Tryptic or Chymotryptic Peptide Mapping Protocol

Procedure:

1. Immunoprecipitate the protein and run it on a preparative gel. Scale up your immunoprecipitation so that you have at least 500 cpm. Transfer the protein to nitrocellulose, Immobilon or Nylon according to Kamps's Western Protocol. Tryptic digestion can be done with either NC, Nylon, or Immobilon. Nitrocellulose seems to give more reproducible yields of peptides.

   Transfer of the protein to a filter is much superior to homogenization of the prep gel and elution. Although some people have concern about inefficient transfer of large proteins and loss of hydrophobic peptides on the filters, we have had good success with essentially every protein we have studied.
   As a general principle, it is valuable to work fast and keep the filter damp. It is generally thought that the longer the protein remains bound to the filter, the lower the yield.

2. Rinse blots with water after transfer. Wrap the membrane in Saran wrap to keep the membrane wet during exposure. Try to keep the membrane damp throughout the procedure. If it dries out, you can rewet NC with water, or Immobilon with methanol and then water, but your recovery will probably go down.

   After exposure, cut out the membrane piece of interest and soak it immediately in 0.5% PVP-360 (Polyvinylpyrrolidone, MW360) in 100mM acetic acid for 30 min. at 37°C. If you don't do this, your yield will be poor. This may result from the protease adsorbing onto the filter.

3. Aspirate the liquid. Wash the membrane extensively with H_2O (5 X 1ml). Then wash with freshly-made 0.05 M NH_4HCO_3 once or twice.

4. Digestion: Add 150 -200 µl fresh 0.05 M NH_4HCO_3 (enough to cover the piece of membrane). Start the digestion by adding 10 µg trypsin or chymotrypsin (Stock is 1 mg/ml in 0.05 M NH_4HCO_3 stored at -70°C). Mix by pipetting up and down and incubate at 37°C for 2 hr.

5. Vortex the sample and add another 10 micrograms of enzyme. Incubate for 2 hr at 37 °C.

6. Add 300 µl (or more) H_2O to each sample and spin in microfuge for 5 minute. Transfer the supe to new tube or remove the membrane. Dry on the speed vac. It takes about 3-4 hours.

7. Oxidation: The phosphorylated tryptic peptides of p56lck do not contain Met or Cys and oxidation is therefore not necessary. If the peptides you're examining contain Met or Cys, or if you don't know, you should oxidize them with performic acid.

    To make performic acid, mix 1 vol. fresh 30% H2O2 with 9 vol. 98 %-100 % formic acid. Incubate the mixture at room temp. for 1 hr to allow performic acid to form. Cool on ice. The newly-made performic acid can be stored on ice for several hours before use.

    Resuspend the dried peptides in 50 µl performic acid by vortexing or pipetting up and down. Incubate on ice for 2hr. (1hr is probably enough. Just be consistent).

8. Add 1ml H_2O to the tube. Dry on Speed-vac. Count samples

9. Resuspend the sample in 10 µl H_2O for an 8.9 map (or appropriate electrophoresis buffer if you're using another buffer for electrophoresis). Load 1 to 2.5 µl on the 0.1 mm thick, 20 cm x 20 cm cellulose thin layer plate from EM. The amount to load depends on how many counts you have, but you should not load more than 30 % of the sample. More than that will cause the sample to smear in the electrophoretic dimension.

10. The location of the origin depends on the pH of the electrophoresis buffer. Peptides are generally positively charged at pH 1.9 or 4.72 and the origin is therefore displaced toward the positive electrode when using these buffers. As diagrammed above, the origin for pH 1.9 or pH 4.72 is 5 cm from the left (positive) edge and 2.5 cm up from the bottom.

    The charge of phosphopeptides at pH 8.9 is less predictable and the origin for these maps is usually placed in the middle of the plate, 2.5 cm up from the bottom.
    For phosphorylated Lck, run pH 8.9 map at 1KV for 27 min. in the 1st dimension.

11. It is important to run marker dyes. The green dye is a 1:1 mixture of Xylene cyanol FF and epsilon-DNP lysine in pH 4.72 buffer. It should be spotted in a position equivalent to that of the sample, but in a portion of the plate that will not contain peptides.

12. After electrophoresis, air dry the plate. It is thought that drying in the oven will cook the peptides irreversibly to the cellulose.

13. Then carry out chromatography. Here too you should use the green marker dye. Spot it off to the side, the same

distance up from the bottom as the sample.

For phosphorylated Lck, do chromatography in Phosphochromo buffer. Recently, chromatography has taken as long as 12 hours. It seems to vary from batch to batch of plates. All you can do is watch the plates and pull them all when the first has its buffer front approximately 1 cm from the top.

14. Then mark the plate with radioactive ink. It is useful to put marks on that can be used to figure out where the origin was and where the marker dyes ran. Don't mark the origin itself. Usually, 3-4 days exposure with a screen and flashed film is necessary to detect samples containing 100-200 cpm of ^{32}P.

Recipes for peptide mapping.

It's sensible to make 2L of the electrophoresis buffers. The volume of chromatography buffer is dictated by the size of your tanks. You need enough to give a depth of approximately 0.5 cm in the bottom of the tank

pH 4.72 buffer. For 2 liters

n-Butanol	100 ml
Pyridine	50 ml
Acetic acid	50 ml
H_2O	1800 ml

The pH should be adjusted to 4.72 using either acetic acid or pyridine. Note that the recipe in Wade's paper is wrong.

pH 8.9 buffer (1% Ammonium carbonate)

Ammonium carbonate	20 g
H_2O	2 L

The pH should be adjusted to 8.90 with either NH_4OH or CO_2

pH 1.9 buffer. For 2 liters

88 % Formic acid	50 ml
Acetic acid	156 ml
H_2O	1794 ml

Don't use the 98% formic acid and don't adjust the pH.

Regular chromatography buffer.

n-Butanol	97 volumes
Pyridine	75 volumes
Acetic acid	15 volumes
H_2O	60 volumes

Phosphopeptide chromatography buffer.

n-Butanol	75 volumes
Pyridine	50 volumes
Acetic acid	15 volumes
H_2O	60 volumes

If you want to read or cite the papers describing this technique, the appropriate references are:

Gibson, W. (1974) Polyoma virus proteins: a description of the structural proteins of the virion based on polyacrylamide gel electrophoresis and peptide analysis.
Virology. **62**(2):319-36.

Beemon, K., and Hunter, T. (1978) Characterization of Rous sarcoma virus src gene products synthesized in vitro.
J Virol.**28**(2):551-66. [abstract]

Luo, K., Hurley, T. R., and Sefton, B. M. (1990) Transfer of proteins to membranes facilitates both cyanogen bromide cleavage and two-dimensional proteolytic mapping.
Oncogene **5**(6):921-3.