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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 CH_3CN 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 NH_4HCO_3 / CH_3CN (1:1 v/v) for 15 min.
- Remove the supernatant.
- If the pieces are still stained, repeat the NH_4HCO_3 / CH_3CN washes.
- Add 200 ≠1 CH_3CN 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 CH_3CN and wash for a further 15 min, and remove the supernatant.
- Wash the gel pieces with 300 ≠1 100 mM NH_4HCO_3 for 15 min.
- Wash with 300 ≠1 100 mM NH_4HCO_3 / CH_3CN (1:1 v/v) for 15 min.
- Remove the supernatant.
- Add 100 ≠1 CH_3CN 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 ≠1/sample of 10 mM DTT in 100 mM NH_4HCO_3.
- Incubate at room temperature for 30 min, remove the supernatant.
- Add 50 ≠1/gel sample of 55 mM fresh iodoacetamide in 100 mM NH_4HCO_3.
- Incubate at room temperature for 30 min, remove the supernatant.
- Wash the gel pieces with 300 ≠1 100 mM NH_4HCO_3 for 15 min.
- Remove the supernatant.
- Wash the gel pieces with 300 ≠1 20 mM NH_4HCO_3/CH_3CN (1:1 v/v) for 15 min.
- Remove the supernatant.
- Add 100 ≠1 CH_3CN 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 ≠1/gel sample 6 ng/ul promega trypsin in 50 mM NH_4HCO_3 (20 ug trypsin + 200 ≠1 50 mM acetic acid makes 100 ng/≠1; then 5≠1 100 ng/ul trypsin + 40 ≠1 water + 40 ≠1 100 mM NH_4HCO_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_4HCO_3 (minus the trypsin) to cover the pieces.
- Incubate at 37^{o}C, >5 h (or O/N).
- Spin briefly, add 30 ≠1 1% formic acid/2% CH_3CN to the digest.
- Incubate at 30^{o}C for 30 min on shaking platform, or vortex for 10 min.
- Remove the digest supernatant and transfer to a clean tube.
- 1 ≠1 could be spotted on Maldi target and analyze by MALDI-TOF.
- Add 24 ≠1 50% CH_3CN, Vortex for 10 min.
- Transfer supernatant to the tube.
- Repeat the 50% CH_3CN extraction and combine the supernatant.

Analyze digest immediately on MALDI-TOF or store at -20^{o}C until required, e.g. for CapLC. If samples are too diluted, they can be dried to approx. 10 ≠1 for analysis.