In-gel Digestion and Peptide Extraction Protocol

1. A piece of gel containing protein band of interest is cut by a blazer at the centeral area and minced into small pieces.
2. Put the gel piece into a 1.5 mL Eppendorf centrifuge tube (using siliconized tubes, Fisher Cat. #02-681-320).
3. Add 1.0 ml of destaining solution (CH_3OH:H_2O/50:50), heavily shake and keep changing solution (2 – 3 times or more, each time about 0.5 hour) until the gel becomes colorless. The gel staining solution is recommended silver stain (for silver stain just wash twice) or colloidal blue (Novex product, distributed by invitrogen, 11040 Rocelle St., San Diego, CA 92121, Tel: 1 800-456-6839, Cat. # LC6025)
4. Rinse the gel with water twice each time 15 minutes.
5. Transfer the gel to 0.6 mL siliconized Eppendorf centrifuge tube (Fisher Cat. #02-681-311).
6. Rinse the gel with 200 μl of 50 mM NH_4HCO_3 solution for 30 minutes.
7. Dry the gel piece with Kimwipes
8. Punch the gel piece into gellish 'solution' (no visible particle) by a sealed (using a flame) pipet tip (10 μl size). If you see the gel piece is stick to the pipet tip, the gel is still wet and needs dry longer for easily punching. If you see the gel becomes hard and not easy to punch, the gel is over-dried.
9. Add 25 mM NH_4HCO_3 (pH is about 8) buffer to cover the gel particle. If you see some particles stick to the pipet tip, wash down to the tube by 50 mM NH_4HCO_3. Total buffer volume is what you can see just a light layer over the gel (about 30 μl based on the size of the gel pieces).
10. Add 100 nano-gram or 1 μl of trypsin solution (0.1 μg/μl concentration in water). Amount of trypsin added needs to be adjusted according to the amount of proteins in the gel bands judged by the staining color. The rule is that more protein needs more trypsin, less protein needs less trypsin. 100 nano-gram (0.1 μg) is the average. Do not add too much trypsin!
11. Incubate at 37 °C overnight.
12. Add 50 μl of acetonitrile (100%) to stop digestion and centrifuge at 14,000g or higher for 10 minutes. Transfer the supernatant to a 0.6 ml Eppendorf centrifuge tube (Fisher Cat. #02-681-311). Careful, do not transfer any particles to the tube. Do not try to transfer the supernatant completely.
13. Add 10 to 30 μl (just enough to cover the gel) of water to the tube containing the gel. You can see the gel shrinks after acetonitrile extraction and re-hydrates after adding water.
14. Add 50 μl of acetonitrile to extract peptides as in step 12 and combine the two extractions to get a total volume about 100 μl.
15. Evaporate the extracted solution to dryness by a SpeedVac. After dryness, you can see very tiny peptide layer inside the wall of the centrifuge tube.
16. Add 10 to 20 μl of 0.1% formic acid (or acetic acid) to dissolve peptides and stored at – 20 °C or lower refrigerator before analysis by mass spectrometry.
17. Zip-tip clean of the peptide solution according to the Zip-tip protocol.
18. For MALDI-TOF MS, take 0.5 μl of peptide solution, add 0.5 μl of acetonitrile, votex, spin, and then add 1.0 μl of CHCA matrix solution, votex and spin. Load the mixture to the MALDI target.
19. For ESI MS, in the peptide solution remaining after MALDI analysis, add 0.1% formic acid in 50:50 acetonitrile/water and then submit for ESI analysis.
20. When the color of gel band is light, you need to pool multiple lanes of gel bands to get high concentration of sample to ensure the success of ESI-MS/MS.

Cautions:
All the equipment including tubes, pipet tips must be very clean (they must be rinsed by acetonitrile and Millipore water and dried before use). Wear gloves and clean lab. coat through the procedure.

For low-level proteins (<100 fmol), especially those separated by 1-D SDS-PAGE, reduction and alkylation is recommended. These procedures are performed after step 7.

a. Prepare fresh solutions:
   10 mM DTT in 25 mM NH_4HCO_3 (1.5 mg/mL)
   55 mM iodoacetamide in 25 mM NH_4HCO_3 (10 mg/mL)

b. Completely dry gel pieces by SpeedVac. (Modified step 7)

c. Add 25 µL (or enough to cover) 10 mM DTT in 25 mM NH_4HCO_3 to dried gels. Vortex and spin briefly. Allow reaction to proceed at 56 °C for 1 hr.

d. Remove supernatant, add 25 µl 55 mM iodoacetamide to the gel pieces. Vortex and spin briefly. Allow reaction to proceed in the dark for 45 min. at room temperature.

e. Add 4 µL of Thiodiglycol (98%) to react with extra Iodoacetamide.

f. Concentrate the volume to about 10ul by speedvac.

g. For solution Proteins: Use zip-tip (C4) to purify proteins. (1. Add formic acid (1%) to acidify solution, load the solution to column, 3. Wasing with 1% formic acid (3X); 4. 70% acetonitrile to elute proteins, 5. SpeedVac to evaporate ACN. 6, redesolve in 20 mico-litter 25 mM Ammonium bicarbonate. 7. Add 500 nano-gram trypsin. 8. Digestion at 37 C for 10 hours.

h. Remove supernatant (discard). Wash gels with ~100 µl NH_4HCO_3, vortex 10 min, spin.

i. Remove supernatant (discard). Dehydrate gels with ~30µL (or enough to cover) of 25 mM NH_4HCO_3 in 50% ACN, vortex 5 min, spin. Repeat one time with 25 mM NH_4HCO_3 in water.

j. Proceed with step 7.