User Manual

Phosphopeptide Enrichment Spin Columns User Manual

Clontech

United States/Canada
800.662.2566
Asia Pacific
+1.650.919.7300
Europe
+33.(0)1.3904.6880
Japan
+81.(0)77.543.6116

Clontech Laboratories, Inc.
A Takara Bio Company
1290 Terra Bella Ave.
Mountain View, CA 94043
Technical Support (US)
E-mail: tech@clontech.com
www.clontech.com

Cat. Nos. 635634
635635
PT3798-1 (PR56817)
Published 14 June 2005

Table of Contents

List of Figures

List of Tables

I. Introduction & Protocol Overview

The **Phosphopeptide Enrichment Spin Columns** (Cat. No. 635634) and **Phosphopeptide Enrichment Buffer Kit** (Cat. No. 635635) provide a quick and convenient method for isolating phosphorylated peptides. They can be used with trypsin-digested extracts of mammalian cells and tissues, as well as tryptic digests of purified proteins. Peptides that carry a phosphate group on any amino acid—serine, tyrosine, or threonine—are selectively bound by the Phosphopeptide Resin. Non-phosphorylated peptides and other contaminants contained in the columns simply pass through the resin, allowing the enrichment of phosphorylated peptides which can then be eluted from the column.

Significance of phosphopeptide analysis

Protein phosphorylation provides an important mechanism for regulating many essential intracellular processes such as signal transduction, transcription, cell division, and metabolic pathways. Phosphorylation and dephosphorylation of proteins can influence protein interactions, enzymatic activity, and protein turnover. In order to study the dynamics of phosphorylation at specific sites within a protein, phosphoproteins must be digested into peptides and analyzed using mass spectrometry or HPLC (Larsen e*t al.*, 2001; Liao e*t al.*, 1994). Since phosphoproteins are often present in low abundance within a cell, they can be difficult to detect in a cell extract unless an enrichment method is used.

Many current phosphoprotein enrichment methods result in chemical modification of the phosphate group followed by binding to a solid support. In contrast, our method introduces no irreversible chemical changes but rather relies on the selectivity of the resin. This method is utilized in the **Phosphoprotein Enrichment Kit** (Cat. No. 635624) for isolating intact phosphoproteins in their native state from crude cell extracts. The Phosphopeptide Resin in our **Phosphopeptide Enrichment Spin Columns** and and the buffers in our **Phosphopeptide Enrichment Buffer Kit** have been optimized for isolating phosphopeptides directly from crude cell extracts, tissue extracts, or purified proteins digested with trypsin, in a form suitable for downstream applications such as mass spectrometry or HPLC (Posewitz e*t al.*, 1999; Stensballe e*t al.*, 2001).

Phosphopeptide enrichment method

The phosphopeptide enrichment protocol is outlined in Figure 1. Phosphopeptide Enrichment Spin Columns are ready for use after centrifuging to remove the storage buffer. Then the bottom of the column is capped, a trypsin-digested protein sample is diluted with Loading Buffer and loaded onto the column, and the top of the column is capped. The column is placed on a shaker for 10 min to allow the sample to bind to the resin. The PMAC Resin is highly selective for the phosphates on the peptides, allowing other peptides and contaminants to pass through in the flowthrough and washes. Then the enriched phosphorylated peptides are eluted from the column with Elution Buffer. This entire protocol can typically be performed in as little as 30 min.

I. Introduction & Protocol Overview *continued*

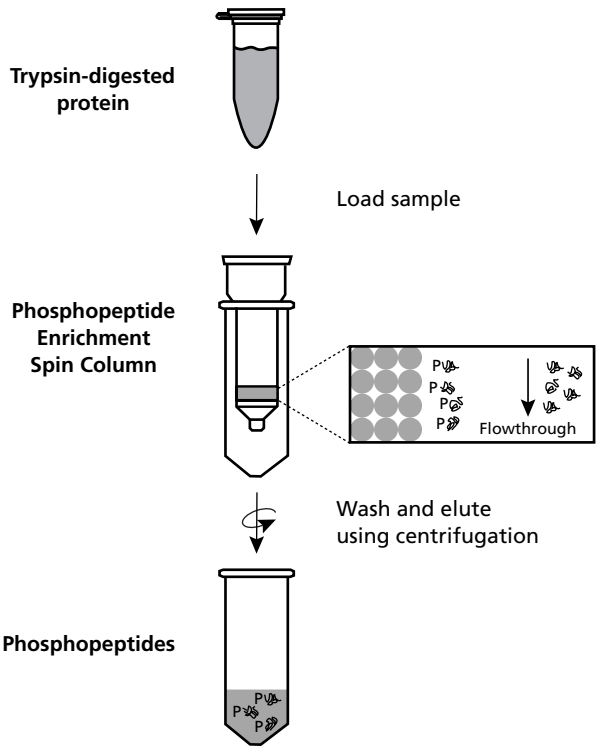

Figure 1. Overview of the Phosphopeptide Enrichment protocol. A simple spin column protocol using buffers that contain no detergent yields enriched phosphopeptides suitable for downstream applications.

This simple protocol is suitable for isolation of phosphopeptides from crude mammalian cell and tissue extracts digested with trypsin, as well as tryptic digests of purified proteins. Most types of cell extracts can be used, provided the extraction buffer contains no phosphatase inhibitors or EDTA, or they are removed prior to trypsin digestion and sample loading.

Each Phosphopeptide Enrichment Spin Column has a maximum binding capacity of 250 μg of phosphopeptide. Sample volumes of up to 0.85 ml can be loaded on the column, which is disposable and fits inside most microcentrifuges, seated inside a 2 ml microcentrifuge tube. Our buffer kit contains detergent-free general purpose loading and elution buffers. These buffers and specified alternative elution buffers, all listed in Section III, are suitable for a variety of downstream applications.

I. Introduction & Protocol Overview *continued*

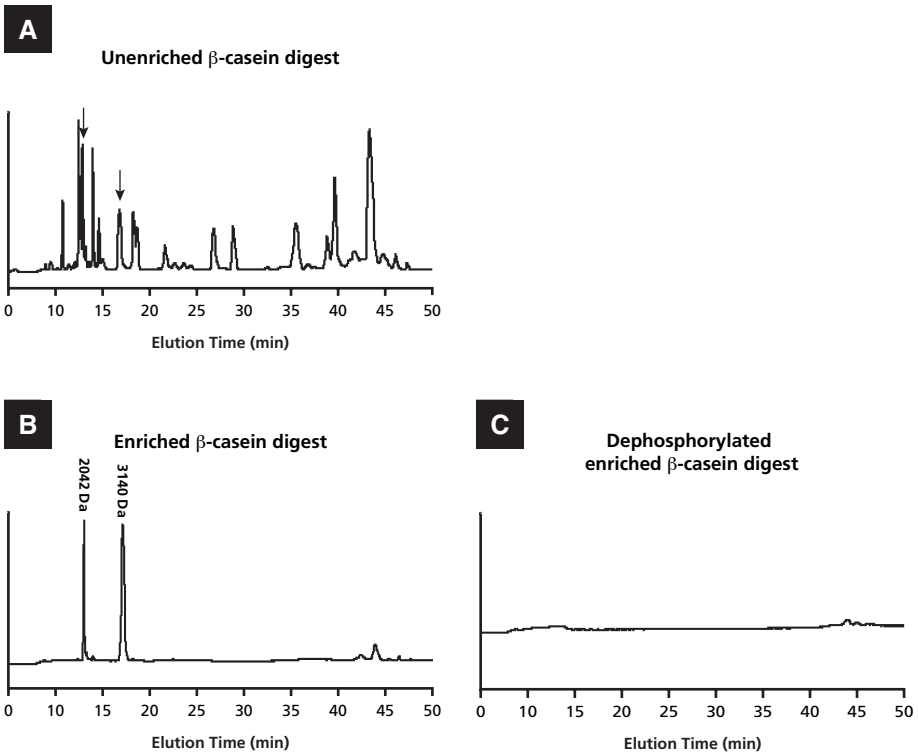

Figure 2. Purification of phosphopeptides using Phosphopeptide Enrichment Spin Columns. β-casein protein was digested with trypsin (sequencing grade), diluted at a ratio of 1:1 with Loading Buffer, and run on a Phosphopeptide Enrichment Spin Column. The enriched fraction was eluted with Elution Buffer, then lyophilized and reconstituted in 0.1% TFA in water (v/v). Reverse phase HPLC (RP-HPLC) data is shown for the crude protein digest (Panel A), the eluate (purified phosphopeptides; Panel B) and the eluate of a dephosphorylated crude protein digest (Panel C). The eluted fractions were separated by RP-HPLC on an XTerra RP18 column (5 µm, 4.6 x 150 mm) with UV detection at 215 nm (Solvent A: 0.1% TFA in water [v/v], Solvent B: 0.1% TFA in acetonitrile [v/v]). The two phosphopeptide fractions collected in Panel B were analyzed using MALDI, identifying the expected phosphorylated proteins (data not shown).

The results obtained from a tryptic digest of β-casein that was purified using a Phosphopeptide Enrichment Spin Column and analyzed via reverse-phase HPLC are shown in Figure 2. The digest produced approximately 16 peptides, of which two are known to be phosphorylated (Liao e*t al.*, 1994). The two phosphorylated peptides were present in the column eluate (Figure 2, Panel B), demonstrating that the column specifically bound these peptides. Further evidence for specific phosphorylated peptide binding is seen in Figure 2, Panel C, where these peptides no longer bound to a column loaded with β-casein that had been dephosphorylated with alkaline phosphatase prior to digestion with trypsin.

II. List of Components

Store spin columns at 4°C.

- 25 Phosphopeptide Enrichment Spin Columns
  (Two 2 ml collection tubes are provided for each column)

III. Additional Materials Required

The following materials are required but not supplied:

Buffers:

The **Phosphopeptide Enrichment Buffer Kit** (Cat. No. 635635), provides the following buffers for use with these spin columns:

— Loading Buffer (250 mM acetic acid, pH 2.5–3.5)

— Elution Buffer (100 mM ammonium bicarbonate buffer, pH 9.0–9.5)

Alternatively, you may prepare the following buffers:

- **Loading/washing buffer: 250 mM acetic acid, pH 2.5–3.5**
- **Elution buffer:** Choose one of the following buffers as suitable for your downstream application.

  — 100 mM ammonium bicarbonate buffer, pH 9.0–9.5

  — 100 mM sodium carbonate buffer, pH 9.3

  — Phosphate buffered saline

Other materials:

- **Trypsin:** Your choice of trypsin vendor and grade will depend on your downstream application (Section V.A).
- **2 ml microcentrifuge tubes**
- **pH paper (with a range suitable for measuring pH 3.5 or less)**
- **Gel Filtration Column PD-10 (GE Healthcare, Cat. No. 17-0851-01)** (optional—needed only for phosphatase inhibitor or EDTA removal)

IV. General Considerations

A. Sample Preparation

The columns may be loaded with trypsin-digested cell and tissue extracts, as well as tryptic digests of purified proteins. For extraction procedures and reagents, refer to the Phosphoprotein Enrichment Kit User Manual (PT3731-1), which may be downloaded from **www.clontech.com/clontech/techinfo/manuals/index.shtml**. See Section V.A of this User Manual for guidelines on digesting a protein sample with trypsin.

B. Phosphatase Inhibitors and EDTA

The protein extraction and/or the protein storage buffer should not contain any phosphatase inhibitors or EDTA. **Phosphatase inhibitors interfere with phosphopeptide binding to the column resin and EDTA causes metal leaching from the resin.** These inhibitors need not be present when enriching phosphopeptides with our columns because of the quickness of the protocol. However, if your application requires the use of phosphatase inhibitors or EDTA, the **inhibitors or EDTA must be removed** by running your sample through a desalting column (See Additional Materials Required & Section V.A) **before** digesting it with trypsin and loading it on a Phosphopeptide Enrichment Spin Column.

C. Column Capacity

Each spin column can bind up to 250 μg of phosphopeptide. A sample volume of up to 0.85 ml can be loaded onto a column.

D. Sample Storage

Samples should be stored at –20°C after trypsin digestion and 4°C or –20°C after phosphopeptide enrichment.

V. Phosphopeptide Enrichment Protocol

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

General

These methods outline general guidelines for preparing trypsin digests from cell extracts, tissue extracts, or purified protein samples, and specific instructions for enriching these digests for phosphopeptides using **Phosphopeptide Enrichment Spin Columns**. The column enrichment protocol can be carried out immediately following trypsin digestion, or the digest can be stored at –20°C.

- The resin in the column will change color as a normal part of the enrichment process.
- Phosphopeptide Enrichment Spin Columns are intended for single use only and cannot be regenerated. **Do not reuse the columns in this kit.**
- Pre-equilibration of the columns is not required. Columns are supplied in loading buffer (250 mM acetic acid, pH 2.5–3.5). The protocol includes a quick centrifugation step to remove the storage buffer from the column before the sample is loaded.
- **The sample should not contain any phosphatase inhibitors or EDTA.**
- Before you begin the column enrichment protocol, bring the following materials to room temperature:
  - — Loading Buffer
  - — Elution Buffer
  - — sterile distilled water
  - — Phosphopeptide Enrichment Spin Columns

A. Preparation of Protein Digest

The optimal conditions for digesting a protein sample with trypsin vary for different protein samples. The following conditions used to prepare the β-casein protein digest in Figure 2 can serve as general guidelines for setting up a protein digest. Table I provides a list of conditions that may be varied and conditions that should remain constant when optimizing a protein digest.

1. Dissolve the protein (1–10mg) in 0.1 M sodium carbonate buffer, pH 8.3. Use a buffer volume between 25 µl and 1 ml.

   Note: If phosphatase inhibitors or EDTA are present in the extraction or storage buffer of a liquid protein sample, remove them before digestion with trypsin by desalting the sample in 0.1 M sodium carbonate buffer, pH 8.3, using a PD-10 column (GE Healthcare, Cat. No. 17-0851-01).

2. Add urea to a final concentration of 8 M to denature the sample and mix for 10 min at room temperature.

V. Phosphopeptide Enrichment Protocol *continued*

TABLE I. OPTIMIZING PROTEIN DIGESTION CONDITIONS

Variable Conditions	Constant Conditions
Incubation time	Dilute 8 M urea to <1 M
Amount of trypsin	Do not heat sample
Type of trypsin	No phosphatase inhibitors or EDTA
Buffer composition	

Notes:

- Avoid heating the sample during denaturation to prevent akaline hydrolysis of phosphoserine and phosphothreonine.
- 8 M urea is preferred over 6 M guanidine for denaturation, since guanidine concentrations of 1 M and above interfere with the binding of phosphopeptides to the column.

3. Dilute the sample with additional buffer (0.1 M sodium carbonate, pH 8.3.) to bring the urea concentration below 1 M before trypsin treatment.
4. Add trypsin (Promega Sequencing Grade Modified Trypsin, Frozen, Cat. No. V5113) in a 1:100 ratio (w/w) of trypsin:protein. Incubate the sample at 37°C overnight.

   Note: Your choice of trypsin vendor and grade will depend on your downstream application.
5. Use the digested sample immediately or store it as such at –20°C. If necessary, it may be lyophilized for storage at –20°C.

V. Phosphopeptide Enrichment Protocol *continued*

B. Column Enrichment

1. Column Preparation

a. Place the column inside a 2 ml collection tube and allow it to stand at room temperature in an upright position until the resin settles.

b. Remove the column top cap and then snap off the bottom tip. Save the top cap.

c. Return the column to the 2 ml collection tube, centrifuge at 4,000 rpm for 20 sec to remove the storage buffer, and discard the flowthrough. Recentrifuge if any buffer remains above the resin surface.

Note: The column storage buffer and the washes have a low pH (<3.5). Discard them according to the required disposal methods.

2. Sample Preparation

a. Dissolve a dry sample (from Step V.A.5) in 0.1–0.85 ml (the maximum sample volume) of Loading Buffer. If the sample is already in solution, dilute it 1:1 with Loading Buffer. For samples smaller than 0.1 ml, raise the volume to 0.1 ml with Loading Buffer.

b. Using pH paper, measure the sample pH, which should be <3.5 to ensure efficient binding. If it is higher, add Loading Buffer in 25 µl increments up to a final volume of no more than 0.85 ml and recheck the pH.

Note: For sample volumes >0.4 ml, the pH can be decreased by adding concentrated acetic acid. Add acetic acid with caution, as this may cause the sample to precipitate, reducing the yield.

c. If the sample is cloudy or contains a precipitate, centrifuge briefly to remove the insoluble material.

3. Sample Loading and Binding

a. Insert the bottom end of the column into the bottom cap and return it to the 2 ml collection tube.

b. Load the sample into the top reservoir of the column.

c. Insert the top cap into the column.

d. To bind the sample to the resin, mix the column contents on a shaker for 10 min or invert it every two minutes for a total of 10 min.

Note: The binding and elution steps are carried out at room temperature. Collected fractions should be stored on ice.

e. Remove the column top cap and then the bottom cap. Save the caps.

Note: If any sample enters the bottom cap, collect it with a pipette and return it to the column before centrifuging.

f. Return the column to the 2 ml collection tube and centrifuge at 4,000 rpm for 20 sec to collect the flowthrough.

V. Phosphopeptide Enrichment Protocol *continued*

Note: After each subsequent centrifugation, transfer the collection tube contents into a different tube if the flowthrough or washes are required for further analysis. If not, discard the collection tube contents after each step prior to elution. The fractions have a low pH (<3.5) and should be discarded according to the required disposal methods.

4. Washing the Column

a. Add 400 µl of Loading Buffer and centrifuge at 4,000 rpm for 20 sec to collect the first wash.

Note: For best results, insert the bottom cap before adding the Loading Buffer, then insert the top cap. Incubate for 5 min with mixing, then remove and save the caps before centrifuging. This will ensure removal of any unbound material.

b. Add 400 µl of Loading Buffer and centrifuge at 4,000 rpm for 20 sec to collect the second wash.

c. Repeat Step 4.b two more times (for a total of four washes). Additional washes are recommended for highly concentrated samples.

d. Add 400 µl of water and centrifuge at 4,000 rpm for 20 sec to collect the final wash.

5. Eluting from the Column

a. Cap the bottom of the column and add 300 µl of Elution Buffer. Cap the top of the column.

b. Mix the column contents on a shaker or by inverting for 5 min.

c. Remove both column caps and discard them.

d. Place the column in a collection tube and centrifuge at 4,000 rpm for 20 sec to collect Eluate 1.

Note: Use a fresh 2 ml collection tube provided in the kit to collect Eluate 1 and additional 2 ml collection tubes (not supplied) to collect the remaining three eluates.

e. Add 300 µl of Elution Buffer and centrifuge at 4,000 rpm for 20 sec to collect Eluate 2.

f. Repeat Step 5.e two more times (for a total of four eluates). Eluate 1 is most likely to have the highest concentration of phosphorylated peptides. The samples are now ready for further analysis and may be concentrated using lyophilization.

VI. Analysis of Results & Troubleshooting Guide

A. Yield lower than expected

Phosphatase inhibitors were added to the samples during the extraction	Samples must be run over a desalting/buffer exchange column or dialyzed before digestion with trypsin (see Additional Materials Required and Section V.A).
EDTA is present in the sample	Desalt before digestion.
Inadequate exposure of sample to resin	Increase sample binding incubation time to ensure complete mixing with resin.
Elution not performed at room temperature	Perform elution step with all materials at room temperature (19–23°C).

B. Incomplete separation of phosphorylated peptides from other peptides

Phosphopeptides or yellow color in flowthrough	Do not add phosphatase inhibitors or EDTA to any buffers that come in contact with the resin.
Unphosphorylated peptides in eluate	Inadequate washing or overloading of column. Perform additional washes before eluting. Do not load more than 250 μg of phosphopeptide for optimal results.

C. Column clogging

Cellular debris in sample	Centrifuge the sample for 1 min to remove insoluble material before loading the column.
High-viscosity sample	If the sample appears to be highly viscous during the extraction, addition of DNase I will often reduce the viscosity and prevent clogging.

D. Problems with trypsin digestion

Digestion is incomplete	Verify that the residual urea or guanidine concentration is <1 M following denaturation. Increase incubation time with trypsin. Optimize trypsin concentration and incubation time.
Alkaline hydrolysis of phosphoserine & phosphothreonine	Avoid heating the sample during denaturation, when it is present in an alkaline buffer.

VI. Analysis of Results & Troubleshooting Guide *continued*

E. Purified sample is too dilute

Sample needs to be concentrated for downstream applications	Lyophilize the sample and reconstitute it in the recommended buffer. Do not use a speed vac for concentrating samples, as heating the sample under alkaline conditions leads to hydrolysis.

VII. References

Phosphopeptide Enrichment Spin Columns. (July 2004) *Clontechniques* **XIX**(3):14.

Larsen, M. R., Sorensen, G. L., Fey, S. J., Larsen, P. M. & Roepstorff, P. (2001) Phospho-proteomics: Evaluation of the use of enzymatic de-phosphorylation and differential mass spectrometric peptide mass mapping for site specific phosphorylation assignment in proteins separated by gel electrophoresis. *Proteomics* **1**:223–238.

Liao, P., Leykam, J., Andrews, P. C., Gage, D. A. & Allison, J. (1994) An Approach to Locate Phosphorylation Sites in a Phosphoprotein: Mass Mapping by Combining Specific Enzymatic Degradation with Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry. *Anal. Biochem.* **219**:9–20.

Posewitz, M. C. & Tempst, P. (1999) Immobilized Gallium(III) Affinity Chromatography of Phosphopeptides. *Anal. Chem.* **71**:2883–2892.

Stensballe, A., Andersen, S. & Jensen, O. N. (2001) Characterization of phosphoproteins from electrophoretic gels by nanoscale Fe(III) affinity chromatography with off-line mass spectrometry analysis. *Proteomics* **1**:207–222.

VIII. Related Products

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

Products	Cat. No.
• Phosphopeptide Enrichment Buffer Kit	635635
• Phosphoprotein Enrichment Kit	635624
• TALON® Products	
Purification Kit	635515
Metal Affinity Resin	635501 635502 635503 635504
Superflow Metal Affinity Resin	635506 635507
TALONspin Columns	635601 635602 635603
2 ml Disposable Gravity Columns	635606
CellThru	635509 635510
Buffer Kit	635514
HT 96-Well Purification Plate	635622
xTractor Buffer Kit	635623
xTractor Buffer	635625
• Glutathione Resins Products	
Uniflow Resin	635610 635611
Superflow Resin	635607 635608
• Thiophilic Resins	
Uniflow Resin	635613 635614
Superflow Resin	635616 635617
• CHROMA SPIN™ Columns	many