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INSTRUCTIONS

PhosPro™
Phosphoprotein enrichment kit

Product Number **P 5012**
Store at RT

This phosphoprotein enrichment and exclusion of unphosphorylated proteins provides advanced chance in detecting protein phosphorylation in gels with non-radiolabeling method(eg. Staining with fluorescence dye) and enables quantitative comparison between cells.

INTRODUCTION

Protein phosphorylation is one of the most frequently occurred posttranslational modifications and plays a critical role in cellular regulatory events. Most cellular processes are in fact regulated by the reversible phosphorylation of proteins on serine, threonine and tyrosine residues. In fact, phosphorylation of proteins plays a key role in oncogenesis, cell signaling, apoptosis and immune disorders[1]. Despite the importance and widespread occurrence of this modification, profiling of phosphoproteins in cells is still a challenge, due to the low copy of phosphorylated proteins in cell and the relative amount of phosphoproteins compared to unphosphorylated proteins.

Radiolabeling by ^{32}P labeling is frequently used conventional method for investigation of phosphoprotein profile in conjunction with 2-DE or 1-D gel electrophoresis and autoradiogram. Alternatively, western blot analysis probed by phosphoprotein-specific antibody is also used for this purpose.
Mass spectrometry has been shown to be a reliable and routine tool to identify proteins in a high throughput manner. However, the identification of phosphorylation by mass spectrometry is not a trivial matter and to this day is not routine also due to the low copy of phosphorylated proteins in cells.

Kit contents

	10 reactions
LYSIS BUFFER	10ml x 1
DILUTION BUFFER	30ml x 1
Native Homogenation Buffer	30ml x 1
SOLUTION A	3ml x 1
SOLUTION B	5ml x 1
DISSOLVING SOLUTION	8ml x 1
DELIPIDATION SOLUTION	10ml x 1

Additional Materials Required

- Methanol
- Ultrapure water

Detecting Phosphorylated Proteins

This phosphoprotein enrichment kit was optimized for the protein solution in denatured condition, for example, the samples prepared for 2-DE and can be applied to native proteins. Enriched phosphorylated proteins could be detected by staining commercially available staining method using fluorescent dye[2] or by probing with antibodies, specific for phosphorylated proteins.

Procedure Summary

1. Protein extraction
2. Phosphoprotein specific complex forming
3. Precipitation of phosphoprotein complex
4. Dedelipidation and recovering of phosphoprotein

Procedure for phosphoprotein enrichment from cell lysate
(Denatured protein condition)

1. Add 300~600μl LYSIS SOLUTION to the cells or tissue and disrupt the cells and tissue by sonication or motor driven homogenation. (Adjust the volume of LYSIS SOLUTION in order the final concentration of extracted protein to be above 4mg/m) Vortex the cell lysate for 15min and centrifuge for 20min. at 12,000 x g and save the supernatant. Assay the protein concentration and dilute 2mg protein with DILUTION SOLUTION to be the final volume of 3ml. (Use 5ml tube)

Alternatively, protein solution prepared for two-dimensional gel electrophoresis could be directly used for enrichment by appropriately diluted with DILUTION SOLUTION.

2. Add 240μl of SOLUTION A and rapidly mix by vortex vigorously for a few seconds then incubate for 15min.by inverting or gentle vortexing. After subsequent adding 360μl SOLUTION B and brief mixing, incubate for 15min. by gentle vortexing then stand still for 5~10 min. for the aggregated materials to be settled down. And discard about 4ml of upper clear solution.

3. Transfer the remaining aggregate suspension to 1.5ml microcentrifuge tube and centrifuge the suspension at 12,000rpm for 5min.. Discard the supernatant and save the aggregate in hard pellet.

This aggregate can be stored for several days.

4. Add 0.7ml DISSOLVING SOLUTION and dissolve the pellet by pipetting several times(*caution : at this time CO_2 gas will be formed. Open lid and degas sufficiently before vortexing) and vortex for 5 min.

5. Add 750 μl of delipidation soln. (methanol:chloroform=600:150) and vortex vigorously for 5 min and centrifuge at 12,000rpm for 10 min for phase separation of solution. Recover the middle phase protein disk and discard lower and upper phase solution completely. Then wash the protein disk with sufficient (~1ml) methanol for two times.

6. Dry the protein pellet in air or oven completely and dissolve the protein pellet with the solution for 2-DE electrophoresis or 1-D SDS PAGE.

Alternative procedure for phosphoprotein enrichment from cell lysate
(Native protein condition)

If you want to isolate the phosphorylated protein from cell lysate in native conformation of proteins, omit step 1 using LYSIS SOLUTION. Instead, prepare the cell lysate with Native Homogenation Buffer or appropriate buffer solution except solution including phosphate.

1. Add 1~3ml Native Homogenation Buffer to the cells or tissue and disrupt the cells and tissue by sonication or motor driven homogenation in order the final concentration of extracted protein to be 2~30mg/ml. Centrifuge for 20min. at 12,000 x g and save the supernatant. (Use1 or 5ml tube)

2. Add 80μl of SOLUTION A per 1ml protein solution and mix gently for 15min by inverting or gentle vortexing. After subsequent adding 120μl SOLUTION B per 1ml original solution, incubate for 15min by gentle inverting then centrifuge briefly for 5~10 min for the aggregated materials to be settled down. And discard upper clear solution.

3. Add one fourth volume of Native Homogenation Buffer to the pelletted phosphoprotein complex and wash the residual non-phosphorylated protein solution by resuspension the pellet and recover the pellet

by brief centrifugation. Repeat this washing one time and save the aggregate in hard pellet.

4. Add 0.7ml DISSOLVING SOLUTION and dissolve the pellet by pipetting several times(*caution : at this time CO_2 gas will be formed. Open lid and degas sufficiently) and stand for 10 min. the solution to be clear.

This solution contains enriched phosphoprotein in 250mM salt solution containing EDTA. Dialysis the enriched phosphoprotein solution with appropriate buffer solution.

Or skip to the next step for preparation of 1D or 2D electrophoresis samples.

5. Add 750 μl of delipidation soln. (methanol:chloroform=600:150) and vortex vigorously for 5 min and centrifuge at 12,000rpm for 10 min for phase separation of solution. Recover the middle phase protein disk and discard lower and upper phase solution completely. Then wash the protein disk with sufficient (~1ml) methanol for two times.

6. Dry the protein pellet in air or oven completely and dissolve the protein pellet with the solution for 2-DE electrophoresis or 1-D SDS PAGE.

Optimization of Results

When you start with cell lysate at higher concentration and smaller volume of proteins, use SOLUTION A and SOLUTION B, 80μl and 120μl per 1ml protein solution respectively.

References

1. Philip Cohen, *Eur. J. Biochem*., 568*,* 2001-2010 (2001)

2. Alein, L. *et al*. Proteomics, 6, 2157–2173 (2006).

Related Products Product Code

Phospep Phosphopeptide enrichment kit