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 ±1 MilliQ water for 15 min.
- Add 300 ±1 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 ±1 100 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 ±1 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 ±1 Milli-Q water for 15 min to remove acetic acid.
- Add 50 ±1/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 ±1 Milli-Q water for 15 min.
- Add 300 ±1 CH₃CN and wash for a further 15 min, and remove the supernatant.
- Wash the gel pieces with 300 ±1 100 mM NH₄HCO₃ for 15 min.
- Wash with 300 ±1 100 mM NH₄HCO₃ / CH₃CN (1:1 v/v) for 15 min.
- Remove the supernatant.
- Add 100 ±1 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 \( \pm 1 \) sample of 10 mM DTT in 100 mM NH\(_4\)HCO\(_3\).
- Incubate at room temperature for 30 min, remove the supernatant.
- Add 50 \( \pm 1 \) gel sample of 55 mM fresh iodoacetamide in 100 mM NH\(_4\)HCO\(_3\).
- Incubate at room temperature for 30 min, remove the supernatant.
- Wash the gel pieces with 300 \( \pm 1 \) 100 mM NH\(_4\)HCO\(_3\) for 15 min.
- Remove the supernatant.
- Wash the gel pieces with 300 \( \pm 1 \) 20 mM NH\(_4\)HCO\(_3\)/CH\(_3\)CN (1:1 v/v) for 15 min.
- Remove the supernatant.
- Add 100 \( \pm 1 \) CH\(_3\)CN 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 \( \pm 1 \) gel sample 6 ng/ul promega trypsin in 50 mM NH\(_4\)HCO\(_3\) (20 ug trypsin + 200 \( \pm 1 \) 150 mM acetic acid makes 100 ng/\( \pm 1 \); then 5\( \pm 1 \) 100 ng/ul trypsin + 40 \( \pm 1 \) water + 40 \( \pm 1 \) 100 mM NH\(_4\)HCO\(_3\) (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 NH\(_4\)HCO\(_3\) (minus the trypsin) to cover the pieces.
- Incubate at 37\(^\circ\)C, >5 h (or O/N).
- Spin briefly, add 30 \( \pm 1 \) 1% formic acid/2% CH\(_3\)CN to the digest.
- Incubate at 30\(^\circ\)C for 30 min on shaking platform, or vortex for 10 min.
- Remove the digest supernatant and transfer to a clean tube.
- 1 \( \pm 1 \) could be spotted on Maldi target and analyze by MALDI-TOF.
- Add 24 \( \pm 1 \) 50% CH\(_3\)CN, Vortex for 10 min.
- Transfer supernatant to the tube.
- Repeat the 50% CH\(_3\)CN extraction and combine the supernatant.

Analyze digest immediately on MALDI-TOF or store at -20\(^\circ\)C until required, e.g. for CapLC. If samples are too diluted, they can be dried to approx. 10 \( \pm 1 \) for analysis.