In Gel Protein Digestion

For Protein Identification by Mass Spectroscopy.

In gel digestion procedures are an amalgamation of procedures described from a variety of laboratories.

General Gel Handling Procedures

Remember to wear powder free nitrile gloves that have been washed under the tap followed by a rinse with distilled water (avoid latex gloves, as natural rubber contains significant amounts of keratin and other proteinaceous materials).

* If making your own SDS PAGE gel, try to prepare it ~ 18 – 24 hours ahead of time to allow maximal polymerization.

Remove glass plates from the gel in a clean place (preferably a laminar flow hood) and do not lean over the gel or touch with exposed skin (to reduce keratin contamination). During staining and de-staining (for coomassie blue gels) use fresh clean reagents and perform in a covered container to reduce dust contamination.

For band excision, use a new scalpel or razor to excise the bans or spots of interest. Cut as close to the band as possible to minimize the volume of gel to be processed. Clean the blade between bands/spots to avoid cross contamination (wash with water, then with methanol). Transfer the gel slice to a clean glass plate/ Petrie dish and dice into smaller pieces (~mm^3). Transfer the gel pieces into a labelled Eppendorf tube. Add enough milli-Q water or 0.5% acetic acid until the sample is ready for further handling.

Procedures

I. Destaining.

Coomassie stained gels (or Sypro-Ruby stained gels)

(use larger volumes if the gel bits are not completely covered)

1. Wash the gel with 50 mM ammonium bicarbonate buffer for 5 min (with shaking) at ambient temperature. Discard liquid into waste.
2. Incubate gel pieces in 70% acetonitrile (in water) for 15 min. Gel may go cloudy or opaque as it becomes dehydrated. Discard liquid into waste.
3. Repeat steps 1 and 2 until the gel slice is completely colourless.
4. Completely dehydrate gel by adding 100% acetonitrile and incubate for at least 5 minutes. Discard liquid into waste.
5. Dry gel pieces under vacuum (~5 min).

Silver Stained Gels Bands

(use larger volumes if the gel bits are not completely covered)

1. The silver-stained band/spot is incubated with 100 µL of a fresh mixture (v 1: 1) of 30 mM potassium ferricyanide (98 mg/10ml H2O) and 100mM sodium thiosulfate (248 mg/10ml H2O) for 15 minutes. Discard liquid into waste.
2. Wash with 50 µL deionized water. Vortex mix and let stand for 5 minutes. Discard liquid into waste.
3. Wash the gel with 50 µL of 50 mM ammonium bicarbonate. Vortex mix and let stand for 5 minutes. Discard liquid into waste.
4. Completely dehydrate gel by adding 100% acetonitrile and incubate for at least 10 minutes. Discard liquid into waste.
5. Dry gel pieces under vacuum (~5 min).

II. Reduction and Alkylation.

(use larger volumes if the gel bits are not completely covered)

6. Swell the gel fragments in 30 µL of 50 mM Ammonium Bicarbonate, 10 mM DTT (or 2 mM TCEP) and incubate at 55C (-60C) for 15 minutes. Remove the sample from heat and sit at ambient temperature for 15 minutes.
7. Centrifuge and discard supernatant.
8. Add 30 µL 100 mM Iodoacetamide (freshly prepared), 50 mM ammonium bicarbonate and let stand for 15 minutes in the dark at ambient temperature.
9. Centrifuge and discard supernatant.
10. Wash the gel with 100 µL of 50 mM ammonium bicarbonate. Vortex mix and let stand for 10 minutes. Discard liquid into waste.
11. Completely dehydrate gel by adding 100% acetonitrile and incubate for at least 10 minutes. Discard liquid into waste.
12. Dry gel pieces under vacuum (~5 min).

III. Digestion.

13. Prepare enough Trypsin to digest all samples. Prepare 15 ng/uL proteomic grade trypsin in 50 mM Ammonium Bicarbonate.
14. Add 28 µL of the enzyme solution from step 13 to each tube. Let tubes stand in ice for 10 minutes. Add more if gel fragments still appear dry. Let sit for another 5 minutes, then remove excess enzyme solution.
15. Add 10 µL 25 mM Amm. Bicarb. (Solution E) to each tube and incubate at 370 C for at least 3 hrs (tubes can be left o/n at this stage).
16. After digestion, add 100 µL of 1% formic acid, vortex and shake for 20 min. This extraction will contain the more hydrophilic peptides.
17. Remove the 1% formic acid wash and transfer to a new, washed, silanised Eppendorf.
18. To the gel piece add 200 µL of 50% acetonitrile containing 1% formic acid, vortex and shake for 20 min. This wash will contain most of the tryptic peptides. Add this supernatant to the 1% formic acid extraction.
19. Speed vac the samples down to >40 µL (avoid complete drying!) and store at 4ºC until analysis.