2. Phos binding reagent (Phosbind) Biotin Protocol

1. Solutions for Phosbind Biotin (PB-B) Method

Tris buffered saline	**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
Tween 20 solution	**Sol. B**: 10% (v/v) Tween 20 solution (50 mL) Tween 20 ----------5 mL distilled water ----------45 mL
TBS-T	**Sol. C**: 1xTBS-T (1 L) Sol. A ----------100 mL Sol. B ----------10 mL distilled water ----------890 mL
PB-B methanol solution	**Sol. D**: Phosbind Biotin methanol solutions Phosbind Biotin (MW: 767)----------10 mg Methanol----------0.13 mL 【Storage】Stored in a dark place at 4°C.
PB-B solution	**Sol. E**: Phosbind Biotin solution (10 mmol/L) Sol. D----------0.13 mL Sol. C----------1.17 mL 【Storage】Stored in a dark place at 4°C.
$Zn(NO_3)_2$ aqueous solution	**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 $ZnCl_2$.)
Streptavidin-conjugated Horseradish Peroxidase solution	**Sol. G**: Streptavidin-Horseradish Peroxidase Conjugate (GE Healthcare Bio-Sciences: RPN1231)

2. Preparation of PB-B-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) ---------- 469 µL

Sol. E (Phosbind Biotin solution) *----------1 ~ 10 µL

Sol. F (10 mmol/L $Zn(NO_3)_2$)---------- 2 ~ 20 µL

Sol. G (Streptavidin-conjugated Horseradish Peroxidase) ---------- 1 µL

2) Sol. H is added in a centrifugal filter device cup (NMWL = 30,000, Nanosep^{TM} 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 Phosbind Biotin.

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+}- PB-B-bound Streptavidin-conjugated HRP**).

*: [Phosbind Biotin] >> [Streptavidin-conjugated HRP]

**: PB-B-bound Streptavidin-conjugated HRP in Sol. PB-SH is stable for 30 days at 4 °C.

3. Probing with PB-B-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.

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., LumigenTM-TMA-6, Lumigen).

4. Reprobing the Protein-blotted PVDF Membrane

Tris-HCl buffer	**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
SDS solution	**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
Stripping buffer	**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+}–PB-B 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.

5. Trouble Shooting

1. Phosbind Biotin in Sol. 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 Phosbind Biotin (e.g., 1 μL Sol. E) and Sol. G (1 μL) used. The user should adjust the volume of Sol. D to obtain the required sensitivity or save expenses. If the volume of Sol. E is decreased, it is no need to change the volume of the zinc(II) solution (Sol. F).

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.

3. PVDF membrane is highly recommended for the Phosbind Biotin method.

6、FAQ

1) **Q:** What is the sensitivity level like?

A: It is at the nanogram level. Use a high-luminescence reagent such as ImmunoStar LD.

2) **Q:** Do we need other reagents besides this product?

A: Prepare a Streptavidin-conjugated HRP solution.

3) **Q:** How many times can Phosbind Biotin be used?

A: It depends on the frequency of use. Please refer to the following as a guide.

Phosbind Biotin: 130～1300 times

4) **Q:** Can phosphorylated proteins be assayed?

A: You can do semi-quantitative assay based on the density of bands.

5) **Q:** Is it possible to determine the number of binding phosphate groups?

A: No, it isn't.

6) **Q:** Can I strip the antibodies of Phosbind Biotin?

A: Yes, you can. Mix it with a solution containing 62.5 mM of Tris-HCl (pH 6.8), 2% (w/v) of SDS, and 0.1 M of 2-mercaptoethanol and shake the mixture for 15 minutes. Then, wash the mixture with 1×TBS-T three times for 10 minutes each time. For further details, please contact us.

7) **Q:** What kind of membrane is recommended?

A: We recommend PVDF membranes.

8) **Q:** Does the use of Phosbind Biotin require blocking?

A: No, it doesn't. Blocking causes the sensitivity to drop.