In-gel Trypsin Digestion

(Boisvert Lab, December 2013)

Adapted from Dr Angus Lamond Lab

Remember to wear gloves at all times. Keratin contamination is less of any issue *after* peptide extraction, as whole keratin proteins will not interfere with MS analysis.

Peptides tend to stick to plastic surfaces on storage. We use 1.5 ml Eppendorf Protein LoBind tubes (cat no. 2243108-1) that are designed for storing peptides at low concentrations.

When preparing stock solutions, clean spatulas thoroughly with water and ethanol. Even better, simply tip the solid chemical into clean Eppendorf tubes.

All solutions and reagents used (Water, acetonitrile, NH_4HCO_3) should be MS-grade quality. Acetonitrile (CH_3CN) is light sensitive, so store in a dark bottle or tube wrapped in foil.

Stage 1: If you are doing IP, first elute your IP samples from beads

NOTE: To improve elution of proteins from beads and to save time during the digestion, we now elute in SDS and then reduce and alkylate the proteins prior to running them on a gel.

1. *Elute proteins from beads (sepharose, agarose, dynabeads, etc.)* Add 1 volume of 1% SDS to the beads (e.g. 50 μl of 1% SDS to 50 ul of beads) and boil for 10 min at 95oC. Add 4 volumes of dH2O (e.g. 200 μl of dH2O to 50 μl of beads) and vortex well to elute proteins. Pellet the beads and collect the supernatant. This results in a more efficient release of proteins from the beads.
Note: Save the beads and add sample buffer directly (e.g. 20 μl dH2O plus 20 μl of 4X sample buffer) to elute any remaining proteins (can then run them on the same gel to see how much did not elute).
2. *Speedvac the supernatant* back down to the original volume (e.g. 50 μl), which will take the SDS concentration back to 1%.
3. Alternatively, concentrate samples using microcon filters.

Stage 2: Reduction and Alkylation

Reduction and alkylation of cysteine residues using DTT and IAA, respectively, improves the recovery of cysteine-containing peptides from in-gel digests and minimizes the appearance of unknown masses in MS analysis due to disulfide bond formation and side chain modification.
These steps can be performed in SDS-PAGE loading buffer (we usually use 4X NuPAGE LDS sample buffer from Invitrogen (NP0007) and then do the DTT and IAA as described below, avoiding step 3.

1. *Reduce the sample.* Add DTT to a final concentration of 10 mM and boil for 1–2 min.
(e.g. for 50 μl, add 0.5 μl of 1M DTT stock).
2. *Alkylate the sample.* Add iodoacetamide (Sigma I1149) to a final concentration of 50 mM and incubate at room temperature in the dark for 30 min.
(e.g. for 50 μl, add 2.5 μl of 1M IAA stock).
3. Add 4X NuPAGE LDS sample buffer (a few μl is fine).

Stage 3: Separating proteins on gels and excising bands

1. *Separate your protein sample by 1D PAGE*. We routinely use 4–12% gradient Novex precast gels. For a complicated sample run them all the way (200V for 50 min) and cut the gel into 12-16 slices. For less complicated samples (e.g. IPs), we use straight percentage gels (usually 10% or 12%), run them halfway down (200V for 25 min) and cut the gel into 5–6 slices.

2. *Stain the gel with Coomassie blue.* We routinely use the SimplySlue SafeStain solution from Invitrogen (LC6060; protocol on the bottle). To minimize contaminants, do all staining steps in a sterile 14-cm tissue culture dish. Destain the gel thoroughly in dH2O (overnight is fine). The gel can also alternatively be silver-stained.
DO NOT USE METHANOL/ACETIC ACID BASED COOMASSIE STAINING.

3. *Scan the gel* before cutting out the bands. To do that, put the gel into the cover of the 14-cm dish and scan it. Print out the scanned image so that you can mark on it where you cut the bands. The gel can be returned to the dH2O-filled dish until ready to excise the bands.

4. *Excising the bands from the gel.* For this step, we transfer the gel to a clean 14-cm tissue culture dish and cut away the unnecessary parts (top, bottom, MW marker lanes) with a clean razor blade or scalpel, leaving only the lanes in which you are interested. If you want to identify proteins in a single Coomassie-blue-stained band, excise the gel as close to the band as possible, with no excess around the band (to ensure that proteins you identify are from that one band).

5. Mincing the gel bands. Once you have the slices cut out for a particular sample lane (and marked on the printout of the scanned gel), cut each slice into cubes 2 x 2 mm pieces using a fresh scalpel and transfer each slice into a 1.5 ml Lobind Eppendorf tube.
The bands can be stored for later use at –20°C.
Note: If your gel is silver stained, add 50 μl/band of 15 mM potassium ferricyanide/50 mM sodium thiosulphate (Farmers reagent - made fresh from 2X stock solutions) for 5–10 min until the band pieces go clear (i.e. until all the silver is removed).

Stage 4: Destaining the gel bands

1. *Wash the band pieces* with 300 μl of H_2O for 15 min. Add 300 μl of CH_3CN Acetonitrile and wash for a further 15 min.

2. *Remove the supernatant* (Use a P1000 tip with a P10 tip on the end, it is necessary because your gel pieces may be lost through the blue tips).

3. *Wash the band pieces* with 300 μl of 20 mM NH_4HCO_3 (1M aliquots at –20°C that are to be diluted in MS water) for 15 min. Discard the supernatant.

4. *Wash the band pieces* with 300 μl of 20 mM NH_4HCO_3 / CH_3CN (50:50 v/v) for 15 min. The gel pieces should shrink and look opaque. Discard the supernatant.

5. *If the band pieces are still blue, repeat the NH_4HCO_3 and NH_4HCO_3 / CH_3CN washes*. (On adding NH_4HCO_3, the gel pieces should be restored to the original sizes and look transparent again.)

6. *Add 150 μl of CH_3CN to dehydrate* the band pieces for 5 min. The gel pieces should shrink and look completely white. Discard the supernatant. If not completely dehydrated, wash again with 100 μl of CH_3CN

7. *Dry the band pieces* in a Speedvac for 5 min.

Stage 4.1: Reduction /Alkylation of band pieces (optional)

This stage is only necessary if you did **NOT** reduce/alkylate your sample prior to running the SDS-PAGE.

1. Add 50 ul/band of 10 mM DTT in 20 mM NH_4HCO_3.

2. Incubate at 56°C for 1 hour. Discard the supernatant.

3. Add 50 ul/band of either 50 mM freshly prepared iodoacetamide in 20 mM NH_4HCO_3.

4. Incubate at room temperature for 30 min. Discard the supernatant.

5. Wash the band pieces with 300 ul of 20 mM NH_4HCO_3 for 15 min. Discard the supernatant.

6. Wash the band pieces with 300 ul of 20 mM NH_4HCO_3 / CH_3CN (50:50 v/v) for 15 min. Discard the supernatant.

7. Add 150 ul of CH_3CN to dehydrate the band pieces for 5 min. Discard the supernatant.

8. Dry the band pieces in a Speedvac for 5 min.

Stage 5: Digestion of band pieces

Trypsin is a serine protease that specifically cleaves peptide bonds on the carboxyl side of lysine and arginine residues. However, cleavage can be blocked or slowed by proximal acidic, aromatic or proline residues, proline having the most significant effect. Peptide fragments with one missed cut are common and should be taken into consideration during mass analysis.

1. *Add 50-75 μl/band of 12.5 ng/ml of modified trypsin in 20 mM NH_4HCO_3.* We use Trypsin Gold from Promega (V5280), which is supplied as 100 μg of powder. We resuspend it at 1 μg/ μl as our stock solution (i.e. add 100 μl of 50mM acetic acid (see note) to the 100 μg powder in the vial, and store it at –80oC in 10 μl aliquots). Trypsin is not stable especially when pure and in non-acidic condition, so we dilute down from this stock solution just before use. We routinely use 50-75 μl/band of a freshly prepared 12.5 ng/ml stock in 20 mM NH_4HCO_3.
(Resuspend a 10 μl trypsin aliquot in 800 μl of 20 mM NH_4HCO_3 to obtain 12.5 ng/ μl).

Note: *Preparation of 50mM acetic acid: We use MS grade acetic acid (100%). The molecular weight of acetic acid is 60.05 g / mol, and the density is 1.051 g / ml. Hence 57.13 ml = 60.05g = 1 mol, so 57.13 ml in 1 litre is 1M. To prepare a 50 mM solution, add 71.4 μl in 25 ml of MS-water.*

2. *Allow bands to rehydrate in trypsin digestion buffer for 30 min*. The gel pieces should be restored to the original sizes, and there should be JUST enough trypsin solution to cover all the gel pieces.

3. *Incubate at 30°C overnight* (>16h).

Stage 6: Extraction of peptides

Perform all the gel washing extraction steps on a Thermo Mixer at approximately 850 RPM to ensure complete extraction of peptides.

1. *Add an equal volume (e.g. 50-75 μl)* of CH_3CN to the digest.

2. *Incubate at 30°C for 30 min*.

3. Transfer supernatant to a new clean Eppendorf LoBind tube. This supernatant contains the peptides you are going to analyze.

4. *Add the same 50-75 μl of 1% formic acid to the gel pieces.* Incubate for 20 min.
Prepare the 1% formic acid solution fresh in the fume hood, by adding 0.5 ml of 100% formic acid (BDH cat. no. 101155F) to 49.5 ml of dH_2O.

5. *Transfer supernatant* to the tube at step 3.

6. *Repeat steps 4 and 5* once more.

7. *Add 150* Incubate for 20 min.*l of CH_3CN to the gel pieces*. The gel pieces should shrink and turn white. Incubate for 10 min.

8. *Transfer supernatant* to the tube at step 3. If not completely dehydrated, repeat step 7.

9. *Speedvac to dry the peptides* in the tube at step 3 completely (60°C is fine).

10. *Resuspend the (invisible) pellet with 0.1% TFA*.

11. Clean samples using ZipTips