Magnetic Phosphopeptide Enrichment Kit User Manual

PT3950-1 (PR732185)
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I. Introduction

A. Protocol Overview

The Magnetic Phosphopeptide Enrichment Kit provides a quick and convenient method for isolating phosphorylated peptides from tryptic digests (*Clontechniques,* April 2007). Protein samples or extracts are prepared by denaturing with urea followed by digestion with trypsin to render a solution of mixed peptides. Phosphopeptide enrichment is then performed on the sample using Phospho Magnetic Beads (Figure 1). Peptides that carry a phosphate group on any amino acid—serine, tyrosine, or threonine—are selectively bound to the beads, while nonphosphorylated peptides and other unbound contaminants are removed during subsequent bead washing steps. Bound phosphorylated peptides are finally eluted in an enriched solution.

B. Binding Capacity

Phospho Magnetic Beads have a binding capacity of 1–2 pmol of phosphate per µg of beads, and are provided as a 5% solution (50 mg/ml). Therefore, 100 µl of the bead suspension contains 5 mg of beads and will bind 5–10 µmol phosphate.

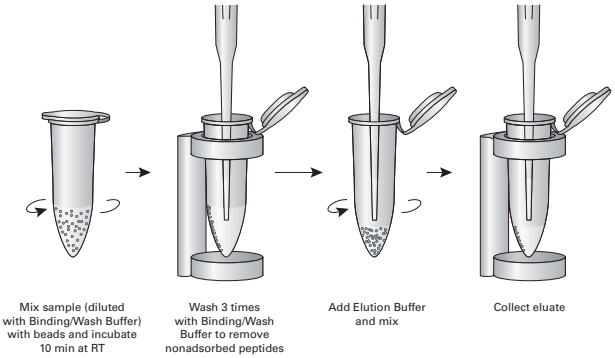

Figure 1. The Magnetic Phosphopeptide Enrichment Kit protocol. Phosphopeptide enrichment is carried out in a single microfuge tube, using a simple 30 min protocol. Phospho Magnetic Beads that have been equilibrated with Binding/Wash Buffer are mixed with the sample (diluted with Binding/Wash Buffer) for 10 min at room temperature (RT). Then the mixture is placed on a magnetic separator to collect the beads and allow removal of buffer containing unbound peptides. The separator is used to wash the beads three times with Binding/Wash Buffer and once with distilled water to remove remaining nonadsorbed material and unphosphorylated peptides. Phosphopeptides are finally eluted in as little as 20 µl of Elution Buffer.

II. List of Components

Store all components at 4°C.

Magnetic Phosphopeptide Enrichment Kit (Cat. No. 635643)

- 2 x 1.0 ml Phospho Magnetic Beads
- 45 ml Binding/Wash Buffer; pH 3.0
- 15 ml Elution Buffer (100 mM ammonium bicarbonate buffer; pH 9.0–9.5)

Other

- User Manual (PT3950-1)

Visit our Protein Expression and Purification product pages at **www.clontech.com** for a current list of products available for protein research.

III. Additional Materials Needed

A. Required

- Magnetic separator for 1.5 ml tubes
- Urea
- Trypsin or immobilized trypsin. Choose a vendor and grade to suit your protein or downstream application.
- pH paper (with a range suitable for measuring pH 3.5 or less)

B. Optional

- PD-10 Desalting Columns (GE Healthcare, Cat. No. 17–0851–01). These are needed only to remove phosphatase inhibitors or EDTA from the protein sample prior to digestion and enrichment.
- Alternative elution buffers
  - 100 mM sodium carbonate buffer, pH 9.3
  - Phosphate buffered saline (PBS; 250 mM sodium phosphate, 0.5 M sodium chloride, pH 7.2)

IV. Phosphopeptide Enrichment

A. Using the Phospho Magnetic Beads

This section lists important things to know about using the Phospho Magnetic Beads.

- Phospho Magnetic Beads are intended for single use only and cannot be regenerated. Do not reuse the beads.
- Samples should not contain EDTA or phosphatase inhibitors other than sodium fluoride.
- 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.
  - During washing and elution procedures, ensure that the beads are adhering to the side of the tube toward the magnet before removing the supernatant. A microcentrifuge can also be used to pellet the beads, but this provides less efficient washing and elution, and may result in loss of beads over successive washes.

B. Preparing Peptides by Tryptic Digestion

The optimal conditions for preparing tryptic peptides from a protein sample will depend on the trypsin used and on the protein being digested. In general, follow the digestion protocol provided with your trypsin preparation. To prevent alkaline hydrolysis of phosphate groups, do not heat protein samples to high temperature during protein denaturation with 8 M urea.

NOTE: If phosphatase inhibitors or EDTA are present in the extraction or storage buffer of a protein sample, remove them before digesting with trypsin by desalting the sample with 0.1 M sodium carbonate buffer, pH 8.3, and a PD-10 Desalting Column (GE Healthcare, Cat. No. 17-0851-01), per the manufacturer's protocol. Only sodium fluoride (NaF) is known to be compatible with the beads.

IV. Phosphopeptide Enrichment continued

Protocol
30 min

C. PROTOCOL: Phosphopeptide Enrichment Using Phospho Magnetic Beads

Phosphopeptide enrichment can be carried out immediately following trypsin digestion, or after the digest has been stored at –20°C. The protocol for a single tube consists of the following steps:

Phospho Magnetic Bead Equilibration

1. Aliquot 100 µl of bead suspension into a 1.5 ml microfuge tube. Different amounts of beads may be used, depending on the initial peptide concentration of the sample.
2. Place the tube on a magnetic separator for 1 min and remove storage buffer.
3. Add 200 µl 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 200 µl of Binding/Wash Buffer.
7. Repeat Steps 4 and 5, and proceed to Phosphopeptide Enrichment.

Phosphopeptide Enrichment

1. Dilute the solution of digested peptides (from Section B) at least 1:4 with Binding/Wash Buffer to ensure a pH of 3.0. Add the diluted peptide solution to the washed and equilibrated beads. V

   NOTE: Trypsin digestion requires pH 8.0. Verify that the pH is 3.0 with pH paper, before adding the peptide solution to the beads.
2. Mix on a rotary shaker or end-to-end shaker for 10 min at room temperature.
3. Place on a magnetic separator and collect the supernatant, which contains nonadsorbed material.
4. Add 200 µl of Wash Buffer.
5. Mix thoroughly and let it stand for a minute before placing on a magnetic separator and collecting the first wash.
6. Repeat Steps 4 and 5 twice to collect the second and third washes.
7. To elute the protein, add 25 µl of Elution Buffer. (The volume of Elution Buffer can be varied depending on the amount of beads.)
8. Mix for 2 min and use the magnetic separator to collect Eluate 1.

   NOTE: If necessary, peptides can be eluted in 100 mM carbonate buffer, pH 9.3.
9. Add another 25 µl of Elution Buffer
10. Mix for 2 min and collect Eluate 2.
11. If necessary, Steps 7 and 8 can be repeated twice to ensure maximal peptide recovery.
12. Prior to storage at 4°C or –20°C, neutralize the fractions by adding 1–2 µl of concentrated acetic acid. Samples before and after enrichment can be stored at 4°C for short term storage and at –20°C for long term storage. Neutralize eluted fractions with 1–2 µl of concentrated acetic acid.

V. Troubleshooting Guide

Table I. Troubleshooting Guide for Magnetic Phosphopeptide Enrichment

Description of Problem	Explanation	Solution
Phosphopeptide yield is lower than expected/phosphopeptides in flowthrough	Phosphatase inhibitors present in the sample during enrichment	Samples containing phosphatase inhibitors (other than NaF) must be treated with a desalting or buffer exchange column prior to digesting with trypsin (see Section III, Additional Materials Required, and Section V.A).
	1. EDTA is present in the sample; 2. pH > 3.5	1. Desalt sample before digestion 2. Further dilute sample with Binding/Wash Buffer or add acetic acid to ensure pH < 3.5. Check with pH paper.
	Inadequate exposure of sample to the beads	Increase sample incubation time with the beads to ensure complete peptide binding. If incubating longer than 30 min, perform at 4°C.
Incomplete separation of peptides: unphosphorylated peptides in eluate.	Inadequate washing or overloading of beads.	Perform additional washes before eluting. Decrease the amount of sample loaded onto the beads.
Beads and sample is viscous. Beads fail to migrate to magnetic separator.	Cellular debris in sample	Centrifuge sample to remove insoluble material before adding to the beads.
Problems with trypsin digestion.	Digestion incomplete	Verify that the residual urea or guanidine concentration has been diluted to <1 M before digestion step. Increase trypsin incubation time. Optimize trypsin concentration and incubation time.
Alkaline hydrolysis of phosphoserine and phosphothreonine	Denaturation temperature too high in the presence of alkaline buffer.	Avoid excessively high temperatures during sample denaturation. Room temperature denaturation for longer periods (2–6 hr) is adequate for most proteins.
Eluted phosphopeptide solution is too dilute	Concentration of phosphopeptides may have been low in original sample	Decrease elution buffer volume or lyophilize the eluted peptides and reconstitute in buffer suitable for downstream applications

VI. References

Liao, P. *et al.* (1994) *Anal. Biochem.***219**(1):9–20.

Magnetic Phosphopeptide Enrichment Kit (2007) *Clontechniques* **XXII**(2):1–2.

Appendix A: Sample Results and Analysis

Phospho Magnetic Beads demonstrate high binding specificity and affinity for diverse phosphopeptides, irrespective of the site of phosphorylation. The specific enrichment of two phosphoserine peptides from β-casein, as well as that for a synthetic phosphotyrosine peptide, are shown in Figure 2. The β-casein protein was digested with immobilized trypsin to generate 16 peptides, two of which are known to contain phosphoserine (Liao, P. *et al.*) (Figure 2, Panel A). After performing phosphopeptide enrichment, the two phosphorylated peptides were present in the enriched fraction eluted from the beads and could be resolved by reverse phase HPLC (Figure 2, Panel B). The specific binding and recovery of a synthetic phosphotyrosine (pY) peptide is also shown in Figure 2 (Panels C & D). Details of the phosphopeptides are provided in Table II.

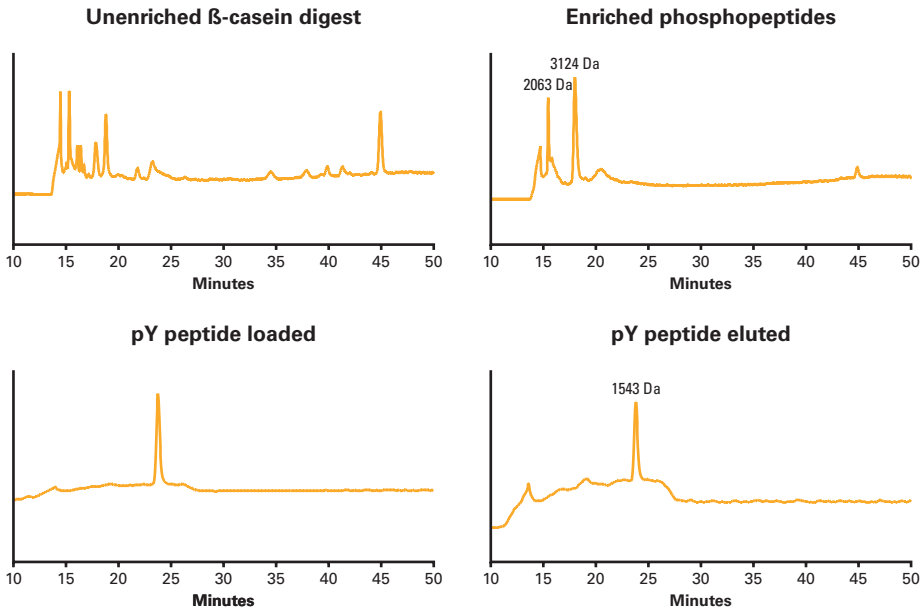

Figure 2. Phosphopeptide purification with the Magnetic Phosphopeptide Enrichment Kit. Panels A & B. ß-casein tryptic peptides were generated by denaturing the protein with 8 M urea (2 hr at RT) and digesting it with immobilized trypsin (overnight at 37°C). The peptide mixture was diluted with Binding/Wash Buffer and mixed with Phospho Magnetic Beads at room temperature for 10 min. After washing, the enriched fraction was eluted with Elution Buffer. Reverse phase-HPLC (RP-HPLC) analysis was performed on a Waters Breeze HPLC, XTerra® RP18 column (5 m, 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]). RP-HPLC data are shown for the crude protein digest in **Panel A** and for the eluate containing enriched phosphopeptides in **Panel B. Panels C & D.** A synthetic pY peptide was dissolved in Binding/Wash Buffer (**Panel C**) mixed with Phospho Magnetic Beads. After washing, bound phosphopeptide was eluted from the beads with Elution Buffer and subjected to RP-HPLC analysis (**Panel D**).

Table II: Phosphopeptides Enriched using the Magnetic Phosphopeptide Enrichment Kit

Peptide	$[M+H]^+$ Da	Amino acid sequence
pS Monophosphopeptide, T6 (1P) from ß-casein (bovine)	2062.96	Phe-Gln-pSer-Glu-Glu-Gln-Gln-Gln-Gln-Thr-Glu-Asp-Glu-Leu-Gln-Asp-Lys
pS Tetraphosphopeptide, T1-2(4P) from ß-casein (bovine)	3123.92	Arg-Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-pSer-pSer-pSer-Glu-Glu-Ser-Ile-Thr-Arg
pY peptide pp60c-src Fragment 521-533	1543.48	Thr-Ser-Thr-Glu-Pro-Gln-pTyr-Gln-Pro-Gly-Glu-Asn-Leu