Procedure for Preparation of Coomassie G-250-stained 2-Dimensional Gel Spots for In-gel Trypsin Digestion and MALDI-TOF Analysis

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1. The second dimension (SDS-PAGE) gel should only be stained with high quality Coomassie Brilliant-Blue G-250. Use only ultrapure water for destaining and reagent preparation.
2. Once gel is destained and background is minimal, excise stained spots, mince into 1 mm^3 pieces and transfer into a clean 1.5 mL microcentrifuge tube. (Spot pickers can be obtained from www.gelcompany.com).
3. Wash gel with 500 μL of wash solution (50 % acetonitrile, 50 mM ammonium bicarbonate) and incubate at room temperature for 15 min with gentle agitation (vortex mixer on lowest setting). Remove solution with a pipette.
4. Wash gel two more times with 500 μL of wash solution (15 min each) or until the Coomassie dye has been completely removed (usually 4 washes with G-250 coomassie).
5. Discard wash solution and dehydrate gel in 500 μL 100% acetonitrile briefly. Remove with pipette and discard, taking care not to pull gel piece(s) into pipette tip. Add 500 μL 100 % acetonitrile and incubate for 20 minutes.
6. Discard the second acetonitrile wash and completely air-dry each gel piece. Usually the cap can just be left open with tube resting on its side; but care should be observed when handling the tube once the gel is dry because it will "jump" out due to static electricity.
7. While gel is drying prepare protease digestion solution. [Typically, this is modified sequencing grade trypsin (Product number V5111, Promega, Madison, WI)]. Resuspend lyophilized trypsin (20 μg/vial) in 20 μL of the 50 mM acetic acid solution provided with trypsin, yielding a 1 μg/μL stock solution. Dilute that stock to 1 μg/50 μL with 50 mM ammonium bicarbonate (50 fold dilution), keeping in mind the number of gel pieces you have to digest. Store the remaining trypsin stock at –70° C. Do not freeze-thaw trypsin stock solutions more than once.
8. Rehydrate the gel with a minimal volume of trypsin protease digestion solution. Use 20 μL for small gel pieces, 30-40 μL for larger gel pieces. Add more if gel pieces absorb all the liquid.
9. Incubate at 4° C (on ice) for 1 hour. At this point most of the trypsin will be absorbed into the gel pieces.
10. Remove the trypsin solution after 1 hour and replace with 30 μL of 50 mM ammonium bicarbonate to keep gel pieces hydrated throughout the digest. Digest overnight at 37° C.
11. Spin down sample by centrifugation (12,000xg for 30 sec).
12. 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.
13. 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 prepared by combining 600 μL ACN, 300 μL fresh dd H_2O and 100 μL of a 10 % TFA stock)
14. Spin down sample by brief centrifugation (12000xg for 30 sec).
15. Transfer supernatant (containing additional tryptic peptides) to tube from step 12.

16. 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.
17. Spin down sample and transfer supernatant to tube from step 12.
18. Freeze pooled supernatants using liquid nitrogen.
19. Once frozen, immediately dry the pooled extracted peptides by lyophilization.
20. When dry add MilliQ water (~50 μL) to resuspend the peptides, freeze in liquid nitrogen, and lyophilize again. (*2nd lyophilization usually obviates the need for ZIP tip clean up*)
21. Add 3-5 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.
22. 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).
23. Allow spots to dry completely. Load plate into Voyager.
24. Calibrate using internal tryptic peaks of 842.5 and 2211.1 Da.

Notes

- This protocol does not contain a reduction and alkylation step, and assumes these steps have been performed during IPG strip equilibration, prior to running the second dimension.
- 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.

More information regarding this procedure: [1, 2]

1. Havlis J, Thomas H, Sebela M, Shevchenko A: Fast-response proteomics by accelerated in-gel digestion of proteins. *Analytical Chemistry* **75**: 1300-1306 (2003).
2. Jiménez CR, Huang L, Qiu Y, Burlingame AL: In-gel digestion of proteins for MALDI-MS fingerprint mapping. In: Coligan JE (ed) Current Protocols in Protein Science, pp. 16.4.1-16.4.5. John Wiley & Sons, Inc., Brooklyn, N.Y. (1998).