In-gel Digestion of Proteins Separated by SDS-PAGE

Stage 1

Coomassie/Sypro stained bands

- Place the gel on a clean glass or plastic plate, excise gel band or spot as close to the band/spot as possible.
- Cut into 1 mm square pieces with a clean scalpel and place into a tube.
- Wash the band pieces with 300 µl MilliQ water for 15 min.
- Add 300 µl CH₃CN and wash for 15 min.
- Remove the supernatant (Use a P1000 tip with a P10 tip on the end to prevent).
- Wash with 300 µl 100 mM NH₄HCO₃ / CH₃CN (1:1 v/v) for 15 min.
- Remove the supernatant.
- If the pieces are still stained, repeat the NH₄HCO₃ / CH₃CN washes.
- Add 200 µl CH₃CN and incubate for 5 min, remove the supernatant.
- Dry the band pieces in a Speedvac for 2-3 min.
- If you want to do reduction/alkylation proceed to Stage 2 otherwise to Stage 3.

Silver Stained bands

- Excise gel bands or spots as above.
- Wash gel pieces with 300 µl Milli-Q water for 15 min to remove acetic acid.
- Add 50 µl/band of 15 mM potassium ferricyanide/50 mM sodium thiosulphate for 5 - 10 min until the gel pieces go clear (i.e. until all the silver is removed).
- Remove the supernatant (Use a P1000 tip with a P10 tip on the end).
- Wash the gel pieces with 300 µl Milli-Q water for 15 min.
- Add 300 µl CH₃CN and wash for a further 15 min, and remove the supernatant.
- Wash the gel pieces with 300 µl 100 mM NH₄HCO₃ for 15 min.
- Wash with 300 µl 100 mM NH₄HCO₃ / CH₃CN (1:1 v/v) for 15 min.
- Remove the supernatant.
- Add 100 µl CH₃CN to dehydrate the band pieces for 5 min.
- Remove the supernatant
- Dry the band pieces in a Speedvac for 5 min.
- If you want to do reduction/alkylation proceed to stage 2 otherwise to stage 3.
Stage 2

Reduction/Alkylation of band pieces

- Add 50 µl/sample of 10 mM DTT in 100 mM NH₄HCO₃.
- Incubate at room temperature for 30 min, remove the supernatant.
- Add 50 µl/gel sample of 55 mM fresh iodoacetamide in 100 mM NH₄HCO₃.
- Incubate at room temperature for 30 min, remove the supernatant.
- Wash the gel pieces with 300 µl 100 mM NH₄HCO₃ for 15 min.
- Remove the supernatant.
- Wash the gel pieces with 300 µl 20 mM NH₄HCO₃/CH₃CN (1:1 v/v) for 15 min.
- Remove the supernatant.
- Add 100 µl CH₃CN to dehydrate the gel pieces for 5 min.
- Remove the supernatant, dry the gel pieces in a Speedvac for 2-3 min.

Stage 3

Digestion of Proteins

- Add 25 µl/gel sample 6 ng/ul promega trypsin in 50 mM NH₄HCO₃ (20 ug trypsin + 200 µl 50 mM acetic acid makes 100 ng/µl; then 5µl 100 ng/ul trypsin + 40 µl water + 40 µl 100 mM NH₄HCO₃) (0.1% n-octyl glucoside can be included).
- Allow band pieces to rehydrate in digestion buffer for 30 min.
- If required, add more 50 mM NH₄HCO₃ (minus the trypsin) to cover the pieces.
- Incubate at 37°C, >5 h (or O/N).
- Spin briefly, add 30 µl 1% formic acid/2% CH₃CN to the digest.
- Incubate at 30°C for 30 min on shaking platform, or vortex for 10 min.
- Remove the digest supernatant and transfer to a clean tube.
- 1 µl could be spotted on Maldi target and analyse by MALDI-TOF.
- Add 24 µl 50% CH₃CN, Vortex for 10 min.
- Transfer supernatant to the tube.
- Repeat the 50% CH₃CN extraction and combine the supernatant.

Analyse digest immediately on MALDI-TOF or store at -20°C until required, e.g. for CapLC. If samples are too diluted, they can be dried to approx. 10 µl for analysis.