

In-gel Digestion Protocol for LC-MSⁿ Analysis of an SDS-PAGE Separated, Coomassie-Stained Gel

Materials

Stained polyacrylamide gel containing protein(s) of interest
25 mM ammonium bicarbonate (NH₄HCO₃; 1.98 mg/mL)/50% (v/v) acetonitrile
10 mM dithiothreitol (DTT, 1.54 mg/mL) in 25 mM NH₄HCO₃
55 mM iodoacetamide (10.2 mg/mL) in 25 mM NH₄HCO₃
50 mM acetic acid
25 mM NH₄HCO₃, pH 8
10 µg/mL trypsin in 25 mM NH₄HCO₃, pH 8
Promega Trypsin Gold (V5280); one vial contains 100 µg trypsin. Add 900 µL 50 mM HOAc to 10 non-stick 1.5 mL tubes and chill to 4°C. Add 1 mL 50 mM HOAc to trypsin vial. Aliquot 100 µL trypsin solution to each chilled tube. Store at -70°C until use. Once a vial is taken from -70°C storage, aliquot 20 µL into 50 non stick 600 µL tubes (on ice), and store at -20°C.
5% aqueous trifluoroacetic acid (TFA)
Acetonitrile
0.5 mL non-stick tubes (*e.g.*, VWR *SuperSlik*), silanized, washed with ethanol and water (2x)
37 ° and 56°C dry baths
Ice Bucket

NOTE: To avoid or reduce contamination with human keratins, one MUST wear gloves and preferably work in a dust free area.

Excise and wash gel fragments

1. Excise protein bands/spots of interest from a stained polyacrylamide gel using the a gel coring tool, and place into a 0.5 mL siliconized tube (VWR *SuperSlik* microcentrifuge tubes).

Small gel particle size facilitates the removal of SDS (and Coomassie) during the washes, and improves enzyme access to the gel. For Coomassie stained proteins, three gel particles per tube using the smallest coring tool is usually sufficient. If the band is faint, more can be used, or the band can be sliced out with a razor and chopped into smaller pieces.

2. Add 200 µL of 25 mM NH₄HCO₃/50% acetonitrile (or enough to immerse the gel particles) and vortex overnight (12 h) on a low setting (more like shaking). Use gel loading pipet tips to remove the solution (pale blue in the case of Coomassie staining) and discard. Repeat this wash step one additional time (2 hr mixing). Resulting gel particles should be clear.

3. Dehydrate gel pieces by adding acetonitrile (100 µL). At this point the gel pieces should shrink and become an opaque-white color.

4. Remove acetonitrile and let air-dry for 5-10 minutes.

Perform reduction and alkylation of cysteine residues (optional). *Steps 5 to 9 should be included when maximum protein coverage is required or when digesting a band from a one dimensional gel. Proteins separated by two dimensional gel electrophoresis may already be reduced and alkylated, so these steps can be omitted. Proteins without cysteines do not need to go through these steps.*

5. Add 30 µL of the freshly prepared 10 mM DTT solution to cover the gel pieces, and reduce for 30-45 min at 37°C.

6. Replace the DTT solution with roughly the same volume of freshly prepared 55 mM iodoacetamide (30 µL). Incubate for 45 min at 56 °C.

7. Remove the iodoacetamide solution and wash gel pieces with ~200 μL of 25 mM NH_4HCO_3 pH 8, for 10 min while vortexing. Remove wash solution and repeat wash procedure.

8. Remove wash solution and dehydrate with ~200 μL acetonitrile. The gel pieces should shrink and become an opaque-white color. If they do not, remove the acetonitrile and repeat the washing-dehydration cycle until they do.

9. Remove the acetonitrile and dry the gel pieces in the air for 5-10 minutes.

Digest protein sample

10. Rehydrate gel particles in 10 μL trypsin solution (or volume necessary to cover the expanding gel pieces) and place on ice for 10-15 minutes.

One can usually get two digestions per trypsin vial.

11. Remove excess trypsin solution and overlay the rehydrated gel particles with 30 μL of 25 mM NH_4HCO_3 to keep them immersed throughout digestion.

12. Incubate 12 to 16 hrs at 37°C.

Sample processing

13. Add 5 μL of 5% aqueous TFA to halt the digestion.

14. Shake the tubes containing gel pieces for about 15 minutes and centrifuge briefly to bring the liquid to the bottom of the tube.

15. Place labeled tube in freezer until analysis.

It has been documented that samples can be stored for several months at -20 °C with no detectable change in results.