

In-gel digest (Silver stained) with trypsin

Adapted from:

Shevchenko, A., Wilm, M., Vorm, O and Mann, M. (1996). Mass spectrometric sequencing of proteins from silver stained polyacrylamide gels. *Anal. Chem.* 68, 850-858.

A. Excision of protein bands from polyacrylamide gels

- A1. Wash the gel with water (2 changes, 10 minutes each)
- A2. Excise the spots of interest from the gel. Cut the excised piece into 1 mm²-cubes.
- A3. Transfer the gel cube to a 0.5 ml Eppendorff tube.

B. Washing of gel pieces

- B1. Wash the gel particles with 50 µl wash buffer (10 mM NH₄HCO₃, 50% ACN) for 15 min.
- B2. Remove all remaining liquid and add 200 µl Silver destain solution.
- B3. Gel pieces should be destained within 5 min.
- B4. Remove the solution and wash the gel pieces with 50 µl 10 mM NH₄HCO₃.
Repeat this step until gel pieces is colorless. (~ 2 times)
- B5. Remove all remaining liquid and add 100 µl ACN to cover the gel particles.
- B6. After the gel pieces have shrunk, remove the ACN.
- B7. Dry down gel particles in a vacuum centrifuge or air dry.

C. Reduction and alkylation

- C1. Add 50 µl reduction solution (10 mM DTT/ 10 mM NH₄HCO₃ (freshly prepared).
- C2. Incubate for 15 min at 56 °C, then chill tubes to room temperature.
- C3. Remove liquid and replace it quickly by roughly the same volume as above of freshly prepared 50 µl alkylation solution (55 mM IAA/ 10 mM NH₄HCO₃). * light sensitive!!
- C4. Incubate for 20 min at room temperature in the dark.
- C5. Remove alkylation solution.
- C6. Wash the gel with 50 µl wash buffer for 15 min, 1 or 2 times.
- C7. Add 100 µl ACN to cover the gel particles.
- C8. After the gel pieces have shrunk, remove the ACN.
- C9. Dry down the gel particles in a vacuum centrifuge or air dry.

D. In-gel digestion

- D1. Add 3 µl freshly prepared enzyme solution (20 ng/µl of trypsin, in 10 mM NH₄HCO₃) to cover the gel.
- D2. Incubate at 4 °C for 60 min.
- D3. Add 3 µl 10 mM NH₄HCO₃ to keep the gel wet overnight, but avoid excess liquid.
- D4. incubate at 37 °C overnight or at 56 °C for 60min.

E. Extraction of peptides

- E1. Make sure that about 3 µl of liquid is in the tube.
- E2. Add 2 µl of 50% ACN with 1% TFA or formic acid.
- E3. Sonify 10 min sonication, recover the supernatant.
- E4. Repeat the step E2 and E3.
- E5. pool the supernatant.

Mass spectrometric peptide analysis:

Preferably, use an AnchorChip target for sample preparation.
If purification is necessary, perform microcolumn purification.

General remarks size of gel pieces:

Cut as close to protein band as possible to reduce the amount of “background” gel.
Avoid to use gel pieces much bigger than 1 mm × 1 mm.
Excise a gel piece of roughly the same size from a non-protein containing region of the gel for use as a control.

Solvent amounts:

Unless otherwise specified all the solvent volumes used in the washing steps should roughly equal twice the gel volume.

Solvent purity:

All solvent should be as pure as possible. Make sure that the bottles remain close when not in use.

Lab material purity:

Scalpel, pipette tips and tubes should be clean. Use original Eppendorff bubes.

Contamination by keratin:

Keratin contamination should be avoided. Avoid keratin to fall on the gel, in bottles or on pipette tips. It might help to use clean gloves.

Prepare the trypsin solution:

Sequencing grade, modified trypsin is recommended. The solid powder is solved in 1 mM hydrochloride acid (for Roche Diagnostic, Mannheim trypsin) or 50 mM acetic acid (for Promega trypsin) giving a concentration of 100 ng/µl, store 10 µl aliquots at -20 °C.