

Digestion of Excised Protein Spots (Coomassie Stained Gels)

1. Washing/Destaining

- a. Excise bands/spots (cut as close to the band/spot as possible to minimize excess Polyacrylamide gel material), and cut into 1 mm cubes and place them in an eppendorf tube.
- b. Incubate the gel pieces at room temperature in 100 μ l of ddH₂O for 15 min.
- c. Pull off the solution and discard, add 50 μ l of 50% ACN and incubate for 15 mins at room temperature (Note: Use a thermo-mixer/shaker when performing all incubations at room temp).
- d. Pull off solution and discard, add 50 μ l of 50% ACN, incubate at room temp until gel pieces are white and sticky (this usually takes 5-10 min).
- e. Pull off solution and discard, add 50 μ l of 100 mM Ambic, incubate at room temp for 5 min.
- f. Add 50 μ l of acetonitrile to make a solution of Ambic and ACN, incubate at room temp for 15 min.
- g. Pull off solution and discard, dry samples in a speed vacuum at around 56~60°C for about 45 mins, the gel pieces must shrink and be completely dehydrated.

2. Reduction/Alkylation

- a. Remove samples from the speed vacuum and let cool.
- b. Add 40 μ l of 10 mM DTT/100 mM Ambic and incubate at 56°C in a water bath or thermocycler for 45 min.
- c. Remove samples and let cool.
- d. Pull off solution and discard, immediately add 40 μ l of 55 mM IAA/100 mM Ambic, then incubate at room temperature for 30 min in the dark.
- e. Pull off solution and wash with 40 μ l of 100 mM Ambic, then incubate at room temperature for 5 min.
- f. Add 40 μ l of acetonitrile to make 1:1 solution of Ambic and ACN, then incubate at room temperature for 15 min.
- g. Pull off solution and discard, dry gel pieces in speed vacuum as in 1h).

3. Digestion/Extraction

- a. Add 40 μ l or enough trypsin solution to cover gel pieces and incubate at 4°C for 45 mins (use ice bath or thermocycler, add more solution if pieces absorb all the liquid).
- b. Pull off excess solution and discard, add 40 μ l (or enough to cover gel pieces) same buffer but without trypsin and incubate at 37°C for 16 hrs (overnight).
- c. Pull off supernatant and save at 3°C.
- d. To gel add 20 μ l of 25 mM Ambic and incubate at room temperature for 15 min.
- e. Add 20 μ l acetonitrile to make 1:1 solution of Ambic/ACN and incubate for 15 min.
- f. Pull off the supernatant and combine it with the one from c).
- g. To gel pieces add 20 μ l of 5% Formic Acid, then incubate at room temperature for 15 min.
- h. Add 20 μ l acetonitrile to make 1:1 solution of ACN/Formic Acid, then incubate for 15 min.

- i. Repeat 3f), 3g), 3h), 3f).
- j. To pooled supernatant add 10 mM DTT to give final concentration of 1 mM DTT.
- k. Completely dry the supernatant, which is the digested extracts in speed vacuum.
- l. Resuspend the digested extracts in 15 μ l~20 μ l of 5% Formic Acid for MS or MS/MS analysis.

Note: Larger spots/bands may require more solution. Please adjust the volume accordingly.