Enrichment of Low-molecular-weight Phosphorylated Molecules

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

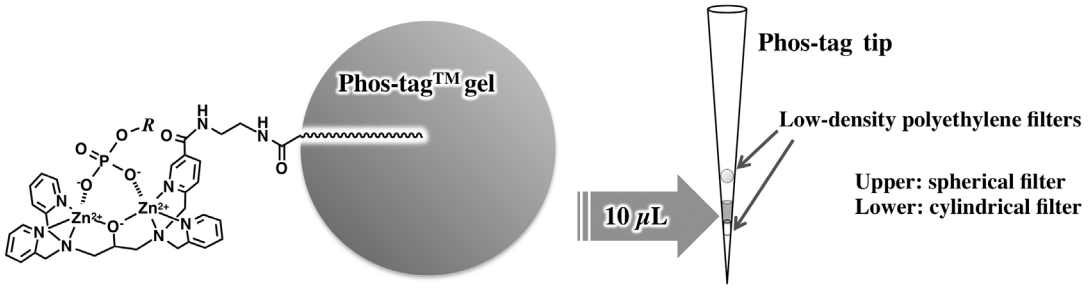
Ver. 4 (2025/4)

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(pyridin-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_3^{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 = 20-30 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)



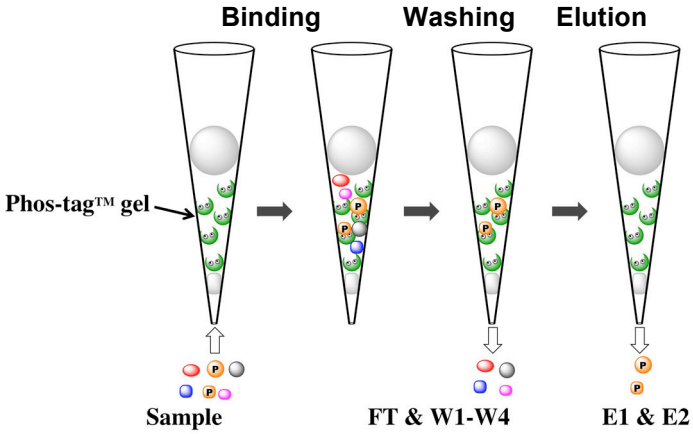
3. Warning and Limitations

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 $HP_2O_7^{3-}$) and various phosphomonoester. dianions (*R*OPO_3^{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	20 mL
# distilled water for preparation of the 100 mL solution	a proper quantity

Sol. B: Binding/Washing buffer:

0.10 mol/L Bis-tris–CH_3COOH + 0.10 mol/L CH_3COONa (pH 6.8, **100 mL**)

# Bis-tris (MW = 209, 10 mmol)	2.1 g
# CH_3COONa (FW = 82, 10 mmol)	0.82 g
# aqueous 1.0 M CH_3COOH (4.6 mmol) solution	4.6 mL (4.6 g)
# distilled water for preparation of the 100 mL solution	a proper quantity

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 CH_3COOH) can be used instead of CH_3COONa.

Sol. C: Elution buffer 1: 0.10 M $Na_4P_2O_7$–0.10 M CH_3COOH buffer (pH 7.0, **100 mL**)

# $Na_4P_2O_7 \cdot 10H_2O$ (FW = 446, 10 mmol)	4.46 g
# aqueous 1.0 M CH_3COOH solution (10 mmol)	10.0 mL (10.0 g)
# distilled water for preparation of the 100 mL solution	a proper quantity

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)	100 mL
# aqueous 10 M NaOH solution (a commercial product) for adjustment of pH to 7.0 ± 0.1 by using a pH meter.	a proper quantity

Sol. E: Reactivation buffer 2:

0.10 mol/L Bis-tris–CH_3COOH + 10 mmol/L $Zn(CH_3COO)_2$ (pH 6.8, **100 mL**)

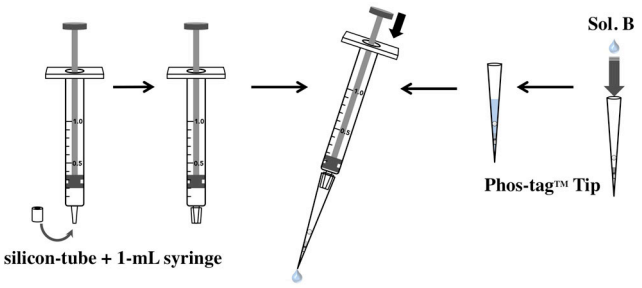
# Bis-tris (MW = 209, 10 mmol)	2.1 g
# aqueous 0.10 mol/L $Zn(CH_3COO)_2$ solution (a commercial product)	10 mL
# distilled water	70 mL
# aqueous 1.0 M CH_3COOH solution for adjustment of pH to 6.8 ± 0.1 by using a pH meter.	a proper quantity
# distilled water for preparation of the 100 mL solution	a proper quantity

7. Basic Protocol for Phosphate-affinity Enrichment

Preparation of Phos-tag™ Tip

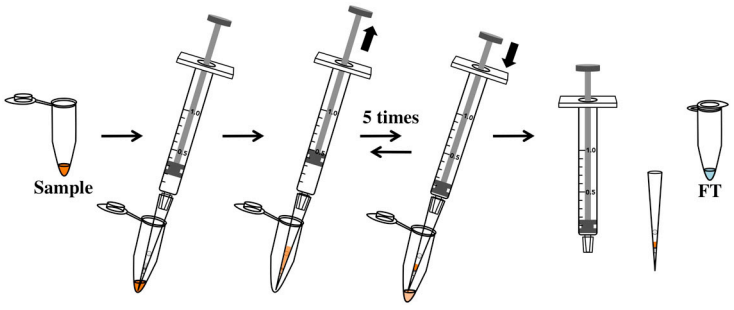
1) Wash the outside of Phos-tag™ Tip and the inside space over the upper filter using distilled water to remove the preservation solution.
2) After addition of 100 µL of Sol. B into the tip from the top, attach a 1-mL syringe with a silicon-tube adapter to the tip.
3) Dispense the liquid out with air 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)

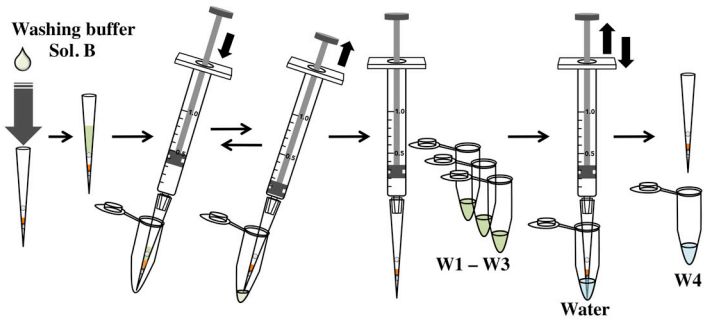
5) Prepare a sample solution (30 ~ 100 µL in a microtube) containing ≤50 nmol phosphorylated species in Sol. B: The sample solution pH should be in ranged between 6 and 8.
6) Draw the sample solution **gently** into the tip by using the 1-mL syringe.
   All the sample solution is passed through the Phos-tag™ 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)

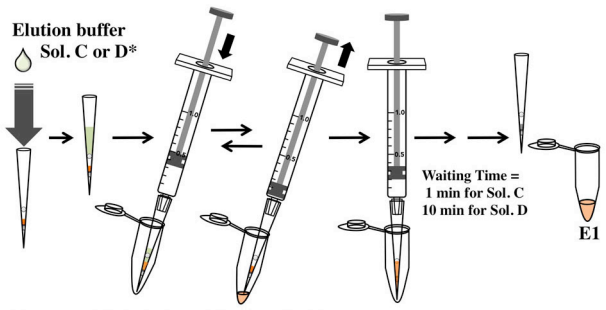
9) After remove the syringe, 100 µL of Sol. B is added into the tip from the top.
10) Attach a 1-mL syringe to the tip and the washing buffer is **gently** pushed into the space between two filters.
11) The phosphate-bound Phos-tag™ gel is resuspended in the space by **gently** moving the syringe piston 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. Aqueous 5% NH_3 solution or aqueous 0.10 M HCl can be used as an elution buffer with a waiting time of 1 min.

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, Zn^{2+}–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 Zn^{2+}–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% NH_3 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 Zn^{2+} 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. Separation of Riboflavin-*5′*-phosphate (FMN) and Riboflavin

- **Sample solution:** 15 nmol FMN and 15 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 3: E1, E2, and E3)

The separation experiment was conducted according to the basic protocol at room temperature. The recoveries of FMN (λ_{max} = 445 nm, ε = 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 (80%), washing fractions W1–W3 (20%) and W4 (0%), FMN molecules were selectively eluted in the elution fractions E1 (82%), E2 (12%), and E3 (6%). 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 the 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 one year, kept in a bottle containing 20%(v/v) 2-propanol/water solution.

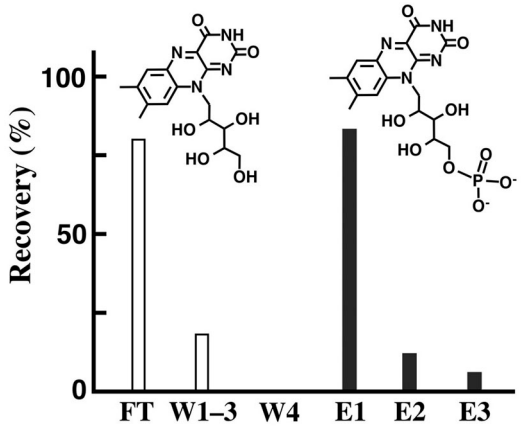


Fig. 1. Recoveries of riboflavin (white columns) and riboflavin-*5′*-phosphate (black columns).

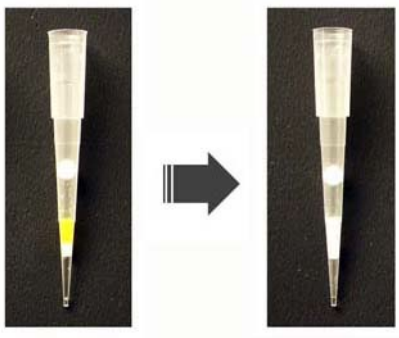


Fig. 2. Phos-tag™ Tip after washing step (left) (the yellow part is FMN-bound Phos-tag™ gel) and after elution step (right).

10. Selective Separation of Phosphorylated Peptide Derived from β-Casein

- **Gel volume:** 10 µL
- **Sample solution:** Tryptic digest of 5 nmol β-casein in 60 µL of Sol. B*
  + 5 nmol diphosphorylated MAP kinase substrate (P2) in in 60 µL of Sol. B*
- **Washing solution:** Sol. B (100 µL x 3: W1–W3)
- **Elution solution:** Sol. C (60 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 was analyzed by a reverse phase HPLC (see Fig. 3). Both the phosphorylated peptides (P1 and P2) were preferentially eluted in the E1 fraction.

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

P2: Asp-His-Thr-Gly-Phe-Leu-**pThr**-Gul-**pTyr**-Val-Ala-Thr-Arg

*This digest contains less amount of the β-casein tetra-phosphorylated peptide.

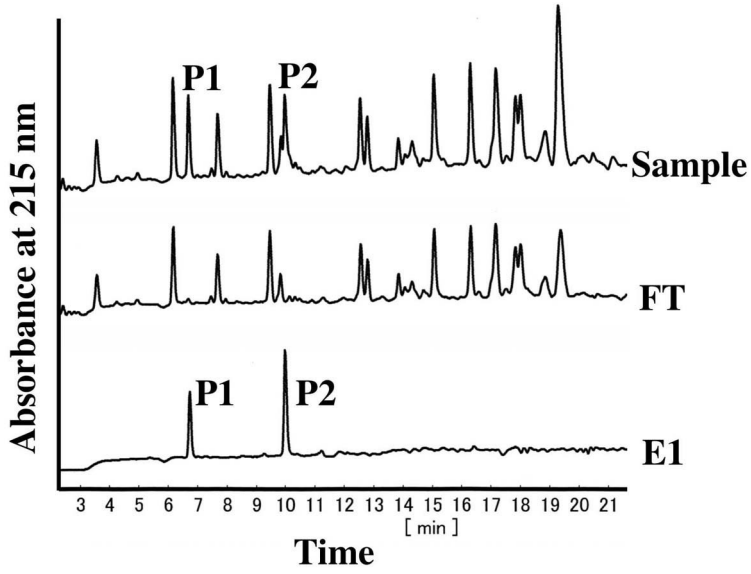
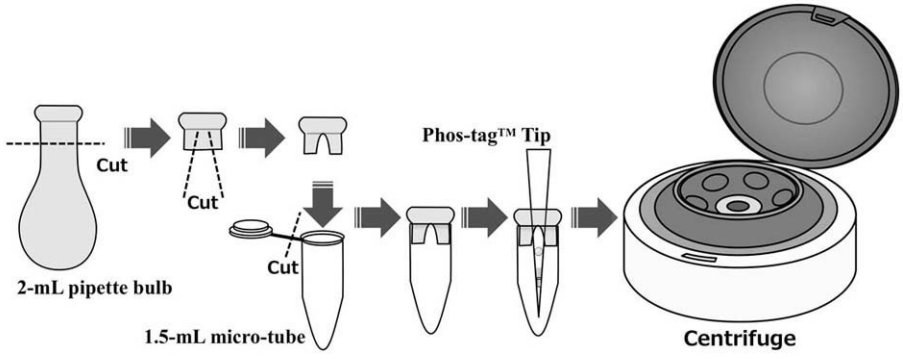


Fig. 3. Chromatographic result with a tryptic digest of β-casein:
FT is flow-through fraction, E1 is the first elution fraction.

Appendix:

To reduce residual washing and elution fractions in a Phos-tag tip, we recommend a use of a micro-centrifuge (*ca.* 20 sec) and 1.5-mL micro-tubes attached with a silicon adaptor. The adaptor can be made from a commercially available 2-mL pipette bulb.



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