Western Blot Analysis of Phosphorylated Proteins

- Chemiluminescent Detection using Biotinylated Phos-tag™ -

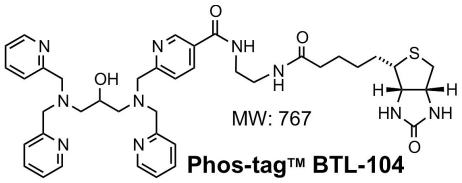
Ver. 8 (2013/4)

1. Introduction

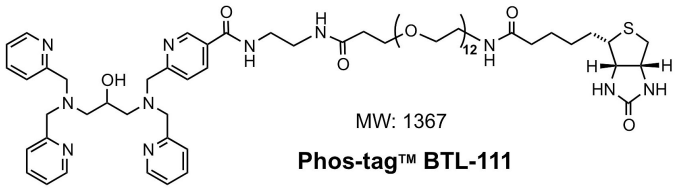
Phosphorylation is a fundamental covalent post-translational modification that regulates the function, localization, and binding specificity of target proteins. Methods for determining the phosphorylation status of proteins (*i.e.,* phosphoproteomics) are thus very important with respect to the evaluation 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 demonstrate a novel application of Phos-tag™ for chemiluminescence detection of whole phosphorylated proteins on an electroblotting PVDF membrane using biotinylated Phos-tag™ zinc(II) complex (dinuclear zinc complex of Phos-tag™ BTL).

2. Description of Phos-tag™ BTL-104 and BTL-111

The Phos-tag™ ligands, BTL-104 and BTL-111, provide a sensitive method for detection of phosphorylated proteins on a PVDF membrane. This method needs streptavidin-conjugated horseradish peroxidase (HRP) and chemiluminescent detection reagents. The products are supplied as odorless solid, which have no irritant effect on the skin. Phos-tag™ BTLs should be stored in a refrigerator (4˚C).



Phos-tag™ BTL-104



Phos-tag™ BTL-111

3. Warning and Limitations

The Phos-tag™ ligands are not for use in human diagnostic and the therapeutic procedures. Do not use internally or externally in human or animals. For research use only. The products should be handled only by those persons who have been trained in laboratory techniques. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water.

4. Advantages of Phos-tag™ Method

The radioactivity is avoided.

The blocking treatment of a PVDF membrane is unnecessary.

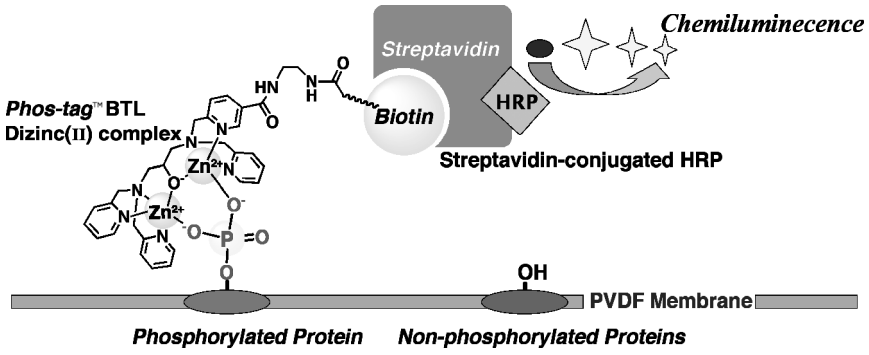
The binding specificity of Phos-tag™ is independent on amino acid and sequence context.

Downstream procedures such as antibody reprobing and MS analysis are applicable.

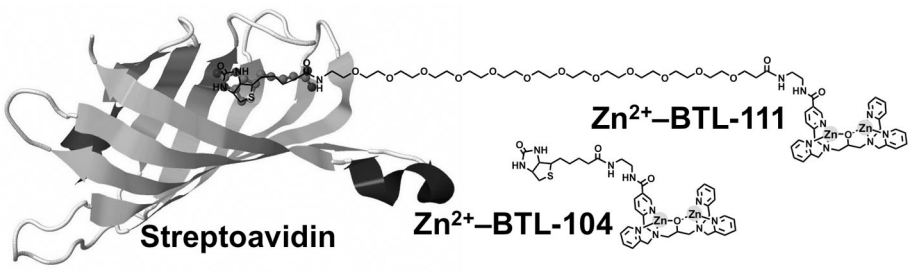
Phos-tag™ BTL in solution is stable at room temperature for at least 6 months.

The procedure is almost the same as that with an HRP-conjugated antibody.

5. Principle of Phos-tag™ Method



Note: BTL-111 has a long hydrophilic spacer, which provides the highest sensitivity in the method.



6. Solutions for Phos-tag™ Method

Sol. A: Tris buffered saline (10xTBS, 1 L, pH 7.5)

# Tris (0.10 mol/L)12.1 g	
# NaCl (1.0 mol/L)	58.4 g
# distilled water	0.9 L
# 2 mol/L aqueous HCl for pH adjustment at 7.5	a proper quantity
# distilled water for preparation of the 1 L solution	a proper quantity

Sol. B: 10% (v/v) Tween 20 solution (50 mL)

# Tween 20	5 mL
# distilled water	45 mL

Sol. C: 1xTBS-T (1 L)

# Sol. A	100 mL
# Sol. B	10 mL
# distilled water	890 mL

Sol. D: Phos-tag™ BTL-104 solution (Stored in a dark place at 4˚C)

A commercially available aqueous solution (1 mmol/L Phos-tag™ BTL-104)

Sol. E: Phos-tag™ BTL-111 solution (Stored in a dark place at 4˚C)

A commercially available aqueous solution (1 mmol/L Phos-tag™ BTL-111)

Sol. F: 10 mmol/L $Zn(NO_3)_2$ aqueous solution (50 mL)

# $Zn(NO_3)_2(H_2O)_6$ (FW. 297)	0.15 g
# distilled water	50 mL

(An alternative is an aqueous solution of 10 mmol/L $ZnCl_2$.)

Sol. G: Streptavidin-conjugated Horseradish Peroxidase solution

Streptavidin-Horseradish Peroxidase Conjugate (GE Healthcare Bio-Sciences: RPN1231)

7. Preparation of Phos-tag™ BTL-bound Streptavidin-conjugated HRP

1) After mixing of the following solutions, the obtained solution (Sol. H) is allowed to stand for 30 min at room temperature.

# Sol. C (1xTBS-T)	479 μL
# Sol. D **or** Sol. E (Phos-tag™ BTL solution)[¶]	2 ~ 10 μL
# Sol. F (10 mmol/L $Zn(NO_3)_2$)	5 ~ 10 μL
# Sol. G (Streptavidin-conjugated Horseradish Peroxidase)	1 μL

2) Sol. H is added in a centrifugal filter device cup (NMWL = 30,000, Nanosep™ 30K, Pall Life Sciences). Seal with the attached cap.

3) Centrifuge (14,000 x*g*) for 20 min at room temperature to remove the excess Phos-tag™ BTL.

4) The remaining solution (<10 μL) in the cup is diluted with 30 mL of Sol. C (1xTBS-T), which is Sol. PB-SH (a solution of Zn^{2+}–Phos-tag™ BTL-bound Streptavidin-conjugated HRP).[§]

[§]Phos-tag™ BTL-bound Streptavidin-conjugated HRP in Sol. PB-SH is stable for 30 days at 4 ˚C.
[¶][Phos-tag™ BTL] >> [Streptavidin-conjugated HRP]

« See Troubleshooting 1 »

8. Probing with Phos-tag™-bound Streptavidin-conjugated HRP

1) A protein-blotted PVDF membrane is soaked with Sol. C (1xTBS-T) in a Tupperware.
Use plastic gloves in this procedure. The membrane is gently rocked for at least 1 h.
Confirm that the membrane does not stick to the Tupperware.
Be careful not to dry the membrane.

« See Troubleshootings 2 & 3 »

2) The membrane is incubated with Sol. PB-SH (*ca.* 1 mL/5 cm^2) in a plastic bag.
The bag is gently rocked for 30 min.

3) The membrane is taken out of the bag and washed twice with Sol. C (*ca.* 10 mL/5 cm^2) in a Tupperware for 5 min each time at room temperature (the Tupperware is gently rocked).
Confirm that the membrane does not stick to the Tupperware. Be careful not to dry the membrane.

4) The chemiluminescence is observed using an X-ray film or an image analyzer with an appropriate amount of a chemiluminescence reagent kit (*e.g.*, Lumigen™-TMA-6, Lumigen)

9. Reprobing the Protein-blotted PVDF Membrane

Sol. K: 0.5 mol/L Tris-HCl buffer (1 L, pH 6.8)

# Tris	60.6 g
# distilled water	0.8 L
# 6 mol/L aqueous HCl for pH adjustment at 6.8	a proper quantity
# distilled water for preparation of the 1 L solution	a proper quantity

Sol. L: 10% (w/v) aqueous SDS solution (1 L)

# SDS	100 g
# distilled water for preparation of the 1 L solution	a proper quantity

Sol. M: Stripping buffer (1 L)

# Sol. K	125 mL
# Sol. L	200 mL
# 2-mercaptoethanol	7 mL
# distilled water	668 mL

1) A protein-blotted PVDF membrane probed with Zn^{2+}–Phos-tag™ BTL and Streptavidin-conjugated HRP is soaked with Sol. C (1xTBS-T, 25 mL/5 cm^2) in a Tupperware.
The membrane is gently rocked for at least 1 h. Be careful not to dry the membrane.
If the membrane is dry, the membrane is soaked with methanol before this stripping treatment.

2) The membrane is soaked with Sol. M (Stripping buffer, 25 mL/5 cm^2) in a Tupperware.
The membrane is gently rocked for 20 min at room temperature. There is no need of heating.
Confirm that the membrane does not stick to the Tupperware.

3) The membrane is soaked with Sol. C (1xTBS-T, 25 mL/5 cm^2) in a Tupperware.
The membrane is gently rocked for 1 h at room temperature.
Confirm that the membrane does not stick to the Tupperware. The washing solution is removed.

4) The membrane is soaked with Sol. C (1xTBS-T, 25 mL/5 cm^2) in the Tupperware.
The membrane is gently rocked for 1 h at room temperature.
Confirm that the membrane does not stick to the Tupperware. The washing solution is removed.

5) The membrane is soaked with Sol. C (1xTBS-T, 25 mL/5 cm^2) in the Tupperware.
The membrane is gently rocked for 1 h at room temperature.
Confirm that the membrane does not stick to the Tupperware.
Be careful not to dry the membrane.

6) The membrane is blocked with an appropriate protein and then reprobed with an appropriate antibody.
The chemiluminescent analysis is conducted.

10. Dot-blotting Analysis with Phos-tag™ BTL-104

Sample Proteins

Nonphosphorylated Proteins:

Bovine Serum Albumin, Human Serum Albumin, Carbonic Anhydrase, β-Galactosidase

Phosphorylated Proteins:

α-Casein, β-Casein, Ovalbumin, Pepsin

Dephosphorylated Proteins (by Alkaline Phosphatase):

α-Casein, β-Casein, Ovalbumin, Pepsin

Blotting Membrane

Polyvinylidene Fluoride (PVDF) membrane（Hybond P, GE Healthcare Bio-Sciences）

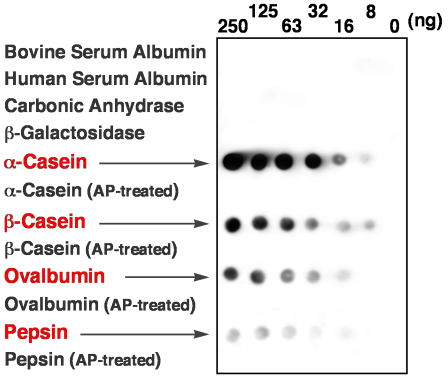
Chemiluminescence Reagent Kit

Western blotting detection reagent, Lumigen™-TMA-6, (Lumigen)

Apparatus

LAS 3000 Image Analyzer (Fujifilm)

Chemiluminescence Image



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The phosphorylated proteins spotted on a PVDF membrane were specifically detected at ng levels. No signal was detected on the spots of the corresponding dephosphoryated proteins and the nonphosphorylated proteins.

11. Western Blotting 2D (IEE/SDS-PAGE) Analysis with Phos-tag™ BTL-104

Sample Proteins (each at 50 μg)

Lysate of A431 human epidermoid carcinoma cells **before** EGF-stimulation

Lysate of A431 human epidermoid carcinoma cells **after** EGF-stimulation

Blotting Membrane

Polyvinylidene Fluoride (PVDF) membrane（Hybond P, GE Healthcare Bio-Sciences）

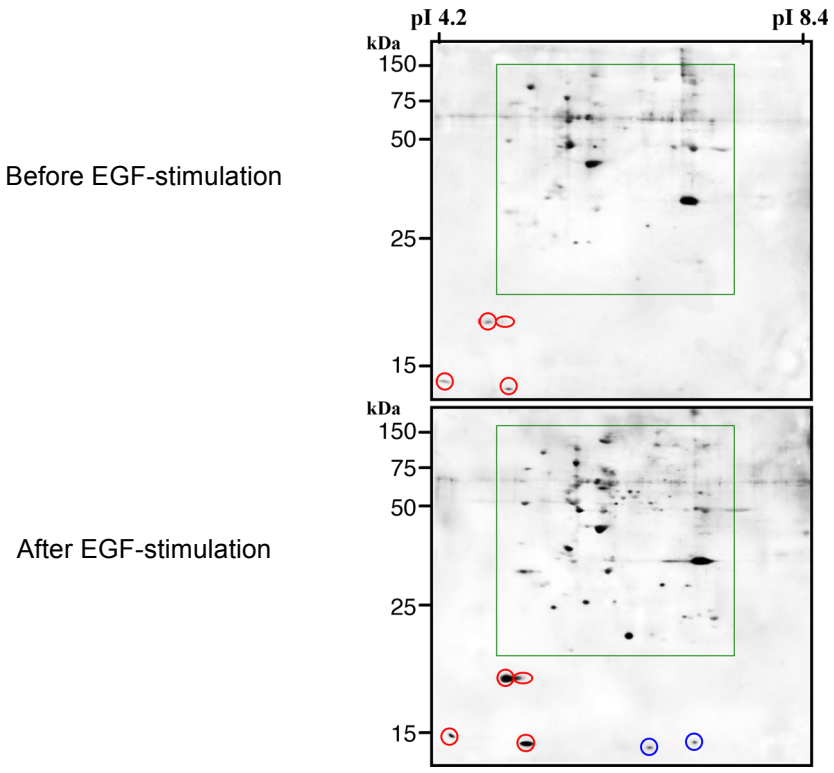
Chemiluminescence Reagent Kit

Western blotting detection reagent, Lumigen™-TMA-6, (Lumigen)

Apparatus

LAS 3000 Image Analyzer (Fujifilm)

Chemiluminescence Image



Before EGF-stimulation

After EGF-stimulation

The signal intensity (*e.g.,* red circles, MW <20 kDa) and number of spots (*e.g.,* blue circles, MW <20 kDa) on the membrane for EGF-stimulated lysate remarkably increased in comparison with those before EGF-stimulation.

12. Western Blotting 2D (IEE/SDS-PAGE) Analysis with Phos-tag™ BTL-104

Sample

Above-mentioned membrane for Lysate of A431 cells after EGF stimulation (after stripping)

Antibody and Antibody-bound HRP

HRP–anti-pTyr antibody (PY20, GE Healthcare Bio-Sciences)

anti-pSer antibody (Rabbit-Polyclonal, Zymed Laboratories)

HRP–anti-IgG antibody (GE Healthcare Bio-Sciences)

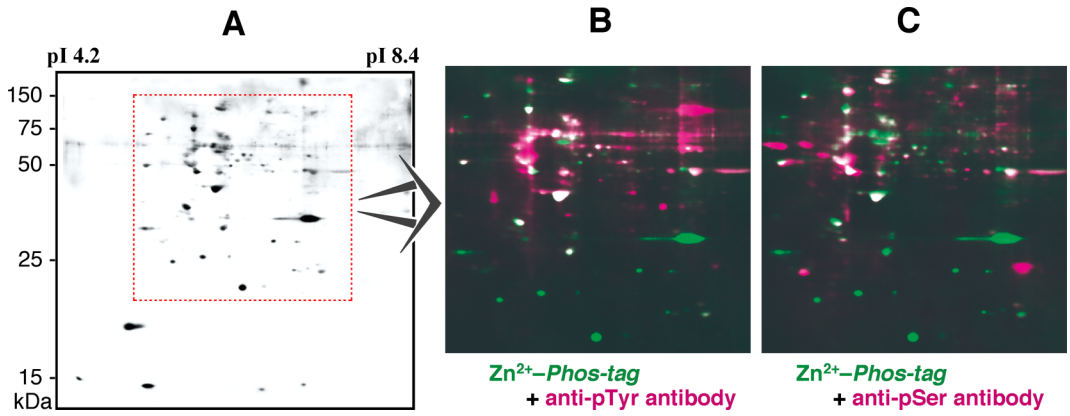
Chemiluminescence Reagent Kit

Western blotting detection reagent, Lumigen™-TMA-6, (Lumigen)

Apparatus

LAS 3000 Image Analyzer (Fujifilm)

Chemiluminescence Image (A) and Superimposed Images (B & C)



A: Chemiluminescence using Phos-tag™ BTL-104
B: A + Chemiluminescence using HRP–anti-pTyr antibody
C: A + Chemiluminescence using anti-pSer antibody/HRP–anti-IgG antibody
Green spots in B and C are using Phos-tag™ BTL-104.
Magenta spots in B and C are using antibodies.
Proteins detected by both methods appear as white spots in B and C.

13. Comparative Analysis by Using Phos-tag™ BTL-111 and Phos-tag™ BTL-104

Sample

Phosphorylated Proteins:

β-Casein, Ovalbumin, Pepsin

Dephosphorylated Proteins (by Alkaline Phosphatase, AP):

β-Casein, Ovalbumin, Pepsin

Blotting Membrane

Polyvinylidene Fluoride (PVDF) membrane（Hybond P, GE Healthcare Bio-Sciences）

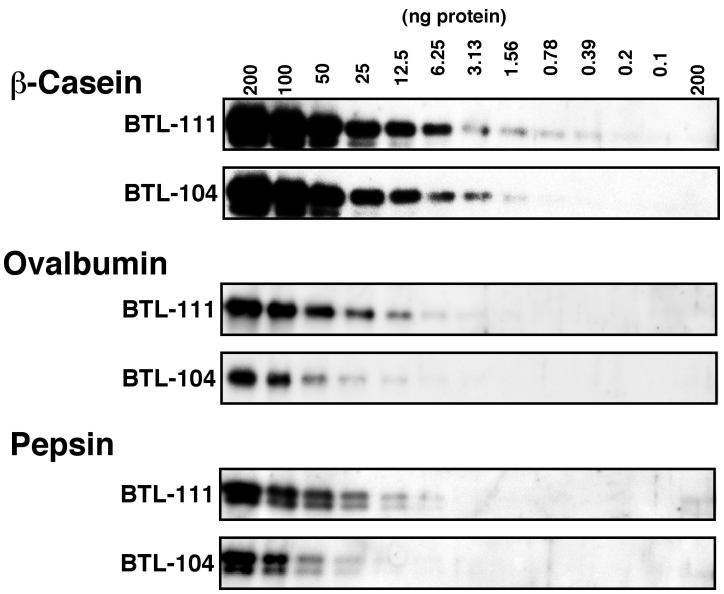
Chemiluminescence Reagent Kit

Western blotting detection reagent, Lumigen™-TMA-6, (Lumigen)

Apparatus

LAS 3000 Image Analyzer (Fujifilm)

Chemiluminescence Images



Upper: β-Casein (200–0.1 ng) and β-casein treated with AP for 12 h (200 ng, rightmost lane).
Center: Ovalbumin (200–0.1 ng) and ovalbumin treated with AP for 12 h (200 ng, rightmost lane).
Lower: Pepsin (200–0.1 ng) and pepsin treated with AP for 12 h (200 ng, rightmost lane).

These results show that the length of the spacer linking the Phos-tag and biotin moieties affects access to the phosphorylated targets in Western blotting analysis (see **5. Principle of Phos-tag™ Method** described above).

14. Western Blotting 1D (SDS-PAGE) Analysis with Phos-tag™ BTL-111

Sample Proteins (each at 5 μg)

Lysate of A431 cells (lane 2)
AP-treated lysate of A431 cells (lane 3)
Lysate of EGF-stimulated A431 cells (lane 4)
AP-treated lysate of EGF-stimulated A431 cells (lane 5)
Lysate of pervanadate-stimulated A431 cells (lane 6)
AP-treated lysate of pervanadate-stimulated A431 cells (lane 7)
Lysate of HeLa cells (lane 8)
AP-treated lysate of HeLa cells (lane 9)
Lysate of PMA-stimulated HeLa cells (lane 10)
AP-treated lysate of PMA-stimulated HeLa cells (lane 11)

Blotting Membrane

Polyvinylidene Fluoride (PVDF) membrane（Hybond P, GE Healthcare Bio-Sciences）

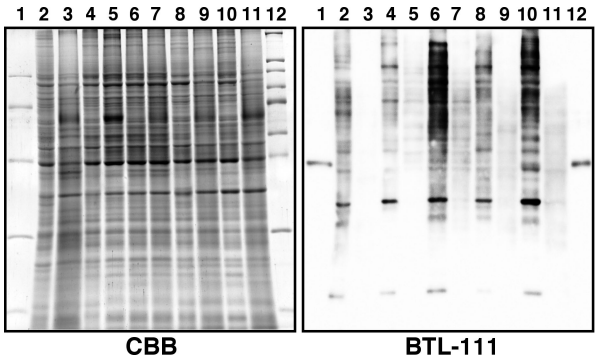
Chemiluminescence Reagent Kit

Western blotting detection reagent, Lumigen™-TMA-6, (Lumigen)

Apparatus

LAS 3000 Image Analyzer (Fujifilm)

CBB-staining and Chemiluminescence Images



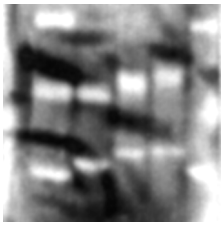
Ten samples of various lysates (lanes 2–11) and two molecular-weight markers (lanes 1 and 12). Lanes 1 and 12 correspond to APRO™ marker low range (APRO Science) and Amersham marker broad range (GE Healthcare Bio-Sciences), respectively. Phos-tag™ BTL-111 permits specific visualization of phosphorylated proteins on a blotting membrane. The blocking treatment of the PVDF membrane was also unnecessary for the analysis of the lysate samples using Phos-tag™ BTL-111.

« Troubleshooting 1 »

Phos-tag™ BTL in Sol. D or E (10 μL) is large excess amount against Streptavidin-conjugated Horseradish Peroxidase in Sol. G (1 μL). We obtained almost the same result using smaller amount of Phos-tag™ BTL (*e.g.,* 1 μL Sol. E) and Sol. G (1 μL) used. The user should adjust the volume of Sol. D or E to obtain the required sensitivity or save expenses. If the volume of Sol. D or E is decreased, it is no need to change the volume of the zinc(II) solution (Sol. F). Difference in the detection efficiency (due to the spacer effect) between BTL-104 and BTL-111 was observed in this protocol using the commercially available phosphorylated proteins and the Streptavidin-conjugated Horseradish Peroxidase (GE Healthcare Bio-Sciences).

« Troubleshooting 2 »

If the membrane is not thoroughly soaked with Sol. C, the background signal is high and spotted. Furthermore, the protein signals are not observed (*i.e.*, white spots in the right-side figure). Confirm that the membrane does not repel Sol. C.



« Troubleshooting 3 »

PVDF membrane is highly recommended for the Phos-tag™ BTL method.

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