In-Gel digestion protocol

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Reagents: 25 mM Ammoniam Bicarbonate(AB), 10mM DTT, 10mM IAA, Acetonitrile,
MS gradeTrypsin: 20ug+ 100ul 0.01% formic acid-stock ; take 1ul from stock +300ul 25 mM AB

- Cut each gel slices/bands into 1 x 1 mm pieces using a fresh scalpel and transfer each slice/band into a 1.5ml LoBind Eppendorf tube. Try to work as quickly as possible as the gel becomes stickier as it dries out. The slices/bands can be stored at -20°C for later use.
- De-stain gel pieces with 300µl dH20 for 15 minutes. Add 300µl of 100% Acetonitrile and wash for a further 15 minutes. Remove supernatant and discard.
- Wash gel pieces with 300µl of 25mM NH4HCO3, 1M aliquots at -20°C that are to be diluted in MS water for 15 minutes. Discard supernatant.
- Wash gel pieces with 300µl of 25mM NH4HCO3 / Acetonitrile (50:50 v/v) for 15 minutes. The gel should shrink and look opaque. Discard supernatant.
- If gel pieces are still blue, repeat steps 3 & 4. On adding NH4HCO3, the gel pieces should be restored to original size and look transparent.
- Add 100µl of Acetonitrile to dehydrate the gel pieces for 5 minutes. Discard the supernatant.
- Add 300ul of 10mM DTT to each tube incubate for 45 minutes at 50C .
- Add an equal volume of Acetonitrile and incubate for 15 minutes for gels to shrink and remove supernatant.
- Add 300ul of 100mM IAA to each tube and incubate for 30 minutes at RT .
- Add an equal volume of Acetonitrile and incubate for 15 minutes for gels to shrink.

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- Dry gel pieces in a speedvac for 5 minutes to remove solvent.
- Add 300µl/band of Trypsin 12.5µg/ml of modified trypsin in 25mM NH_4HCO_3 (1:300).
- Allow bands to rehydrate in trypsin digestion buffer for 30 minutes. The gel pieces should be restored to the original sizes, and there should be just enough trypsin solution to cover all the gel pieces. If required add more 25mM NH_4HCO_3 (minus the trypsin)
- Incubate overnight at 30°C (>16 hours; no shaking).
- Perform all the gel washing extraction steps on a shaking platform to ensure complete extraction of peptides.
- Add an equal volume of Acetonitrile to the digest.
- Incubate for 30 minutes at 30°C.
- Transfer supernatant to a new clean Eppendorf LoBind tube. This supernatant contains peptides you will analyse.
- Speedvac to dry the peptides in the tube at step 14 completely (60°C is fine).
- Re-suspend in MS loading buffer