

Procedure for Preparation of Silver-stained 1-Dimensional Gel Band for In-gel Trypsin Digestion and MALDI-TOF Analysis*

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1. Excise silver-stained gel band(s) and place into a 1.5 mL tube. Mince into 1 mm³ pieces.
2. Wash minced gel band with 500 µL ddH₂O (3 x 15 min) at room temperature with agitation on a Vortex mixer set to lowest speed.
3. Reduce the silver stain in the gel pieces with 250 µL of freshly prepared aqueous solution containing 50 mM sodium thiosulfate and 15 mM potassium ferricyanide for 5 minutes at room temperature with gentle agitation [1]. (Reagent is prepared by mixing equal volumes of a stock 16 mg Na₂S₂O₃/mL ddH₂O and a stock 10 mg K₃Fe(CN)₆/mL ddH₂O)
4. Wash gel pieces with 500 µL ddH₂O (3 x 5 min) at room temperature with agitation on a Vortex mixer set to lowest speed to remove silver reducing agents.
5. Equilibrate gel pieces with 500 µL 100 mM ammonium bicarbonate (aq) buffer for 20 minutes at room temperature with gentle agitation.
6. Wash gel pieces with 500 µL 50 mM ammonium bicarbonate (aq)/50% acetonitrile.
7. Rinse gel pieces briefly with 500 µL 100 % acetonitrile and discard. Dehydrate the gel pieces with 500 µL 100 % acetonitrile for 20 minutes at room temperature with gentle agitation. Discard acetonitrile and air-dry.
8. Reduce the in-gel protein with dithiothreitol (150 µL 10 mM DTT in 100 mM ammonium bicarbonate) for 30 minutes at 56° C.
9. Cool the sample to room temperature, remove and discard DTT solution.
10. Alkylate the in-gel protein with iodoacetamide (100 µL 50 mM IAA in 100 mM ammonium bicarbonate) in the dark at room temperature for 30 minutes. Remove and discard IAA solution.
11. Wash sample at room temperature for 15 minutes with 500 µL 50 mM ammonium bicarbonate (aq)/50% acetonitrile.
12. Rinse gel pieces briefly with 500 µL 100 % acetonitrile and discard. Dehydrate the gel pieces for 20 minutes at room temperature with 500 µL of 100 % acetonitrile.
13. Rehydrate gel pieces for 60 minutes at 4° C (on ice) in 30 µL 0.02 µg/µL trypsin (Promega modified porcine TPCCK-treated) in 50 mM ammonium bicarbonate (aq) [6]. (Be sure that enough volume is added to ensure complete rehydration of gel pieces)
14. After one hour, remove trypsin solution and replace with 30-50 µL 50 mM ammonium bicarbonate (aq). Incubate at 37° C for 16-18 hours (overnight).
15. Transfer supernatant (containing tryptic peptides) to 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 25-50 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. (Extraction solution is made by combining 600 µL 100 % ACN, 300 µL fresh ddH₂O and 100 µL of a 10 % TFA stock solution)
17. Spin down sample by brief centrifugation (12,000xg for 30 seconds).
18. Transfer supernatant (containing additional tryptic peptides) to tube from step 15.
19. Extract the gels again with an additional 25-50 µL of extraction solution. Agitate gel pieces by sonicating in a waterbath for 10 min or with gentle vortexing.

20. Spin down sample and transfer supernatant to tube from step 15.
21. Freeze the pooled gel extraction solutions using liquid nitrogen.
22. Once frozen, immediately dry the peptides by centrifugal evaporation to near dryness. Do not use heat. Do not dry for extended time.
23. Add 5 μL of resuspension solution (50 % acetonitrile, 0.1 % TFA) to each tube and sonicate tube in water bath or gently agitate on a vortex at lowest setting.
24. Spin down sample and spot 0.5 μL on MALDI plate followed by 0.5 μL of alpha-cyano-4-hydroxycinnamic acid matrix (10 mg/mL in 50% acetonitrile, 0.1% TFA).
25. Allow spots to dry completely. Load plate into Voyager.
26. Calibrate using internal tryptic peaks of 842.5 and 2211.1 Da.

Notes

- After peptide extraction mass spec analysis should be performed as soon as possible.
- Gel staining and preparation of peptides must be performed with labware that has never been in contact with nonfat milk, BSA, or any other protein blocking agent to prevent carryover contamination.
- Always use non-latex gloves when handling samples, keratin and latex proteins are potential sources of contamination.
- Never re-use any solutions, abundant proteins will partially leach out and contaminate subsequent samples.

* Adapted from [3, 5]

More information regarding this procedure: [2, 4]

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6. Shevchenko A, Wilm M, Vorm O, Mann M: Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Analytical Chemistry* **68**: 850-858 (1996).