



Phosphoamino acid analysis (Mark Kamps's method)

1. Label your protein with ^{32}P i. Then subject the protein to SDS polyacrylamide gel electrophoresis and transfer your gel-fractionated protein to Immobilon-P.

Neither nitrocellulose nor nylon will work! Keep the membrane wet and wrap in Saran wrap. Add radioactive markings for orientation of film later. Expose to film.

2. Cut out band of interest, re-wet in methanol, rinse well in water and place in a screw-cap tube containing 150 μl 5.7 N HCl or enough to submerge your piece of membrane.
3. Incubate in 110°C oven for 60 min.
4. Microfuge sample full speed for at least 1 min.
5. Transfer supernatant to new tube and lyophilize on Speedivac. (It takes about 3 hr.)
6. Resuspend in H_2O , and microfuge 5 min. Spotting more than 3 μl is tedious, so don't use much H_2O . On the other hand, I wouldn't use much less than 6 μl so as to have good recovery.
7. Spot 1 μl PAA standards (about 0.3 μg each of P Ser , P Thr , and P Tyr) on a cellulose thin layer chromatography plate and then spot your sample. We use 0.1 mm EM cellulose plates

You can spot the whole sample if you are skillful and have no choice because you don't have many counts.

Spot 0.25-0.30 μl at a time and dry with house air, blown through plugged pipet, between applications. How do I wet the plate? We use a blotter that has four, 2 cm, circular holes cut out of it, one for each origin. We cut the holes with a sharp cork borer. The blotter can be made from a good grade of blotting paper, or two layers of Whatmann 3MM sewn together. It should be wet, but not dripping. For the first dimension, you wet the plate with pH 1.9 buffer.

8. Electrophorese at pH 1.9, 1.5 KV, 20 min in the first dimension.

9. Let plate air dry well.

How do I wet the plate for the second dimension? Use three rectangular pieces of Whatmann 3MM that have been wet with pH 3.5 buffer. Wet the bottom of the plate below the lower two origins. Keep the paper at least 1 cm away from where the PAAs are. Then wet the area between the 4 origins. Finally wet the top of the plate. The blotters should be quite damp, but not soaking wet.

10. Rotate 90° counter clockwise. Electrophorese at pH 3.5, 1.6 KV, 13 min in the second dimension.

The above electrophoresis times are appropriate when you are using a Salk/TVL/MBVL type electrophoresis rig. A knock off of this is sold by CBS scientific. If you are using another rig, you will have to optimize your electrophoresis times.

11. Dry plate in oven.

12. Spray plate with ninhydrin soln for several seconds. Incubate in oven until can see purple spots of PAA standards. 15 min should be plenty.

13. Mark the plate with radioactive ink so that you can extrapolate where the origins were and can align the film unambigously with the plate and the PAA markers.

14. Expose the plate to flashed film with a screen a -70°C. Film is much preferable to the phosphorimager because it is transparent and is exactly the same size as the plate and this facilitates alignment of the film and the plate. In the rare case that you need quantification, you can use the phosphorimager.

15. After developing the film, trace the locations of the PAA standards and the radioactive ink marks onto a Xerox transpency and save this in your notebook

Recipes: In general, you will need 2 liters of each buffer.

pH 1.9 buffer.

88% Formic acid	50 ml
Acetic acid	156 ml
H ₂ O	1794 ml

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

pH 3.5 buffer

Pyridine	10 ml
Acetic acid	100 ml
100 mM EDTA	10 ml
H ₂ O	1880 ml

Don't bother to adjust the pH.

* There have been occasional problems with badly smeared PAAs during electrophoresis at pH 3.5. Something seems to leach out of the wicks and make the PAAs relatively insoluble. Tony thinks it is aluminum. Bart thinks that it's calcium. In any case, this problem can be prevented by including 0.5 mM EDTA in the 3.5 buffer.

** This two dimensional technique was originally developed, in large part by Tony Hunter, for analysis of cell extracts or proteins eluted from ground up gels. Like Bill Welch before him, Mark Kamps tired of grinding up gel pieces and found that you could hydrolyze proteins bound to filters.

*** If you want to refer to the original references for this technique, cite:

Hunter, T. R., and Sefton, B. M. (1980) Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. *Proc Natl Acad Sci U S A* 1980 **77**:1311-1315.

Kamps, M. P., and Sefton, B. M. (1989) Acid and base hydrolysis of phosphoproteins bound to immobilon facilitates analysis of phosphoamino acids in gel-fractionated proteins. *Anal Biochem* **176**:22-7