

Mobility Shift Detection of Phosphorylated Proteins

- Phosphate Affinity SDS-PAGE using Acrylamide-pendant Phos-tag™ -

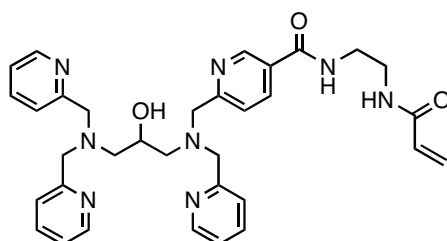
Ver. 8 (2010/8)

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 for detection of phosphorylated proteins in SDS-PAGE using an analogous Phos-tag™ complex with two manganese(II) ions, Mn^{2+} -Phos-tag™ (a dinuclear manganese complex of acrylamide-pendant Phos-tag™ ligand).

2. Description of Acrylamide-pendant Phos-tag™

The acrylamide-pendant Phos-tag™ ligand (Phos-tag™ AAL-107) provides a phosphate affinity SDS-PAGE for mobility shift detection of phosphorylated proteins. This method requires only a general mini-slab PAGE system. The product is supplied as light yellow viscous oil (each at 10 mg in an airtight plastic tube), which has no irritant effect on the skin. Below 4°C, the product is stable for at least 1 year.



Phos-tag™ AAL-107

Mol. Wt.: 595

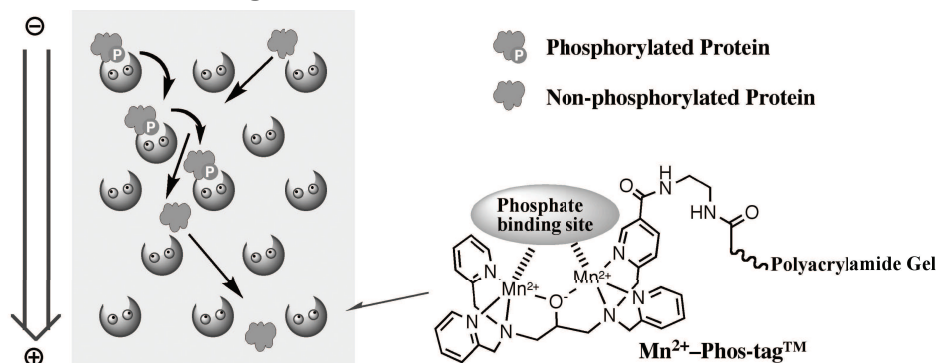
3. Warning and Limitations

Phos-tag™ AAL-107 is not for use in human diagnostic and the 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 skin or eyes. In the case of contact with skin or eyes wash immediately with water.

4. Advantages of Phos-tag™ SDS-PAGE

- # Radioactive and chemical labels are avoided.
- # Phosphoprotein isotypes can be detected as multiple migration bands in the same lane.
- # The procedure is almost the same as that for the general SDS-PAGE.
- # The binding specificity of Phos-tag™ is independent on amino acid and sequence context.
- # Downstream procedures such as Western blot analysis and MS analysis are applicable.
- # Phos-tag™ AAL-107 dissolved in distilled water is stable for at least 3 months.
- # The time-course ratio of phosphorylated and non-phosphorylated proteins can be determined.
- # Separation of phosphoprotein isotypes having the same number of phosphate groups is possible.

5. Principle of Mn²⁺-Phos-tag™ SDS-PAGE



6. Solutions for Phos-tag™ SDS-PAGE

Sol. A: 30% w/v acrylamide solution (100 mL, stored at ca. 4°C in the dark)

# acrylamide	29.0 g
# <i>N,N'</i> -methylene-bisacrylamide	1.0 g
# distilled water for preparation of the 100 mL solution	a proper quantity

Sol. B: 1.5 mol/L Tris-HCl buffered solution (0.4% w/v SDS, 100 mL)

# Tris	18.2 g
# SDS	0.40 g
# distilled water	80 mL
# 6 mol/L aqueous HCl for pH adjustment at pH 8.8	a proper quantity
# distilled water for preparation of the 100 mL solution	a proper quantity

Sol. C: 0.50 mol/L Tris-HCl buffered solution (0.4% w/v SDS, 100 mL)

# Tris	6.1 g
# SDS	0.40 g
# distilled water	90 mL
# 6 mol/L aqueous HCl for pH adjustment at pH 6.8	a proper quantity
# distilled water for preparation of the 100 mL solution	a proper quantity

Sol. D: 5.0 mmol/L Phos-tag™ AAL Solution containing 3% (v/v) MeOH

# Phos-tag™ AAL-107	10 mg
# methanol	0.10 mL
# distilled water	3.2 mL

The oily product, Phos-tag™ AAL-107 (10 mg) placed in a small plastic tube is completely dissolved in 0.10 mL methanol. The solution is diluted with 3.2 mL distilled water by pipetting. If a trace amount of insoluble material appeared as white fine powder (impurity) in the solution, it can be separated by centrifuging (2000xg, 10 min) using two 2-mL microtubes. Store the solution in the 2-mL microtubes at 4°C in the dark. From the supernatant solution, 45 mini-slab gels (50 μmol/L Phos-tag™, 1-mm-thick, 9-cm-wide, 9-cm-long) can be prepared.

Sol. E: 10 mmol/L MnCl₂ solution (50 mL, stored at room temperature)

# MnCl ₂ (H ₂ O) ₄ (FW. 197.9)	0.10 g
# distilled water	50 mL

The MnCl₂ solution is stable for at least 6 months. Do not use the other anion salt, such as Mn(NO₃)₂.

Sol. F: 10 % w/v diammonium peroxydisulfate solution (0.30 mL)	
# (NH ₄) ₂ S ₂ O ₈ (FW. 228.2)	30 mg
# distilled water	0.30 mL
The solution should be freshly prepared just before the acrylamide polymerization.	
Sol. G: Electrode buffer (0.5 L, pH is near 8.4, stored at room temperature)	
# Tris (25 mmol/L)	1.50 g
# SDS	0.50 g
# glycine (192 mmol/L)	7.2 g
# distilled water	0.50 L
Sol. H: Sample buffer 3x (10 mL, stored at -20°C)	
# bromophenol blue (BPB, a tracking dye)	1.5 mg
# SDS	0.60 g
# glycerol	3.0 mL
# Sol. C	3.9 mL
# 2-mercaptoethanol	1.5 mL
# distilled water for preparation of the 10 mL solution	a proper quantity
Sol. I: Acidic solution for fixation of proteins (1 L)	
# acetic acid	0.10 L
# methanol	0.40 L
# distilled water	0.50 L
Sol. J: CBB staining solution (0.5 L)	
# Coomassie Brilliant Blue (CBB)	1.25 g
# methanol	0.20 L
# acetic acid	50 mL
# distilled water	0.25 L
After dissolving CBB in methanol, acetic acid and water are added into the solution.	
Sol. K: Washing and destaining solution (1 L)	
# methanol	0.25 L
# acetic acid	0.10 L
# distilled water	0.65 L
Resolving gel solution (7 mL: e.g., 100 µmol/L Phos-tag™ & 10% w/v acrylamide)	
# Sol. A	2.33 mL
# Sol. B	1.75 mL
# Sol. D	0.14 mL
# Sol. E (the same volume of Sol. D)	0.14 mL
# distilled water	2.52 mL
# TEMED (tetramethylethylenediamine)	20 µL
# Sol. F	0.10 mL

Stacking gel solution (2 mL: e.g., 4.5% w/v acrylamide)

# Sol. A	0.30 mL
# Sol. C	0.50 mL
# distilled water	1.08 mL
# TEMED (tetramethylethylenediamine)	20 μ L
# Sol. F	0.10 mL

7. Casting Gels

- 1) Set up the casting apparatus (e.g., ATTO AE-6500 mini-slab gel system).
- 2) Prepare the resolving gel solution by mixing the solutions (see above, except for the catalysts Sol. F and TEMED). Degas the mixed solution less than 40 mmHg (0.05 atm) under stirring for 15 min.

« See Troubleshooting 1 »

- 3) Add appropriate volume of Sol. F and TEMED (e.g., 50 μ L and 10 μ L, respectively) into the degassed solution and mix gently. The volumes must be adjusted for the Phos-tag electrophoresis.
- 4) Transfer the resolving gel solution (6.3 mL) between the plates, pore distilled water (1 mL) on top of the resolving gel solution, and then allow the gel to polymerize for ca. 30 min.
- 5) Stacking gel solution (see above) is prepared by a similar manner for the resolving gel.
- 6) Rinse the top of the resolving gel with distilled water and dry off the residual liquid with a paper towel.
- 7) Pore the stacking gel solution (1.8 mL) on top of the resolving gel and then insert the comb.
- 8) Allow the gel to polymerize for 1 h.

8. Sample Preparation

- 1) Mix sample with 3 μ L Sol. H (+ a proper amount of distilled water) in a microcentrifuge tube and heat at 95 °C for 5 min (total volume of 9 μ L).
- 2) Allow the solution to cool to room temperature.
- 3) Load the sample solution (e.g., 1.5 μ L/well) using a micropipette.

Phosphorylated proteins:

# 1.8 mg/mL phosphorylated protein (e.g., α -casein, β -casein, or ovalbumin)	1.0 μ L
# Sample buffer 3x (= Sol. H)	3.0 μ L
# distilled water	5.0 μ L

Dephosphorylated proteins:

# 0.3 mg/mL dephosphorylated protein (e.g., α -casein, β -casein, or ovalbumin) (alkaline phosphatase-treated proteins)	6.0 μ L
# Sample buffer 3x (= Sol. H)	3.0 μ L

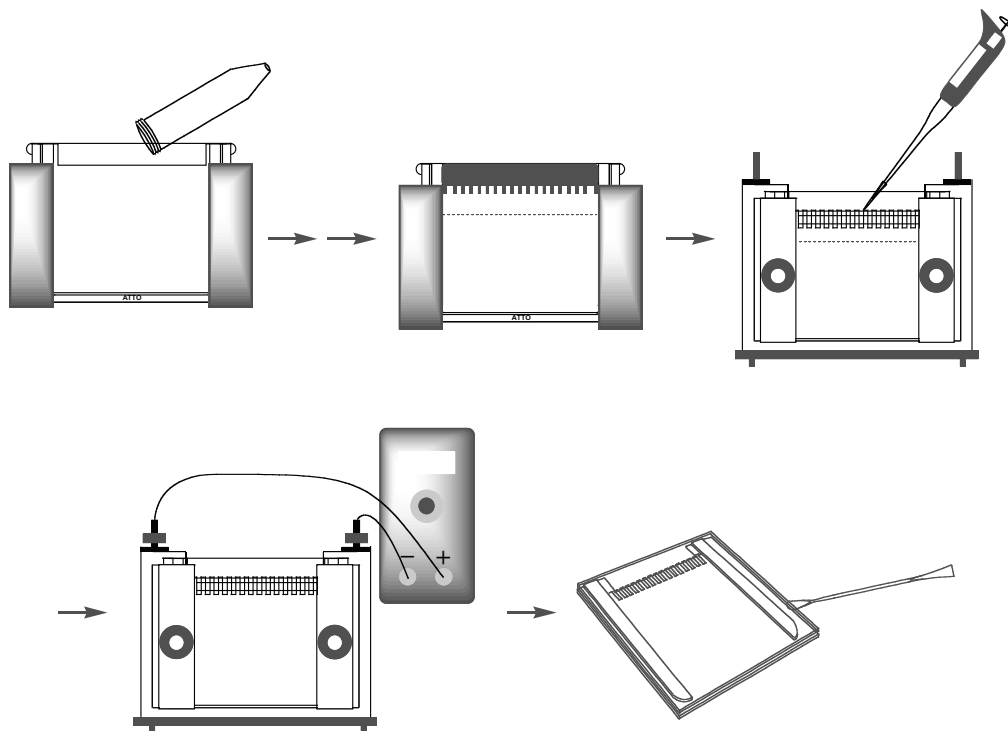
Reagents for the dephosphorylation at 37 °C:

# 10 mg/mL phosphorylated protein solution	50 μ L
# 0.50 M Tris-HCl buffer (pH 9.0) containing 0.10 M MgCl ₂	10 μ L
# Sterilized water	39 μ L
# Alkaline phosphatase (Sigma-Aldrich)	0.33 unit
# Reaction stop solution (= Sol. H)	3.0 μ L/6.0 μ L sample

9. Electrophoresis

- 1) Assemble the electrophoresis equipments (e.g., ATTO AE-6500 mini-slab gel system) and fill the electrode chambers with the electrode buffer (= Sol. G).
- 2) Gently remove the comb from the stacking gel and load the samples into the wells using a micropipette.
- 3) Attach the leads to power supply (e.g., ATTO AE-8750 Power Station 1000XP). Run the gels under a constant current condition (30 mA/gel) until the BPB reaches the bottom of the resolving gel.

« See Troubleshooting 2 »



10. CBB Staining

- 1) Fix the proteins in the gel by soaking in Sol. I (50 mL) for ca. 10 min with gentle agitation.
- 2) Stain the gel by soaking in the staining solution (50 mL Sol. J) for ca. 2 h with gentle agitation.
- 3) Wash the gel in the destaining solution (e.g., 50 mL x 3 Sol. K) to remove excess stain until the background is sufficiently clear. Take a photograph of the gel.

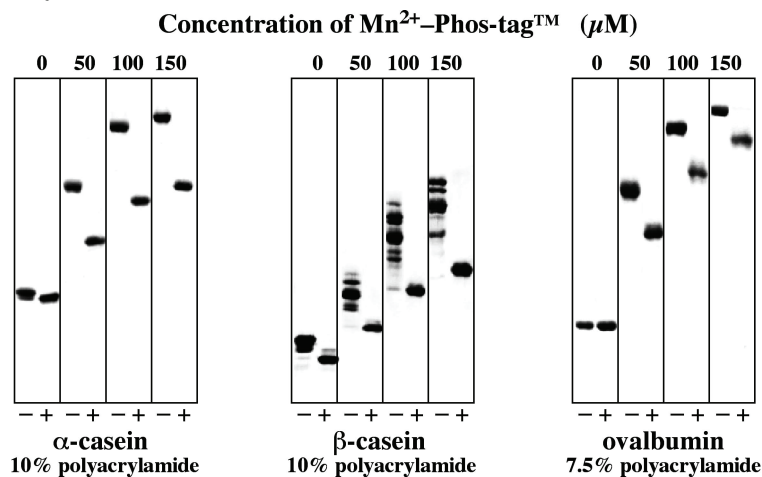
The more sensitive staining methods (e.g., silver staining and SYPRO Ruby staining) and other detection methods (e.g., immunoblotting) would be available.

11. Hints for Western Blotting

Elimination of the manganese ion from the gel is necessary before electroblotting. Just after the electrophoresis, the gel is soaked in a general transfer buffer containing 1 mmol/L EDTA for 10 min with gentle agitation. Next, the gel is soaked in a general transfer buffer without EDTA for 10 min with gentle agitation. These handling increase the transfer efficiency of the phosphorylated and dephosphorylated proteins onto a PVDF membrane. A wet-tank method is strongly recommended for the effective protein transfer from the Mn²⁺-Phos-tag™ acrylamide gel to the PVDF membrane. The blotting conditions, such as time and temperature, must be optimized for the target phosphoprotein in the Phos-tag gel.

12. Mn²⁺-Phos-tag™ (0, 50, 100, and 150 μmol/L) SDS-PAGE

Signals in left and right lanes for each run of electrophoresis are phosphorylated proteins and dephosphorylated proteins, respectively. The R_f values for all phosphorylated proteins are smaller than those for corresponding dephosphorylated proteins. In the absence of Mn²⁺ ion (*i.e.*, Phos-tag™ ligand only), no mobility shift was observed.



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- : Phosphorylated proteins (octa-, penta-, and di-phosphorylated, respectively)

+ : Dephosphorylated proteins (AP-treated proteins)

A commercially available β-casein contains partially dephosphorylated proteins.

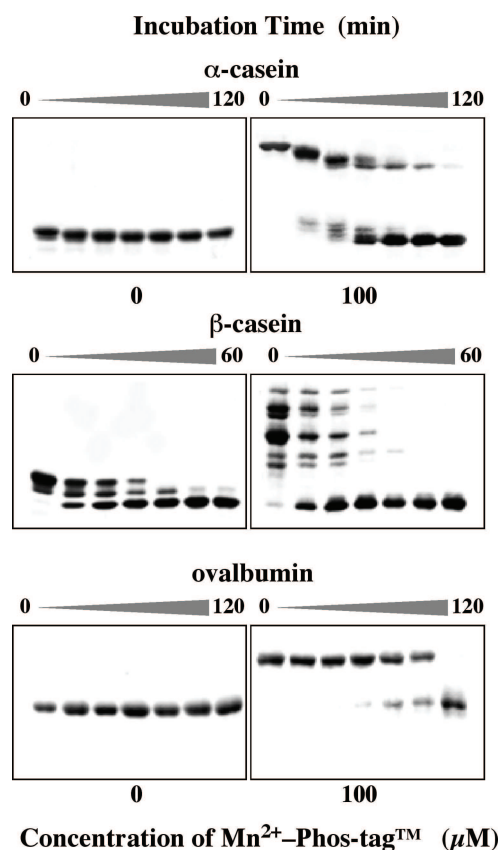
13. Phosphatase Assays by Mn²⁺-Phos-tag™ SDS-PAGE and CBB Staining

The left and right gels are normal SDS-PAGE (*i.e.*, without Mn²⁺-Phos-tag™) and 100 μM Mn²⁺-Phos-tag™ SDS-PAGE, respectively. The incubation time is 0 – 120 min. A similar assay for the kinase reaction (*i.e.*, tyrosin phosphorylation) using Abltide-GST and Abl kinase was reported (E. Kinoshita-Kikuta *et al.* 2007). The SDS-PAGE results show that Mn²⁺-Phos-tag™ preferentially captures phospho- monoester dianions (-OPO₃²⁻) bound to proteins. Thus, Mn²⁺-Phos-tag™ SDS-PAGE can identify the time-course ratio of phosphorylated and corresponding dephosphorylated proteins in a polyacrylamide gel.

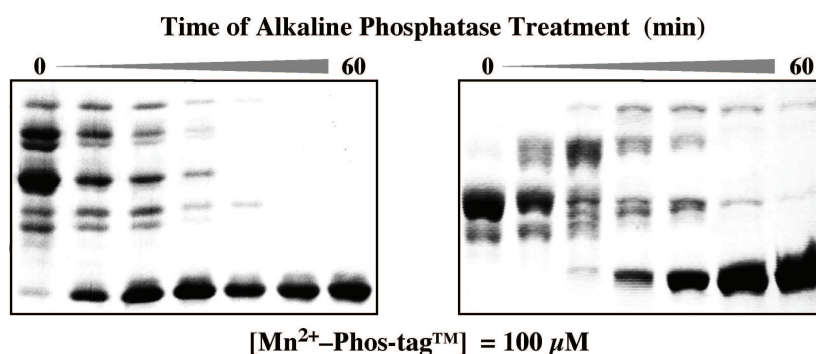
α-casein: 10% w/v acrylamide

β-casein: 10% w/v acrylamide

ovalbumin: 7.5% w/v acrylamide

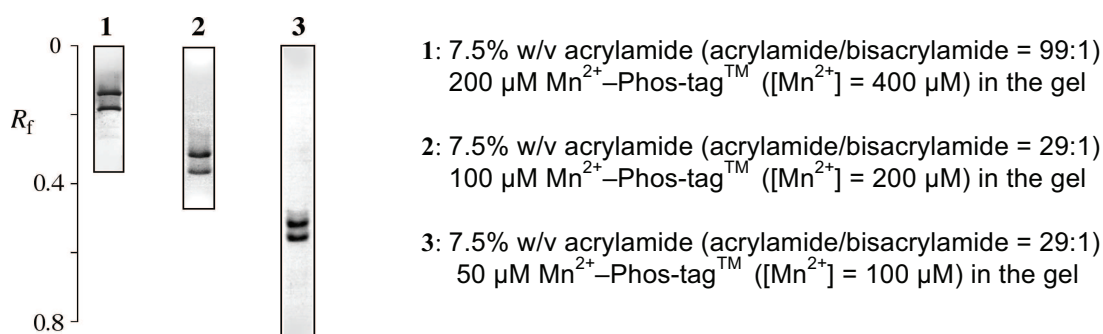


14. Purity Check of β -Casein (Penta-phosphorylated Protein Sold Commercially)



A product of β -casein (the left PAGE) appears as multi-bands at 0 min, indicating the existence of at least eight isotypes with a different number (and/or position) of phosphorylated serine residues. Another β -casein (the right PAGE) shows less bands at 0 min, indicating a high content of the penta-phosphorylated isotype. The phosphorylated β -casein decreases time-dependently, while the fastest migration band (*i.e.*, completely dephosphorylated β -casein) increases.

15. Separation of a Phosphorylated-Histidine Protein by $\text{Mn}^{2+}\text{-Phos-tag}^{\text{TM}}$ SDS-PAGE



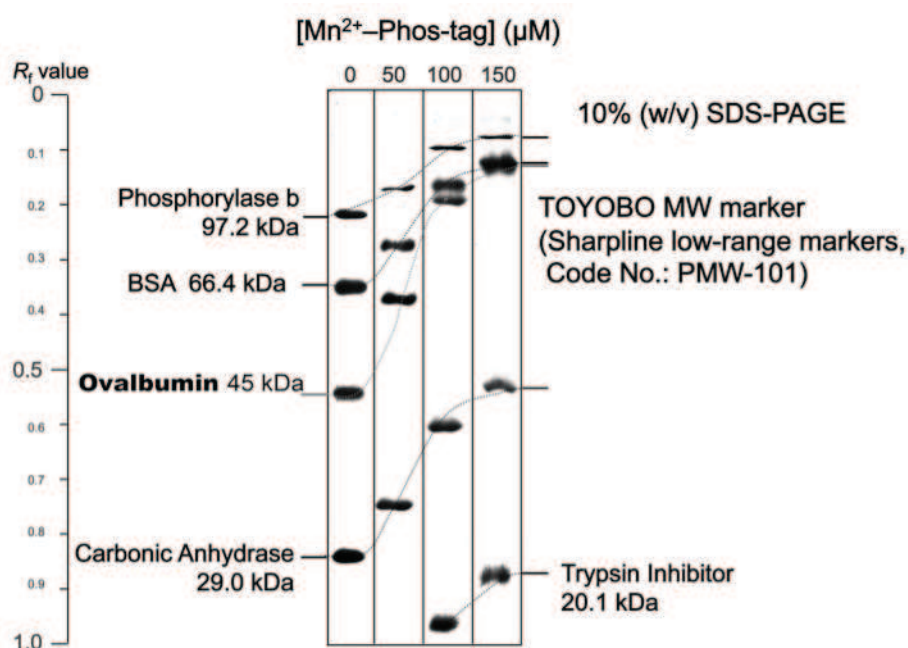
The slower and faster migration bands are a phosphorylated and non-phosphorylated histidine kinase (*i.e.*, an auto-phosphorylation kinase, MW = 41 kDa). The gels were stained using SYPRO Ruby (Invitrogen). The total amount of the protein per lane is 0.27 μg . When the kinase reaction was conducted using $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$, the upper band was detected by autoradiography. Since the separation efficiency depends on the gel composition, an appropriate $\text{Mn}^{2+}\text{-Phos-tag}^{\text{TM}}$ SDS-PAGE condition (*e.g.*, concentration of $\text{Mn}^{2+}\text{-Phos-tag}^{\text{TM}}$) should be optimized for each target protein.

16. Separation of Phosphoprotein Isotypes by 2D Phosphate-affinity Electrophoresis

Users interested in two-dimensional-electrophoresis applications can consult the original article (by E. Kinoshita *et al.*, 2009). By the novel 2D procedure, the separation of phosphoprotein isotypes should be improved relative to the 1D method.

« Troubleshooting 1 »

Generally, the R_f values of all proteins (*i.e.*, both phosphorylated and dephosphorylated proteins) in Mn^{2+} -Phos-tag™ SDS-PAGE are smaller than those in normal SDS-PAGE. Please determine the best electrophoresis conditions, such as percentage of acrylamide gel and concentration of Mn^{2+} -Phos-tag™ (*e.g.*, 100 μM), for the sufficient separation between phosphorylated and dephosphorylated proteins. In particular, a dilute Mn^{2+} -Phos-tag™ (5 ~ 25 μM) polyacrylamide gel should be used for a complex sample such as cell lysate containing various phosphorylated and non-phosphorylated proteins. A phosphorylated protein, ovalbumin (45 kDa) in a molecular-weight protein marker can be used for the gel-shift check of Mn^{2+} -Phos-tag™ SDS-PAGE. In this gel check procedure, prestained protein markers should be avoided. Some stained proteins interact with the Phos-tag gel resulting in broad and/or distorted bands.



« Troubleshooting 2 »

Various contaminants (*e.g.*, EDTA, inorganic salts, surfactant) in the sample proteins solution often disorder the electrophoresis bands (*i.e.*, waving and/or tailing). In order to minimize the disorder, the desalting of the sample is recommended before the sample loading. For example, a dialysis filtration is used to decrease the amount of the low molecules in the sample.

Before the pH measurement for the buffer solutions, the pH-electrode system should be calibrated using the two pH buffer solutions (*e.g.*, pH 4 and 7). The pH of the electrophoresis buffers is one of the most important factors for the separation of phosphoprotein isotypes.

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