In Gel Digestion Protocol

Solutions

a) 25 mM NH_4HCO_3/ 50% Acetonitrile
b) 0.1 M NH_4HCO_3/ 10 mM DTT (Dithiothreitol)
c) 0.1 M NH_4HCO_3/ 55 mM IAA (Iodoacetamide)
d) 25 mM, 50 mM, 0.1M NH_4HCO_3
e) 5% formic acid/ 60% Acetonitrile
f) Digestion buffer: 20 ng/µL MS Sequencing Grade Trypsin in 50 mM NH_4HCO_3
g) Silver destain solution (for silver stain samples only): 0.01 g Potassium ferricyanide, 0.16 g Sodium Thiosulfate in 1 mL MilliQ Water

Procedures

1. Wash the gel band with MilliQ water/LCMS grade water in 1.5 mL Eppendorf tubes by vortexing briefly.
2. Cut the gel band into small cubes (1mm). Transfer the fragments into a new tube.
3. a. For the ***Coomassie Blue*** Stained gel band, wash the gel fragments with 25 mM NH_4HCO_3/ 50% Acetonitrile. Add enough solution to cover gel pieces and shake the tubes on the mixer for 15 mins at room temperature. Centrifuge the tube and remove the solution. Repeat the wash 2 times or until the gel fragments become colorless. Remove all the remaining liquid.

   b. For ***Silver Stain*** gel bands, add 200 µL silver destain solution. The gel piece should be destained within 5 mins. Remove the solution and wash the gel with 25 mM NH_4HCO_3. Repeat until the gel piece become colorless. Remove all the remaining liquid.
4. Shrink the gel fragments in 3-4 times gel volumes of Acetonitrile for 15 mins at room temperature. The gel fragment should become cloudy and sticky. Briefly centrifuge the tube and discard the supernatant. Dry the fragments in a vacuum centrifuge for about 5 mins without heat. If necessary, you can use the vacuum centrifuge in BioCRF for drying upon permission.
5. Swell the gel pieces in 2-3 gel volumes of 0.1 M NH_4HCO_3/ 10 mM DTT. Incubate at 55 °C for 45 mins with occasional vortexing. Discard the liquid.

6. Add 2-3 gel volume of 0.1 M NH_4HCO_3/ 55 mM IAA. Incubate at room temperature in a dark place for 45 mins with occasional vortexing. Discard the supernatant.
7. Wash the gel fragments in 100 µL of 0.1 M NH_4HCO_3 with shaking on a mixer at room temperature for 15 mins.
8. Remove the washing solution and shrink the gel fragments using Acetonitrile as in Step 4. The gel fragments should be completely dried.
9. Swell the gel fragments in 2-3 volumes of digestion buffer on ice for 30 mins. Add more solution if the initially added buffer is completely absorbed by the gel pieces. Make sure that the gel pieces are covered by the digestion buffer.
10. Remove the excess digestion buffer and add a sufficient 50 mM NH_4HCO_3 solution to cover the gel pieces. Incubate at 37 °C overnight.
11. Briefly centrifuge the tubes and transfer the supernatants to clean tubes.
12. Add a sufficient 25 mM NH_4HCO_3 solution to cover the gel pieces for digested peptides extraction. The tubes are shaken on the mixer for 15 mins at room temperature. Briefly centrifuge the tube and combine the extracts with the supernatants collected in Step 11.
13. Continue the extraction of peptides using 5% formic acid/ 60% Acetonitrile as described in Step 12. Repeat this extraction 2 times.
14. Dry the combined extracts in a vacuum centrifuge (no heat) and store them at -20 °C for further processing (Desalting and MS analysis). If necessary, you can use the vacuum centrifuge in BioCRF for drying upon permission.

Remarks

i) Wear clean gloves and a long-sleeved lab coat to reduce keratin contamination.
ii) Do not use autoclaved tubes or tips for in-gel digestion. High-quality tubes and tips from Eppendorf are recommended.
iii) Use a clean blade to cut the gel bands.
iv) Use HPLC grade or LCMS grade Acetonitrile.
v) Trypsin can be dissolved in 10 mM acetic acid at 1 µg/µL as stock and store at -20 °C. To prepare the digestion solution, the stock trypsin solution is diluted to 20 ng/µL using 50 mM NH_4HCO_3 to reach a final pH of 7-8.

   Recommended sources for Trypsin:

   1. Thermo Scientific™ Pierce™ Trypsin Protease, MS Grade, Catalog number: 90058
   2. Promega Trypsin Gold, Mass Spectrometry Grade, Catalog number selected: V5280