

In-gel Digestion Protocol for Mass Spec

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Materials

- Stained polyacrylamide gel containing protein(s) of interest
- 50 mM ammonium bicarbonate (NH_4HCO_3 ; 3.96 mg/mL)/50% (v/v) acetonitrile
- 10 mM dithiothreitol (DTT, 1.54 mg/mL) in 50 mM NH_4HCO_3
- 55 mM iodoacetamide (10.2 mg/mL) in 50 mM NH_4HCO_3
- 0.1% TFA
- 50 mM NH_4HCO_3 , pH 8
- HPLC Grade Acetonitrile
- 0.5 mL non-stick tubes

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 a clean razor, and place into a 0.5 mL siliconized tube (VWR *SuperSlik* microcentrifuge tubes).
2. Add 200 μL of 50 mM NH_4HCO_3 /50% acetonitrile and vortex for 30 minutes 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).

5. Add 30 μL of the freshly prepared 10 mM DTT solution to cover the gel pieces, and reduce for 30-45 min at 55°C.
6. Replace the DTT solution with roughly the same volume of freshly prepared 55 mM iodoacetamide (30 μL). Incubate for 45 min at room temperature.
7. Remove the iodoacetamide solution and wash gel pieces with ~200 μL of 50 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.
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.
11. Remove excess trypsin solution and overlay the rehydrated gel particles with 30 μL of 50 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 1% 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. Transfer supernatant (containing tryptic peptides) to a clean 0.5 mL centrifuge tube and keep supernatant on ice. Before solution transfer, poke one small hole into cap using a needle.
16. Add 50 μL of extraction solution (60 % acetonitrile, 1 % TFA) to gel pieces and sonicate in ultrasonic waterbath for 10 min. Alternatively, agitate gently by vortexing at lowest setting.
17. Transfer supernatant (containing additional tryptic peptides) to tube from step 15.
18. Extract the gels with an additional 25-50 μL of extraction solution. Agitate gel pieces by sonicating in a waterbath for 10 min or with gentle vortexing.
19. Spin down sample and transfer supernatant to tube from step 12.
20. Dry the pooled extracted peptides by centrifugal evaporation to near dryness. Do not dry for extended time.
21. Add 15 μL of resuspension solution (0.1 % TFA) to each tube and sonicate tube in water bath or gently agitate on a vortex at lowest setting.
22. Transfer supernatant to the vial for LC-MS/MS analysis.