

PROTEIN IN-GEL DIGESTION PROTOCOL

Reagents

- Ammonium bicarbonate (Sigma)
- Dithiothreitol (Sigma)
- Iodoacetoamide (Sigma)
- Trypsin (Sigma)
- Water (Sigma)
- Acetonitrile (Sigma)
- Formic acid (Sigma)
- Trifluoroacetic acid
- H₂¹⁸O (Sigma Chemicals), only used if subsequent manual *de novo* interpretation of spectra of tryptic peptides or their relative quantification by this method is intended

Equipment

- Laminar flow hood
- Incubator
- Bench-top centrifuge 5415D
- 0.65 ml thin-walled PCR tubes
- Pipettes
- Vacuum centrifuge RC 1022

Reagent setup

- **Trypsin** Prepare a solution of 13 ng μl^{-1} trypsin in 10 mM ammonium bicarbonate containing 10% (vol/vol) acetonitrile. Dissolve the content of 20 μg vial in 1.5 ml of the buffer.
Critical Make shortly before use; discard unused volume. If only a small volume of trypsin buffer is required, the lyophilized enzyme can be redissolved in 1 mM HCl and 10 μl aliquots stored at $-20\text{ }^{\circ}\text{C}$ before use. Note that after thawing frozen aliquots, pH should be adjusted by adding 1.5 μl of 50 mM ammonium bicarbonate shortly before use.
- 100 mM ammonium bicarbonate in water
Critical Make ammonium bicarbonate buffer daily in large (50–100 ml) volumes and discard after use.
- 10 mM DTT in 100 mM ammonium bicarbonate.
Critical Make shortly before use.
- 55 mM iodoacetamide in 100 mM ammonium bicarbonate.
Critical Make shortly before use.
- 5% formic acid in water (vol/vol)
- **Processing of bands (spots) from one- or two-dimensional gels** Upon electrophoresis, proteins should be fixed within a polyacrylamide matrix by incubating the entire gel in 5% (vol/vol) acetic acid in 1:1 (vol/vol) water:methanol. Staining with Coomassie, at the same time, fixes proteins, whereas a separate fixation step should precede colloidal Coomassie or silver staining. The in-gel digestion procedure is, in principle, compatible with any convenient silver staining protocol. However, the reagents used to improve staining sensitivity and contrast must not modify proteins covalently. Thus, avoid treating gels with crosslinking reagents (such as glutaraldehyde) or strong oxidizers, such as chromates or permanganates. Note that the abundance of silver-stained spots (bands) strongly depends, among other factors, on the time of gel exposure to the developing solution. If possible, allocate two lanes on a one-dimensional gel for protein standards (e.g., 50 and 200 fmol of bovine serum albumin). Semiquantitative estimation of the amount of protein available for sequencing helps to choose optimal sample loading. Do not start with silver staining of gels of unknown protein preparations. First stain them with Coomassie and then, if required, directly by silver without prior destaining. Note that, especially in femtomole sequencing, controls (blank gel pieces excised and processed in parallel with the experimental

bands or spots) are usually unreliable and do not accurately represent actual patterns of keratin peptides and related contaminations. Instead, contaminating precursors should be identified by database searching.

PROCEDURE

Excise protein bands (spots) **Timing: ~5 min per band per spot**

1. Rinse the entire slab of a one- or two-dimensional gel with water for a few hours, put a plastic tray with the gel onto a light box and excise bands (spots) of interest with a clean scalpel. Excise a gel piece of similar size from a nonprotein region of the gel and treat it identically to the protein band as a control throughout the in-gel digestion protocol and subsequent mass spectrometry analysis.

Critical step Cut as close to the protein as possible to reduce the amount of background. Take special care to prevent massive keratin contamination of the samples.

2. Cut excised bands (spots) into cubes (ca. 1 × 1 mm). Note that smaller pieces could clog pipette tips.
3. Transfer gel pieces into a micro-centrifuge tube. Wash the gel pieces for 15 min with H₂O/acetonitrile mixture (1:1). Centrifuge quickly (1sec) to transfer liquid from the sides of the tube to the bottom. Remove the liquid using a pipette with a clean pipette tip.
4. Add acetonitrile to completely cover the gel pieces until they have shrunk and turned sticky white, remove the acetonitrile.
5. **In-gel reduction, alkylation and destaining of proteins.** Additional reduction/alkylation step is only performed for processing of silver-stained bands (spots) or to prepare samples for manual or automated *de novo* sequencing (option A). If rapid identification of Coomassie-stained bands (spots) is intended, skip reduction/alkylation and proceed directly with the steps described in option B. **Timing: 60 min**

A. Processing silver-stained bands (spots) or samples for *de novo* sequencing

- i. Add 500 µl of neat acetonitrile and incubate tubes for 10 min until gel pieces shrink (they become opaque and stick together).
- ii. Spin gel pieces down, remove all liquid.
- iii. Add 30–50 µl of the DTT solution to completely cover gel pieces. Incubate 30 min at 56 °C in an air thermostat.
- iv. Chill down the tubes to room temperature (ca. 22 °C), add 500 µl of acetonitrile, incubate for 10 min and then remove all liquid.
- v. Add 30–50 µl of the iodoacetamide solution (the volume should be sufficient to cover the gel pieces) and incubate for 20 min at room temperature in the dark.
- vi. Shrink gel pieces with acetonitrile and remove all liquid.

B. Destain gel pieces excised from Coomassie-stained gels **Timing: 30 min**

- i. Add ca. 100 µl of 100 mM ammonium bicarbonate/acetonitrile (1:1, vol/vol) and incubate with occasional vortexing for 30 min, depending on the staining intensity.
- ii. Add 500 µl of neat acetonitrile and incubate at room temperature with occasional vortexing, until gel pieces become white and shrink and then remove acetonitrile. Although the bulk of Coomassie staining should be removed, it is not necessary to destain the gel pieces completely. **Pause Point** Samples are now ready for in-gel digestion. Alternatively, they can be stored at –20 °C for a few weeks.

Saturate gel pieces with trypsin **Timing: 120 min**

6. Add enough trypsin buffer to cover the dry gel pieces (typically, 50 µl or more, depending on the volume of a gel matrix) and leave it in an ice bucket or a fridge.
7. After ca. 30 min, check if all solution was absorbed and add more trypsin buffer, if necessary. Gel pieces should be completely covered with trypsin buffer.
8. Leave gel pieces for another 90 min to saturate them with trypsin and then add 10–20 µl of ammonium bicarbonate buffer to cover the gel pieces and keep them wet during enzymatic cleavage.

Critical step Although after ca. 30 min dried gel pieces do not absorb any more buffer, the yield of tryptic

peptides increases substantially while extending the incubation time, presumably because of slow diffusion of the enzyme into a polyacrylamide matrix³.

9. **Digestion** Place tubes with gel pieces into an incubator and incubate samples overnight at 37 °C for analyses performed at the limit of instrument sensitivity, which require maximal peptide recovery. Otherwise (typically, for the rapid identification of Coomassie stainable spots (bands) by MALDI mass fingerprinting), the acceptable digestion yield—exceeding, on average, 75% of the yield of overnight cleavage—can be achieved in 30 min at 55 °C.
Critical step It is important to avoid a temperature gradient between the bottom and the lid of the tube to prevent condensation of water at the inner surface of the lid and, consequently, premature dehydration of the gel pieces.
10. **Withdraw an aliquot from the digest for the protein identification by MALDI peptide mass mapping** Chill tubes to room temperature, spin down gel pieces using a microcentrifuge and withdraw 1–1.5 µl aliquots of the supernatant directly from the digest without further extracting the gel pieces¹⁵. As a typical volume of the digestion buffer is approximately 50 µl, this leaves ample peptide material for the subsequent MS/MS analysis, if required. **Pause Point** Non-extracted digests can be stored at –20 °C for a few months until it is decided if further LC MS/MS analysis is required.
11. **Extract peptide digestion products** **Timing: 15 min** Add 100 µl of extraction buffer (1:2 (vol/vol) 5% formic acid/acetonitrile) to each tube and incubate for 15 min at 37 °C in a shaker. For samples with much larger (or smaller) volume of gel matrix, add the extraction buffer such that the approximate ratio of 1:2 between volumes of the digest and extraction is achieved.
Critical step To withdraw the supernatant, use a pipette with fine gel loader tip to prevent clogging the needle of autosampler injector or nanoLC MS/MS column. Collect the supernatant into a PCR tube, dry down in a vacuum centrifuge. (Do not discard extracted gel pieces.) **Pause Point** Dried extracts can be safely stored at –20 °C for a few months.
12. **Redissolve tryptic peptides for further analysis** For further LC MS/MS analysis, add 10–20 µl of 0.1% (vol/vol) trifluoroacetic acid into the tube, vortex and/or incubate the tube for 2–5 min in the sonication bath and centrifuge for 15 min at 6.7g (10,000 r.p.m.) at the bench-top centrifuge and withdraw the appropriate aliquot for further analysis. Dry down the rest in a vacuum centrifuge and store at –20 °C as contingency.

TIMING

Excise protein bands (spots): ~5 min per band or spot
 In-gel reduction and alkylation of proteins: 60 min
 Destain gel pieces excised from Coomassie-stained gels: 30 min
 Saturate gel pieces with trypsin: 120 min
 Digestion: 30 min to overnight
 Extract peptide digestion products: 15 min

Extraction of peptide digestion products

Do not discard extracted gel pieces: if, for any reason, the digestion failed, it can be repeated with the same gel pieces using the same enzyme (trypsin) or using another enzyme. Note that, in the latter case, strong peptide background may be encountered because of the digestion of residual intact trypsin.