubator.

5. Extraction of Peptides

a) Briefly spin down tubes in a centrifuge at low speed and recover supernatant from overnight incubation. Place supernatant in new 1.5 mL tube labeled accordingly.

b) Add sufficient volume of 50% acetonitrile, 0.3% formic acid to cover gel pieces (approximately 60 µL), incubate 15 min.

c) Recover supernatant and place in corresponding tube.

d) Add the same volume used in step (b) of 80% acetonitrile, 0.3% formic acid, incubate 15 min. Recover supernatant and place in corresponding tube.

e) Freeze pooled extracts in -80 °C freezer 30 minutes, then dry in speed vac.

f) Proceed to zip tip/stage tip protocol (for desalting) or store at -80 °C prior to submission for mass spec analysis.