Procedure of In-Gel Digestion for Protein ID

Silver staining of SDS-PAGE gel

Use a clean glass tray to stain the gel and **DO NOT TOUCH THE GEL WITH YOUR HANDS**.

A large gel will require 250 to 500 ml of each solution. All washes and rinses should be performed while gently rocking the glass tray.

1. Fix gel in 50% methanol, 10% acetic acid for 20 minutes. Repeat once.
2. Rinse gel in 20% ethanol for 10 minutes.
3. Rinse gel in water for 10 minutes.
4. Reduce the gel with sodium thiosulfate (0.2 g/litre) for 1 minute.
5. Rinse twice with water - 20 seconds each wash.
6. Incubate in silver nitrate (2.0 g/litre) for 30 minutes at 40C.
7. Rinse once with water for 20 seconds.
8. Wash the gel once with developing solution (50 to 75 ml) for 30 seconds, discard the wash and develop to desired intensity with the remainder of developing solution. Developing solution contains sodium carbonate (30 g/litre), formaldehyde (1.4 ml of 37% solution/litre), and sodium thiosulfate (10 mg/litre).
9. Stop the reaction by exchanging the developing solution with 1 % acetic acid. Incubate for a minimum of 20 minutes.

Excising the bands of interest and sample preparation

ALWAYS WEAR GLOVES - RINSE THE GLOVES WITH WATER TO REMOVE TAL7 December, 2007E CONTAMINATION BY SKIN, HAIR, OR DUST.

Make all solutions fresh using only high grade reagents. Always use new tubes, tips, and clean tube racks etc.

1. Rinse the gel with water and excise the bands of interest with a clean scalpel. Keep the gel volume to a minimum by cutting as close to the band of interest as possible. Cut the gel slice into 1 mm x 1 mm cubes and place into a 0.5 ml eppendorf tube. The eppendorf tubes should be free of dust and untouched by human hands.
2. To store the gel slices for a short period of time - up to 2 days, add 10 to 20 μl of 1% acetic acid and refrigerate. To store the gel band for an extended period of time freeze the samples at -70°C.
3. Wash the gel particles with 100 - 150 μl of HPLC grade water (5 minutes with occasional mixing). Briefly centrifuge and remove liquid.

4. Add 200 µl of acetonitrile (approximately 3 to 4 times the volume of the gel particles) and incubate at room temperature 10 to 15 minutes with occasional mixing. The gel particles should become white and should stick together. A second acetonitrile wash may be required to completely shrink the gel particles. Briefly centrifuge and remove all the liquid.
5. Reduction and Alkylation: - Reduce the gel slices by covering them with 100 mM ammonium bicarbonate containing 10 mM dithiothreitol and incubating at 50°C for 30 minutes.
6. Briefly centrifuge and remove all the liquid. Add acetonitrile to shrink the gel particles (same as step 4) and remove the excess liquid.
7. Alkylate the gel particles by covering them with 100 mM ammonium bicarbonate containing 55 mM iodoacetamide and incubation for 20 minutes at room temperature in the dark.
8. Briefly centrifuge and remove all the liquid. Wash the gel particles with 150 to 200 µl of 100 mM ammonium bicarbonate for 15 minutes with occasional mixing.
9. Briefly centrifuge and remove all the liquid. Add acetonitrile to shrink the gel particles (same as step 4) and remove the excess liquid.
10. Briefly centrifuge and remove all the liquid. Dry the gel particles by Speedvac for 1 minute.

Additional Steps for Coomassie stained gel slices

1. If the gel particles are still blue, rehydrate with 100µl to 150µl 100 mM ammonium bicarbonate and after 10 to 15 minutes incubation add an equal volume of acetonitrile and mix for 15 to 20 minutes.
2. Briefly centrifuge and remove all the liquid. Add acetonitrile to shrink the gel particles (same as step 4) and remove the excess liquid.
3. Briefly centrifuge and remove all the liquid. Dry the gel particles by Speedvac for 1 minute.
4. If the gel particles are still blue repeat steps 1 to 3.

In gel digestion with trypsin

Trypsin solution

Dissolve 25 µg Roche unmodified sequencing grade trypsin in 250 µl of 1 mM HCl and aliquot 15 µl per tube. Trypsin solutions can be stored at -70°C.

Digestion buffer

50 mM ammonium bicarbonate, 5 mM $CaCl_2$.

1. Add ~200 µl digestion buffer and trypsin solution in ratio of 1:20 to amount of protein by weight. Incubate the gel particles on ice for 30 to 45 minutes. The gel slices should be fully rehydrated with digestion buffer

containing trypsin on ice. Remove the excess trypsin solution and add 10 to 15 µl digestion buffer to ensure the gel particles remain hydrated during digestion.

2. Incubate the samples at 37°C overnight. Use of an incubator is recommended to reduce the amount of water condensation on the lid of the tube.
3. Add 1 µl TFA to quench the reaction.

Extraction of tryptic fragments

1. Briefly centrifuge and transfer all the liquid to a clean microcentrifuge tube (0.5 ml). Add 100 µl of 60% acetonitrile and extract peptides by sonicating for 10 minutes.
2. Briefly centrifuge and pool the liquid with the liquid from step 1. Add 100 µl of 60% acetonitrile and extract peptides a second time by sonicating for 10 minutes.
3. Briefly centrifuge and pool with the liquid from steps 1 and 2.

Peptide Mass Fingerprint by MALDI-TOF MS

1. If there is sufficient amount of protein, it can be submitted directly for MALDI analysis (consult with the personnel at mass spec lab).
2. If there is not enough sample, preconcentrate by drying the sample down in a Speedvac to a smaller volume (~5µl) and submit for MALDI analysis.
3. If the sample needs to be further concentrated and purified, sample from step 2 should be zip tipped for MALDI analysis by the procedure below.

Purification of tryptic fragments by C18 zip tip

Prior to zip tip, add 45µl of 1% acetic acid in water to the solution of Step 2.

1. Prepare a C18 zip tip by equilibrating it with 65% acetonitrile/1% acetic acid about three cycles, and then 2% acetonitrile/1% acetic acid about three cycles.
2. Allow sample to bind to zip tip by repeatedly drawing and dispensing sample through zip tip (slowly, about 3-5 times).
3. Wash zip tip slowly with 2% acetonitrile/1% acetic acid about 3 times.
4. Elute sample slowly from zip tip with 5 µl of 65% acetonitrile/1% acetic acid into a clean 0.5 ml microcentrifuge tube.
5. The eluate is now ready for MALDI-MS analysis.

Information about zip tip suppliers can be found at following web site:
http://www.millipore.com/catalogue.nsf/docs/C5737

Peptide Sequencing by LC/MS/MS

After the tryptic peptides are extracted from gel, the sample should be dried down in Speedvac for peptide sequencing by LC/MS/MS.