User Manual

TALON® PMAC Phosphoprotein Enrichment Kit User Manual

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I. Introduction & Protocol Overview

The **TALON PMAC 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, threonine, or tyrosine— 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 (Rishi, *et al.,* 2006).

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 TALON PMAC Phosphoprotein Enrichment Kit ideal for use with downstream applications such as mass spectrometry, two-dimensional PAGE, and antibody microarray analyses.

The TALON PMAC Phosphoprotein Enrichment Procedure

Phosphoprotein enrichment using the TALON PMAC Phosphoprotein Enrichment Kit 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*

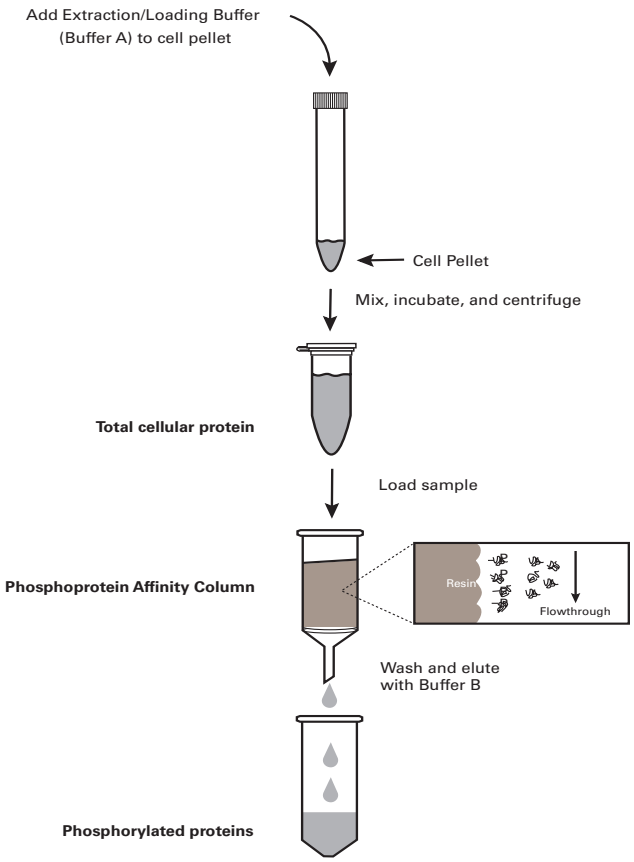

Figure 1. Overview of the TALON PMAC Phosphoprotein Enrichment Procedure. Extraction/Loading Buffer contains a mild, nonionic detergent for efficient, nondenaturing extraction of cellular protein.

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 WITH THE TALON PMAC PHOSPHOPROTEIN ENRICHMENT KIT**

Cell Line	Protein Loaded (mg)	Flowthrough (mg)	Washes (mg)	Eluate (mg)	Percentage of Protein Eluted
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).

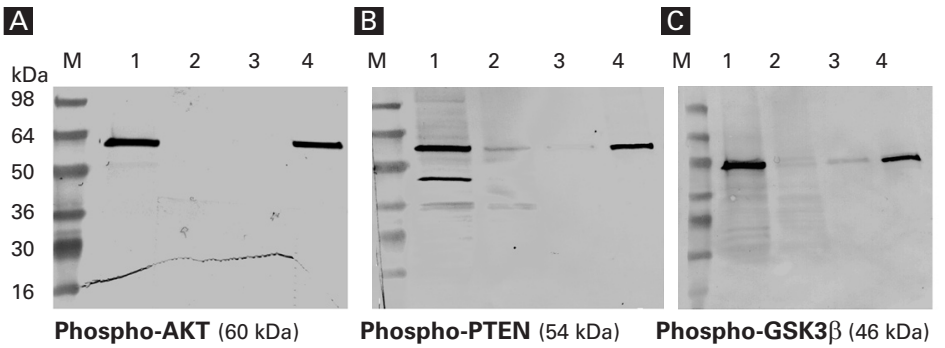

Figure 2. Highly effective enrichment of phosphorylated proteins using the TALON PMAC Phosphoprotein Enrichment Kit. A Phosphoprotein Affinity Column was loaded with ~3 mg of total protein from HEK 293 cells. The extract (Lanes 1), flowthrough (Lanes 2), wash (Lanes 3), and eluate (Lanes 4) were then 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=molecular weight standards..

I. Introduction & Protocol Overview *continued*

TALON PMAC Magnetic Phospho Enrichment Kit

The TALON PMAC Magnetic Phospho Enrichment Kit provides a simple, metal affinity-based method for microscale purification of phosphorylated proteins from mammalian cells and tissues under native conditions. It combines the phosphospecificity of our current TALON PMAC Phosphoprotein Enrichment Kit with convenience of magnetic bead separation, offering the following advantages:

- Quick and easy enrichment of phosphoproteins from any cell or tissue sample
- Nondenaturing protocol maintains protein conformation and solubility
- Eluted samples are ideal for small volume applications

This kit contains TALON PMAC resin-based Phospho Magnetic Beads, which specifically bind phosphoproteins. Magnetic particles in the beads facilitate quick and easy separation of microgram quantities of phosphoproteins when placed on a magnetic separator.

Each kit supplies a complete set of buffers along with our Phospho Magnetic Beads for group-specific enrichment of all types of phosphoproteins, both cytosolic and membrane-bound, that contain a phosphorylated amino-acid side chain—including serine, threonine, or tyrosine. The Extraction/Loading Buffer contains a non-ionic detergent to extract cellular proteins. Wash and Elution Buffers are detergent-free. Proteins can be eluted in small sample volumes (50–200 µl) to yield concentrated samples. If desired, phosphoproteins can be eluted in pH 9.0 buffer, which is more suitable for downstream applications such as mass spectrometry.

Phospho Magnetic Beads are supplied in a 2 x 1 ml format, as a 5% suspension in 25% ethanol. These beads have a binding capacity of 400 µg of α-casein per ml of suspension. When peforming assays using single tubes, 100–200 µl of beads may be used per assay. Smaller amounts of beads may be used, but there may be difficulties in handling the beads in small buffer volumes.

Only a small percentage of all cellular proteins are phosphorylated, so it is often necessary to enrich for this fraction before beginning an analysis. The TALON PMAC Magnetic Phospho Enrichment Kit can be used with any mammalian cell type or tissue sample. The yield of phosphorylated protein varies with different cell lines (Table II). The enrichment procedure is highly efficient, as demonstrated by Western blotting (Figure 3).

I. Introduction & Protocol Overview *continued*

TABLE II. **YIELDS OF PHOSPHORYLATED PROTEIN OBTAINED WITH THE TALON PMAC MAGNETIC PHOSPHO ENRICHMENT KIT**[1]

Cell Line	Protein Loaded (mg)	Flowthrough + Washes (mg)	Eluate (mg)	Percentage of Protein Eluted
HEK 293	0.192	0.146	0.034	18%
Jurkat	0.183	0.133	0.033	18%
Cos-7	0.150	0.113	0.025	17%
NIH 3T3	0.176	0.132	0.027	15%
HeLa	0.174	0.139	0.023	13%

[1] 200 µl of a 5% suspension of Phospho Magnetic Beads was used for each purification.
Total protein in each fraction was determined with BCA Protein Assay Reagent (Pierce Biotechnology, Cat. No. 23225).

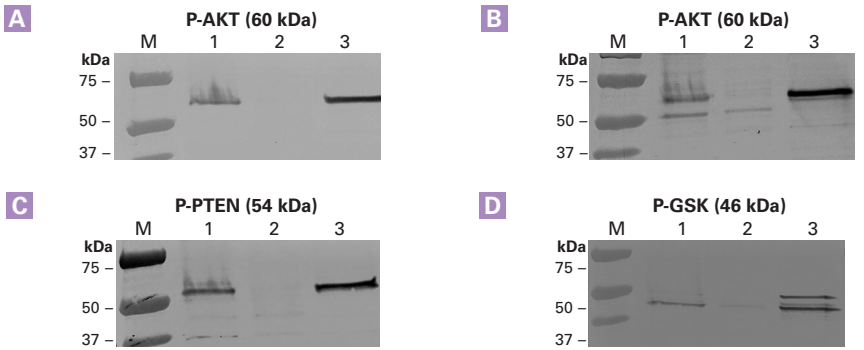

Figure 3. Highly effective enrichment of phosphorylated proteins using the TALON PMAC Magnetic Phospho Enrichment Kit. Proteins extracted from HEK 293 cells in Extraction/Loading Buffer containing protease inhibitors (without EDTA) and a phosphatase inhibitor (10 mM sodium fluoride), were mixed with 200 µl of a 5% suspension of Phospho Magnetic Beads at room temperature for 30 min, followed by washing and elution. The extract (Lanes 1), flowthrough (Lanes 2), and eluates (Lanes 3) were then analyzed by Western blotting using antibodies specific for phosphorylated AKT (Ser 473) in **Panel A,** P-AKT (Thr 308) in **Panel B,** P-PTEN (Ser 380) in **Panel C,** and P-GSK3ß (Ser 9)in **Panel D;** the band at 51 kDa is due to cross-reactivity of the antibody with P-GSK3α. Please note that samples were neither diluted nor concentrated before loading on the gel. M=molecular weight standards.

II. List of Components

Store all components in the following kits at 4°C.

Phosphoprotein Enrichment Starter Kit (Cat No. 635666)

The following reagents are suitable for 1 purification:

- 1 Phosphoprotein Affinity Column (1 ml, disposable)
- 40 ml Buffer A (Extraction/Loading Buffer)
- 10 ml Buffer B (Elution Buffer)
  (20 mM sodium phosphate, 0.5 M sodium chloride)
- TALON PMAC Phosphoprotein Enrichment Kit
  User Manual (PT3731-1)

TALON PMAC 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)
- TALON PMAC Phosphoprotein Enrichment Kit
  User Manual (PT3731-1)

TALON PMAC Magnetic Phospho Enrichment Kit (Cat. No. 635641)

- 2 x 1 ml TALON Phospho Magnetic Beads
  (5% suspension in 25% ethanol))
- 110 ml Extraction/Loading Buffer (same as Buffer A)
- 110 ml Wash Buffer (Buffer A without detergent)
- 80 ml Elution Buffer (250 mM sodium phosphate, 0.5 M NaCl, pH 7.2)
- TALON PMAC Phosphoprotein Enrichment Kit
  User Manual (PT3731-1)
- TALON PMAC Magnetic Phospho Enrichment Kit
  Protocol-at-a-Glance (PT3905-2)

Note: If any of these buffers contains a precipitate, allow it stand at room temperature, and, if necessary, mix to dissolve the precipitate.

III. Additional Materials Required

A. The following materials are required but not supplied for the **TALON PMAC Phosphoprotein Enrichment Kit:**

- Phosphate Buffered Saline (PBS), pH 7.0–7.5
- 2 ml microcentrifuge tubes
- 5 ml tubes
- pH meter or pH paper
- micropipettor
- BCA Protein Assay Reagent Kit (Pierce Biotechnology; Cat. No. 23225)

  Provides a detergent-compatible BCA reagent for quantifying total protein. Pierce's BCA Protein Assay Reagent Kit should be used for allTALON PMAC 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. 265497)

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 mM)
- gel filtration column (a desalting column for phosphatase inhibitor removal or buffer exchange)

  PD-10, (GE Healthcare, Cat. No. 17-0851-01)
- microconcentrators for sample concentration (not usually necessary)
  - — Millipore 4 ml centrifugal filter and tube (Cat. No. UFC8 010 96)
  - — Millipore 0.5 ml centrifugal filter and tube (Cat. No. UFV5 BCC 00)

III. Additional Materials Required *continued*

B. The following materials are required but not supplied for the **TALON PMAC Magnetic Phospho Enrichment Kit:**

- magnetic separator for 1.5 ml tubes

Optional additional materials include:

- protease inhibitors (should not contain EDTA)
- phosphatase inhibitors
  - — sodium fluoride (10 mM)
  - — sodium orthovanadate (1–2 mM) [must be removed by running the sample through a desalting column (see Section A) before incubating with Phospho Magnetic Beads]
- 100 mM carbonate buffer, pH 9.3
- alumina needed for extraction from tissue samples
- mortar & pestle for alumina extraction
- 1.5 ml and 0.5 ml microfuge tubes

IV. General Considerations

A. Sample Preparation

- For typical results achieved using the procedures outlined in this manual, refer to Table I (for the Phosphoprotein Enrichment Kit) and Table II (for the Magnetic Phospho Enrichment Kit), both in Section I.
- Section V contains procedures for extracting phosphoproteins from cells (V.A) or tissues (V.B) to be purified (V.C) using the Phosphoprotein Enrichment Kit. 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 (Extraction/Loading Buffer) before loading on a phosphoprotein affinity column. Additional Buffer A is available separately (Cat. No. 635626).
- If buffer exchange is necessary, we recommend using a desalting column (see Section III).
- Although any amount of total protein can be run over a phosphoprotein affinity column in any concentration, we recommend that a range of 2–8 mg of total protein be loaded per column.
- Section VI contains procedures for extracting phosphoproteins from cells (VI.A) or tissues (VI.B) to be purified (VI.C) using the Magnetic Phospho Enrichment Kit.
- For Phospho Magnetic Beads, different amounts of beads may be used, depending on the initial amount of protein in the sample. For general guidelines see Table III.

TABLE III. RECOMMENDED AMOUNTS OF PHOSPHO MAGNETIC BEADS FOR DIFFERENT AMOUNTS OF PROTEIN

Amount of Beads (µl)	Amount of Protein (µg)
200	200
100	100
50	50
25	25

- Although different sample volumes may be used, we recommend sample volumes ranging from 200 µl to 1 ml. If the sample volume is less than 100 µl, when incubating the beads with the sample, add sufficient Extraction/Loading Buffer to bring the total sample volume up to at least 100 µl (a volume of 200 µl is optimal). Elution may still be carried out in small volumes (50–200 µl) to yield concentrated samples.

IV. General Considerations *continued*

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 Section III 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 ~1 mg/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 or –70°C.
- If longer storage of enriched samples is necessary, phosphatase inhibitors should be added (See Appendix A and Section III), or samples can be stored frozen at –20°C or –70°C.

D. Phosphatase Inhibitors

- The phosphatase Inhibitor sodium fluoride (10 mM final concentration) can be used with PMAC Resin and Phospho Magnetic Beads. The phosphatase inhibitor sodium orthovanadate interferes with phosphoprotein binding to PMAC resin and magnetic beads.
- You may find that phosphatase inhibitors are not necessary when enriching phosphoproteins with PMAC resin or Phospho Magnetic Beads because of the quickness and mildness of the procedure. However, if you require phosphatase inhibitors for your experiments, use sodium fluoride. **If sodium orthovanadate is used, then it must be removed** by running the sample through a desalting column (see Section III and Appendix A) before loading on a PMAC resin or incubating with Phospho Magnetic Beads.

E. Protease Inhibitors

Proteins can be extracted in presence of protease inhibitors specially designed for purification of metal binding proteins since they do not contain EDTA.

V. Phosphoprotein Enrichment Kit Protocols

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 (Extraction/Loading Buffer) before 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 (Extraction/Loading Buffer)
  - 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 for 5 min in a preweighed 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 (Extraction/Loading Buffer) 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 Kit Protocols *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 assays and other analysis. Proceed to Section C: Column Enrichment.

    Note: Use the BCA Protein Assay Reagent Kit (See Section III) for protein quantification.

B. Extracting Proteins from Crude Tissues

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

- 5 ml Buffer A (Extraction/Loading Buffer)
- one mortar & pestle
- two 2 ml microcentrifuge tubes
- one 5 ml tube

Then perform protein extraction as follows:

1. Transfer 100–200 mg of frozen tissue to a prechilled 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 prechilled Buffer A (Extraction/Loading Buffer) .
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 prechilled 2 ml microcentrifuge tube.
7. While holding the pestle over the mortar, rinse the pestle with 2 ml of Buffer A (Extraction/Loading Buffer) .
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 prechilled 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 for protein assays and other analysis. Proceed to Section C: Column Enrichment.

    Note: Use the BCA Protein Assay Reagent Kit (See Section III) for protein quantification.

V. Phosphoprotein Enrichment Kit Protocols *continued*

C. Phosphoprotein Enrichment using Columns

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 (Extraction/Loading Buffer) 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 (Extraction/Loading Buffer) .
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: The following steps can be performed 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 (Extraction/Loading Buffer) and allowing it to flow through. Repeat this wash three more times for a total of four 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. Magnetic Phospho Enrichment Kit Protocols

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 (Extraction/Loading Buffer) before incubating with Phosphomagnetic Beads (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.

- Phosphomagnetic Beads are intended for single use and cannot be regenerated. Do not reuse the beads 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 Phosphomagnetic Beads**. See Appendix A if you wish to use phosphatase inhibitors.

A. Extracting Proteins from Cells

1. Wash 25–50 mg of cells three times with 20 volumes of Phosphate Buffered Saline (PBS) by centrifuging at 500 x g for 5 min in a preweighed centrifuge tube.

   Note: We find that one 150 mm culture plate containing 80–90% confluent cells yields ~70 mg of cells. Smaller or larger cell pellets can be used.

2. After washing, centrifuge cells as in Step 1, 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 an –80°C freezer.
5. Resuspend the cell pellet in 20 µl of Extraction/Loading Buffer for each mg of cells. (e.g., if your sample consists of 50 mg of cells, add 1 ml of Extraction/Loading Buffer.)

   Optional: Add 1 µl of 1 U/µl DNAse I solution.

   Notes:

   - If a smaller sample volume is desired, scale the amount of the pellet correspondingly—i.e., resuspend 12 mg of cells in 240 µl of Extraction/Loading Buffer. Scale all volumes correspondingly.
   - The phosphatase inhibitor NaF (10 mM) can be added during extraction and incubation (Section C.9). Sodium orthovanadate is not compatible with magnetic phosphoprotein enrichment and if used during incubation will result in no phosphoprotein binding to the beads, unless removed beforehand using a desalting column (see Section III).

VI. Magnetic Phospho Enrichment Kit Protocols *continued*

6. Mix pellet by gently pipetting up and down approximately 20 times.
7. Incubate at 4°C for 10 min with additional mixing (inverting tube) approximately every minute. Transfer cell lysate to a microcentrifuge tube.
8. Centrifuge the cell extract at 10,000 x g for 20 min at 4°C to pellet any insoluble material.
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 assays and other analyses. Then proceed to Section C to perform phosphoprotein enrichment using Phospho Magnetic Beads.

    Note: We recommend using the Pierce BCA Protein Assay Reagent Kit (Section III) for protein quantification. The detergents in the Extraction/Loading Buffer may interfere with the Bradford assay.

B. Extracting Proteins from Crude Tissues

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

- one mortar & pestle
- three 2.0 ml microcentrifuge tubes

Prepare the tissue extract as follows:

1. Transfer 50 mg of frozen tissue to a prechilled mortar (a smaller amount of tissue sample can be used).

   Note: If a smaller sample volume is desired, scale the amount of the pellet correspondingly—i.e., resuspend 12 mg of cells in 240 µl of Extraction/Loading Buffer. Scale all volumes correspondingly.
2. Add 0.125 g of alumina to the mortar.
3. Use the pestle to grind the tissue until a paste is formed.
4. Add 1 ml of prechilled Extraction/Loading Buffer.

   Note: The phosphatase inhibitor NaF (10 mM) can be added during extraction and incubation (Section C.9). Sodium orthovanadate is not compatible with magnetic phosphoprotein enrichment and if used during incubation will result in no phosphoprotein binding to the beads, unless removed beforehand using a desalting column (see Section III).
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 prechilled 2.0 ml microcentrifuge tube.
7. While holding the pestle over the mortar, rinse the pestle with 1 ml of Extraction/Loading Buffer.
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.

VI. Magnetic Phospho Enrichment Kit Protocols *continued*

10. While taking care not to disturb the pellet, transfer the supernatant to a prechilled 2 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,and proceed to Section C to perform phosphoprotein enrichment using Phospho Magnetic Beads.

    Note: We recommend using the Pierce BCA Protein Assay Reagent Kit (Section III) for protein quantification. The detergents in the Extraction/ Loading Buffer may interfere with the Bradford assay.

C. Phosphoprotein Enrichment using Phospho Magnetic Beads

General guidelines for working with magnetic beads are as follows:

- Use a pipette to mix buffer thoroughly with the beads to make a homogenous suspension.
- If needed, magnetic beads can be mixed using a vortexer.
- If there is a great deal of liquid/buffer adhering to the sides of the tube, centrifuge the tubes using a microfuge before placing them on a magnetic separator.
- Ensure that the beads are adhering to the sides to the magnet before removing the supernatant.

The protocol for magnetic bead-based purification in a single tube consists of the following steps:

1. Aliquot 200 µl of bead suspension into a 1.5 ml microfuge tube. Different amounts of beads may be used, depending on the initial amount of protein in the sample (see Table III).
2. Place the tube on a magnetic separator for 1 min and remove storage buffer.
3 Add 0.5 ml of deionized water to the beads.
4. Mix the liquid and the beads thoroughly using a pipette.
5. Place the tube on a magnetic separator and remove the supernatant.
6. To equilibrate the beads, add 0.5 ml of Extraction/Loading Buffer.
7. Repeat Steps 4 and 5.
8. Add the cell lysate (from Sections A or B) to the beads.

   Note: If the cell lysate volume is less than 100 µl, add sufficient Extraction/Loading Buffer to bring the volume up to at least 100 µl (200 µl is optimal). This is necessary to ensure thorough mixing of beads with the cell lysate, for optimal binding.

VI. Magnetic Phospho Enrichment Kit Protocols *continued*

9. Mix on a rotary shaker or end-to-end shaker for 30 min at room temperature.

   Notes:

   - If the protein is vulnerable to degradation at room temperature, incubate at 4°C for 1 hr. Protease inhibitors that do not contain EDTA can also be added during incubation.
   - The phosphatase inhibitor NaF (10 mM) can be added during extraction (Sections A and B) and incubation. Sodium orthovanadate is not compatible with magnetic phosphoprotein enrichment and if used during incubation will result in no phosphoprotein binding to the beads, unless removed beforehand using a desalting column (see Section III).

10. Place on a magnetic separator and collect the supernatant, which contains non-adsorbed material.
11. Add 0.5 ml of Wash Buffer.
12. Mix thoroughly and let it stand for a minute before placing on a magnetic separator and collecting the first wash.
13. Repeat Steps 11 and 12 twice to collect the second and third washes.
14. To elute the protein, add 100 µl of Elution Buffer. (The volume of Elution Buffer can be varied depending on the amount of beads. 100 µl of Elution Buffer can be used for eluting from 200 µl of bead suspension. Most of the protein will elute in this fraction. Smaller volumes, such as 50 µl, can be used if a concentrated sample is needed. Volumes below 50 µl may be difficult to handle.)
15. Mix for 5 min and collect Eluate 1.

    Note: If necessary, proteins can be eluted in 100 mM carbonate buffer, pH 9.3.

16. Add another 100 µl of Elution Buffer
17. Mix for 5 min and collect Eluate 2.
18. If necessary, Steps 14 and 15 can be repeated twice to ensure that protein recovery is maximized. In a specific instance, when using 200 µl of bead suspension, 85% of the total protein was eluted in first 100 µl fraction and 15% in the second fraction.
19. Use spectrophotometric and SDS-PAGE analyses to determine which eluate fractions contain the bulk of the protein.

    Note: We recommend using the Pierce BCA Protein Assay Reagent Kit (Section III) for protein quantification. The detergents in the Extraction/Loading Buffer may interfere with the Bradford assay. Either run the original lysate and non-adsorbed fraction at a 1:5 dilution or use BCA assay for the original lysate and non-adsorbed fraction.

VII. Analysis of Results and Troubleshooting Guide

A. Yield lower than expected

Column/beads were not equilibrated to pH 6.0 with Buffer A (Extraction/Loading Buffer) before loading	Equilibrate column/beads with Buffer A (Extraction/Loading Buffer) before loading samples. Additional Buffer A (Cat. No. 635626) can be purchased if necessary.
The phosphatase inhibitor sodium orthovanadate was added to the samples during the extraction.	Samples must be run through a desalting/buffer exchange column (see Section III) before loading on the column.
Samples allowed to incubate at room temperature for a long time before loading	Run samples on column/beads 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), or freeze the the samples at –20°C or –70°C.
Inadequate exposure of sample to resin/beads	Increase binding incubation time; ensure complete mixing with resin/beads.
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 (Extraction/Loading Buffer)	Desalt or dialyze sample into Buffer A (Extraction/Loading Loading Buffer). 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 the phosphatase inhibitor sodium orthovanadate to any buffers that come in contact with the resin.
Unphosphorylated proteins in eluate	Inadequate washing or saturation of column/beads capacity. For phosphoprotein column, possible protein clogging of column may have occurred. Do not load more than 8 mg total protein on the column for optimal results. For Phosphomagnetic Beads, see Table III for the optimum amount of protein.

VII. Analysis of Results and Troubleshooting Guide *cont...*

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 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.

D. Beads fail to migrate to magnet

High-viscosity sample	If sample appears to be highly viscous during the extraction, and beads fail to migrate to the magnet, add sufficient DNase I and dilute the sample with Extraction/Loading Buffer.

VIII. References

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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 wish to use phosphatase inhibitors during your extraction, sodium fluoride can be used at a final concentration of 10 mM. If sodium orthovanadate it used, it must be removed before loading on the PMAC column or incubating with Phosphomagnetic Beads because it interferes with 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:

A. 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 (Extraction/Loading Buffer) . 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 V.C) or incubating it with Phosphomagnetic Beads (Section VI.C), remove the phosphatase inhibitors by running the samples through a desalting/buffer exchange column (See Section III). If only sodium fluoride is used, it is compatible with the PMAC column/beads and desalting/buffer exchange is not required.

B. 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

A. Mass Spectrometry

- For Phosphoprotein Enrichment Column

  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 spectrometry analysis.

- For Phosphomagnetic Beads

  Elution Buffer (250 mM sodium phosphate, 0.5 M sodium chloride) does not contain detergents. Intermediate washes with detergent-free Wash Buffer prevent the carryover of residual detergent. Enriched samples may require dialysis/buffer exchange to remove excess phosphate and salt if they produce interference for mass spectrometry analysis.

B. 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.

This document has been reviewed and approved by the Clontech Quality Assurance Department.