Purification of Phosphorylated Peptide using Phos binding reagent (Phosbind) Biotin LC & Streptavidin-Agarose

1. Additional Materials Required

Zinc(II) Acetate Solution	0.50 mM Zn(CH3COO)2 containing 5.0 mM Tris-acetate (pH 7.4)
Tris acetate Buffer	5.0 mM Tris-acetate solution (pH 7.4)
Balancing Buffer (pH 7.4)	5.0 mM Tris-acetate containing 10 μM Zn(CH3COO)2
Washing Buffer (pH 7.4)	5.0 mM Tris-acetate containing 0.50 M NaNO3
Elution Buffer (optional)	1.0 mM NaH2PO4-NaOH (pH 7.0) containing 0.50 M NaNO3
Centrifugal filtration units	Membrane pore size: 0.22 ~ 0.45 μm Sample volume: 0.40 ~ 0.50 mL
Micropipette for 0.30 mL	
Centrifuge for 2,000×g	
Streptavidine-agarose (Sigma-Aldrich Fine Chemicals)	

2、Sample Preparation

1) Competing anions (e.g., inorganic phosphate, thiolate) and metal chelating agents (e.g., EDTA) should be removed from the sample as much as possible.

Note: The protease inhibitors except of thiol compounds are not effective on the purification. The large amount of surfactant (e.g., SDS) lowers the efficiency of the purification.

2) The sample is dissolved in the 5.0 mM Tris-acetate buffer (pH 7.4, 0.30 mL) at room temperature.

3) The final concentration of phosphorylated compounds in the sample solution (0.3 mL) should be below 15 nmol/mL.

3、Purification Procedure

1) Apply ca. 0.3 mL of Streptavidine Agarose (in suspension) in the sample reservoir of the centrifugal filtration unit.

2) Centrifuge the unit at 2,000×g for 15 sec and the filtrate is discarded.

3) Apply 0.30 mL of the 5.0 mM Tris-acetate buffer in the sample reservoir. Centrifuge the unit at 2,000×g for 15 sec and the filtrate is discarded. (The operations are repeated 5 times).

4) Apply 0.30 mL of 0.12 mM Phosbind Biotin LC containing 0.50 mM Zn(CH3COO)2 and 5.0 mM Tris-acetate (pH 7.4) in the sample reservoir.

5) Equilibrate for 5 min.

6) Centrifuge the unit at 2,000×g for 15 sec and the filtrate is discarded.

7) Apply 0.30 mL of the 5.0 mM Tris-acetate buffer in the sample reservoir. Centrifuge the unit at 2,000×g for 15 sec and the filtrate is discarded. (The operations are repeated 5 times).

8) Apply 0.30 mL of 10 μM Zn(CH3COO)2 containing 5.0 mM Tris-acetate (pH 7.4) in the sample reservoir.

9) Centrifuge the unit at 2,000×g for 15 sec and the filtrate is discarded.

10) Add 0.3 mL of the sample solution into the sample reservoir.

11) Equilibrate for 5 min in order to sufficiently bind phosphorylated compounds to Phosbind Biotin LC.

12) Centrifuge the unit at 2,000×g for 15 sec. The filtrate contains non-phosphorylated compounds.

13) Add 0.30 mL of the washing buffer into the sample reservoir. Centrifuge the unit at 2,000×g for 15 sec. The filtrate contains non-phosphorylated compounds. (The operations are repeated 3 times).

14) Add 0.30 mL of the elution buffer into the sample reservoir. Centrifuge the unit at 2,000×g for 15 sec and the filtrate contains phosphorylated compounds. (The operations are repeated 2~4 times)

15) Analyze the obtained solutions containing phosphorylated compounds.