PhosphoProtein Purification Protocol

Important notes before starting

- For most downstream applications, concentration of the separated protein fractions is required. The PhosphoProtein Purification Kit contains six Nanosep ultrafiltration devices that enable easy buffer exchange and concentration of dilute protein samples. The ultrafiltration columns have a molecular weight cut off (MWCO) of 10 kDa. It is recommended that the most concentrated phosphorylated protein fraction (usually the third elution fraction) be concentrated by a factor of 10 for SDS-PAGE and subsequent Coomassie® staining or western blot analysis (i.e., the 500 µl elution volume should be reduced to a volume of 50 µl). For SDS-PAGE followed by silver staining, the eluates can be used without concentration.
- For 2-D gel analysis, both phosphorylated and unphosphorylated protein fractions must be concentrated and desalted. This can be achieved by reducing the volume of the eluate fraction to 50 µl by ultrafiltration; adding 450 µl 10 mM Tris·Cl, pH 7.0; reducing the volume to 50 µl once more by ultrafiltration; adding a further 450 µl 10 mM Tris·Cl, pH 7.0; and reducing the volume for a third time by ultrafiltration. The concentrated, desalted protein fraction can then be taken up in 2-D gel loading buffer and loaded onto the gel.
- Although lysis is carried out at 4°C the protein purification procedure is performed at room temperature. A protease inhibitor mix prevents proteolytic degradation of the proteins. Under the lysis buffer conditions used, no phosphatase activity is measurable, ensuring that the phosphorylated protein fraction that binds to the PhosphoProtein Purification Column accurately reflects the in vivo phosphorylation profile. Performing the purification at 4°C is not recommended as this significantly reduces the yield, and results in incomplete purification of phosphorylated proteins.

Procedure

1. **Add 875 µl CHAPS Stock Solution (10% [w/v]) to 35 ml of PhosphoProtein Lysis Buffer and 75 µl CHAPS Stock Solution (10% [w/v]) to 3 ml of PhosphoProtein Elution Buffer to yield a final concentration of 0.25 % (w/v) CHAPS in both buffers.**
2. **Add 1 Protease inhibitor tablet and 10 µl of Benzonase stock solution to a 5 ml aliquot of PhosphoProtein Lysis Buffer containing CHAPS prepared in step 1. Mix.**
3. **By gentle pipetting, resuspend a cell pellet corresponding to 10^7 cells in the 5 ml of lysis buffer containing protease inhibitors and Benzonase prepared in step 2.**
4. **Incubate for 30 minutes at 4°C. Vortex briefly every 10 minutes.**
5. **After 30 minutes incubation, centrifuge the cell lysate at 13,000 rpm and 4°C for 30 minutes.**

Protocol

6. **During centrifugation, prepare a PhosphoProtein Purification Column by detaching the top cap, breaking of the bottom closure, and allowing the storage buffer to flow out. Apply 4 ml PhosphoProtein Lysis Buffer containing 0.25% CHAPS (prepared in step 1) to equilibrate the column and allow the buffer to flow out.**
7. **Harvest the supernatant and determine the protein concentration.***
8. **Take a volume of lysate containing approximately 2.5 mg of total protein, and adjust the protein concentration to 0.1 mg/ml by adding PhosphoProtein Lysis Buffer containing 0.25% CHAPS. This will yield a final volume of 25 ml of lysate.**
9. **Pour half the lysate (12.5 ml) into the upper reservoir of the PhosphoProtein Purification Column. When almost all the lysate has entered the gel bed, add the second half of the lysate and allow to pass through the column. This step will take approximately 50 minutes and allows enough time for complete binding of any phosphorylated protein to the affinity column. Collect the flow-through fraction if analysis of unphosphorylated proteins in the lysate is desired.**

   Note: This and all subsequent steps should be performed at room temperature.
10. **Apply 6 ml of PhosphoProtein Lysis Buffer containing 0.25% CHAPS to wash the column, and allow to flow through.**
11. **Apply 500 µl PhosphoProtein Elution Buffer containing 0.25% CHAPS to the column, and collect the eluted fraction.**
12. **Repeat step 11 four times. The highest concentrations of phosphorylated proteins will be found in elution fractions 3 and 4.**
13. **Determine protein concentration in all eluate fractions (e.g., using the Bradford method) to determine the most concentrated fraction.**

* *Protein concentration can be quickly and easily determined using the Bradford method. Bradford assay dye reagent is available commercially, e.g., Bio-Rad Protein Assay Dye Reagent Concentrate, cat. no. 500-0006.*