BD™ Phosphoprotein Enrichment Kit User Manual

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I. Introduction & Protocol Overview

The **BD™ Phosphoprotein Enrichment Kit** provides materials for isolating phosphorylated proteins from mammalian cells and tissues. Proteins that carry a phosphate group on any amino acid—including serine, tyrosine, or threonine—are selectively bound by the Phosphate Metal Affinity Chromatography (PMAC) Resin. Non-phosphorylated proteins simply pass through the resin, along with other contaminants, so that an enriched solution of phosphorylated proteins elutes from the column.

Significance of phosphoprotein analysis

Signal transduction, transcriptional regulation, and cell division are just three examples of the many metabolic processes regulated by the phosphorylation and dephosphorylation of proteins by kinases and phosphatases. Despite the broad occurrence of phosphorylation in regulatory mechanisms, only a small percentage of all cellular proteins are phosphorylated at any given time (Alberts, *et al.,* 1994; Ficarro, *et al.*, 2002). Enrichment of the phosphorylated fraction may be necessary before starting an analysis, in order to reduce the background and increase the sensitivity of an assay. After enrichment, rare and perhaps novel phosphoproteins are less likely to escape detection.

In the past, many phosphoprotein enrichment methods have involved chemical modification of the phospho group followed by binding to a solid support. In contrast, our method introduces no chemical changes but rather relies on the selectivity of the PMAC resin. The nondenaturing protocol maintains protein conformation and solubility, and thus activity. In addition, the elution buffer is detergent-free, so activity is not lost during a time-consuming buffer exchange step. These factors make the Phosphoprotein Enrichment Kit ideal for use with downstream applications such as mass spectroscopy, two-dimensional PAGE, and antibody microarray analyses.

Phosphoprotein enrichment method

The phosphoprotein enrichment procedure is outlined in Figure 1. Extraction/ Loading Buffer (Buffer A) is added to the cell or tissue pellet, and the proteins are extracted either by grinding or by freezing and then thawing the cells. After centrifuging to remove insoluble cellular debris, the cellular extract is loaded on a Phosphoprotein Affinity Column. The PMAC Resin is highly selective for the phosphates on the proteins, allowing other proteins and contaminants to pass through in the flowthrough and wash. Then the phosphorylated proteins are eluted from the column with Buffer B. This entire procedure can typically be performed in as little as 2 hours.

This simple procedure can be used with any mammalian cell or tissue type. It has been tested with several different mammalian cell lines (Table I). Any lysate can be used as long as the sample has been extracted, desalted or dialyzed in Buffer A, which has been formulated for the phosphoprotein affinity columns. In addition, the procedure usually does not require dilution of the loaded extract nor subsequent concentration of the eluted fractions—two extra steps that would contribute to sample loss.

I. Introduction & Protocol Overview *continued*

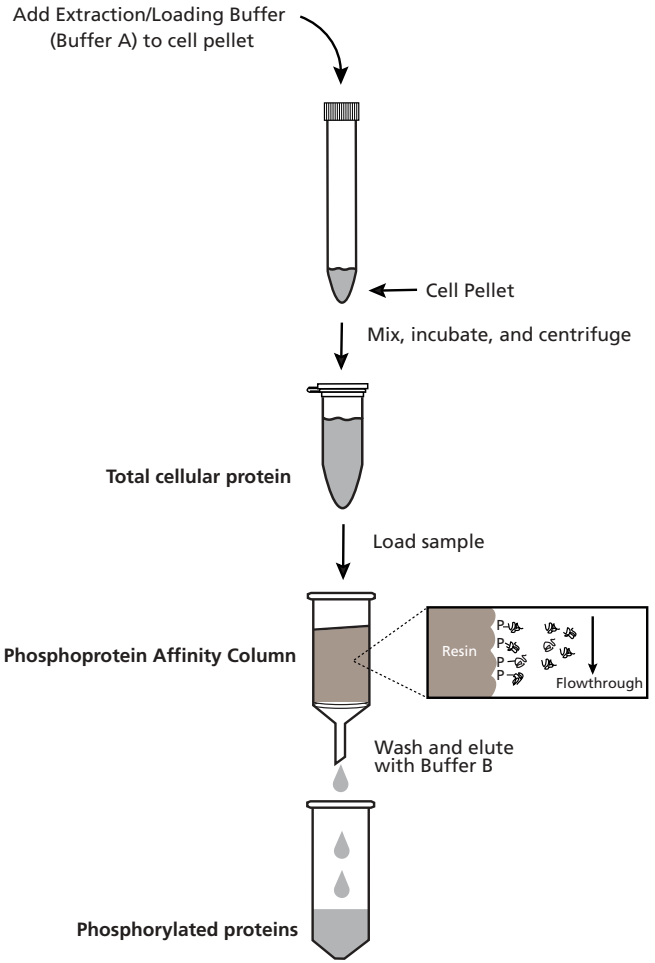

Figure 1. Overview of the Phosphoprotein Enrichment Procedure. Extraction/Loading Buffer contains a mild, nonionic detergent for efficient, nondenaturing extraction of cellular protein.

Each Phosphoprotein Affinity Column has a maximum binding capacity of 4 mg of phosphorylated protein/column. Although any amount of total protein can be run over a phosphoprotein affinity column in any concentration, we recommend that sample in the range of 2–8 mg of total protein be loaded per column. If you desire to load more than 10 mg, additional wash steps may be necessary to achieve best results. The resin specifically captures more than 90% of the phosphorylated protein, when used according to specifications.

I. Introduction & Protocol Overview *continued*

TABLE I. **YIELDS OF PHOSPHORYLATED PROTEIN OBTAINED**

Cell line	Loaded (mg)	Flowthrough (mg)	Washes (mg)	Eluate (mg)	% of total
HEK 293	2.5	1.9	0.23	0.41	16%
Jurkat	3.3	2.4	0.3	0.52	16%
Cos-7	3.1	2.4	0.26	0.47	15%
NIH 3T3	2.7	1.9	0.21	0.45	17%
HeLa	3.4	2.5	0.24	0.46	14%

The data described in Table I indicate typical results obtained from an extraction of approximately 100 mg of mammalian cells at 80–90% saturation, using the methods described in this User Manual. The use of a single buffer for the extraction, loading and washing steps saves time and prevents sample degradation and dephosphorylation. Following the procedures outlined in this manual, extracts of ~1 mg/ml total protein concentration are obtained. After loading the clarified extract on the phosphoprotein affinity column, the non-phosphorylated proteins and other cellular debris are removed in one wash step. The eluted fraction is a concentrated, highly-purified solution of phosphoproteins (Figure 2). We usually obtain concentrations of 0.15–0.20 mg/ml phosphoprotein in the eluted fractions following the procedures outlined in this manual. Typical yields with this method are generally 10–20% of the total cellular protein loaded on the column (Table I).

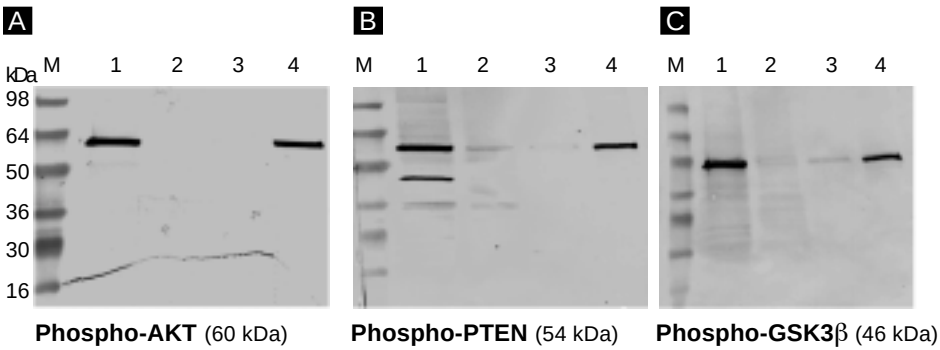

Figure 2. Highly effective enrichment of phosphorylated proteins. A Phosphoprotein Affinity Column was loaded with ~3 mg of total protein from HEK 293 cells. The extract (Lanes 1), flowthrough (Lanes 2), wash (Lanes 3), and eluate (Lanes 4) were then analyzed by Western blotting using antibodies specific for phosphorylated AKT (**Panel A**), PTEN (**Panel B**), and GSK3β (**Panel C**) proteins. The phosphorylated proteins were clearly detected in the eluate fraction. Please note that samples were not diluted, nor concentrated before loading on the gel. M = Mol. Wt. Standards.

II. List of Components

Store all components at 4°C.

Phosphoprotein Enrichment Kit (Cat No. 635624)

The following reagents are suitable for 6 purifications.

- **6** **Phosphoprotein Affinity Columns** (1-ml, disposable)
- **220 ml** **Buffer A** (Extraction/Loading Buffer)
- **45 ml** **Buffer B** (Elution Buffer)
  20 mM sodium phosphate, 0.5 M sodium chloride

PhosphoProtein Kit-Buffer A (Cat. No. 635626)

- **500 ml** **Buffer A** (Extraction/Loading Buffer)

III. Additional Materials Required

The following materials are required but not supplied:

- **Phosphate Buffered Saline**
- **2-ml microcentrifuge tubes**
- **5-ml screw-cap centrifuge tubes**
- **pH meter or pH paper**
- **Micropipettor**
- **BCA Protein Assay Reagent Kit** (Pierce Biotechnology; No. 23225) Provides a detergent-compatible BCA reagent for quantifying total protein. Pierce's BCA Protein Assay Reagent Kit should be used for all Phosphoprotein Enrichment Kit analyses. Using other protein assays or BCA reagents (or kits) could lead to errors in protein estimation, since PMAC buffers contain substances known to interfere with protein assays. Pierce's Kit has been tested by our scientists and approved for use with PMAC procedures and reagents.

Required for tissue extraction:

- **Mortar and Pestle**
- **Alumina** (Sigma Cat. no. A2039)

The following materials may be required depending on your purification:

- **Sterile Syringes and syringe filters (0.45 μm)** for filtering lysates
- **Phosphatase Inhibitors** (if phosphatase inhibitors are desired)

  Sodium orthovanadate (1–2 mM)

  Sodium fluoride (10–50 mM)
- **Gel Filtration Column** (for phosphatase inhibitor removal or buffer exchange)

  PD-10, (Pharmacia Cat. No. 17-0851-01)
- **Microconcentrators** for sample concentration (not usually necessary)

  Millipore 4 ml centrifugal filter and tube (Cat. No. UFV4BGC00) and

  Millipore 0.5 ml centrifugal filter and tube (Cat. No. UFV5BCC00)

IV. General Considerations

A. Sample Preparation

For typical results achieved using the procedures outlined in this manual, refer to Table I in Section I. Introduction and Protocol Overview.

These methods outline procedures for extracting phosphoproteins from cells (V.A), or tissues (V.B). This procedure works with extracts that were obtained by other methods, as long as the protein has been extracted, desalted or dialyzed in Buffer A before loading on a phosphoprotein affinity column. Additional Buffer A is available (Cat. No. 635626).

If Buffer exchange is necessary, we recommend using a desalting column.

Although any amount of total protein can be run over a phosphoprotein affinity column in any concentration, we recommend that the range of 2–8 mg of total protein be loaded per column.

B. Protein Concentration

Since it is not necessary to dilute samples before running on PMAC resin, we find that it is not normally necessary to concentrate proteins. However, if you desire to concentrate enriched fractions, please refer to Additional Materials Required for a list of recommended microconcentrators.

Protein solutions of any concentration can be loaded on the PMAC resin; however, samples of higher concentrations will be more likely to clog the columns. Using the extraction methods outlined in Section V, we usually obtain protein solutions of ~1mg/ml total cellular protein.

For polyacrylamide gel electrophoresis (PAGE) and Western blot analysis, a concentration step is generally not necessary.

C. Sample Storage

Samples should be stored on ice or at 4°C. Therefore, cell extracts and lysates, as well as all fractions should be kept on ice at all times.

For gel electrophoresis and Western blot analysis, samples should be boiled with SDS sample buffer and then stored frozen at –20°C.

If longer storage of enriched samples is necessary, phosphatase inhibitors should be added (See Appendix A and Additional Materials Required).

D. Phosphatase Inhibitors

Phosphatase inhibitors interfere with phosphoprotein binding to PMAC Resin. You may find that phosphatase inhibitors are not necessary when enriching phosphoproteins with PMAC Resins because of the quickness and mildness of the procedure. However, if you require the use of phosphatase inhibitors for your experiments, the **inhibitors must be removed** by running the sample through a desalting column (See Additional Materials Required, and Appendix A) before loading on a PMAC resin column.

V. Phosphoprotein Enrichment Procedure

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

General

These methods outline procedures for extracting phosphoproteins from cells (Section A), or tissues (Section B). If starting with extracts that were obtained by other methods, the protein should be in Buffer A before proceeding with loading on a phosphoprotein affinity column (Section C). See Section IV.A on sample preparation for more details if your protein extracts are not in Buffer A. In order to preserve phosphorylation, proceed with column enrichment procedure immediately following protein extraction.

- The resin in the column will change color as a normal part of the enrichment process.
- Phosphoprotein affinity columns are intended for single use and cannot be regenerated. Do not reuse the columns in this kit.
- Due to the ease and brevity of this enrichment procedure, protease inhibitors are not normally necessary with proper storage of samples. **Phosphatase inhibitors interfere with phosphoprotein binding to the PMAC columns.** See Appendix A if you wish to use phosphatase inhibitors.
- Before you start, bring the following materials to room temperature:
  - — 35 ml Buffer A
  - — 5 ml Buffer B
  - — 1 Phosphoprotein Affinity Column

A. Extracting Proteins from Cells

1. Wash 50–150 mg of cells three times with 20 volumes of Phosphate Buffered Saline (PBS) by centrifuging at 500 x g in a pre-weighed centrifuge tube.

   Note: We find that two 150-mm culture plates of 80–90% confluent cells yield ~150 mg of cells.
2. After washing, centrifuge cells as above, and then decant the supernatant and aspirate the residual liquid.
3. Centrifuge the tube again (for ~2 min), and aspirate any residual traces of liquid. Then reweigh the tube to determine the weight of the cell pellet.
4. Freeze your samples by placing them in liquid nitrogen or in a –80°C freezer.
5. Resuspend the cell pellet (approx. 100 mg) in 30 µl of Buffer A for each mg of cells (e.g., if your sample comprises 100 mg of cells, add 3 ml of Buffer A).
6. Mix pellet by gently pipetting up and down approximately 20 times.
7. Incubate at 4°C for 10 min with mixing by inverting tube approximately every minute. Transfer cell-lysate to a microcentrifuge tube.

V. Phosphoprotein Enrichment Procedure *continued*

8. Centrifuge the cell extract at 10,000 x g for 20 min at 4°C to pellet any insoluble material.

   Note: Start preparing the column (Section C) while centrifuging the samples.
9. Transfer the supernatant to a clean tube without disturbing the pellet. This is the clarified sample.
10. Reserve a small portion of the clarified sample at 4°C for protein assay and other analysis. Proceed to Section C: Column Enrichment.

    Note: Use the BCA Protein Assay (Pierce Cat. No. 23235) for protein quantitation (See Additional Materials Required).

B. Extracting Protein from Crude Tissue

Before starting, chill the following items on ice or at 4°C:

- 5 ml Buffer A
- one mortar & pestle
- two 2-ml microcentrifuge tubes
- one 5-ml tube

1. Transfer 100–200 mg of frozen tissue to a pre-chilled mortar.
2. Add 0.25–0.5 g of Alumina to the mortar.
3. Use the pestle to grind the tissue until a paste is formed.
4. Add 2 ml of pre-chilled Buffer A.
5. Mix the buffer into the paste using the pestle. When you finish, use a micropipette tip or sterile instrument to scrape any paste that adheres to the pestle back into the mortar.
6. Transfer the extract to a pre-chilled 2 ml microcentrifuge tube.
7. While holding the pestle over the mortar, rinse the pestle with 2 ml of Buffer A.
8. Combine the rinse with the original extract in a 2-ml tube. (Use a second 2-ml tube if the volume exceeds the tube's capacity.)
9. Centrifuge the suspension at 10,000 x g for 20 min.

   Note: Start preparing the columns (Section C) while centrifuging the samples.
10. While taking care not to disturb the pellet, transfer the supernatant to a pre-chilled 5-ml tube.
11. Gently invert the tube to mix the lysate.

    Note: If extract or lysate is not translucent, you can clarify the sample by passing it through a 0.45 μm filter or filter paper.
12. Reserve a small portion of the clarified sample at 4°C for later analysis. Proceed to Section C: Column Enrichment.

    Note: Use the BCA Protein Assay (Pierce Cat. No. 23235) for protein quantitation (See Additional Materials Required).

V. Phosphoprotein Enrichment Procedure *continued*

C. Column Enrichment

1. Allow the column to stand at room temperature in an upright position until the resin to settles out of suspension.
2. Remove the column top cap and then the end cap, and allow the storage buffer to drain out until it is flush with the top of the Resin bed.
3. Wash the column with 5 ml of distilled water.
4. Add 5 ml of Buffer A to equilibrate the column and allow the buffer to flow through.
5. Repeat Step 4.
6. Collect and measure the pH of the last 2 ml of flowthrough. If the pH is not less than or equal to 6.0, then continue washing with Buffer A.
7. Close the column with the end cap.
8. Add your clarified sample to the column.

   Notes:

   - We recommend maximum sample load of 8 mg of total protein over a single column. If loading higher amounts, additional washing steps should be performed.
   - Up to 5 ml of extract can be added to the column at a time. If your sample volume is larger than 5 ml, add the extract in steps.
9. Close the column with the top cap.
10. Gently agitate column with sample at 4°C for 20 min on a platform shaker to allow the phosphorylated proteins to bind to the column.

Important: Perform the following steps at room temperature.

11. Let the column stand for 5 min in the upright position to allow the resin to settle out of suspension.
12. Remove the column top cap and then the end cap and allow non-adsorbed material to flow through. Collect the non-adsorbed material, if analysis of non-phosphorylated proteins is necessary.
13. Wash the column by adding 5 ml of Buffer A and allowing it to flow through. Repeat this wash three more times for a total of 4 X 5 ml washes.
14. Add 1 ml of Buffer B (elution buffer) and collect the fraction, on ice.
15. Repeat step 14 four times with 1 ml of Buffer B each time (collect fractions every time). Store all fractions on ice immediately.

    Note: The enriched phosphorylated proteins are generally present in the second and third fractions—approximately 2 ml of elution volume.
16. Run a BCA analysis to determine protein concentration in the cell extract as well as the eluted fractions. Eluted fractions 2 and 3 will most likely have the highest concentration of phosphorylated protein.

VI. Analysis of Results and Troubleshooting Guide

A. Yield lower than expected

Column was not equilibrated to pH 6.0 with Buffer A before loading.	Equilibrate column with Buffer A before loading samples. Additional Buffer A can be purchased if necessary (Cat. No. 635626).
Phosphatase inhibitors were added to the samples during the extraction.	Samples must be run over a desalting/buffer exchange column (see additional materials required) before loading on the column.
Samples allowed to incubate at room temperature for a long time before loading.	Run samples on column immediately after extracting, or store samples on ice. If there will be more than 1 hr between extraction and loading, then use phosphatase inhibitors (Appendix A).
Inadequate exposure of sample to resin	Increase Binding incubation, ensure complete mixing with resin.
Elution not performed at room temperature	Perform elution step with all materials at room temperature (19–23°C).
High phosphatase activity in sample	Try using phosphatase inhibitors before and after PMAC enrichment (Appendix A).
Sample not in Buffer A	Desalt or dialyze sample into Buffer A. Additional Buffer A can be purchased if necessary (Cat. No. 635626).

B. Incomplete separation of phosphorylated proteins from other proteins

Phosphoproteins in flowthrough	Do not add EDTA or phosphatase inhibitors to any buffers that come in contact with the resin.
Unphosphorylated proteins in eluate	Inadequate washing or possibly protein clogging of column. Do not load more than 8 mg total protein for optimal results.

C. Column clogging

Cellular debris in sample	If sample is not clarified after centrifuging, filtering the sample will often prevent column clogging (see Section III. Additional Materials Required for recommended filters).
High-viscosity sample	If sample appears to be highly viscous during the extraction, addition of DNase I will often reduce the viscosity, and prevent clogging.

VII. References

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VIII. Related Products

For a complete listing of all BD Biosciences Clontech products, please visit **www.bdbiosciences.com/clontech**

	Cat. No.	New Cat. No.
• BD TALON™ Products		
Purification Kit	K1253-1	635515
Metal Affinity Resin	8901-1	635501
	8901-2	635502
	8901-3	635503
	8901-4	635504
Superflow Metal Affinity Resin	8908-1	635506
	8908-2	635507
TALONspin Columns	8902-1	635601
	8902-2	635602
	8902-3	635603
	8902-4	635604
2 ml Disposable Gravity Columns	8903-1	635606
CellThru	8910-1	635509
	8910-2	635510
Buffer Kit	K1252-1	635514
HT 96-Well Purification Plate	K1254-1	635622
xTractor Buffer Kit	K1255-1	635623
xTractor Buffer	N/A	635625
• Glutathione Resins Products		
Uniflow Resin	8912-1	635610
	8912-2	635611
Superflow Resin	8911-1	635607
	8911-2	635608
• Thiophilic Resins		
Uniflow Resin	8913-1	635613
	8913-2	635614
Superflow Resin	8917-1	635616
	8917-2	635617
• BD Clontech™ Antibody Microarray	K1847-1	631785
• BD CHROMA SPIN™ Columns	Many	Many

Appendix A: Use of Phosphatase Inhibitors

Due to the ease and quickness of the procedures that are outlined in this user manual, we have found that phosphatase inhibitors are usually not necessary during the extraction and enrichment procedures. However, if you desire to use phosphatase inhibitors during your extraction, they must be removed before loading on the PMAC column because they interfere with the phosphoprotein binding to the PMAC resin.

Phosphatase inhibitors will be beneficial in preserving phosphorylation under the following circumstances:

- If samples will be stored for 1 hour or more between extraction and loading on a PMAC column
- If the extraction materials are known to be unusually high in phosphatase activity
- If storing enriched fractions for extended periods of time

Procedures for use of phosphatase inhibitors:

1. Extraction of samples:

   Add sodium orthovanadate to a final concentration of 1 mM and sodium fluoride to a final concentration of 10 mM to 5 ml of Buffer A. Use this buffer to extract proteins as described in Sections V.A and V.B. Before loading the extracted protein solution on a PMAC column (Section C), remove the phosphatase inhibitors by running the samples through a desalting/buffer exchange column (See Section III. Additional Materials Required).

2. Storage of enriched samples:

   Add sodium orthovanadate to a final concentration of 1 mM and sodium fluoride to a final concentration of 10 mM to the eluted fractions before storage of samples.

Appendix B: Notes on Downstream Applications

1. Mass Spectroscopy

Although Buffer B (Elution Buffer; 20 mM sodium phosphate, 0.5 M sodium chloride) does not contain any detergents, some residual detergents may be present from the previous washing steps. Therefore, enriched samples might require dialysis to remove residual amounts of phosphate and detergent from samples if those compounds produce interference for mass spectroscopy analysis.

2. 2-Dimensional Gel Analysis

It may be necessary to desalt samples by dialysis or desalting column before PAGE or 2-Dimensional PAGE analysis because the Buffer B salt concentration is higher than the concentration in many electrophoresis buffers.