

In-Gel Digestion Procedure Suitable for Mass Spectrometry

If possible, all work prior to extracting the peptides should be done in a laminar flow hood to avoid contamination from keratins (skin proteins). Wearing gloves and sleeve protectors, wipe down ALL surfaces in the hood with methanol/water moistened lint-free cloth, including the outside of all your tubes (make sure you don't wipe off the labeling!), the outside and inside of the Speed Vac and centrifuge, tube racks, bottles, etc. Wipe razor blades with methanol-soaked lint-free cloth.

Reagents and solutions

- 1 M NH_4HCO_3 stock (FW 79.06): 3.95 g NH_4HCO_3 /50 mL H_2O .
- 50 mM ammonium bicarbonate (0.5 mL of 1 M NH_4HCO_3 + 9.5 mL H_2O)
- 25 mM NH_4HCO_3 (Add 5 mL of 50 mM NH_4HCO_3 to 5 mL H_2O)
- 25 mM NH_4HCO_3 in 50% ACN (Add 5 mL of 50 mM NH_4HCO_3 to 5 mL acetonitrile)
- 50% Acetonitrile/0.1% TFA in water
- 12.5 ng / μL trypsin (~0.5 μM) in 25mM NH_4HCO_3 (Prepare immediately before use). Alternatively, reconstitute 20 μg Promega trypsin in 1.6 mL of 25mM NH_4HCO_3 , aliquot and freeze (-80 deg recommended). Avoid repeated freezing and thawing the enzyme.
- Trifluoroacetic acid, TFA, Pierce 28901
- Trypsin porcine, sequencing grade, Promega, cat # V5111.
- Ammonium Hydrogen Carbonate (Ammonium Bicarbonate), Fluka, cat # 09830
- DL-Dithiothreitol, Sigma, cat # D0632
- Iodoacetamide, Sigma, cat # I6125

Tubes

- Use colorless Eppendorf brand tubes if possible. Recommended 0.5 ml PCR clean, Eppendorf cat# 22 36 371-9 (for small gel bands) and 1.5 ml, Eppendorf cat# 22 36 321-2 (for large gel pieces).
- Wash all tubes with methanol prior to use if preparing Silver or Sypro Ruby stained spots.

Coomassie Stain and SDS Removal

1. Dice each gel slice into small pieces (~0.5-1 mm^3) and place into tubes.
2. Add 200 μL of 25 mM NH_4HCO_3 /50% ACN and vortex for 10 min.
3. Using gel loading pipet tip, extract the supernatant and discard.
4. Repeat steps 3 and 4 until the gel pieces are colorless.
NOTE: Gel pieces can be left in supernatant overnight to avoid long de-stain times. Change supernatant until it no longer turns blue and then leave gel pieces to vortex overnight.
5. Remove supernatant (discard). Add 100% acetonitrile to cover the gel pieces. Let stand for a few minutes until the gel pieces shrink and turn white.
6. Remove acetonitrile (discard). Speed Vac the gel pieces to complete dryness (~10 min). Do not heat during the Speed Vac process. Proceed with trypsin digest for 2D gel pieces or perform Reduction and Alkylation Procedures for 1D gel bands.

*For proteins separated by 1-D SDS-PAGE, reduction and alkylation (of cysteines) is recommended. These procedures are performed after step 6, see **Reduction and Alkylation Procedures** (at the end of the Protocol). For proteins prepared by other methods and/or already S-alkylated, continue with step 7.*

Digestion

7. Add an excess of trypsin solution to completely cover gel pieces. This volume will vary from sample to sample, but on average 25 μL is sufficient.
8. Allow gel pieces to re-hydrate with trypsin on ice or at 4 $^{\circ}\text{C}$ for 60 min. After 60 minutes, aspirate any trypsin solution which has not absorbed into the gel and discard. Add 25 mM NH_4HCO_3 as needed to cover the gel pieces. This will prevent the gel from drying out overnight.
NOTE: Longer incubation period will ensure that trypsin penetrates into the gel completely. Removal of excess trypsin will minimize autolysis cleavage products from trypsin.
9. Spin briefly and incubate at 37 $^{\circ}\text{C}$ overnight (12-16 hrs).

Extraction of Peptides

10. Spin down and aspirate supernatant (water extract) into 0.5 mL Eppendorf tube.
11. Add 20-30 μL of 50% ACN/ 0.1% TFA (MALDI) or 0.1% FA (ESI) in water to the gel pieces, vortex 15 min., & spin. Aspirate supernatant (organic extract) and combine with the water extract taken in the previous step.
12. Repeat step 11.
13. Speed Vac combined peptide extracts to reduce volume to approximately 10 μL . **Do not Speed Vac to dryness.** At this point peptide extracts can be stored at -80 $^{\circ}\text{C}$ or -20 $^{\circ}\text{C}$ until mass spectrometric analysis.

Reduction and Alkylation Procedures

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), **protect from light.**

- a) Re-hydrate gel pieces in ~ 40 μL of 10 mM DTT (ensure full gel coverage). Vortex and spin briefly. Allow reaction to proceed at 56 $^{\circ}\text{C}$ for 45 min.
- b) Remove supernatant and add 40 μL of 55 mM iodoacetamide to the gel pieces. Vortex and spin briefly. Allow reaction to proceed **in the dark** at room temperature for 30 min.
- c) Remove supernatant (discard). Wash gel pieces with 100 μL of 25 mM NH_4HCO_3 , vortex 10 min, & spin.
- d) Remove supernatant (discard). Add 200 μL of 100% acetonitrile, vortex briefly, and let it stand for few minutes until the gel pieces shrink and turn white. If gel pieces do not look opaque, remove acetonitrile and repeat the step.
- e) Remove the acetonitrile (discard). Speed Vac the gel pieces to complete dryness (~10 min). Proceed with trypsin digestion at step 7.

References:

Shevchenko *et al.* Anal. Chem.(1996) 68:850-858

Havlis *et al.* Anal. Chem. (2003) 75:1300-1306.