
Directions for in-gel digestion of proteins separated by SDS-PAGE

Note:

It is particularly important during this procedure to avoid contaminating your gels or your spots with protein. The followings are some of very important practices to avoid contaminations.

- 1. Any gel manipulation prior to trypsin digestion should be done in a BSC or laminar flow hood.*
- 2. To avoid Keratin, a human skin protein, you should wear nitrile (not latex) gloves when handling any instruments or containers that will come into contact with your sample or solutions. Also tie back your hair and avoid getting dust on your experiment.*

Procedure:

Coomassie/Sypro stained gel bands preparation

1. For each spot/band you intend to cut, label a 1.5 mL tube and fill it with 300 μ L of NanoPure water. Carefully transfer the gel on to a clean glass plate. Remove gel band or spot using a clean scalpel as close to the band/spot as possible.
2. Move the band/spot to clean area on the plate, cut into pieces (about 2mm) with scalpel and then transfer these pieces into its labelled 1.5 mL tube.
3. After you have cut each spot and placed it in a tube, remove the water by pipetting.
4. Wash gel pieces with 300-500 μ L of a mix of 40% CH_3CN and 60% 50mM NH_4HCO_3 . Place it in the shaker or vortex for 15 min.
5. Spin in centrifuge. Remove the supernatant. Repeat the NH_4HCO_3 / CH_3CN washes until the stain is completely gone.
6. Discard remaining solvents, shrink pieces with neat acetonitrile and incubate for 5min. Remove the supernatant.
7. Place tubes open, in the Speedvac. Turn on the cold trap. Spin 1-3 minutes; just until the gel pieces are dry.

Reduction/Alkylation of band pieces

1. Add 100 μ L of 10mM dithiothreitol stock (15.4 mg DTT in 10 mL of 50 mM NH_4HCO_3)
2. Incubate for 30 min at 80 $^\circ\text{C}$.
3. Add 100 μ L /gel sample of 55 mM fresh iodoacetamide (IAA) stock (102mg IAA in 10 mL of 50mM NH_4HCO_3). Incubate 20 min at room temperature in the dark.

4. Discard excess solvent mix and wash the gel pieces with 300 μL 100 mM NH_4HCO_3 for 15 min.
5. Wash the gel pieces with 300 μL 20 mM $\text{NH}_4\text{HCO}_3/\text{CH}_3\text{CN}$ (1:1 v/v) for 15 min. Remove excess
6. Add 100 μL CH_3CN to dehydrate the gel pieces for 5 min. Remove acetonitrile and swell in 300 μL NH_4HCO_3 . Repeat this step.
7. Shrink gel pieces with 200 μL CH_3CN , remove excess acetonitrile and dry gel pieces with tubes open.

Digestion of Proteins

1. To each tube, add 25 μL digestion buffer [6 ng/ μL promega trypsin in 50 mM NH_4HCO_3 (20 μg trypsin + 200 μL 50 mM acetic acid makes 100 ng/ μL ; then 5 μL 100 ng/ μL trypsin + 40 μL water + 40 μL 100 mM NH_4HCO_3]. Gel pieces should be just covered, but not in a large excess volume.
2. Put samples in the 37 $^\circ\text{C}$ oven on the top shelf. Leave up to 12 hours.
3. Following digestion, centrifuge the vials and then add 30 μL 1% formic acid/ 2% CH_3CN to the digest.
4. Incubate at 30 $^\circ\text{C}$ for 30 min on shaking platform, or vortex for 10 min.
5. Spin down. Transfer the supernatant of each sample into a LC vials.
6. Add 50 μL neat CH_3CN to the gel pieces, Vortex for 10 min. then repeat step 5.
7. Place sample vials in the SpeedVac with the cold trap on, spin them until the liquid has evaporated leaving a tiny pellet. Re-suspend in LC buffer (0.2% formic acid, 2% acetonitrile in water)
8. The samples can now be stored in -20-degree freezer, or ZipTipped prior to LCMS analysis

Above protocol is based on the procedure from "Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry" Wilm, M., Shevchenko, A., Houthaeve, T., Breit, S., Schweigerer, L., Fotsis, T., & Mann, M. (1996). *Nature*, 379(6564), 466.