In-Gel digestion protocol for Mass Spec analysis

* Wear gloves at all times and work in a laminar flow hood.

* Do not use polymeric detergents (Tween, Triton, etc) for cleaning flasks & glass plates for electrophoresis.

Always use LC-MS grade water (Ultrapure) for making solutions (such as 100mM Ammonium Bicarbonate). Always store solutions/solvents in acid-cleaned glassware. It is advisable to make up solutions fresh prior to running this protocol. Never use sealing film and avoid plastic bottles as polymers such as PEG and Phthalates and slip agents strongly interfere with MS analyses.

Excise gel bands or spots

1) Cut band(s) from the gel using a **clean** scalpel.
2) Cut each band into 1 mm x 1 mm cubes. Do **not** cut smaller as this could block pipette tips.
3) Transfer gel pieces to a microcentrifuge tube and spin them down with a bench-top centrifuge.

Reduction, alkylation and destaining of proteins.

4) Add 500 µl of neat acetonitrile (ACN) and incubate for 10 min until gel pieces shrink, become opaque and stick together.
5) Spin gel pieces down and remove all liquid.
6) Add 30-50 µl of DTT solution (10 mM DTT in 100 mM ammonium bicarbonate – **critical**: make just before use) – ensure that enough is used to completely cover the gel pieces. Incubate for 30 min at 60°C in an air thermostat.
7) Allow tubes to return to room temperature (~21°C), add 500 µl ACN, incubate for 10 min and then remove all liquid.
8) Add 30-50 µl of IAA solution (55 mM IAA in 100 mM ammonium bicarbonate – **critical**: make just before use) – ensure that gel pieces are covered. Incubate for 20 min **in the dark**.
9) Wash gel pieces with 100 µl of 100 mM ammonium bicarbonate/100% ACN (1:1 vol/vol) and incubate with occasional vortexing for up to 30 mins, depending on the staining intensity.
10) Add 500 µl of 100% ACN to dehydrate the gel pieces, incubating at room temperature with occasional vortexing. When gel pieces become white and shrink, then remove supernatant ACN.

Gel pieces can now be stored at -20°C if desired.

Digestion

** This stage is the most significantly different from most online protocols. This is due to us **not** aliquoting the 20 µg vials of Trypsin but adding 100 µl of 0.01% FA to the original vial and storing **that** at -20 C, and subsequently using 1 µl of this for each sample in the following protocol.

NOTE: a ratio of 1:100 – 1:200 of trypsin:substrate is desirable. As typical gel loading is in the range of 5 – 25 µg protein (lysates etc.,), 1 µl (200ng Trypsin) is more than sufficient for the digestion of a single band. For reduced protein loading (<1 µg), and/or weak gel bands/spots, a dilution is required.

11) Add 25 µl of 25 mM ammonium bicarbonate (enough to completely cover the gel pieces) to each sample, and then add the required trypsin solution to each and place in 4°C for 30 min.
12) Check if gel pieces are still completely covered, add more 25 mM ammonium bicarbonate if required.
13) Leave for a further 90 min to completely saturate with Trypsin, and afterwards add a further 10-20 µl of 25 mM ammonium bicarbonate to ensure they stay wet through enzymatic cleavage. **This step substantially increases peptide yield, do not skip it.**
14) Place tubes in 37°C air circulated incubator **overnight**.
15) Chill on ice for 10 mins, then spin down each tube (10 mins @ 10k rpm) and pipette the supernatant in to a fresh clean tube for analysis. **Do not throw the old tube away – a double digest may be needed.**

Additionally, if possible, pass the supernatant holding the peptides through a MWCO 10k Da filter to remove any undigested protein and gel particles which may block columns.

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