VISUAL PROTEIN

Manual

PhosPRO™ Phosphoprotein Purification Kit

PP05-2C/PP05-6C/PP05-5E

V2.0

Store at 2-8 °C
For Research Use Only

Introduction

PhosPRO™ Phosphoprotein Purification Kit is a new version of immobilized metal affinity chromatography (IMAC) based purification kit. With our proprietary chromatographic technology, **PhosPRO™ Phosphoprotein Purification Kit** delivers excellent efficacy of phosphoprotein purification, as compared with other commercial kit. Almost none of non-phosphoproteins are copurified in the elution fraction. **PhosPRO™ Phosphoprotein Purification Kit** has been successfully demonstrated to purify phosphoproteins from different biological materials, such as animals, plants, or micro-organisms and to apply in 2-DE.

Product Components

PhosPRO™ Phosphoprotein Purification Kit (PP05-2C)

PhosPRO™ Resin Pre-packed Column	0.5 mL	2 columns
Lysis Buffer	2 mL	1 bottle
System Buffer	250 mL	1 bottle
Elution Buffer	100 mL	1 bottle
Stringent Buffer Stock	100 mL	1 bottle
User's manual		

PhosPRO™ Phosphoprotein Purification Kit (PP05-6C)

PhosPRO™ Resin Pre-packed Column	0.5 mL	6 columns
Lysis Buffer	2 mL	1 bottle
System Buffer	250 mL	1 bottle
Elution Buffer	100 mL	1 bottle
Stringent Buffer Stock	100 mL	1 bottle
User's manual		

PhosPRO™ Phosphoprotein Purification Kit (PP05-5E)

PhosPRO™ Resin	3 mL	1 bottle
Lysis Buffer	2 mL	1 bottle
System Buffer	250 mL	1 bottle
Elution Buffer	100 mL	1 bottle
Stringent Buffer Stock	100 mL	1 bottle
User's manual		

Safety Information

Please wear gloves, lab coat and goggles while operating. Prevent contact product directly. In case of contacting, wash with large amount of water.

Storage

PhosPRO™ Phosphoprotein Purification Kit should be stored at 2-8 °C. Expiration date is labeled on the bottle or box.

Materials needed but not provided

1. Column clamps and stands
2. 1.5 mL microcentrifuge tubes
3. PBS buffer: 10 mM KH_2PO_4, 150 mM NaCl, pH 7.4

Instruction

A. Sample preparation

1. Collect cells ($5x10^7$, T75 flask) by centrifugation at 250 x g for 5 minutes. For adherent cells, scrape cells in PBS and then spin down (3,000 rpm for 5 minutes) to pellet cells.
2. Wash cells once with 5 mL of PBS and repeat this step 2 times.
3. According to sample volume, add into 2-3 folds volume of Lysis Buffer.
4. Extract total protein by freeze and thaw (repeat 3 times), then centrifugate and harvest the liquid phase.
5. The protein concentration of sample should be adjusted approximately to 5 mg/mL. (The extracted sample can be stored at -20 °C for months without significant decreasing of phosphorylation status of proteins.)
6. Mix 0.4 mL extracted proteins with 3.6 mL System Buffer. Around 2 mg of total extracted proteins with 4 mL volume are now ready to purify.

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NOTE: The capacity of PhosPRO™ Resin might depend on the content of phosphoproteins in individual sample. Users have to adjust the amount of loading proteins according to their specific needs. Trial experiments may be necessary before purification. **PhosPRO™ Phosphoprotein Purification Kit (PP05-5E)** is suggested for evaluation and optimization.

B. Phosphoprotein Purification

- **For PP05-2C/6C**

1. Install a pre-pack column onto a table stand and wash the column with 5 mL ddH_2O.
2. Equilibrate the column with 5 mL System Buffer before loading sample.
3. Load 4 mL of above sample onto the top of the column. Collect the flow through.
4. Recharge the flow through to the resin for 3 more times for maximizing the binding of phosphoproteins.
5. At the last round of sample loading, collect the 4 mL flow through into four centrifuge tubes (1 mL/tube). These unbound fractions should contain approximately 50-90% of the proteins of the loading sample. Label the tubes as N1-N4 respectively.
6. Wash the column with 5 mL System Buffer. Collect the first 2 mL into two centrifuge tubes and label as N5 and N6. discard the rest of wash waste.
7. Wash the column with 5 mL ddH_2O and repeat once.
8. Elute the bound phosphoproteins with 2-4 mL Elution Buffer. Collect the eluants into two centrifuge tubes (1 mL/tube). Label the tubes as B1-B4 respectively.
9. Evaluate above samples as described in section D1.

- **For PP05-5E**

1. Aliquot 50 µL of PhosPRO™ Resin in 1.5 mL centrifuge tube.
2. Wash the resin with 0.5 mL ddH_2O and discard the wash waste by 1,500 rpm of centrifugation for 1 minute.
3. Equilibrate the resin with 0.5 mL System Buffer by rocking for 1 minute, then remove the System Buffer and drain the resin by 1,500 rpm of centrifugation for 1 minute.
4. Load proper amount of samples in System Buffer to the resin. Depending on the content of phosphoproteins in different samples; 50-500 µL of prepared samples (e.g. 100-200 µg of proteins) from section A is recommended.
5. Rock samples with the resin at room temperature for 20 minutes.
6. Separate the resin and the supernatant by 1,500 rpm centrifugation for 30 seconds, aliquot the supernatant and label as N1.
7. Thoroughly wash the resin by 1 mL System Buffer for tive times.
8. Wash the resin by 1 mL ddH_2O for three times.
9. Add 0.2 mL Elution Buffer to the resin and vigorously rock for 3 minutes.
10. Separate the resin and the eluant by 1,500 rpm centrifugation for 30 seconds, aliquot the

B. Phosphoprotein Purification (~continued)

eluant and label as B1.

11. Repeat step 9 and 10 twice. Another two eluants will be collected in tube B2 and B3.
12. Evaluate above samples as described in section D1.

C. Optimization the chromatographic condition (to reduce the binding of non-phosphor-proteins)

C1. Preparation of stringent washing buffer

1. Make different stringent washing buffer by mixing System Buffer with Stringent Buffer Stock as following instructions.

Stringent washing buffer	S1	S2	S3	S4	S5
System Buffer (mL)	4	3	2	1	0
Stringent Buffer Stock (mL)	1	2	3	4	5
Total volume (mL)	5	5	5	5	5

C2. Evaluatation for washing stringency

- **For PP05-2C/6C**

1. Column installation and sample loading procedures are identical as described in Section B (for PP05-2C/6C), step 1-5. Labeled the unbound flow through in tubes as N1-N4.
2. Wash the column with 5 mL System Buffer and discard the rest of wash waste.
3. Wash the column with 5 mL S1 buffer. Collect the first 1 mL into a centrifuge tube and label as S1. Discard the rest of wash sample.
4. Replace previous buffer with another stringent washing buffer (S2-S5) and repeat step 3.
5. Wash the column with 5 mL of ddH_2O and repeat once.
6. Elute the bound phosphoproteins with 2-4 mL Elution Buffer. Collect the eluants into two centrifuge tubes (1 mL / tube). Label the tubes as B1-B4 respectively.
7. Evaluate above samples as described in section D1.

- **For PP05-5E**

1. Resin preparation and sample loading procedures are identical as described in Section B (for PP05-5E), step 1-6. Labeled the unbound flow through in tubes as N1.
2. Thoroughly wash the resin by 1 mL System Buffer for five times.
3. Wash the column with 0.2 mL S1 buffer. Collect the supernatant and label as S1.
4. Replace previous buffer with another stringent washing buffer (S2-S5) and repeat step 3.
5. Wash the column with 1 mL of ddH_2O for three times.
6. Add 0.2 mL Elution Buffer to the resin and vigorously rock for 3 minutes.
7. Separate the resin and the eluant by 1,500 rpm centrifugation for 30 seconds, aliquot the eluant and label as B1.
8. Repeat step 9 and 10 twice. Another two eluants will be collected in tube B2 and B3.
9. Evaluate above samples as described in section D1.

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NOTE: Find the best stringent buffer from S1 to S5 that removes most non-phosphoproteins without elutes phosphoproteins. This stringent washing buffer should be used as washing buffer in your further experiments.

D. Result evaluation

D1. Phosphoprotein stain on 1-D gel

1. Analyze N1-N4 and B1-B4 by SDS-PAGE. 10 µl of each sample should be sufficient for detection of phosphoproteins. Run protein standard marker containing phosphoprotein in parallel as positive control, such as the Low Molecular Weight Proteinmarker from GE Healthcare.
2. For phosphoprotein staining, Pro-Q™ Diamond phosphoprotein stain (Invitrogen) is suggested.
3. For total protein staining, **VisPRO™ 5 Minutes Protein Stain Kit (VP01-500)** or SYPRO™ Ruby stain (Invitrogen) are suggested.
4. It is possible to use anti-phosphptyrosine, anti-serine or anti-threonine antibody for immunological evaluation. However, due to the unexpected quality of available anti-serine or anti-threonine antibodies, the result of immunological evaluation by above antibodies should not be conclusive.

D2. Preparation of phosphoprotein containing fraction for 2-DE

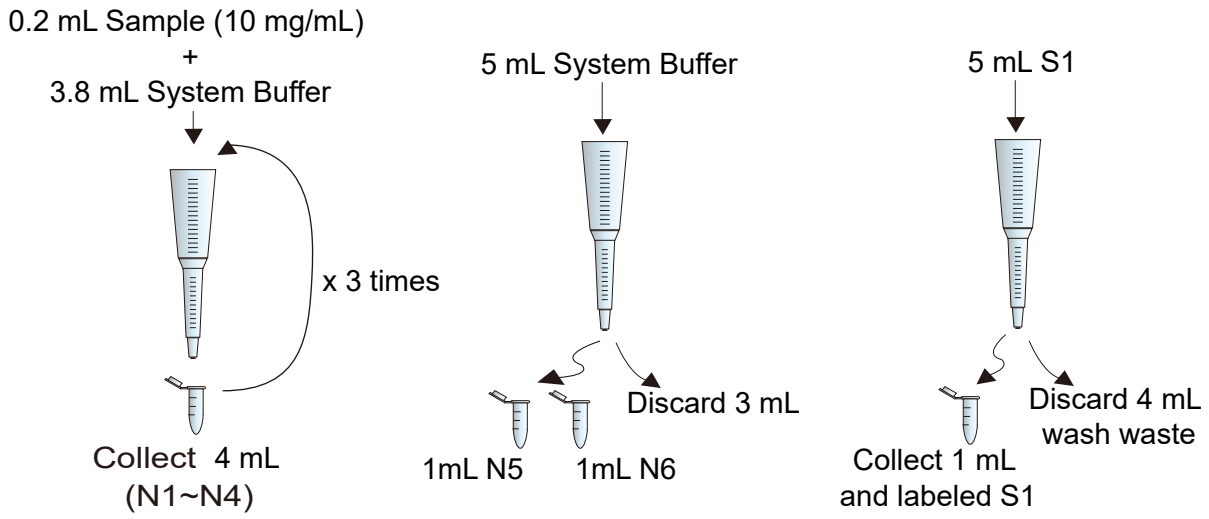
1. The phosphoprotein containing fractions (B1-B4) can be concentrated by proper methods, such as TCA protein precipitation. Acetone precipitation is not recommended.
2. Add proper amount 2-DE Lysis Buffer or rehydration buffer into the tube. Solublize the protein by sonication at 0 °C. The sample can be directly analyzed by 2-DE.

Troubleshooting

Problem	Possible cause	Remedy
No phosphoprotein is observed in all fractions	Endogenous phosphatases dephosphorylate phosphoproteins during the sample preparation	Including more pan/specific phosphatases inhibitors into the System Buffers
Significant amount of phosphoprotein is found in the flow through (N1-N4)	Phosphate or nucleic acid in the sample might prevent the binding of phosphoprotein to chromatographic media	Performing desalting or protein precipitation before diluting into System Buffer
	Sample might contain more phosphoprotein than expected	Reducing the amount of loading protein
Non-phosphoprotein is found in the bound fraction (B1-B4)	Some nonphosphoproteins might interact with the chromatographic media through functional groups other than phosphates	Utilizing the stringent buffer stock to optimize the washing condition as described
	Aging of the chromatographic media	Avoiding using the expired kits

Appendix

Figure 2. Optimization Condition Protocol For PhosPRO™ Phosphoprotein Purification Kit.



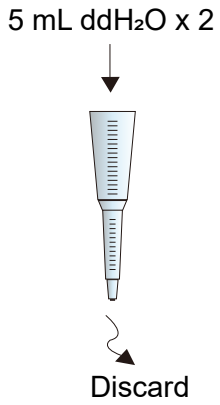
Repeat **STEP 3** with 5 mL

S2, S3, S4, S5

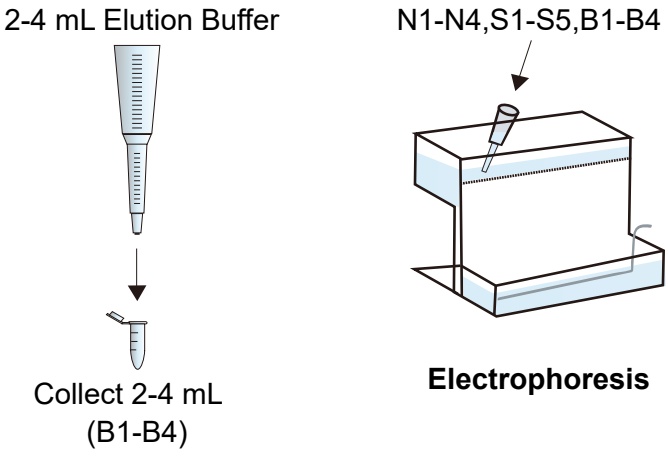
S2 1 mL | S3 1 mL | S4 1 mL | S5 1 mL

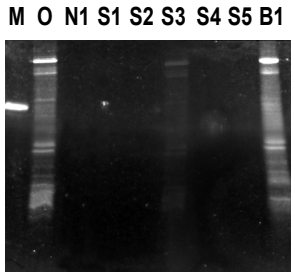
Stringent washing buffer	S1	S2	S3	S4	S5
System Buffer (mL)	4	3	2	1	0
Stringent Buffer Stock (mL)	1	2	3	4	5
Total volume (mL)	5	5	5	5	5

STEP 4



STEP 5





Pro-Q Diamond stained gel

S2 is the best stringent washing buffer for further experiments.S3 and S4 are too stringent and will elute phosphoprotein.

STEP 8

Energenesis Biomedical Co., Ltd.
6F.-3, No.21, Ln. 583, Ruiguang Rd., Neihu Dist., Taipei City 114, Taiwan
Website: www.visualprotein.com
Tel: +886-2-2627-0835