In-gel digestion of immunoprecipitated proteins separated by SDS-PAGE

(Lamond Lab / April 2008)

- Perform all the pipetting steps in a laminar flow hood. We routinely do our digestions in our TC room hoods. Remember to wear gloves at all times, and don't scratch your head or any other part of your body during the procedure (if you do, change your gloves!) 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 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.
- Acetonitrile (CH_3CN) is light sensitive, so store in a dark bottle or tube wrapped in foil.

Stage 1: Eluting 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 ul of 1% SDS to 50 ul of beads) and boil for 10 min at 95C. Add 4 volumes of dH2O (e.g. 200 ul of dH2O to 50 ul 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**

ul dH2O plus 20 ul 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 ul), which will take the SDS concentration back to 1%. 45 min at 45ºC is a good approximation.

Stage 2: Reduction and Alkylation

- Reduction and alkylation of cystine residues using DTT and IAA, respectively, improves the recovery of cystine-containing peptides from in-gel digests and minimizes the appearance of unknown masses in MS analysis for disulfide bond formation and side chain modification.

1. *Reduce the sample.* Add DTT to a final concentration of 10 mM (we use BDH 443553B) and boil for 1-2 min.

(e.g. for 50 ul, add 0.5 ul of 1M DTT stock).

2. *Alkylate the sample.* Add iodoacetamide (We use Sigma I1149) to a final concentration of 50 mM and incubate at room temperature in the dark for 30 min.

(e.g. for 50 ul, add 2.5 ul of 1M IAA stock).

3. Let cool and add dye (a few ul of 4X sample buffer is fine).

Stage 3: Separating proteins on gels and excising bands

1. *Separate your protein sample by 1D PAGE*. We routinely use Novex precast gels. For a complicated sample we use 4-12% gradient gels, run them all the way (200V for 50 min) and cut the gel into 10-12 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 Novex colloidal blue staining kit (Invitrogen LC6025). To minimize contaminants, do all fixing and staining steps in a sterile 14 cm tissue culture dish. When gel is finished running, fix for 10 min in fixing solution (40 ml dH2O + 50 ml Methanol + 10 ml Acetic acid) and then shake in staining solution (55 ml dH2O + 20 ml Methanol + 20 ml Stainer A) for 10 min. Add 5 ml Stainer B and stain for 3 hours. Destain the gel thoroughly in dH2O. The gel can be silver-stained at this stage.

3. *Scan the gel* before cutting out the bands. To do that, we sandwich the gel between two pieces of acetate (that have been washed well in dH2O), and put the sandwich on a scanner. 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 sterile scalpel (e.g. Swann-Morton disposable scalpels, Cat no. 0511), 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). We routinely cut the entire lane into 5-12 slices, regardless of the Coomassie staining profile, in order to analyze the entire protein constituent of the sample.

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 1 x 1 mm pieces and place into a 1.5 ml Lobind Eppendorf tube. Use a fresh scalpel to cut up each slice, and transfer the cut up slices to the tube using this same scalpel. Try to work as quickly as possible, because the gel becomes stickier as it dries out. *Note: If your gel is silver stained, add 50 ul/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 ul of dH_2O water for 15 min. Add 300 ul of CH_3CN (Acetonitrite; Sigma A3396) 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 ul of 20 mM NH_4HCO_3 (Sigma A6141) for 15 min. Discard the supernatant.

4. *Wash the band pieces* with 300 ul 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.) You may crush the band pieces with a teflon stick, but we found this step not essential in most case, and it may increase keratin contamination.

6. *Add 100 ul of CH_3CN to dehydrate* the band pieces for 5 min. The gel pieces should shrink and look completely white. Discard the supernatant.

7. *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 20 ul/band of 12.5 ug/ml of modified trypsin in 20 mM NH_4HCO_3.* We use Trypsin Gold from Promega (V5280), which is supplied as 100 ug of powder. We resuspend it at 0.5 ug/ul as our stock solution (i.e. adding 200 ul of 20 mM NH_4HCO_3 to the 100 ug powder in the vial). 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 0.5 ug of trypsin per band, or 20 ul/band of a freshly prepared 12.5 ug/ml stock in 20 mM NH_4HCO_3. For larger slices we use 50 ul/band.

(for 1.5 ml of trypsin digestion buffer, add 19 ul of Trypsin Gold stock to 1.481 ml of 20 mM NH_4HCO_3)

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. If required, add more 20 mM NH_4HCO_3 (minus the trypsin) to cover the band pieces. Keep a record of how much liquid you add per band, because you will need to know this for the first step of the extraction protocol (i.e. when you add an equal volume of CH_3CN to start extracting the peptides).

3. *Incubate at 30^oC overnight* (>16h).

Stage 6: Extraction of peptides

- Perform all the gel washing extraction steps on a shaking platform to ensure complete extraction of peptides.

1. *Add an equal volume* (e.g. 20 or 50 ul) of CH_3CN to the digest.

2. *Incubate at 30^{o}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 50 ul (or enough volume to cover the gel pieces) of 1% formic acid to the gel pieces.* 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. Incubate for 20min.

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

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

7. *Add 150 ul 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.

9. *Speedvac to dry the peptides* in the tube at step 3 completely.

10. *Resuspend the (invisible) pellet with 1% formic acid*. To optimize solubilization of peptides, first add 10 ul of 5% formic acid, vortex, and then add 40 ul of dH_2O. Peptides should now be stored in the freezer.

11. For injection on the MS, thaw the peptides, spin hard to pellet any crap (e.g. 13,000g for 10 min) and transfer 15-20 ul to a crimp vial (Kinesis ref. 20413) with a lid. Store the rest in the freezer.