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PhosphoSeek™ Phosphoprotein Enrichment Kit

02/16

(Catalog # K1402-2, -6; Store at 4°C)

Introduction:

I. The investigation of protein phosphorylation is of key interest in current proteomics. It has been estimated that phosphoprotein accounts for 10-20% of total cellular proteins. Consequently, enrichment of phosphoproteins may increase 5-10 folds in abundance of the phosphoproteins, and thus facilitate the detection of some minor phosphoproteins. A number of strategies have been developed to separate phosphoproteins or phosphopeptides from non-phosphoproteins or non-phosphopeptide.

PhosphoSeek™ Phosphoprotein Purification Series is based on immobilized metal affinity chromatography (IMAC). It is thought that the phosphate groups on proteins or peptides interact with the metal ions immobilized to the chelating ligand, such as iminodiacetic acid (IDA) or nitrilotriacetic acid (NTA) on the chromatographic media. Employing metal ion, such as ferric ($Fe3^+$), gallium ($Ga3^+$) or zinc ($Zn3^+$) or other metal ions charged IMAC resin, numbers of studies have successfully enriched phosphoproteins or phosphopeptides from model proteins or biological samples. However, even though IMAC based method is one of the most convenient and cost effective method for enriching phosphoproteins; the practical utilization of it in proteomic study remains limited. Its major challenging would be the ubiquitous observation in the binding of non-phosphoproteins to the chromatographic media, which might reduce the enriching effect of phosphoproteins significantly. BioVision now provides a new version of IMAC based enrichment kit, PhosphoSeek™ Phosphoprotein Enrichment Kit, delivering excellent enrichment result for phosphoproteins.

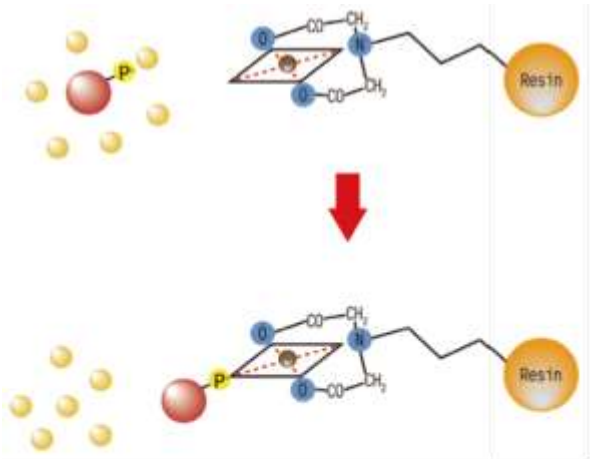


Figure 1. Strategy of immobilized metal affinity chromatography (IMAC)

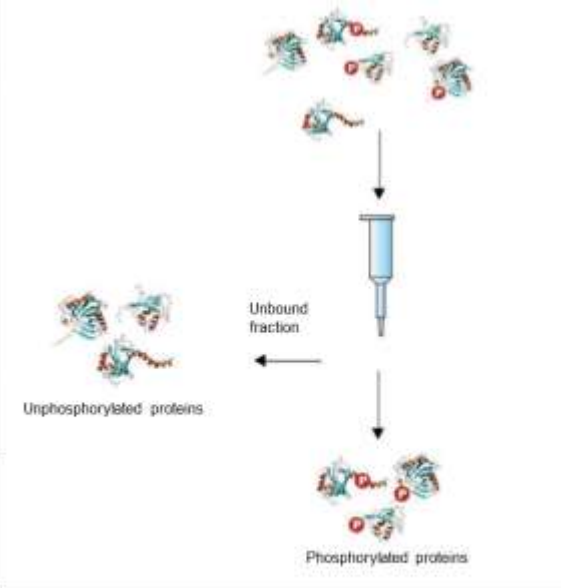


Figure 2. Phosphoprotein Purification procedure

II. Application:

PhosphoSeek™ Phosphoprotein Enrichment Kit is based on IMAC (immobilized metal affinity chromatography), can isolate the most amount of phosphoprotein from different biological materials, such as animal, plant and microorganism. Isolated phosphoprotein can be Ideal for use in many downstream applications, including mass spectrometry and 2D-PAGE.

III. Kit Contents:

Components	K1402-2	K1402-6	Part Number
	2 assays	6 assays	
Pre-packed column	2	6	K1402-(x)-1
Lysis buffer	2ml	2ml	K1402-(x)-2
System buffer	250ml	250ml	K1402-(x)-3
Elution buffer	100ml	100ml	K1402-(x)-4
Stringent buffer stock	100ml	100ml	K1402-(x)-5

IV. User Supplied Reagents and Equipment:

Column clamps and stands
1.5 ml centrifuge tubes
PBS buffer: 10 mM NaH2PO4, 130 mM NaCl, pH 7.0

V. Storage:

PhosphoSeek™ Phosphoprotein Purification Kit can be stored at 4°C for 12 months. Please perform the phosphoprotein purification before the kit expires. It has been found that nonspecific binding of non-phosphoproteins might increase for expired kit.

VI. PhosphoSeek™ Phosphoprotein Purification Protocol:

A. Sample Preparation:

1. Collect cells ($5x10^7$, T75 flask) by centrifugation at 600 g for 5 minutes at 4° C. 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. Repeat 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 centrifuge and collect the liquid phase.
5. The protein concentration of sample should be adjusted approximately to 10 mg/ml. (The extracted sample can be stored at -20° C for months without significant decreasing of phosphorylation status of proteins).
6. Mix 0.1 ml extracted proteins with 1.9 ml system buffer. Around 1 mg of total extracted proteins with 2 ml volume is now ready to enrich.

B. Phosphoprotein Purification:

1. Install a pre-pack column onto a table stand. All purification procedures can be carried out at room temperature.
2. Equilibrate the column with 5 ml system buffer before loading sample.
3. Load 2 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 2 ml flow through into two 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~N2 respectively.
6. Wash the column with 5 ml system buffer. Collect the first 2 ml into two centrifuge tubes and label as N3 and N4. Discard the rest of wash waste.
7. Perform additional wash with 5 ml system buffer.
8. Wash the column with 5 ml of distilled water.
9. Perform additional wash with 5 ml distilled water.
10. Elute the bound phosphoproteins with 2 ml elution buffer. Collect the eluents into two centrifuge tubes. Label the tubes as B1 and B2 respectively.
11. Perform another elution with 2 ml elution buffer. Collect and label the eluents as B3 and B4.
12. Evaluate above samples as described in section D1.

C. Optimization the chromatographic condition (to reduce the binding of non-phosphoproteins):

Due to the complexity of biological samples, it is possible to observe the binding of non-phosphoproteins in B1-B4 fraction. To reduce the binding of non-phosphoproteins, users might make more stringent washing buffers (S1-S5) from the provided stringent buffer stock for their specific needs.

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
Total volume (ml)	1	2	3	4	5
Stringent buffer stock (ml)	5	5	5	5	5

C2. Evaluation for washing stringency:

1. Column installation and sample loading procedures are identical as described in Section B, step 1-4. Labeled the unbound flow through in tubes as N1 and N2.
2. Wash the column with 5 ml system buffer. Collect the first 2 ml into two centrifuge tubes and label as N3 and N4. Discard the rest of wash waste.
3. Perform additional wash with 5 ml system buffer.
4. Wash the column with 5 ml S1 buffer. Collect the first 2 ml into centrifuge tube and label as S1. Discard the rest of wash sample.
5. Wash the column with 5 ml S2 buffer. Collect the first 2 ml into a centrifuge tube and label as S2. Discard the rest of wash sample.

6. Wash the column with 5 ml S3 buffer. Collect the first 2 ml into centrifuge tube and label as S3. Discard the rest of wash sample.
7. Wash the column with 5 ml S4 buffer. Collect the first 2 ml into centrifuge tube and label as S4. Discard the rest of wash sample.
8. Wash the column with 5 ml S5 buffer. Collect the first 2 ml into centrifuge tube and label as S5. Discard the rest of wash sample.
9. Wash the column with 5 ml of distilled water.
10. Perform additional wash with 5 ml distilled water.
11. Elute the bound phosphoproteins with 2 ml elution buffer. Collect the eluents into two centrifuge tubes (1 ml / tube). Label the tubes as B1 and B2 respectively.
12. Perform another elution with 2 ml elution buffer. Collect and label the eluents as B3 and B4.
13. Evaluate above samples as described in section D1.
14. 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 is sufficient for detection of phosphoproteins. Run protein standard marker (Low Molecular Weight Protein) containing phosphoprotein in parallel as positive control

D2. Preparation of phosphoprotein containing fraction (B1-B4) 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. Solubilize the protein by sonication at 0°C. The sample can be directly analyzed by 2-DE.

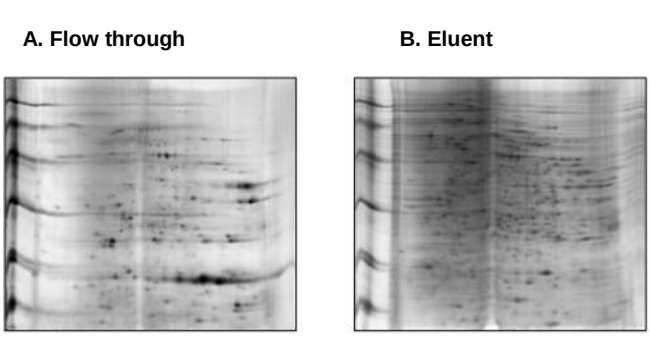
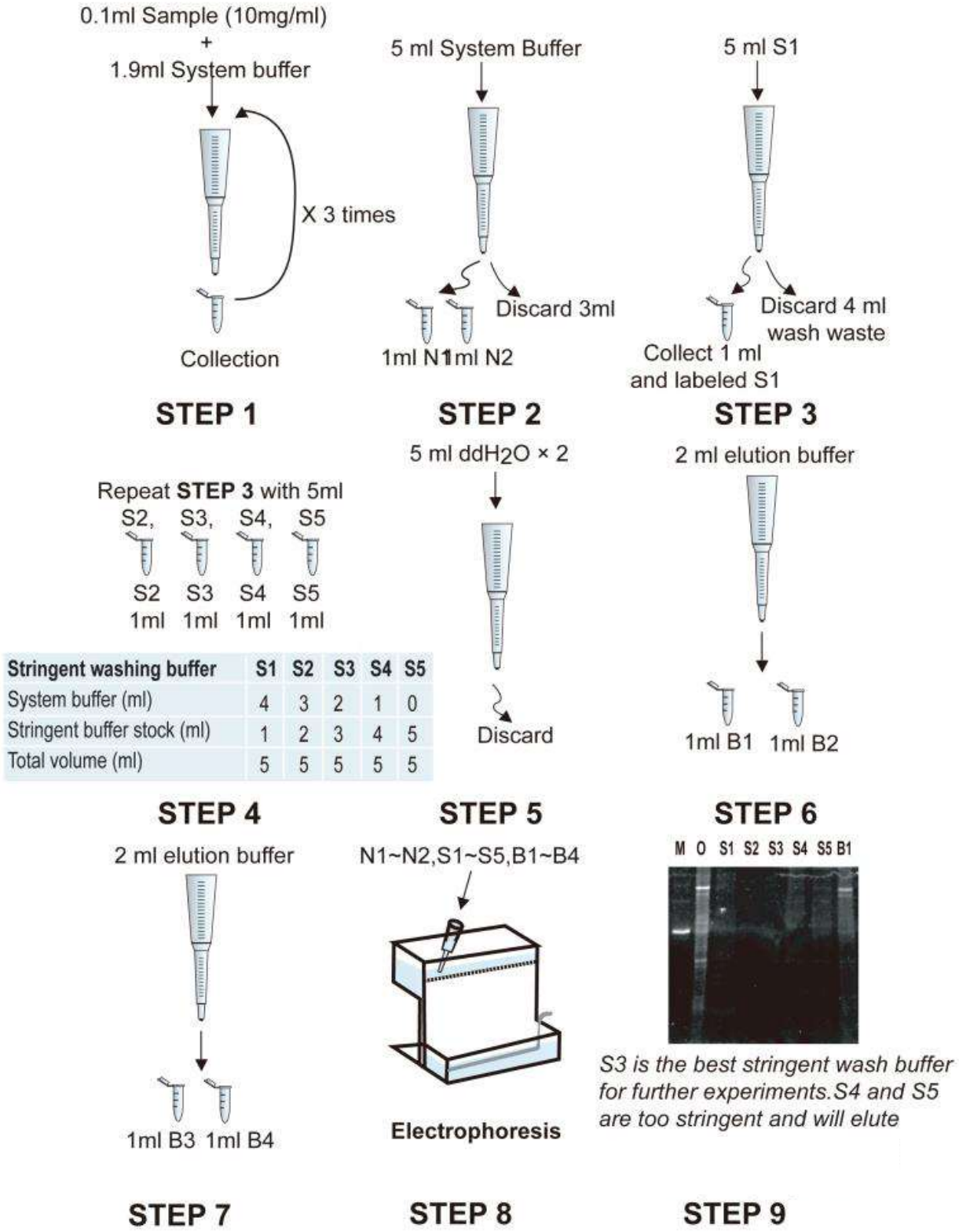


Figure 3. Enrichment efficacy of SP2 cell line and separated by 2-DE. (A) Flow through fraction after enriching; (B) Eluent fraction after enriching.

Figure 4. Enrichment efficacy of biological materials. Biological materials including Human hepatocellular liver carcinoma cell line (HepaG2), mouse myeloma cell line NS-1 (NS-1), rat mesangial cell (RMC), mouse myeloma cell line SP2 (SP2) were used for test. **F:** flow through; **E:** eluent.

General Troubleshooting Guide:

Problems	Cause	Solution
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 non-phosphoproteins 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.



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

Figure 5. Optimization Condition Protocol For PhosphoSeek™ Phosphoprotein Purification Kit.

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