Sample Preparation and In Gel Tryptic Digestion for Mass Spectrometry Experiments

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48.1 Introduction and Purpose

Once the protein lysates are separated on polyacrylamide gels (SDS-PAGE), the interested proteins can be excised from gels, digested with trypsin, and identified by Mass Spectrometry (MS). The protocol described here applies to one- or two-dimensional gels stained with Coomassie brilliant blue R250 or G250 or with silver [1, 2]. The method is compatible with downstream MS experiments; by including $H_2{}^{18}O$ into the digestion buffer [3–5] or by mixing SILAC-labeled protein mixtures before separation, it enables quantification of the digestion products [6, 7].

Careful attention should be paid in casting the gels and handling the excised spots or bands of interest in order to avoid the risk of contaminating samples with keratin and enhanced chemical noise in analyzed samples. (Measures to minimize this contamination are described in paragraph "Protein Handling" in Chap. 36).

48.1.1 Reagents[1]

- *Double-distilled water*
- *30 mM Potassium ferricyanide*
- *100 mM Sodium thiosulfate*
- *100 mM Ammonium bicarbonate*
- *100 mM Ammonium bicarbonate/Acetonitrile* (50:50, v/v)
- *10 mM DTT in 100 mM Ammonium bicarbonate*
- *50 mM Iodoacetamide in 100 mM Ammonium bicarbonate*

[1] If not specified, all the buffers are made in distilled water.

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- *20 mM Ammonium bicarbonate/Acetonitrile* (50:50, v/v)
- *Trypsin* (e.g. Promega)
- *60% Acetonitrile, 1% Trifluoroacetic acid (TFA)*

48.1.2 Equipment

- *Adjustable pipettes, range*: 2–20 µl, 20–200 µl, 100–1,000 µl
- *Scalpel*
- *1.5 ml tubes*
- *Teflon stick*
- *Speed vacuum centrifuge*
- *Light box*

48.1.3 Method

- Rinse the entire slab of a one- or two-dimensional gel with water for few hours.
- Put a plastic tray with gel onto a light box.
- Excise manually with a scalpel the selected spots for further MS analysis from the preparative gel[2].
- Place the individual gel samples in a 1.5 ml tube.
- Add 300 µl of double-distilled water[3].
- To remove silver staining, place the samples in a 1:1 mix of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate until the gel pieces turn clear.
- Discard this solution and wash each gel sample with 300 µl of double-distilled water for 15 min. If identification of Coomassie-stained bands (spots) is intended, skip these two steps and proceed with the next one.
- Add 300 µl of 100 mM ammonium bicarbonate into the solution, and incubate the gel pieces for additional 15 min.
- Discard the solution and wash the bands with 100 mM of ammonium bicarbonate/acetonitrile for 15 min.
- Remove this solution and crush the gel samples with a Teflon stick.
- Add 100 µl of acetonitrile for 5 min.
- Remove the acetonitrile and dry the bands in a Speed vacuum centrifuge for 5 min.
- Resuspend the sample in 50 µl of 10 mM DTT in 100 mM ammonium bicarbonate and incubate for 1 h at 56°C.
- Remove the solution and place the samples in 50 µl of 50 mM iodoacetamide in 100 mM ammonium bicarbonate at room temperature for 30 min in the dark.
- Remove the solution and wash the gel samples with 300 µl of 100 mM ammonium bicarbonate for 15 min.
- Place the samples in 300 µl of 20 mM ammonium bicarbonate/acetonitrile (50:50, v/v) for 15 min.
- Replace the previous solution with 100 µl of acetonitrile and leave at room temperature for 5 min.
- Dry the samples in a Speed vacuum centrifuge for 10 min.
- Resuspend with 5 µl of trypsin[4] (0.1 µg/µl) in 100 mM ammonium bicarbonate pH 8.0 for 10 min.
- Add 100 µl of 100 mM ammonium bicarbonate and carry out the digestion overnight at 37°C.
- Collect the supernatant in a second microcentrifuge tube.
- Wash the gel pieces once with 100 µl of double-distilled water and twice with 100 µl of 60% acetonitrile/1% TFA.
- Pool all the washes and add to the previously collected supernatant. Reduce the volume of the solution to 5–10 µl in a Speed vacuum centrifuge and store the sample at 4°C until analyzed with the mass spectrometer.

[2]Only the darkest zone of the spot must be cut-off.

[3]The cut spots can be stored for many weeks at 4°C before performing MS experiments by the specialist group.

[4]Trypsin specifically hydrolyzes peptide bonds at the carboxylic sides of lysine and arginine residues. Unmodified trypsin is subject to autolysis, generating fragments that can interfere with mass spectrometry analysis of the peptides. In addition, autolysis can result in the generation of pseudotrypsin, which has been shown to exhibit an additional chymotrypsin-like specificity. Promega trypsin has been modified by reductive methylation, rendering it extremely resistant to autolytic digestion. It is very important to maintain an adequate enzyme/substrate ratio to maximize the digestion. This ratio is much higher than the one needed for *in-solution* digestion.

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