INSTRUCTIONS for Phos-tag® BTL-105 (Purification of Phosphorylated Peptide using Phos-tag® Biotin & Streptavidin-Agarose)
by the NARD Institute Ltd. & MANAC Incorporated Group (http://www.Phos-tag.com)

Introduction

Phos-tag® (a dinuclear zinc(II) complex) acts as a novel phosphate capture molecule in aqueous solution at physiological pH (6 ~ 8). The anion selectivity indexes of Phos-tag® are as follows: 16,000 for phenyl phosphate dianion, 3 for SO_4^{2-}, 1 for CH_3COO^-, 0.02 for Cl^-, <0.02 for diphenyl phosphate monoanion and alkyl sulfate ($ROSO_3^-$) monoanion. Phos-tag® BTL-105 is a biotin derivative attached with Phos-tag® molecules, which allows the fast and effective purification of phosphate dianions (*e.g.*, phosphorylated peptides, phosphorylated proteines, phosphorylated lipids, *etc*.) using streptavidin-bound agarose (*Sigma-Aldrich Fine Chemicals*). To ensure best performance and trouble-free operation, please read the following instructions before using Phos-tag® BTL-105.

Product Specifications for Phos-tag® BTL-105

Mol. Wt.:	880.1
Binding site:	Phos-tag®/biotine = 1:1
Form:	Zn^{2+}-unbound ligand
Storage place:	at +2 ~ +8 ˚C in the dark

Additional Materials Required

The followings are the materials required for use with Phos-tag® BTL-105.

1) *Zinc(II) Acetate Solution*
   0.50 mM $Zn(CH_3COO)_2$ containg 5.0 mM Tris-acetate (pH 7.4)
2) *Tris acetate Buffer*
   5.0 mM Tris-acetate solution (pH 7.4)
3) *Balancing Buffer* (pH 7.4)
   5.0 mM Tris-acetate containing 10 μM $Zn(CH_3COO)_2$
4) *Washing Buffer* (pH 7.4)
   5.0 mM Tris-acetate containing 0.50 M $NaNO_3$
5) *Elution Buffer* (optional)
   1.0 mM NaH_2PO_4-NaOH (pH 7.0) containing 0.50 M $NaNO_3$
6) Micropipette for 0.30 mL
7) Centrifugal filtration units
   Membrane pore size: 0.22 ~ 0.45 μm
   Sample volume: 0.40 ~ 0.50 mL
8) Centrifuge for 2,000×*g*
9) Streptavidine-agarose (*Sigma-Aldrich Fine Chemicals*)

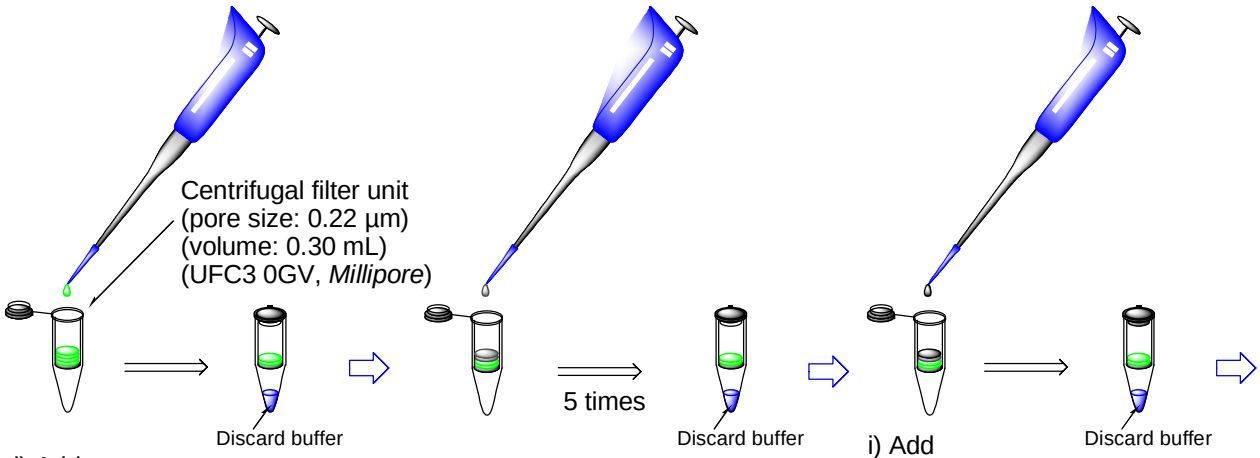
Sample Preparation

1) Competing anions (*e.g.*, inorganic phosphate, thiolate) and metal chelating agents (*e.g.*, EDTA) should be removed from the sample as much as possible.
   Note: The protease inhibitors except of thiol compounds are not effective on the purification. The large amount of surfactant (*e.g.*, SDS) lowers the efficiency of the purification.
2) The sample is dissolved in the 5.0 mM Tris-acetate buffer (pH 7.4, 0.30 mL) at room temperature.
3) The final concentration of phosphorylated compounds in the sample solution (0.3 mL) should be below 15 nmol/mL.

Purification Procedure

1) Apply *ca.* 0.3 mL of Streptavidine Agarose (in suspension) in the sample reservoir of the centrifugal filtration unit.
2) Centrifuge the unit at 2,000×*g* for 15 sec and the filtrate is discarded.
3) Apply 0.30 mL of the 5.0 mM Tris-acetate buffer in the sample reservoir.
   Centrifuge the unit at 2,000×*g* for 15 sec and the filtrate is discarded.
   (The operations are repeated 5 time)
4) Apply 0.30 mL of 0.12 mM Phos-tag® BTL-105 containg 0.50 mM $Zn(CH_3COO)_2$ and 5.0 mM Tris-acetate (pH 7.4) in the sample reservoir.
5) Equilibrate for 5 min.
6) Centrifuge the unit at 2,000×*g* for 15 sec and the filtrate is discarded.
7) Apply 0.30 mL of the 5.0 mM Tris-acetate buffer in the sample reservoir.
   Centrifuge the unit at 2,000×*g* for 15 sec and the filtrate is discarded.
   (The operations are repeated 5 time)
8) Apply 0.30 mL of 10 μM $Zn(CH_3COO)_2$ containing 5.0 mM Tris-acetate (pH 7.4) in the sample reservoir.
9) Centrifuge the unit at 2,000×*g* for 15 sec and the filtrate is discarded.
10) Add 0.3 mL of the sample solution into the sample reservoir.
11) Equilibrate for 5 min in order to sufficiently bind phosphorylated compounds to Phos-tag®.
12) Centrifuge the unit at 2,000×*g* for 15 sec. The filtrate contains non-phosphorylated compounds.
13) Add 0.30 mL of the washing buffer into the sample reservoir. Centrifuge the unit at 2,000×*g* for 15 sec. The filtrate contains non-phosphorylated compounds.
   (The operations are repeated 3 time)
14) Add 0.30 mL of the elution buffer into the sample reservoir. Centrifuge the unit at 2,000×*g* for 15 sec and the filtrate contains phosphorylated compounds.
   (The operations are repeated 2~4 time)
15) Analyze the obtained solutions containing phosphorylated compounds.

Purification Protocol for Phosphorylated Peptides
using Phos-tag®Biotin & Streptavidin-Agarose



i) Add
Streptavidin Agarose
(60~120 nmol (biotin binding site)/mL)
(0.30 mL, in suspension)

ii) Centrifuge
at 2,000 x*g* for 15 sec

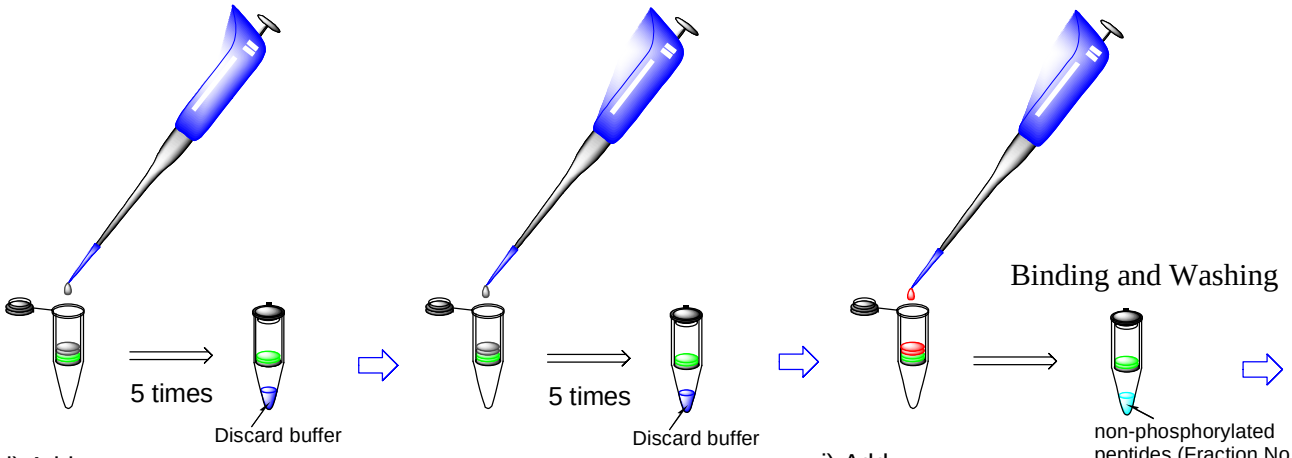
i) Add
5.0 mM Tris-acetate
(pH 7.4, 0.30 mL)

ii) Centrifuge
at 2,000 x*g* for 15 sec

i) Add
120 nmol/mL Phos-tag® BTL
500 nmol/mL $Zn(CH_3COOH)_2$
in 5.0 mM Tris-acetate
(pH 7.4, 0.30 mL)

ii) Equilibrate for 5 min at r.t.

iii) Centrifuge
at 2,000 x*g* for 15 sec



i) Add
5.0 mM Tris-acetate
(pH 7.4, 0.30 mL)

ii) Centrifuge
at 2,000 x*g* for 15 sec

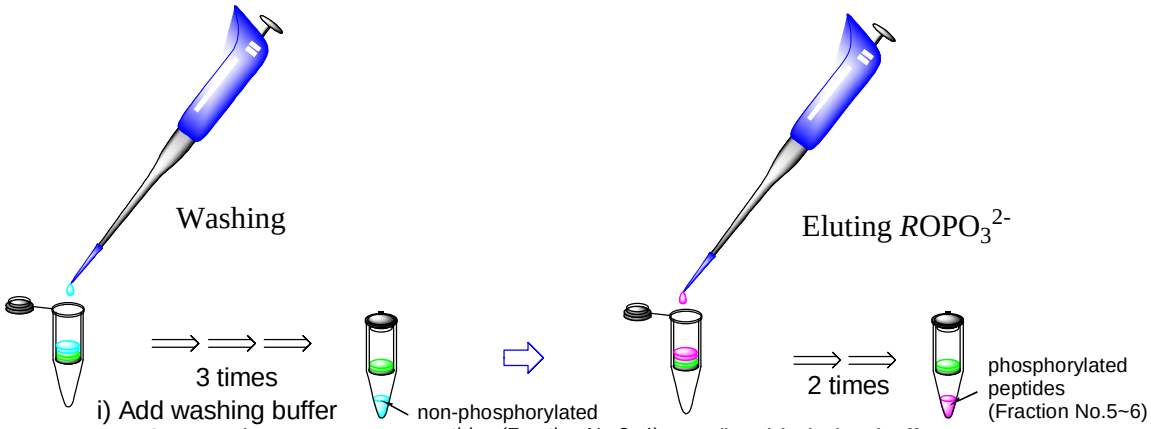
i) Add
10 nmol/mL $Zn(CH_3COO)_2$
in 5.0 mM Tris-acetate
(pH 7.4, 0.30 mL)

ii) Centrifuge at 2,000 x*g* for 15 sec

i) Add
Sample solution ([*R*OPO_3^{2-}] 15 nmol/mL)
in 5.0 mM Tris-acetate (pH 7.4, 0.30 mL)

ii) Equilibrate for 5 min at r.t.

iii) Centrifuge at 2,000 x*g* for 15 sec



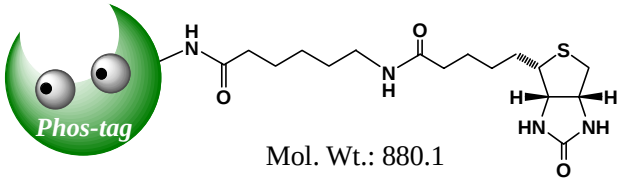
i) Add washing buffer
5.0 mM Tris-acetate
(pH 7.4, 0.50 M $NaNO_3$, 0.30 mL)

ii) Centrifuge
at 2,000 x*g* for 15 sec

i) Add elution buffer
1.0 mM phosphate-NaOH
(pH 7.4, 0.50 M $NaNO_3$, 0.30 mL)

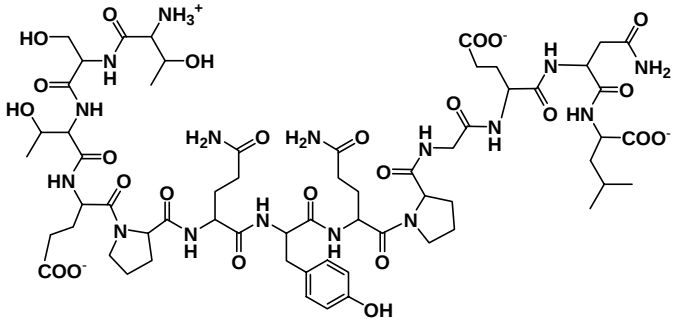
ii) Centrifuge
at 2,000 x*g* for 15 sec

Phos-tag® BTL-105

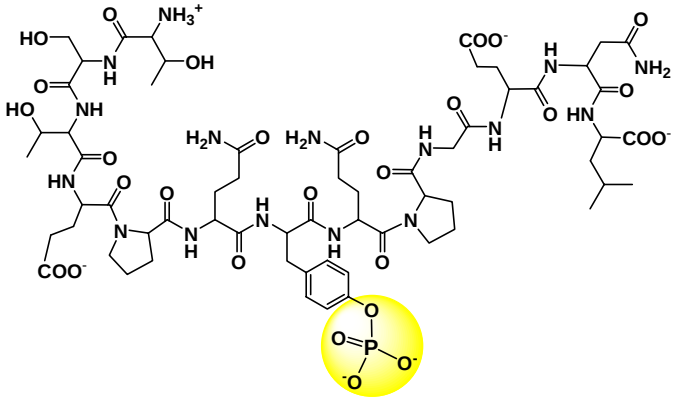


Mol. Wt.: 880.1

Sample solution in 5.0 mM Tris-CH_3COOH (pH 7.4, 0.30 mL)



p60c-*src* peptide 521-533 (p60c)
14 nmol/mL



Phosphorylated p60c-*src* peptide 521-533 (P-p60c)
12 nmol/mL

1) Separation result in the presence of Phos-tag® Biotin BTL-105

Fraction No.	1	2	3	4	5	6
p60c (%)	67	24	9	0	0	0
P-p60c (%)	0	0	0	0	83	17

← Washing → Eluting

2) Separation result in the absence of Phos-tag® Biotin BTL-105

Fraction No.	1	2	3	4	5	6
p60c (%)	75	20	5	0	0	0
P-p60c (%)	83	17	0	0	0	0

← Washing → Eluting

Quantitative analysis of the peptides is conducted by HPLC as follows.

Column: Shiseido Capcell Pak C_{18} type UG80, 150 mm in length, 4.6 mm in ϕ.
Eluent: 0.1%(v/v) trifluoroacetic acid, 14%(v/v) acetonitrile aqueous solution.
Detector: UV at 266 nm. Flow speed: 1 mL/min. Column temperature: 40 ºC.

Retention time: 4.6 min for P-p60c; 12.6 min for p60c

Surface Plasmon Analysis of Phosphorylated β-Casein Using a Phos-tag® Biotin- and Streptavidin-Bound Sensor Chip

Ligand binding procedures

1) A sensor chip, onto which streptavidin was bound (Sensor Chip SA, made by Biacore), was set in Biacore J (made by Biacore).
2) A 10mM aqueous HEPES-sodium hydroxide solution (pH 7.4), containing $5X10^{-3}$%(w/v) Tween 20, 0.20M sodium nitrate, and 10μM zinc nitrate was used as the running buffer. The sensor chip temperature was set at 25°C and the flow rate of the running buffer was set at 30μL/min. The running buffer was made to flow until the surface plasmon resonance value stabilized. This procedure was carried out on flow cell A.
3) As a biotin derivative of Phos-tag®, Phos-tag® BTL-105 was used and this was dissolved in the running buffer ($5X10^{-3}$%(w/v) Tween 20, 0.20M sodium nitrate, and 10μM zinc nitrate in 10mM aqueous HEPES-sodium hydroxide solution (pH 7.4)).
4) Binding onto flow cell A was carried out for 6 minutes at a ligand concentration of 1.0mM, a temperature of 25°C, and a flow rate of 30μL/min.
5) For the interaction of the Phos-tag® biotin and streptavidin, a surface plasmon resonance signal of 240RU was obtained for the maximum binding amount.
6) A sensor chip (Phos-tag® sensor chip), having a Phos-tag®-bound sensor, was prepared by the above procedures.

Surface plasmon analysis of β-casein

1) β-casein (pentaphosphorylated protein, Sigma Co., Ltd.) was used as the sample to be analyzed, and this sample was dissolved in the running buffer ($5X10^{-3}$%(w/v) Tween 20, 0.20M sodium nitrate, and 10μM zinc nitrate in 10mM aqueous HEPES-sodium hydroxide solution (pH 7.4)) (sample solution).
2) The surface plasmon resonance analysis was carried out in flow cell A at a sample concentration of 1.5μM, a temperature of 25°C, a flow rate of 30μL/min, a binding time of 15 minutes and a dissociation time of 10 minutes.
3) After measurement, the sensor chip was reactivated (removal of residual

bound substances) by the addition of a 400mM aqueous phosphate solution (pH 7.0) for 6 minutes, a 200mM aqueous EDTA (pH 8.0) solution for 6 minutes and the abovementioned running buffer for 5 minutes.

4) The measurement results are shown in FIG. 1.
5) The results of FIG. 1 shows that a high RU value is obtained for each flow cell and that the maximum binding amount for flow cell A is 2056.

Interaction of the streptavidin sensor chip and β-casein

1) β-casein (pentaphosphorylated protein, Sigma Co., Ltd.) was used as the sample to be analyzed, and this sample was dissolved in the running buffer ($5X10^{-3}$%(w/v) Tween 20, 0.20M sodium nitrate, and 10μM zinc nitrate in 10mM aqueous HEPES-sodium hydroxide solution (pH 7.4)) (sample solution).
2) The surface plasmon resonance analysis was carried out in flow cell A of a new sensor chip at a sample concentration of 1.5μM, a temperature of 25°C, a flow rate of 30μL/min, a binding time of 15 minutes and a dissociation time of 10 minutes.
3) There was absolutely no interaction of β-casein with streptavidin.

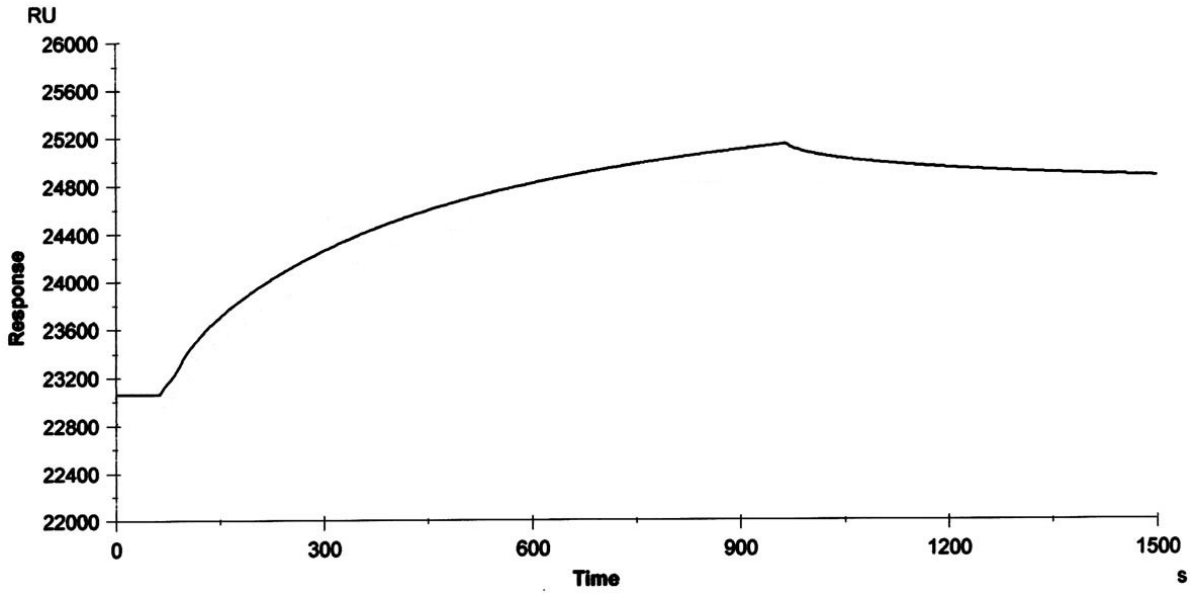


FIG. 1. Surface plasmon analysis of phosphorylated β-casein using Phos-tag® Biotin (BTL-105) and Sensor Chip SA