In-gel Digestion of Excised Protein Spots

All steps at room temperature unless noted otherwise. Adjust volumes as required to cover gel pieces for large bands/spots.

1. Washing and Destaining Coomassie blue stained gels.
    a. Rinse gels 2 times for 15 min in ddH2O.
    b. Excise bands or spots of interest, cut into cubes (< 1mm) and place in low retention microfuge tubes (Siliconized, e.g. Fisher 02-681-311).
    c. Cover pieces with 40 µl 100 mM ammonium bicarbonate (ambic) plus 40 µl acetonitrile and incubate 15 min.
    d. Remove supernatant and cover again with 1:1 acetonitrile/ 100 mM ambic. Use a gel-loading tip to prevent sucking up gel pieces.
    e. Repeat steps 1c and 1d until gel pieces are clear.
2. Reduction and alkylation (Start here for silver stained gels).
    a. Add 40 µl of 10 mM DTT in100 mM ambic and incubate at 56ºC for 45 min.
    b. (optional) Remove supernatant, immediately add 40 µl of 55 mM iodoacetamide in 100 mM ambic and incubate 30 min in the dark.
    c. Remove supernatant and cover pieces with 40 µl 100 mM ambic and incubate 5 min
    d. Add 40 µl of acetonitrile.
    e. Remove supernatant and dry gel pieces completely in a speed vac.
3. Digestion and extraction.
    a. Add 40 µl of trypsin solution and incubate 45 min on ice. Add more solution if all the liquid is absorbed.
    b. Remove excess solution, cover pieces with digestion buffer and incubate 16 h at 37ºC
    c. Remove supernatant and save in a low retention microfuge tube.
    d. Add 20 µl of 25 mM ambic and incubate 15 min.
    e. Add 20 µl of acetonitrile to make 50% solution and incubate 15 min.
    f. Remove supernatant and add to solution from step 3c.
    g. Add 20 µl of 5% formic acid or 1% TFA to gel pieces and incubate 15 min.
    h. Add 20 µl of acetonitrile to make 50% solution and incubate 15 min
    i. Remove supernatant and add to solution from step 3c.
    j. Repeat steps 3g-3i
    k. Add 10 mM DTT to give a final concentration of 1mM.
    l. Dry extracted peptides in a speed vac.
    m. Resuspend in 20 µl of 1% TFA
4. Zip-Tip Purification
    a. Wet Zip-Tip (Millipore ZTC18S096) with 10 µl Wetting/Elution solution, expel. (wait for tip to fill and expel completely to avoid bubbles in the matrix)
    b. Rinse 2X with wash solution. Expel and hold plunger down after 2nd wash.
    c. Bind peptides to tip by filling and expelling resuspended peptides through zip-tip several times (more cycles for more complex mixtures). At the last cycle leave the solution in the tip to allow for preparation of a tube for elution.
    d. Wash tip 2X with wash buffer. Expel and hold plunger after 2nd wash.
    e. Elute peptides with 2 µl of elution solution into the bottom of the fresh tube, flush the solution through the tip 2-3X and expel after the final rinse. Repeat elution step by suspending the droplet on the side of the tube. Combine with the first eluate. The sample is ready for MALDI-TOF analysis. The samples could be concentrated slightly by allowing the solvent to evaporate but this is usually not necessary.

Stock Solutions

1 M ammonium bicarbonate
0.79 g ammonium bicarbonate in 10 ml ddH2O (pH should be ~7.8)
1 M DTT
0.15 g DTT in 1 ml ddH_2O
1 M CaCl2
0.11 g CaCl2 in 1 ml ddH_2O
Trypsin, Sequencing grade
25 mg dissolved in 0.25 ml ddH_2O

Digestion Solutions

Prepare all solutions fresh before performing the digest.

100 mM Ammonium bicarbonate 100 µl 1M ammonium bicarbonate 900 µl ddH_2O	**55 mM iodoacetamide in 100 mM ambic** 10 mg iodoacetamide 100 µl 1M ammonium bicarbonate 900 µl ddH_2O
10 mM DTT in 100 mM ambic 10 µl 1 M DTT 100 µl 1M ammonium bicarbonate 900 µl ddH_2O	**Trypsin solution** (prepare enough to process the number of samples in each expt., 40 µl per sample) 34 µl/sample Digestion buffer 6 µl/sample 0.1 mg/ml Trypsin
Digestion buffer 50 µl 1M ambic 5 µl 1 M CaCl2 945 µl ddH_2O	**10 mM DTT** 10 µl 1 M DTT 990 µl ddH_2O
25 mM ambic 25 µl 1 M ammonium bicarbonate 975 µl ddH_2O	**1% TFA** 10 µl Trifluoroacetic acid 990 µl ddH_2O
Zip-Tip wash solution 100 µl 1% TFA 900 µl ddH_2O	**Zip-Tip wetting/elution solution** 500 µl acetonitrile (HPLC or UV grade) 100 µl 1% TFA 400 µl ddH_2O