Enrichment of Low-molecular-weight Phosphorylated Molecules

- Phosphate-affinity Enrichment using Phos-tag™ Tip -

Ver. 1 (2014/11)

1. Introduction

Phosphorylation is a fundamental covalent post-translational modification that regulates the function, localization, and binding specificity of target proteins. Methods for determining the phosphorylation status of proteins (i.e., phosphoproteomics) are thus very important for the evaluation of diverse biological and pathological processes. In 2002, Prof. Koike's group (Hiroshima University) reported that a dinuclear metal complex (i.e., 1,3-bis[bis(pyrindin-2-ylmethyl)amino]propan-2-olato dizinc(II) complex) acts as a selective phosphate-binding tag molecule, Phos-tag™ in an aqueous solution at a neutral pH (e.g., $K_d = 25$ nM for phenyl phosphate dianion, Ph-OPO$_4^{2-}$). Since then, various methods for phosphoproteome research have been developed using Phos-tag™ derivatives. Here, we introduce a simple and efficient protocol to enrich low-molecular-weight phosphorylated molecules under near-physiological conditions. This method is based on immobilized metal affinity chromatography (IMAC) using a phosphate-binding tag molecule (a dinuclear zinc(II) complex) attached on a highly cross-linked agarose bead.

2. Description of Phos-tag™ Tip

Phos-tag™ Tip (FMN° binding site ≥50 nmol/tip) provides an efficient procedure for separation of low-molecular-weight phosphorylated molecules such as nucleotides and peptides from biological samples at physiological pH. The procedure of the phosphate-affinity enrichment requires a 1-mL syringe attached with a silicon tube adapter and the aqueous buffers for the binding, washing, and elution processes. Phos-tag™ Tip containing 10 µL of swelled Phos-tag™ gel (Zn$^{2+}$-binding form) is supplied in a preservation solution of 20%(v/v) 2-propanol. The Phos-tag™ gel has no irritant effect on the skin. Store the tip in a refrigerator at ca. 4°C. Under this condition, the product is stable for at least one year. *FMN: riboflavin-5′-phosphate (a flavin mononucleotide)

3. Warning and Limitations

Phos-tag™ Tip is not for use in human diagnostic and therapeutic procedures. Do not use internally or externally in human or animals. It's used only for research. Care should be taken to avoid contact with Phos-tag™ gel. In the case of contact with skin or eyes wash immediately with water.

4. Advantages of Phos-tag™ Tip Method

# The total time for the phosphate-affinity enrichment is less than 30 min.
# The method requires no special apparatus and the procedure is simple.
# The buffers for the binding, washing, and elution processes are all under near-physiological conditions.
# Phos-tag™ Tip captures inorganic phosphates (HPO$_4^{2-}$ or HP$_2$O$_7^{3-}$) and various phosphononoester dianions ($ROPO_4^{2-}$) bound to amino acids (Tyr, Thr, Ser, Asp, His, etc.), sugars, and lipids.
# Desalting of the phosphate-enriched sample in the elution fraction can be conducted by a commonly used method using a micro bed of reversed-phase resin.
5. Principle of Phos-tag™ Tip Method

6. Solutions for Phosphate-affinity Enrichment (at ca. 25°C)

**Sol. A:** Preservation solution: 20%(v/v) 2-propanol/water (100 mL)
- # 2-propanol
- # distilled water for preparation of the 100 mL solution

**Sol. B:** Binding/Washing buffer:
- 0.10 mol/L Bis-tris–CH₃COOH + 0.10 mol/L CH₃COONa (pH 6.8, 100 mL)
  - # Bis-tris (MW = 209, 10 mmol)
  - # CH₃COONa (FW = 82, 10 mmol)
  - # aqueous 1.0 M CH₃COOH (4.6 mmol) solution
  - # distilled water for preparation of the 100 mL solution

Note: Check the pH is 6.8 ± 0.1 by using a pH meter. In some cases, a neutral salt such as 0.10 M NaCl (+ 4.0 mL of 1.0 M CH₃COOH) can be used instead of CH₃COONa.

**Sol. C:** Elution buffer 1: 0.10 M Na₄P₂O₇–0.10 M CH₃COOH buffer (pH 7.0, 100 mL)
- # Na₄P₂O₇·10H₂O (FW = 446, 10 mmol)
- # aqueous 1.0 M CH₃COOH solution (10 mmol)
- # distilled water for preparation of the 100 mL solution

Note: Check the pH is 7.0 ± 0.1 by using a pH meter.

**Sol. D:** Elution buffer 2 (Reactivation buffer 1): 0.10 M EDTA–NaOH buffer (pH 7.0, ca. 100 mL)
- # aqueous 0.10 M EDTA–2Na solution (a commercial product)
- # aqueous 10 M NaOH solution (a commercial product) for adjustment of pH to 7.0 ± 0.1 by using a pH meter.

**Sol. E:** Reactivation buffer 2:
- 0.10 mol/L Bis-tris–CH₃COOH + 10 mmol/L Zn(CH₃COO)₂ (pH 6.8, 100 mL)
  - # Bis-tris (MW = 209, 10 mmol)
  - # aqueous 0.10 mol/L Zn(CH₃COO)₂ solution (a commercial product)
  - # distilled water
  - # aqueous 1.0 M CH₃COOH solution for adjustment of pH to 6.8 ± 0.1 by using a pH meter.
  - # distilled water for preparation of the 100 mL solution
7. Basic Protocol for Phosphate-affinity Enrichment

Preparation of Phos-tag™ Tip

1) Wash the outside of Phos-tag™ Tip and the inside space over the upper filter using distilled water to remove the preservation solution.

2) After addition of 100 µL of Sol. B into the tip from the top, attach a 1-mL syringe with a silicon-tube adapter to the tip.

3) Dispense the liquid out with air by using the empty syringe.

4) Steps 2 & 3 are repeated once more.

   Note: Please check that Phos-tag™ gel (10 µL) in the space (ca. 30 µL) between the two filters is compressed on the lower filter.

Flow-through Fraction (FT)

5) Prepare a sample solution (30 ~ 100 µL in a microtube) containing ≤50 nmol phosphorylated species in Sol. B: The sample solution pH should be in ranged between 6 and 8.

6) Draw the sample solution gently into the tip by using the 1-mL syringe.

   All the sample solution is passed through the Phos-tag™ gel and then the liquid is moved above the upper spherical filter.

7) All the liquid in the tip is gently dispensed into the microtube used for the sample preparation.

8) Steps 6 & 7 are repeated five times, and then the liquid is dispensed completely to obtain a flow-through fraction (FT) in the microtube.
**Washing Fractions (W1, W2, W3, and W4)**

9) After remove the syringe, 100 µL of Sol. B is added into the tip from the top.

10) Attach a 1-mL syringe to the tip and the washing buffer is *gently* pushed into the space between two filters.

11) The phosphate-bound Phos-tag™ gel is resuspended in the space by *gently* moving the syringe piston up and down a few times.

12) All the liquid in the tip is *gently* dispensed into a microtube.

13) This washing operation (steps from 9 to 12) is repeated three times and the resulting liquids are collected as a series of washing fractions (W1 ~ W3).

14) Prepare a microtube containing 100 µL of distilled water.

15) The water is *gently* drawn into the tip and then all the liquid is moved above the upper spherical filter.

16) The liquid in the tip is *gently* dispensed into a microtube to obtain a washing fraction (W4).

**Elution Fractions (E1, E2, and more)**

17) To elute the phosphorylated molecules bound to Phos-tag™ gel, 50 ~ 100 µL of Sol. C (or Sol. D) is added into the tip from the top.

18) Attach a 1-mL syringe to the tip and the elution buffer is *gently* dispensed into a microtube.

19) The liquid in the microtube is again *gently* drawn and passed through the Phos-tag™ gel to the upper spherical filter.

20) The Phos-tag™ gel is resuspended by *gently* moving the syringe piston up and down a few times.

21) Keep the suspended state for **1 min** for Sol. C (or **10 min** for Sol. D).

22) The liquid is *gently* dispensed into the microtube to obtain the first elution fraction (E1).

23) If necessary, the steps from 17 to 22 are repeated to obtain an additional elution fraction (E2).

24) As for mass spectrometric analysis, desalting of the phosphate-enriched sample can be conducted by a commonly used method using reversed-phase resin.
Note: The elution method with Sol. C is based on a fast phosphate-exchange reaction at the binding site of a Phos-tag™ moiety in the presence of excess amounts of pyrophosphate anions. The reaction of FMN-bound Phos-tag™ occurs on a timescale of seconds using Sol. C. As for using an alternate elution buffer, Sol. D, the phosphate-release is caused by a slower reaction of demetallation of Phos-tag™ to produce metal-free Phos-tag ligand, Zn²⁺–EDTA complex, and phosphate compounds. Thus, the elution time with Sol. C is much shorter than that with Sol. D. After the elution procedure with Sol. D, the Phos-tag™ Tip can be reactivated by formation of phosphate-free Zn²⁺–complex (see next section 8).

* If a target phosphorylated molecule, such as a phosphopeptide and a phosphorylated sugar, is stable for a few hours in a basic or acidic pH solution, aqueous 5% NH₃ or aqueous 0.10 M HCl can be used as an elution solution in the same procedure with a waiting time of 1 min. After evaporation of the solvent of the basic or acidic eluted solution, the phosphate-enriched residue contains less amount of inorganic salt in comparison with the eluted sample obtained by the basic protocol with Sol. C or Sol. D.

8. Reactivation of Phos-tag™ Tip

1) Dispense the liquid in a used tip with air by using an empty syringe.
2) To eliminate phosphorylated molecules and Zn²⁺ ions from Phos-tag™ gel, 100 µL of aqueous 0.10 M EDTA solution (Sol. D: Reactivation buffer 1) is added into the tip from the top.
3) Attach a 1-mL syringe to the tip and the buffer is gently pushed into the space between two filters.
4) The gel is resuspended in the space by gently moving the syringe piston up and down a few times.
5) Keep the suspended state for over 30 min at room temperature.
6) Dispense the liquid with air by using the empty syringe.
7) Steps from 2 to 6 are repeated once more.
8) Washed the gel by gentle injection of 1 mL of distilled water from the top using a 1-mL syringe.
9) To form the phosphate-free Phos-tag™ Tip (active form), 100 µL of Sol. E (Reactivation buffer 2) is added into the tip from the top.
10) Attach a 1-mL syringe to the tip and the buffer is gently pushed into the space between two filters.
11) The Phos-tag™ gel is resuspended in the space by gently moving the syringe piston up and down a few times.

12) Keep the suspended state for over 30 min at room temperature and leave the solution in the tip.

13) Preserve the tip in a 50-mL bottle containing a few mL of 20%(v/v) 2-propanol/water before use.

Note: If a sample contains large-molecular-weight biomolecules such as protein or polysaccharides, those molecules could partially remain in Phos-tag™ gel even after the washing step. In that case, the elution fraction would include some impurities derived from the macromolecules. As for the peptide analysis of a protein digest, ultrafiltration is recommended before the phosphate-affinity enrichment.

9. Selective Separation of Riboflavin-5′-phosphate (FMN) and Riboflavin

- **Sample solution:** 18 nmol FMN and 16 nmol riboflavin in 100 µL of Sol. B
- **Washing solution:** Sol. B (100 µL x 3: W1, W2, and W3); distilled water (100 µL: W4)
- **Elution solution:** Sol. C (100 µL x 2: E1 and E2)

The separation experiment was conducted according to the basic protocol at room temperature. The recoveries of FMN ($\lambda_{\text{max}} = 445$ nm, $\varepsilon = 12,500$ M$^{-1}$ cm$^{-1}$) and its dephosphorylated counterpart, riboflavin are shown in Fig. 1. The flavin derivatives are yellow in color. While riboflavin molecules were all eliminated in the flow-through (85%), washing fractions W1–W3 (15%) and W4 (0%), FMN molecules were selectively eluted in the elution fractions E1 (86%) and E2 (12%). Even after the washing, the yellow color of the gel remained due to the strong FMN-binding (see Fig. 2). No degradation of FMN and riboflavin was observed. The quantity of the flavin derivatives in each fraction was analyzed by HPLC and visible spectrophotometry. The total time for the separation experiment was within 15 min. The Phos-tag™ Tip is reusable at least 10 times without a decrease in the FMN-binding ability and it is stable for 6 months in 20%(v/v) 2-propanol/water solution (see a reactivation procedure in Section 8).
10. **Selective Separation of Two Phosphorylated Peptides Derived from β-Casein**

- **Sample solution:** Tryptic digest of 6 nmol β-casein in 100 µL of Sol. B + ca. 10 nmol P1
- **Washing solution:** Sol. B (50 µL x 4: W1–W4); distilled water (50 µL: W5)
- **Elution solution:** Sol. C (50 µL x 1: E1)

The separation experiment was conducted in reference to the basic protocol at room temperature. The total time for the separation experiment was within 15 min. The separation result is analyzed by a reverse phase HPLC (see Fig. 3). The sample contains mono- and tetra-phosphorylated peptides (P1 and P2), which are preferentially eluted in the E1 fraction.

P1: Phe-Gln-pSer-Glu-Glu-Gln-Gln-Thr-Glu-Asp-Glu-Leu-Gln-Asp-Lys

![Chromatographic result](image)

**Fig. 3** Chromatographic result for a mixed sample of a tryptic digest of β-casein and P1 peptide, flow-through (FT), and elution (E1) fractions.

**References on Phos-tag™ Chemistry**


The original article for the Phos-tag-based phosphate affinity electrophoresis:


A SNP genotyping method using phosphate-affinity polyacrylamide gel electrophoresis, Analytical Biochemistry, 361, 294-298 (2007), E. Kinoshita, E. Kinoshita-Kikuta, and T. Koike (The phosphate group at DNA-terminial is efficiently captured by Zn⁺-Phos-tag.)


Separation of phosphoprotein isotypes having the same number of phosphate groups using phosphate-affinity SDS-PAGE, Proteomics, 8, 2994-3003 (2008), E. Kinoshita, E. Kinoshita-Kikuta, M. Matsubara, S. Yamada, H. Nakamura, Y. Shiro, Y. Aoki, K. Okita, and T. Koike


Formation of lysophosphatidic acid, a wound-healing lipid, during digestion of cabbage leaves Bioscience, Biotechnology, and Biochemistry, 73, 1293-1300, (2009), T. Tanaka, G. Horuchi, M. Matsuoka, K. Hirano, A. Tokumura, T. Koike, and K. Satouchi


Phos-tag beads as an immunoblotting enhancer for selective detection of phosphoproteins in cell lysates Analytical Biochemistry, 389, 83-85, (2009), E. Kinoshita-Kikuta, E. Kinoshita, and T. Koike

Mobility shift detection of phosphorylation on large proteins using a Phos-tag SDS-PAGE gel strengthened with agarose, Proteomics, 9, 4098-4101 (2009), E. Kinoshita, E. Kinoshita-Kikuta, H. Ujihara, and T. Koike


Genotyping and mapping assay of single-nucleotide polymorphisms in CYP3A5 using DNA-binding zinc(II)
E. Kinoshita, E. Kinoshita-Kikuta, H. Nakashima, and T. Koike

- The DNA-binding activity of mouse DNA methyltransferase 1 is regulated phosphorylation with casein kinase 1ε/δ, *Biochemical Journal*, 427, 489-497 (2010),

E. Kinoshita and E. Kinoshita-Kikuta

- Phos-tag SDS-PAGE systems for phosphorylation profiling of proteins with a wide range of molecular masses under neutral pH conditions, *Proteomics*, 12, 192-202 (2012),
E. Kinoshita, E. Kinoshita-Kikuta, and Tohru Koike

E. Kinoshita-Kikuta, E. Kinoshita, and T. Koike

E. Kinoshita-Kikuta, E. Kinoshita, and T. Koike


E. Kinoshita, E. Kinoshita-Kikuta, and T. Koike

E. Kinoshita, E. Kinoshita-Kikuta, A. Shiba, K. Edahiro, Y. Inoue, K. Yamamoto, and T. Koike

Edited by Emiko Kinoshita-Kikuta, Eiji Kinoshita, and Tohru Koike (Hiroshima University)