Phos-tag™-based Mobility Shift Detection of Phosphorylated Proteins

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

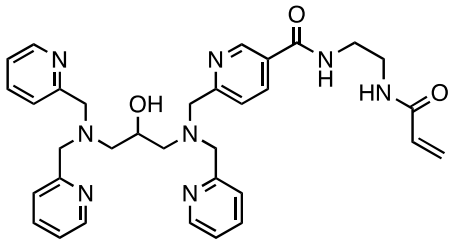
Ver. 12 (2014/8)

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 analytical methods for phosphoproteome research have been developed using Phos-tag™ derivatives. Here, we introduce two electrophoretic procedures for the simultaneous analysis of a phosphoprotein isoform and its non-phosphorylated counterpart: **Method 1) Manganese(II)–Phos-tag™ SDS-PAGE** using the Laemilli's buffer system, and **Method 2) Zinc(II)–Phos-tag™ SDS-PAGE** using a neutral pH buffer system. The methods provide characteristic separation patterns for phosphoprotein isoforms according to the number and/or site of phosphate group.

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 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

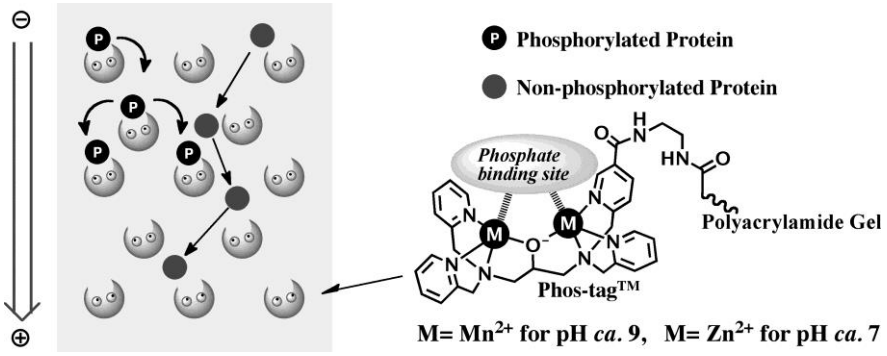
Warning and Limitations

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.

Advantages of Phos-tag™ SDS-PAGE

Radioactive and chemical labels are avoided.
Phosphoprotein isoforms can be detected as multiple migration bands in the same lane.
The procedures are almost the same as that for the general SDS-PAGE using various size gels.
The binding specificity of Phos-tag™ is independent on amino acid and sequence context.
Downstream procedures such as Western blot and MS analysis are applicable.
Time-course ratio of phosphorylated and non-phosphorylated proteins can be determined.
Separation of phosphoproteins having the same number of phosphate groups would be possible.
Shelf life of zinc(II)–Phos-tag™ polyacrylamide gel is more than 3 months at 4˚C.

Principle of Phos-tag™ SDS-PAGE



############################## Method 1 ##############################

Mn^{2+}–Phos-tag™ SDS-PAGE using the Laemmli's buffer system

Mn^{2+}–Phos-tag™ SDS-PAGE is the first phosphate affinity electrophoresis reported in 2006.

Solutions for Method 1:

Sol. A: 30% (w/v) Acrylamide Solution (30% T, 3.3% C)

# acrylamide	29.0 g
# *N*,*N'*-methylene-bisacrylamide	1.0 g

Make to 100 mL with distilled water. Filter and store at 4˚C in the dark.

Sol. B: 1.5 mol/L Tris/HCl Solution, pH 8.8 (4x solution for resolving gel)

# Tris base (FW: 121, pK_a = 8.2 at 20˚C)	18.2 g
# 6.0 mol/L HCl (0.19 equivalents of Tris)	4.85 mL

Make to 100 mL with distilled water. Store at 4˚C.

Sol. C: 0.50 mol/L Tris/HCl Solution, pH 6.8 (4x solution for stacking gel)

# Tris base	6.06 g
# 6.0 mol/L HCl (0.96 equivalent of Tris base)	8.0 mL
# distilled water	90 mL

Carefully adjust to pH 6.8 (non-buffered pH region) with 6.0 mol/L HCl (*ca.* 0.1 mL).
Bring to total volume to 100 mL with distilled water. Store at 4˚C.

Sol. D: 10% (w/v) SDS Solution

Dissolve 10.0 g SDS in 90 mL of distilled water with stirring and bring to total volume to 100 mL with distilled water. Store at 4˚C.

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

# Phos-tag™ AAL-107 （MW = 595）	10 mg
# methanol	0.10 mL
# distilled water	3.2 mL

The viscous oil product, Phos-tag™ AAL-107 (10 mg) placed in a plastic tube is completely dissolved in 0.10 mL methanol. The solution is diluted with 3.2 mL distilled water by pipetting.

Note: If a trace amount of insoluble material appeared as white fine powder (impurity) in the solution, it can be separated by centrifuging 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, 7.7-cm-long) can be prepared.

Sol. F: 10 mmol/L $MnCl_2$ Solution
Dissolve 0.10 g $MnCl_2(H_2O)_4$ (FW: 198) in 50 mL of distilled water.
Note: Do not use the other anion salt, such as $Mn(NO_3)_2$ and $Mn(CH_3COO)_2$.

Sol. G: 10% (w/v) Diammonium Peroxydisulfate Solution
Dissolve 10 mg $(NH_4)_2S_2O_8$ (FW: 228) in 0.10 mL of distilled water.
Note: Freshly prepare prior to use.

Sol. H: Running Buffer, pH 8.3 (10x solution)

# Tris base (0.25 mol/L)	15.1 g
# SDS	5.0 g
# glycine (1.92 mol/L)	72.0 g

Make to 0.50 L with distilled water. Do not adjust pH with acid or base. Store at 4˚C.
Use: Dilute 50 mL of the 10x solution with 450 mL distilled water.

Sol. I: Sample Buffer (3x solution)

# Bromophenol Blue (BPB, a tracking dye)	1.5 mg
# SDS	0.60 g
# glycerol	3.0 mL
# Sol. C: 0.50 mol/L Tris/HCl, pH 6.8	3.9 mL
# 2-mercaptoethanol	1.5 mL

Make to 10 mL with distilled water. Store at –20˚C.
Use: See "Sample Preparation" section.

cf. Sample Buffer (2x solution)

# Bromophenol Blue	1.0 mg
# SDS	0.20 g
# glycerol	3.0 mL
# Sol. C: 0.50 mol/L Tris/HCl, pH 6.8	2.5 mL

Make to 9.5 mL with distilled water.
Use: Add 50 µL 2-mercaptoethanol to 950 µL of the sample buffer prior to use.
Dilute the sample with equivolume of the sample buffer containing 2-mercaptoethanol and heat at 95˚C for 4 min.

Sol. J: Acidic Solution for Fixation of Proteins (1 L)

# acetic acid	0.10 L
# methanol	0.40 L
# distilled water	0.50 L

Sol. K: 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. L: Washing and Destaining Solution (1 L)

# methanol	0.25 L
# acetic acid	0.10 L
# distilled water	0.65 L

Resolving Gel Solution (total 10 mL: *e.g.*, 12% (w/v) acrylamide and 50 µmol/L Phos-tag™ AAL)

# Sol. A: 30% (w/v) Acrylamide Solution	4.00 mL
# Sol. B: 1.5 mol/L Tris/HCl Solution, pH 8.8	2.50 mL
# Sol. E: 5.0 mmol/L Phos-tag™ AAL Solution	0.10 mL
# Sol. F: 10 mmol/L **$MnCl_2$** Solution	0.10 mL
# Sol. D: 10% (w/v) SDS Solution	0.10 mL
# TEMED (tetramethylethylenediamine)	10 µL
# Distilled Water	3.15 mL
——————— Degassing under stirring for 2 min ———————	
# Sol. G: 10% (w/v) Diammonium Peroxydisulfate Solution	20 ~ 50 µL

Stacking Gel Solution (*e.g.*, 4.5% (w/v) acrylamide)	total 10 mL	total 2 mL
# Sol. A: 30% (w/v) Acrylamide Solution	1.50 mL	0.30 mL
# Sol. C: 0.50 mol/L Tris/HCl Solution, pH 6.8	2.50 mL	0.50 mL
# Sol. D: 10% (w/v) SDS Solution	0.10 mL	20 µL
# TEMED (tetramethylethylenediamine)	10 µL	2 µL
# Distilled Water	5.84 mL	1.17 mL
——————— Degassing under stirring for 2 min ———————		
# Sol. G: 10% (w/v) Diammonium Peroxydisulfate Solution	20 ~ 50 µL	4 ~ 10 µL

Note: Prepare any desired volume of the gel solutions by using multiples of the above 10-mL recipe. The volumes of TEMED and Sol. G must be adjusted for individual experiments.
Mn^{2+}–Phos-tag™ SDS-PAGE adopts almost the same gel compositions for Laemmli's method, but SDS may be unnecessary as an additive in the resolving and stacking gels.
In the presence of SDS in the gel, the band of target protein would be rather broad and/or tailing.

Casting Gels

1) Set up the casting apparatus (*e.g.*, a mini-slab gel system, 1-mm-thick).
2) Prepare the resolving gel solution by mixing the solutions (see above recipe, except Sol. G). Degas the mixed solution under stirring for 2 min at less than 0.1 atm.
3) Add Sol. G into the degassed solution and mix under stirring gently.
4) Transfer the resolving gel solution between the glass plates, pore butanol-saturated water on top of the resolving gel solution, and then allow the gel solution to polymerize for 1h at room temperature.
5) Stacking gel solution is prepared by a similar manner for the resolving gel (see above recipe).
6) Rinse the top of the resolving gel with distilled water and remove the residual liquid with a paper towel.
7) Pore the stacking gel solution on top of the resolving gel and then insert a comb.
8) Allow the gel solution to polymerize for 1 h at room temperature.

« See Troubleshooting 1 »

Sample Preparation

1) Mix sample (6.0 µL) with 3.0 µL Sol. I in a microcentrifuge tube and heat at 95˚C for 5 min.
2) Allow the solution to cool to room temperature.
3) Load the sample solution (*e.g.*, 1.5 µL/well) using a micropipette.

Sample solution of phosphorylated protein (*e.g.,* α-casein, β-casein, or ovalbumin)

# Phosphorylated protein (0.3 mg/mL)	6.0 µL
# Sample buffer 3x (= Sol. I)	3.0 µL

Sample solution dephosphorylated proteins (*e.g.,* α-casein, β-casein, or ovalbumin)

# Alkaline phosphatase-treated protein (0.3 mg/mL)	6.0 µL
# Sample buffer 3x (= Sol. I)	3.0 µL

Note: The dephosphorylation can be conducted overnight at 37˚C using the following reagents.

# 10 mg/mL phosphorylated protein	50 µL
# 0.50 M Tris/HCl buffer (pH 9.0) containing 0.10 M $MgCl_2$	10 µL
# Sterilized water	39 µL
# Alkaline phosphatase (Sigma-Aldrich)	*ca*. 0.3 unit / 1 µL

If you want to prepare partially dephosphorylated isoforms, the dephosphorylation reaction is stopped by addition of 3 µL Sol. I (Sample Buffer 3x solution) to 6 µL reaction mixture and heating at 95˚C for 5 min.

Electrophoresis

1) Assemble the electrophoresis equipments (*e.g.*, ATTO AE-6500 mini-slab gel system) and fill the electrode chambers with the electrode buffer prepared from Sol. H.
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 »

CBB Staining

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

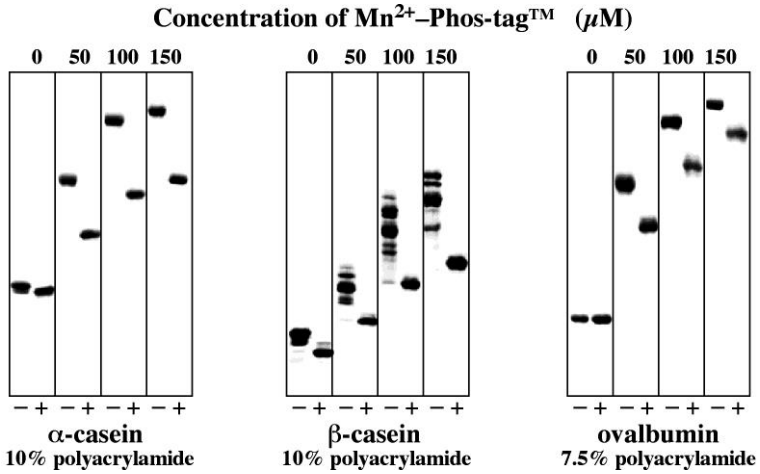
Note: The more sensitive staining methods (*e.g.*, silver staining and SYPRO® Ruby staining) and other detection methods (*e.g.,* immunoblotting) is available.

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 both phosphorylated and non-phosphorylated proteins onto a PVDF membrane. A wet-tank method is recommended for the effective protein transfer from the 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.

Mn^{2+}–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 Mn^{2+} ion (*i.e.,* Phos-tag™ ligand only), no mobility shift was observed.



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

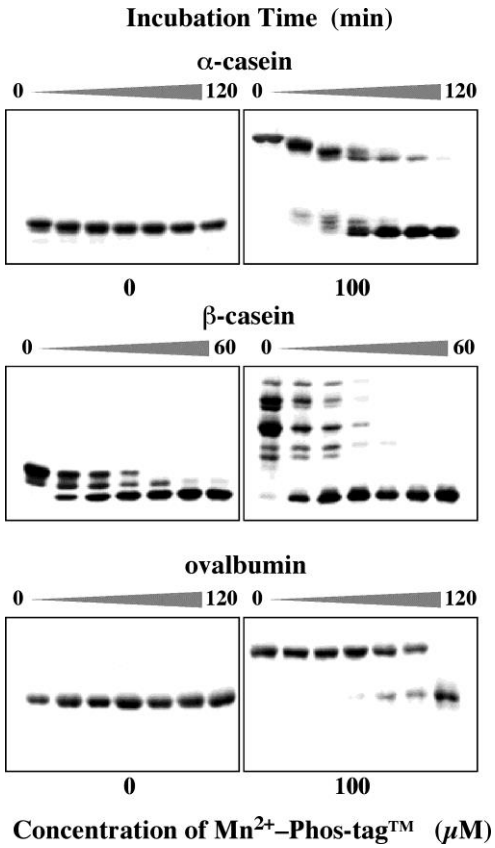
+ : Dephosphorylated proteins (AP-treated proteins)

A commercially available β-casein contains partially dephosphorylated proteins.

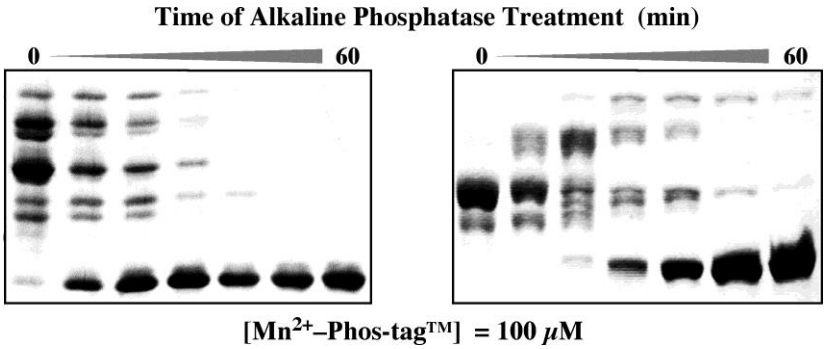
Phosphatase Assays by Mn^{2+}–Phos-tag™ SDS-PAGE and CBB Staining

The left and right gels are normal SDS-PAGE (*i.e.,* without Mn^{2+}–Phos-tag™) and 100 µM Mn^{2+}–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 Mn^{2+}–Phos-tag™ preferentially captures phosphomonoester dianions ($-OPO_3^{2-}$) bound to proteins. Thus, Mn^{2+}–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
ovalbmin: 7.5% (w/v) acrylamide

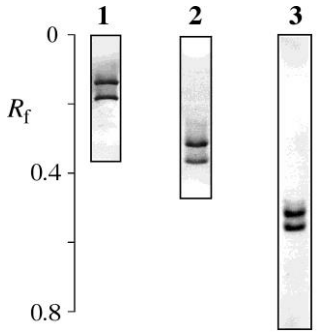


Purity Check of β-Casein (Penta-phosphorylated Protein Sold Commercially)



A product of β-casein (the left PAGE) appears as multi-bands at 0 min, indicating the existence of at least eight isoforms 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 isoform. The phosphorylated β-casein decreases time-dependently, while the fastest migration band (*i.e.,* completely dephosphorylated β-casein) increases.

Separation of a Phosphorylated-Histidine Protein by Mn^{2+}–Phos-tag™ SDS-PAGE



1: 7.5% (w/v) acrylamide (acrylamide/bisacrylamide = 99:1) 200 μM Mn^{2+}–Phos-tag™ ($[Mn^{2+}]_t$ = 400 μM) in the gel

2: 7.5% (w/v) acrylamide (acrylamide/bisacrylamide = 29:1) 100 μM Mn^{2+}–Phos-tag™ ($[Mn^{2+}]_t$ = 200 μM) in the gel

3: 7.5% (w/v) acrylamide (acrylamide/bisacrylamide = 29:1) 50 μM Mn^{2+}–Phos-tag™ ($[Mn^{2+}]_t$ = 100 μM) in the gel

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 (Invitorgen). The total amount of the protein per lane is 0.27 μg. When the kinase reaction was conducted using [γ-^{32}P]-ATP, the upper band was detected by autoradiography. Since the separation efficiency depends on the gel composition, an appropriate Mn^{2+}–Phos-tag™ SDS-PAGE condition (*e.g.,* concentration of Mn^{2+}–Phos-tag™) should be optimized for each target protein.

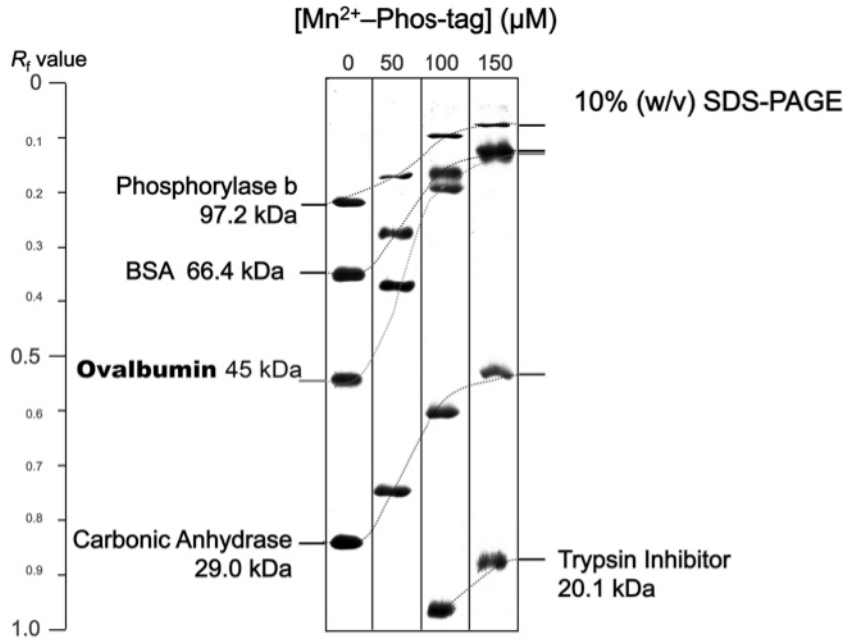
Separation of Phosphoprotein Isoforms by 2D Phosphate-affinity Electrophoresis

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

« Troubleshooting 1 »

Generally, the R_f values of proteins (*i.e.*, both phosphorylated and non-phosphorylated proteins) in Mn^{2+}–Phos-tag™ SDS-PAGE are smaller than those in normal SDS-PAGE. The figure below shows the interaction between Mn^{2+}–Phos-tag™ and SDS-bound proteins resulting short migration distances even for non-phosphorylated proteins. Please determine the best electrophoresis conditions, such as concentrations of acrylamide and Mn^{2+}–Phos-tag™ for the sufficient separation between phosphorylatedand dephosphorylated proteins. For example, 5 – 25 µM Mn^{2+}–Phos-tag™ should be used for a complex sample such as cell lysate containing various phosphorylated and non-phosphorylated proteins.

Note: A phosphorylated protein, ovalbumin (45 kDa) and non-phosphorylated proteins such as BSA and carbonic anhydrase in a molecular-weight protein marker can be used for the gel-shift check of Phos-tag™ SDS-PAGE (Methods 1 and 2). 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 molecular weight compounds in the sample.

Note: 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 isoforms.

Method 2

Zn^{2+}–Phos-tag™ SDS-PAGE using a neutral-pH buffer system

Mn^{2+}–Phos-tag™ SDS-PAGE (Method 1) separates not all phosphoprotein isoforms from the non-phosphorylated counterpart. To overcome this problem, an alternative method using Zn^{2+}–Phos-tag™ (Method 2) was developed in 2010. Since the method employs a neutral pH buffer system, the gel performance is maintained more than 3 months. On the other hand, Mn^{2+}–Phos-tag™ gel should be prepared just before its use. Except the buffer system, the procedure for Zn^{2+}–Phos-tag™ SDS-PAGE is almost the same as that of Method 1.

Solutions for Method 2:

Sol. A: 30% (w/v) Acrylamide Solution (30% T, 3.3% C)

# acrylamide	58.0 g
# *N*,*N*′-methylene-bisacrylamide	2.0 g

Make to 200 mL with distilled water. Filter and store at 4˚C in the dark.

Sol. D: 10% (w/v) SDS Solution

Dissolve 10.0 g SDS in 90 mL of distilled water with stirring and bring to total volume to 100 mL with distilled water. Store at 4˚C.

Sol. E: 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 plastic tube is completely dissolved in 0.10 mL methanol. The solution is diluted with 3.2 mL distilled water by pipetting.

Note: If a trace amount of insoluble material appeared as white fine powder (impurity) in the solution, it can be separated by centrifuging 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, 7.7-cm-long) can be prepared.

Sol. G: 10% (w/v) Diammonium Peroxydisulfate Solution

Dissolve 10 mg $(NH_4)_2S_2O_8$ (FW: 228) in 0.10 mL of distilled water.

Note: Freshly prepare in a sample tube prior to use.

Sol. M: 10 mmol/L $ZnCl_2$ Solution

Dissolve 0.70 g $ZnCl_2$ (FW: 136, purity >98%) in 500 mL of distilled water.

Note: Because zinc(II) chloride is a deliquescent salt, the $ZnCl_2$ solution should be prepared using a fresh product in a new bottle. If an insoluble material such as a small amount of ZnO (impurity) remains in the solution, it should be removed by filtration.

Sol. N: 1.4 mol/L Bis-Tris/HCl Solution, pH 6.8 (4x solution)

# Bis-Tris base (FW: 209, pK_a = 6.5 at 20˚C)	29.9 g
# 6.0 mol/L HCl (0.42 equivalent of Bis-Tris)	10 mL

A measuring glass pipette (10 mL) is used for the HCl solution.

Make to 100 mL with distilled water. Store at 4˚C.

Note: This buffer solution, Sol. N is used for the resolving and stacking gels in Method 2.

On the other hand, Method 1 employs the different pH buffers, Sol. B and Sol. C for the gels, respectively.

Sol. O: 0.5 mol/L sodium bisulfite solution

# $NaHSO_3$ (FW: 106)	5.3 g

Make to 100 mL with distilled water. Store at 4˚C.

Sulfite ion (SO_3^{2-}) is a reducing reagent diminishing O_2 in the electrode buffer and inhibits the oxidation of reduced proteins in the gel. The stock solution should be sealed in a 100-mL glass bottle against access of air.

Sol. P: Running Buffer, pH 7.8 (5x solution)

# Tris base (FW: 121, pK_a = 8.2 at 20˚C, 0.50 mol/L)	30.3 g
# MOPS (FW: 209, pK_a = 7.2 at 20˚C, 0.50 mol/L)	52.3 g
# Sol. D: 10% (w/v) SDS Solution (0.5% (w/v))	25.0 mL

Make to 0.50 L with distilled water. Do not adjust pH with acid or base. Store at 4˚C.

Use: Dilute 100 mL of Sol.P with 5 mL of Sol.O (0.5 mol/L $NaHSO_3$) and 395 mL distilled water prior to use (total volume: 0.50 L).

Resolving Gel Solution (total 10 mL: *e.g.*, 12% (w/v) acrylamide and 50 µmol/L Phos-tag™ AAL)

# Sol. A: 30% (w/v) Acrylamide Solution	4.00 mL
# Sol. N: 1.4 mol/L Bis-Tris/HCl Solution, pH 6.8	2.50 mL
# Sol. E: 5.0 mmol/L Phos-tag™ AAL Solution	0.10 mL
# Sol. M: 10 mmol/L **$ZnCl_2$** Solution	0.10 mL
# TEMED (tetramethylethylenediamine)	10 µL
# Distilled Water	3.24 mL
——————— Degassing under stirring for 2 min ———————	
# Sol. G: 10% (w/v) Diammonium Peroxydisulfate Solution	50 µL

Stacking Gel Solution (*e.g.*, 4.5% (w/v) acrylamide)	total 10 mL	total 2 mL
# Sol. A: 30% (w/v) Acrylamide Solution	1.50 mL	0.30 mL
# Sol. N: 1.4 mol/L Bis-Tris/HCl Solution, pH 6.8	2.50 mL	0.50 mL
# TEMED (tetramethylethylenediamine)	10 µL	2 µL
# Distilled Water	5.94 mL	1.19 mL
——————— Degassing under stirring for 2 min ———————		
# Sol. G: 10% (w/v) Diammonium Peroxydisulfate Solution	50 µL	10 µL

Note: Prepare any desired volume of the gel solutions by using multiples of the 10-mL recipes.

The volumes of TEMED and Sol. G must be adjusted for individual experiments.

SDS is unnecessary as an additive in the resolving and stacking gels.

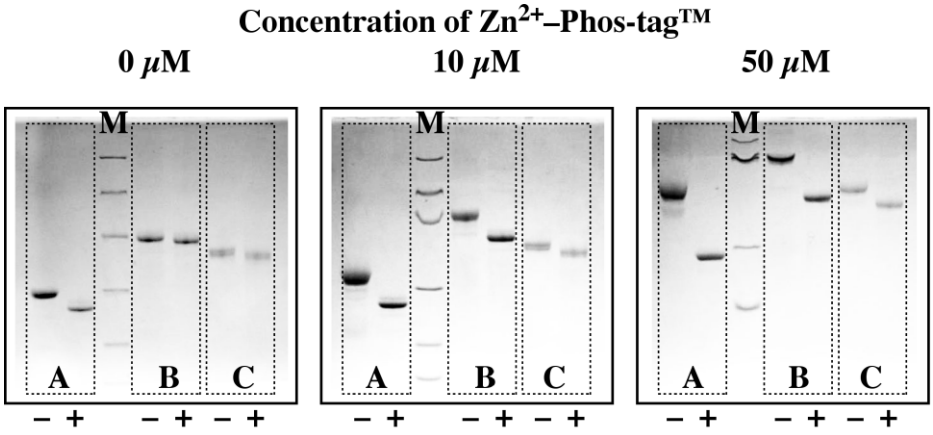
The protocols for "Casting Gels", "Sample Preparation", and "Electrophoresis" are almost the same as those for Mn^{2+}–Phos-tag™ PAGE (see Method 1). Typical results for commercially available phosphoproteins and dephosphorylated counterparts are shown in the next page. The resolving gel is 1-mm-thick, 8.2-cm-wide and 6-cm-long. The electrophoresis was conducted using a Mini-PROTEAN® Tetra Cell system (Bio-Rad Laboratories) at 40 mA/gel under a maximum voltage of 90 V until the BPB reaches the running buffer.

Depend on the sample proteins, "smiling" bands on a Phos-tag gel are observed. One of the reason should be due to metal dissociation from Phos-tag molecule by potent metal-ligands such as thiol, imidazole and carboxylate groups of proteins. To prevent the problem, addition of ca. 1 mM of zinc(II) chloride in the sample buffer would give better electrophoretic resolution.

Zn^{2+}–Phos-tag™ (0, 10, and 50 μmol/L) SDS-PAGE

The running buffer 1 (Sol. P: Tris&MOPS) was used. The R_f values for all phosphorylated proteins are smaller than those for corresponding dephosphorylated proteins. In the absence of Zn^{2+} ion (*i.e.,* Phos-tag™ ligand only), no mobility shift was observed. Pepsin, showing no mobility shift from the non-phosphorylated counterpart in Mn^{2+}–Phos-tag™ gel, can be separated by 50 μM Zn^{2+}–Phos-tag™ PAGE.

Note: The separation width between the phosphoprotein and the non-phosphorylated counterpart is almost twice as long as that by Mn^{2+}–Phos-tag™ PAGE (Method 1) under the same concentration of Phos-tag™. The marker bands of BSA and ovalbumin lie close together in the 50 μM Zn^{2+}–Phos-tag™ PAGE (*cf.* 100 μM Mn^{2+}–Phos-tag™ PAGE in Troubleshooting 1).

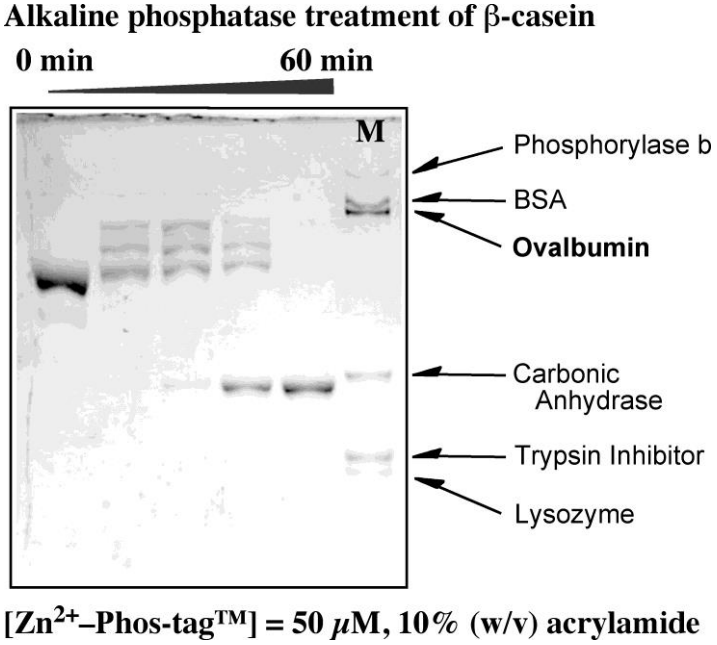


A: β-Casein, B: Ovalbumin, C: Pepsin

M: MW marker

– : Phosphorylated proteins (penta-, d-, and mono-phosphorylated,respectively)

+ : Dephosphorylated proteins (AP-treated proteins)



[Zn^{2+}–Phos-tag™] = 50 μM, 10% (w/v) acrylamide

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