Goodlett Lab

In-gel digestion protocol

Note that this protocol requires extreme care in handling the gel slices to avoid contamination with "finger proteins" (skin keratins). The contamination of gel slices with keratins prior to tryptic digestion overwhelms the spectra generated from the tryptic digestion of the protein of interest, preventing identification by MS.

At a minimum, scrub/wash all surfaces that will touch the gel with ethanol and then water. Always wear gloves. When removing gloves that you plan to re-use, do not touch the finger areas of the gloves with your bare hands. Use a clean glass surface upon which to lay the gel prior to excision of the band with a new razor blade. Do not allow any part of the gel or the gel slice to touch a surface that may have been touched by your or someone else's hands.

Staining/destaining procedure

Coomassie Blue stain: Use Pierce's Imperial Protein Stain (cat # 24615) and follow their directions. This is a non-fixing Coomassie Blue stain. After staining, wash the gel many times with at least 100 mL of pure water to remove all SDS (which will interfere with the MS analysis), wash overnight, and several times the next day prior to gel band excision.

Silver Staining: see the protocol on the Goodlett Lab website. After development of the gel, wash several times in pure water to remove excess formaldehyde as soon as possible to prevent protein cross-linking. In making up solution C, sodium thiosulphate, make it less than five min prior to use in step 4 or the staining procedure will fail.

Destaining a Silver Stain: potassium ferricyanide (III) – $K_3Fe(CN)_6$; 0.2g in 100mL

Reduction and Alkylation

This procedure is optional, but the idea is to reduce any disulfide bonds in your protein to allow the protease to access the protein better. Alkylation is to prevent the disulfide bonds from reforming.

1. Place dry gel pieces in .2mL tubes, add 50-100uL of 20mM DTT (dithiothreitol) in 100mM Ammonium Bicarbonate, and incubate them for 1 h at 60°C
2. Remove DTT solution and add 50-100uL of 55mM IAM (iodoacetamide) in 100mM ammonium bicarbonate
3. Incubate at room temp for 45 min in the **dark**
4. Remove the IAM solution and rinse/vortex the gels for 10 min with 100mM ammonium bicarbonate, and then for another 10 min with ACN (acetonitrile). Repeat this procedure.
5. Dry the gels using the speed vac (~30 min)

Protein digestion

1. After excision of the gel slice with a new, clean razor blade, place it in a 1.5 mL Eppendorf tube. Add 500 uL of 100 mM ammonium bicarbonate and shake/rotate for 15 min at room temp.
2. Discard the liquid and add 500 uL of highly pure acetonitrile (Optima, Fisher, cat # A996-4) and shake/rotate 15 min at room temp. Discard the liquid. Repeat this wash cycle twice more with ammonium bicarbonate and acetonitrile.
3. With a sterile needle, punch several holes in the cap of each Eppendorf tube containing a gel slice, close the tube, and Speedvac at room temp for 45 min to remove all liquid from the gel slices.
4. To a 20 ug vial of Promega Sequencing Grade trypsin (cat # V511) add 1 mL of 50 mM ammonium bicarbonate.
5. Add 50 uL to each gel slice and incubate the tube for 45 min on ice. (Do not chop the gel slice into small pieces since these will be carried over and clog the reverse-phase column used to separate the peptides prior to MS analysis).
6. Add enough 50mM ammonium bicarbonate to cover what will be the expanded gel slice and incubate the tube on a shaker (with Scotch tape over the needle holes to prevent evaporation) overnight at room temperature.
7. Remove the solution to a new tube in the morning. Add 50 uL of 5% acetonitrile/0.1% TFA to the gel slice and shake 15 min, room temp. Repeat this step, pooling each wash with the solution from the overnight digest.
8. Finally add 50 uL of 50% acetonitrile/0.1%TFA to the gel slice for 15 min with shaking. Pool with the prior eluates.
9. Speedvac the eluates to about 10 uL volume but do not take the peptides to dryness since some of the peptides will irreversibly adsorb to the surface of the tube.
10. Transfer the solution to an autosampler vial for the mass spectrometer and store at –80C.

References:

- Shevchekno A, Wilm M, Vorm O, Mann M "Mass-spectrometric sequencing of proteins from silver-stained polyacrylamide gels" *Analytical Chemistry*, **1996** 68, 850-858