FT-FL8630

Phos-Tag products,
for phosphorylated proteins analysis

Summary: Overview of SDS-PAGE technique | Principle | Protocol |
Trouble Shoouting | FAQ | Application data and references
and More Phos-tag products (SuperSep Gels, Biotin, MS Analysis kit, Agarose)

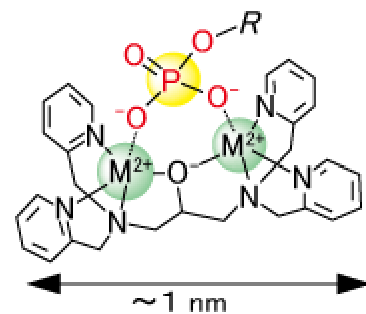
Overview - Principle and Applications

Phos-tag™ is a functional molecule that binds specifically phosphorylated ions. It is applicable for the specific separation of phosphorylated proteins (Phos-tag™ Acrylamide) as well as for the detection using western blot (Phos-tag™ Biotin), purification (Phos-tag™ Agarose), and MALDI-TOF/MS (Phos-tag™ Mass Analytical Kit).

- **Selectivity** of binding of a phosphate ion ($^{2-}$) is much higher than that of other anions.
- **Stable complex** is formed under physiological conditions (pH 5 to 8)

Product	*Purpose of Use*
Phos-tag™ Acrylamide	**Separation**： Separation is possible by SDS-PAGE depending on the degree of phosphorylation.
SuperSep Phos-tag™	**Separation**： Ready-to-Use Precast gel containing 50 µM Phos-tag™ Acrylamide
Phos-tag™ Biotin	**Detection**： A substitute for the anti-phospho antibody used in western blot.
Phos-tag™ Agarose	**Purification**： Phosphorylated proteins are purified by column chromatography.
Phos-tag™ Mass Analytical Kit	**Analysis**： This is used in MALDI-TOF/MS analysis to improve the detection sensitivity of phosphorylated molecules.

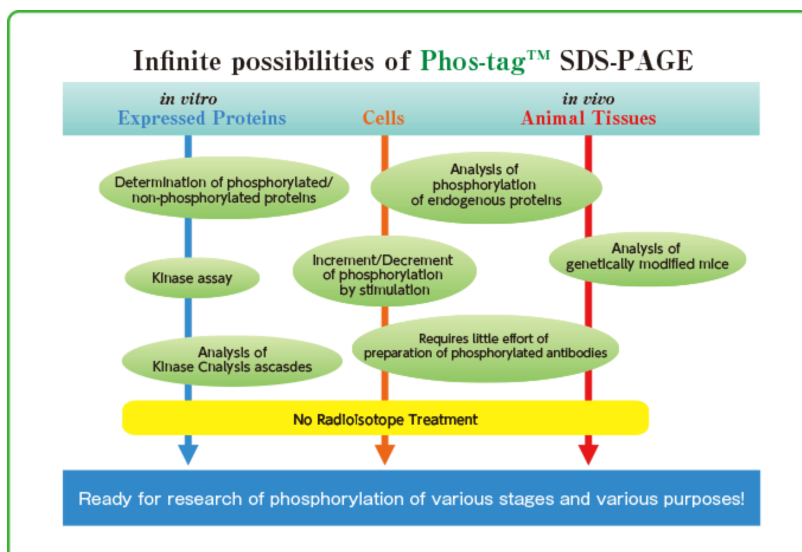
【Basic Structure of Phos-tag™】



M^{2+} : Zinc ion or manganese ion

Product Name	Pkg. Size	Cat. No. (Product #)	Application
Phos-tag™ Acrylamide 5 mM Aqueous Solution	0.3 mL (0.9 mg)	304-93526(AAL-107S1)	Separation of phosphorylated and non-phosphorylated proteins using SDS-PAGE with Phos-tag™ Acrylamide
Phos-tag™ Acrylamide	2 mg	300-93523(AAL-107M)	
	10 mg	304-93521(AAL-107)	

See also SuperSep Phos-tag™ gels



■ Listed products are intended for laboratory research use only, and not to be used for drug, food or human use.

Phos-tag™ was developed by Department of Functional Molecular Science at Hiroshima University.

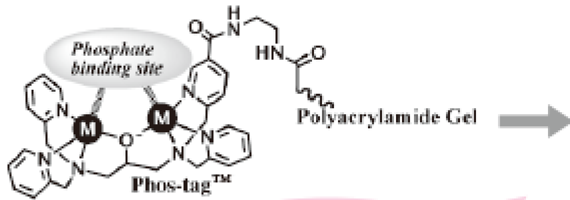
More information on inquire

Contact your local distributor
interbiotech@interchim.com

InterBioTech, powered by interchim
213 Avenue J.F. Kennedy - BP 1140
03103 Montluçon Cedex - France
Tél. 04 70 03 88 55 - Fax 04 70 03 82 60

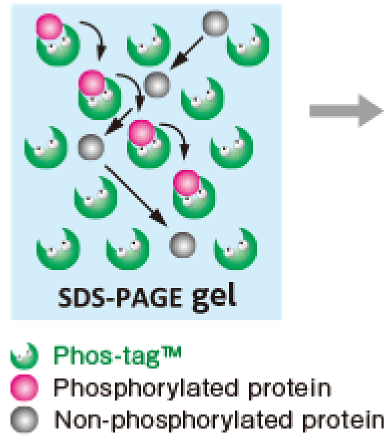
Principle

1) Two metallic ions cooperate to bind a phosphate group



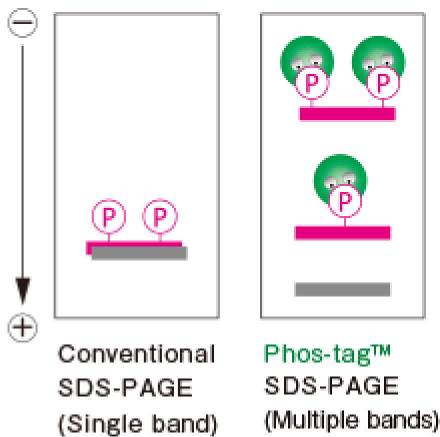
Phosphorylated forms with different numbers and positions of phos-phorylation can also be separated.

2) Phosphorylated proteins move while being bound by Phos-tag™ in the gel



Phos-tag™
Phosphorylated protein
Non-phosphorylated protein

3) Migration speed of phosphorylated proteins decreases and they are separated from non-phosphorylated proteins.



Conventional SDS-PAGE (Single band)

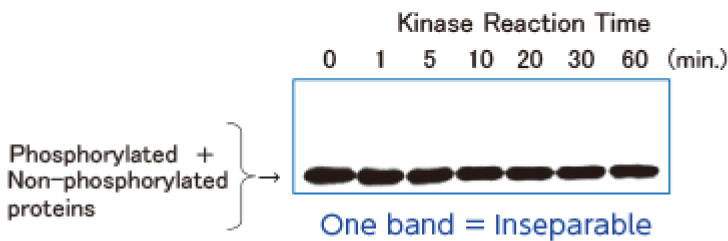
Phos-tag™ SDS-PAGE (Multiple bands)

Application — Time course of phosphorylation by using the Tyrosin Kinase Abl —

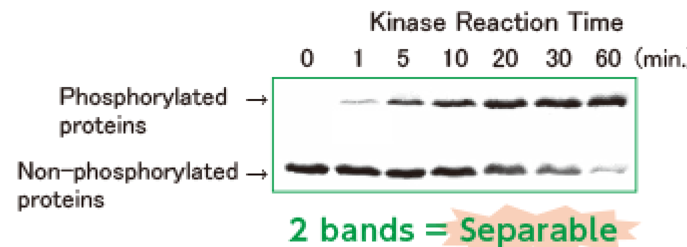
Phosphorylated tyrosine was prepared by GST binding protein of tyrosine kinase Abl and the substrate peptide (Abltide) and separated with conventional SDS-PAGE and Phos-tag™ SDS-PAGE, respectively.

- Conventional SDS-PAGE（CBB staining）

without PhosTag



with PhosTag

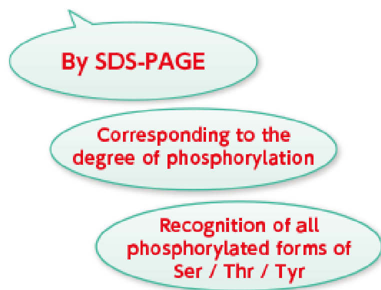


More about Phos-tag™ SDS-PAGE

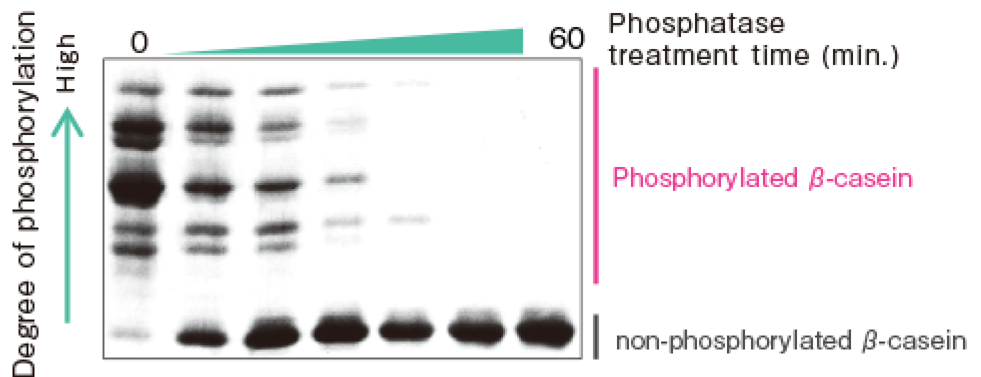
Phos-tag™ Acrylamide is an acrylamide-pendant Phos-tag™ molecule. Phos-tag™ SDS-PAGE, where a polyacrylamide gel containing Phos-tag™ is used, can be prepared by adding Phos-tag™ Acrylamide and MnCl2 in Resolving Gel when preparing SDS-PAGE gels. During migration, the phosphorylated proteins with the phosphate group bound to the divalent metal ions in Phos-tag™. This decreases the migration speed and phosphorylated/non-phosphorylated proteins are separated. After separation, the gel can be utilized for western blotting and mass spectrometry. Various Phos-tag™ SDS-PAGE precast gels are also available. Please see

8. SuperSep Phos-tag™ for the details.

Phosphorylated proteins are easily separable by simply adding Phos-tag™ Acrylamide and MnCl2 to an acrylamide solution when preparing SDS-PAGE gels



Time-course analysis of β-casein using Phos-tag™ SDS-PAGE

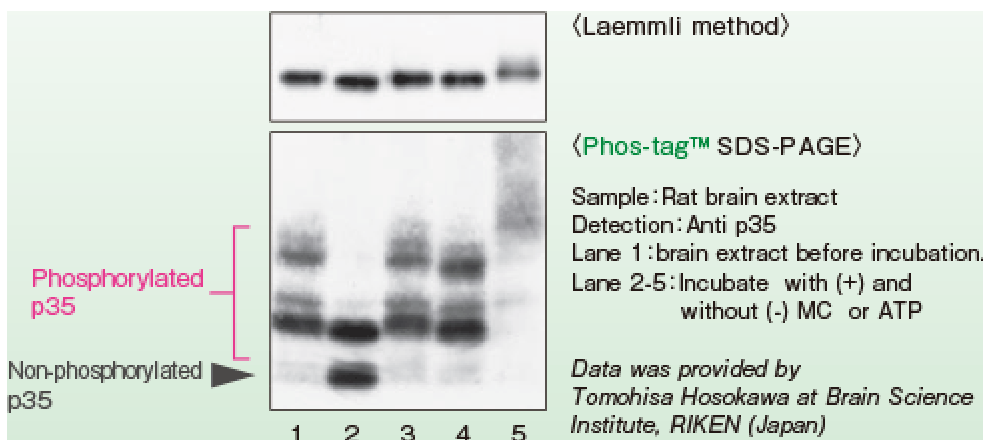


Downstream application of Phos-tag™ SDS-PAGE

By a combination Phos-tag™ SDS-PAGE and various analysis methods, new information of phosphorylated proteins can be obtained.

Western Blotting

Easily-recognizable phosphorylation of your target proteins Simultaneous detection of phosphorylated/non-phosphorylated proteins with a general antibody by their band shift differences. No need to prepare the phosphorylated antibody. Applicable to analysis of phosphorylation of endogenous proteins. Application Data:



Mass Analysis

By separating phosphorylated forms, each phosphorylation site combination can be known.

2D electrophoresis

Phosphorylated forms with the same isoelectric points (same number of phosphorylation sites) can be separated. See Application Data

FT-FL8630

Protocol

[Ⅰ] Mn^{2+}-Phos-tag™ SDS-PAGE
[Ⅱ] Tips for Western blotting of Phos-tag™ SDS-PAGE gels
[Ⅲ] Tips for Mass Spectrometry of Phos-tag™ SDS-PAGE

[Ⅰ] Mn^{2+}-Phos-tag™ SDS-PAGE

Note : Always prepare the gel just before use
Ready to use solutions are available. Please inquire for:

30% Acrylamide Solution(3.3% C)
Separating gel buffer resolving solution (4x) containing SDS
Stacking Gel Buffer Soln X4 containing SDS
10% SDS Soln
10% Ammonium Peroxodisulfate Soln
SDS-PAGE 10X Running Buffer
Sample Buffer Soln (2ME+)
Coomassie Staining solution
Agarose H (High Strenth type)

① Preparation of reagents for Phos-tag™ SDS-PAGE

Acrylamide Solution

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

Acrylamide 29.0 g

N, N'-methylene-bisacrylamide 1.0 g

⇒ Prepare the 100 mL solution by adding distilled water and filter the solution.
【Storage】 Keep at 4℃ in the dark.

Tris-HCl Buffer for Resolving Gel,pH 8.8

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

Tris base (MW : 121, pKa= 8.2 at 20℃) 18.2 g

6.0 mol/L HCl (0.19 equivalents of Tris) 4.85 mL

⇒ Prepare the 100 mL solution by adding distilled water and filter the solution.
【Storage】 Keep at 4℃ in the dark.

Tris-HCl Buffer for Stacking Gel,pH 6.8

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

⇒ Adjust the pH to 6.8 using 6.0 mol/L HCl (ca. 0.1 mL),
then prepare the 100 mL solution by adding distilled water.
【Storage】 Keep at 4℃

SDS Solution

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

SDS 10.0 g

Distilled water 90 mL

⇒ After stirring, prepare the 100 mL solution by adding distilled water
【Storage】 Keep at 4℃

Phos-tag™ AAL Soln.Ready-to-Use Soln.is available. Please see the page #4(Cat. No. 304-93525)

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

※The amount shown in parentheses are required for 2 mg of Phos-tag™ AAL-107

# Phos-tag™ AAL-107 (MW: 595)	10 g(2 g)
# methanol	0.10 mL(0.02 mL)
# Distilled water	3.2 mL(0.64 mL)

The oily product, Phos-tag™ AAL-107 is provided in a small plastic tube and completely dissolved in 0.1 mL methanol.

Phos-tag™ Aqueous Soln. is also prepared. However, it takes more time for compete dissolution than that the methanol soln. is prepared.

The methanol solution should be diluted with 3.2 mL of distilled water by pipetting.

The oily product, Phos-tag™ AAL-107 is provided in a small plastic tube and completely dissolved in 0.1 mL methanol.

Note: If a trace amount of insoluble material appears as white fine powder (impurity) in the solution, it can be separated by centrifuging (2000 x g, 10 min) using 2-mL microtubes.

【Storage】 Wrap the tube with aluminum foil. Keep the soln. in a 2-mL microtube at 4℃ in the dark.

Just after adding water into Phos-tag™ MeOH soln.

$MnCl_2$; Soln.

Sol. F：10 mmol/L $MnCl_2$* Solution

*: Be careful not to confuse manganese with magnesium (Mg)

# $MnCl_2(H_2O)_4$ (MW: 198)	0.10 g
# Distilled water	50 mL

Note: Do not use other anion salts such as $Mn(NO_3)_2$ or $Mn(CH_3COO)_2$. White precipitates ($Mn(OH)_2$) will be formed in basic aqueous solutions and gradually oxidize and turn brown (MnO(OH)), and the gel will be pigmented. Also, the functions of MN_2+ will deteriorate.

APS Solution

Sol. G：10% (w/v) Ammonium Persulfate Solution

# $(NH_4)_2S_2O_8$ (MW: 228)	10
# Distilled water	0.10 mL

⇒ Prepared sol. G can be stored for long period at -20℃ by aliquot in an appropriate amount.

Running Buffer

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

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

⇒ Prepare the 500 mL soln. by adding distilled water.　Avoid to adjust the pH by adding acid or base.
【Storage】 Keep at 4℃.

Just before use, add 450 mL of distilled water to 50 mL of Soln. H.

Sample Buffer

Sol. I：Sample Buffer（3x solution）

Bromophenol Blue (BPB) 1.5mg
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

⇒ Prepare the 10 mL soln. by adding distilled water.
【Storage】 Keep at -20°C.
【Usage of Soln. I】 Please see "④.Sample Preparation".

Fixation Solution

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

acetic acid 0.10 L
methanol 0.40 L
Distilled water 0.50 L

CBB Staining Soln.
Applicable to silver staining and fluorescent staining.

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

⇒ Dissolve CBB in methanol and then add acetic acid and water.

Washing and Destaining Soln.

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

methanol 0.25 L
acetic acid 0.10 L
Distilled water 0.65 L

② Resolving Gel Preparation
Note：Always prepare the gel just before use.

Resolving Gel Solution（0.375 mol/L Tris, 0.1 mmol/L MnCl2 , 0.1% SDS)

(In case of preparation of the 10 mL solution with 12 w/v% polyacrylamide gel and 50 μmol/L Phos-tag™ Acrylamide)

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™ Solution 0.10 mL
Sol. F: 10 mmol/L $MnCl_2$ Solution 0.10 mL ＊1)
Sol. D: 10% (w/v) SDS Solution 0.10 mL
TEMED (tetramethylethylenediamine) 10 μL ＊2)

Distilled Water 3.14 mL
～Dearate with stirring for 2 minutes.～

＊1) For the MnCl2 solution, two times the Phos-tag™ concentration(molar ratio) is added.
＊2) The normally used concentration can be used for TEMED and Sol.G. The above added amounts are examples.

Sol. G: 10% (w/v) Ammonium Persulfate Solution 50 µL ＊2)
＊1) For the MnCl2 solution, two times the Phos-tag™ concentration(molar ratio) is added.
＊2) The normally used concentration can be used for TEMED and Sol.G. The above added amounts are examples.
＊3) Add the agarose after distilled water has been added and thoroughly dissolved in a µwave oven and it is still hot.
＊4) If necessary, preheat the pipette tip and gel preparation table to 40 - 45℃.

Note:Please optimize concentration of Sol. E (Phos-tag™) and Sol. A(Acrylamide). See Optimisation fo PhosTag PAGE Condition.

【Reference】Examples of preparation of 10 mL of resolving gel solution

Phos-tag™ Acrylamide conc.	**20 µM**	**50 µM**	**100 µM**									
Acrylamide conc.	12%	10%	8%	6%	12%	10%	8%	6%	12%	10%	8%	6%
Sol. A(mL)	4	3.33	2.67	2	4	3.33	2.67	2	4	3.33	2.67	2
Sol. B(mL)	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Sol. E(mL)	0.04	0.04	0.04	0.04	0.1	0.1	0.1	0.1	0.2	0.2	0.2	0.2
Sol. F(mL)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Sol. D(mL)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
TEMED(mL)	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Distilled water(mL)	3.2	3.87	4.53	5.2	3.14	3.81	4.47	5.14	3.04	3.71	4.37	5.04
Sol. G	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05

＊1) For the MnCl2 solution, two times the Phos-tag™ concentration(molar ratio) is added.
＊2) The normally used concentration can be used for TEMED and Sol.G. The above added amounts are examples.

② Stacking Gel PreparationM

Stacking Gel Solution （0.125 mol/L Tris, 0.1% SDS）
(In case of preparation of 10 mL (2 mL) of 4.5% polyacrylamide gel.)
※The amount shown in parentheses are required for the 2 mL preparation.

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 (20µL)

TEMED (tetramethylethylenediamine) 10µL (2µL) ＊2)

Distilled Water 5.84 mL (1.17 mL)
～Dearate with stirring for 2 minutes.～

Sol. G: 10% (w/v) Ammonium Persulfate Solution 50µL （10µL) ＊2)

＊2) The normally used concentration can be used for TEMED and Sol.G. The above added amounts are examples.

②’ Preparation of Resolving Gel with low concentration containing agarose

(In case of Separation of 200～350 kDa Phosphorylated Proteins〉
By strengthening gels with 0.5% agarose, low concentration polyacrylamide gel at 3～5% can be prepared.

Resolving Gel Solution（0.375 mol/L Tris, 0.1 mmol/L MnCl2, 0.1% SDS)

(In case of preparation of 10 mL of 20 µmol/L Phos-tag™ Acrylamide containing 3.0% Polyacrylamide gel and 0.5% Agarose)

Sol. A: 30% (w/v) Acrylamide Solution 1.00 mL

Sol. B: 1.5 mol/L Tris/HCl Solution, pH 8.8 2.50 mL

# Sol. E: 5.0 mmol/L Phos-tag™ Solution	0.04 mL
# Sol. F: 10 mmol/L $MnCl_2$ Solution	0.04 mL ＊1)
# Sol. D: 10% (w/v) SDS Solution	0.10 mL
# TEMED (tetramethylethylenediamine)	10 µL ＊2)
# Distilled water	2.93 mL
# 1.5% (w/v) agarose ＊3) ＊4)	3.33 mL
# Sol. G: 10% (w/v) Ammonium Persulfate Solution	50 µL ＊2)

⇨ Pour the agarose directly onto the gel preparation table before it hardens.

＊1) For the MnCl2 solution, two times the Phos-tag™ concentration(molar ratio) is added.
＊2) The normally used concentration can be used for TEMED and Sol.G. The above added amounts are examples.
＊3) Add the agarose after distilled water has been added and thoroughly dissolved in a µwave oven and it is still hot.
＊4) If necessary, preheat the pipette tip and gel preparation table to 40 - 45℃.

③' Preparation of Stacking Gel with low concenration containing agarose

Stacking Gel Solution（0.125 mol/L Tris, 0.1％ SDS）

(In case of preparation of 10 mL (or 2 mL) of 3.0 (w/v)％ polyacrylamide containing 0.5%(w/v) agarose.)

# Sol. A: 30% (w/v) Acrylamide Solution	1.00 mL (0.20 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 (20µL)
# TEMED (tetramethylethylenediamine)	10µL (2µL) ＊2)
# Distilled Water	3.01 mL (602µL)
# 1.5% (w/v) agarose ＊3) ＊4)	3.33 mL (666µL)
# Sol. G: 10% (w/v) Ammonium Persulfate Solution	50µL (10µL) ＊2)

⇨ Pour the agarose directly onto the gel preparation table before it hardens.

＊2) The normally used concentration can be used for TEMED and Sol.G. The above added amounts are examples.

④ Sample Preparation

- 1) Mix sample with 3 µL of Solution I (Sample Buffer) and add an appropriate amount of distilled water to make 9 µL solution in a microcentrifuge tube.
- 2) Heat at 95℃ for 5 minutes, then, allow the solution to cool to room temperature.
- 3) Load the sample solution (eg: 1.5 µL/well) using a micropipette.

  Note: In case of β-casein, load 5～10 µg /well to obtain clear bands.

⑤ Electrophoresis

- 1) Assemble the electrophoresis equipments and fill the electrode chambers with Solution H (Running Buffer).
- 2) Gently remove the comb from the stacking gel and load the sample into each well using a micropipette.
- 3)Attach the leads to the power supply. Run the gel under a constant current condition (25～30 mA/gel) until the BPB reaches the bottom of the resolving gel.

  Note: In case of two gels, run the gels at 50～60 mA.

※When performing Western blotting after electrophoretic migration has occurred, refer to Section［II］below.

Contact your local distributor
interbiotech@interchim.com
InterBioTech, powered by
interchim
213 Avenue J.F. Kennedy - BP 1140
03103 Montluçon Cedex - France
Tél. 04 70 03 88 55 - Fax 04 70 03 82 60

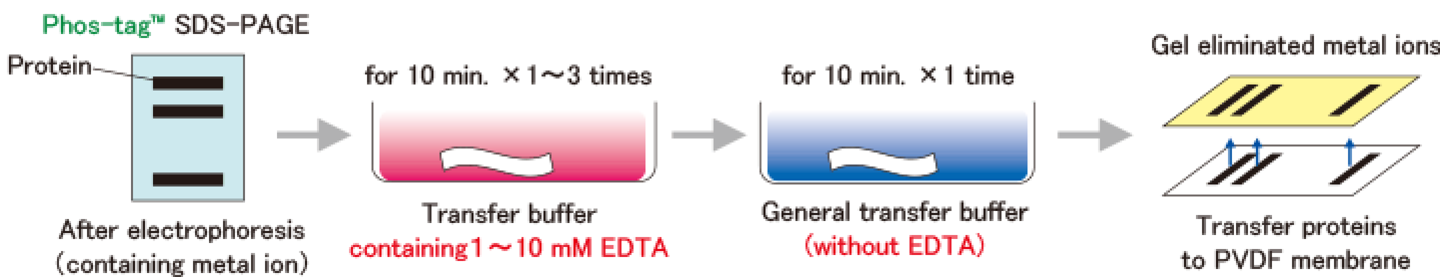
⑥ CBB Staining・Destaining

- 1) Just after electrophoresis, the gel is soaked in 50 mL of the Sol. J (Acidic Solution for Fixation of Proteins) for ca. 10 min. with gentle agitation.
- 2) Stain the gel by soaking in 50 mL of the Sol. K (CBB Staining Solution) for ca. 2 hours with gentle agitation.
- 3) Wash the gel in 50 mL of the Sol. L (Washing and Destaining Solution) 3 times to remove excess stain until the background is sufficiently clear.
- 4) Take a photograph of the gel.

[Ⅱ] Tips for Western blotting of Phos-tag™ SDS-PAGE gels -important-

After electrophoresis, an additional procedure, i.e., elimination of the manganese ion (Mn^{2+}) from the gel using chelating agent (EDTA), is necessary before electroblotting. This procedure increases the transfer efficiency of the phosphorylated and non-phosphorylated proteins onto a PVDF membrane.

- 1） Just after electrophoresis, the gel is soaked in a general transfer buffer containing 1～10 mmol/L EDTA for a minimum of 10 minutes with gentle agitation. （for 10 minutes x 1～3 times）.
  ※ Change the temperature and treatment time with transfer EDTA-buffer according to the gel thickness, etc. （eg: 1.5 mm thick: 20 minute treatment x twice）.
  ※ Besides transfer buffer, 1 x Running buffer can be also used.
- 2） Next, the gel is soaked in a general transfer buffer without EDTA for 10 minutes with gentle agitation （for 10 min. x 1 time）.
  ※ a wet-tank method is strongly recommended for effective protein transfer from the Mn^{2+}-Phos-tag™ acrylamide gel to the PVDF membrane. (The semi-dry method can also be used.)
  ※ The blotting conditions, such as time and temperature, must be optimized for your phosphorylated target protein in the Phos-tag™ gel



[Ⅲ] Tips for Mass Spectrometry of Phos-tag™ SDS-PAGE

No special procedures such as EDTA treatment are necessary.

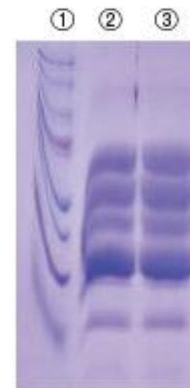
Trouble Shouting

Distortion of bands

The most common complaint for Phos-tag™ SDS-PAGE is "Distortion of bands. Especially, make sure not to contain EDTA in your samples.

- ① Prestained marker: Not only the lane of the marker but also the sample lane may be affected due to a difference in the salt concentration among lanes.

  ⇒ Prestained markers should not be used. (See the right figure.)We recommend to use alkaline phosphatase-treated sample or recombinant protein of your target one as the negative control of phosphorylation instead of using prestained markers.



①Prestained marker
②, ③ β-casein CBB staining

- ② Acidic sample: Bands may be distorted. If the solution is a yellow to orange color even after loading sample buffer, add Tris buffer until it is neutral (violet).

- ③ EDTA (Mn^{2+} is chelated), vanadic acid, inorganic salts, surfactants, etc., cause distortion or tailing of bands.

  ⇒ Desalinate by precipitation with TCA or dialysis.

- ④ Blank lanes: Blank lanes may cause distortion.

  ⇒ Load the same amount of 1 x sample buffer in blank lanes.

- ⑤ Vanadic acid: Competitive binding with phosphoric acid may cause distortion.

  ⇒ Use a different phosphatase inhibitor or remove vanadic acid by precipitation with TCA or dialysis.

- ⑥ Adding $MnCl_2$ to the sample to be applied (eg: 1 mM final concentration) may improve results. If the sample contains an EDTA residue, it is because the added Mn^{2+} is chelated instead of the Mn^{2+} contained in the gel.

Low Resolution

- ① Rise of the molar ratio of $MnCl_2$ to Phos-tag™ Acrylamide may improve the resolution. (eg: 1 : 4)

- ② Adopting Tris-Tricine Buffer as Running Buffer may improve the resolution.

Protein Diffusion

Long-term migration with a constant current will cause decomposition and diffusion of proteins due to excessive heat.

- ① If you want to use a constant current for migration, try techniques such as using a low-temperature room, thoroughly cooling the migration buffer just before use, and wrapping a

cooling agent around the migration tank (but do not use ice because it may cause electric shock).

- ② When a constant voltage can also be used, migrate with a constant voltage (eg: 200 V). The migration speed will slow down but the generation of heat will be suppressed.

Easy breaking of the gel

The gel is softened due to the low concentration of acrylamide.

- ① 5% or higher：Increasing the N,N'-methylene-bisacrylamide to acrylamide ratio (eg: 24：1) will strengthen the gel.

- ② Add 3～5% of agarose to strengthen gels. Refer to "Preparing a Low-Concentration Gel Containing Agarose" in "3. Protocol" and "Separation" in "7. FAQ."

Trouble with Transfer onto the membrane

- ① Treatment with EDTA may be insufficient. Increase the treatment time, frequently exchange the buffer containing EDTA (eg: 20 min. x twice), or fully agitate during EDTA treatment.

- ② To intensify the electric current may improve efficiency (eg: 200 mA)

- ③ Stainings other than negative staining such as CBB staining may diminish the transfer efficiency.

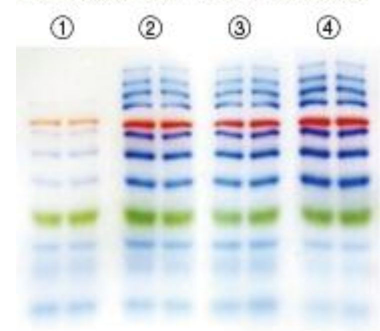
- ④ Using a low-concentration gel will improve the transfer efficiency.

- ⑤ Take steps such as using a thick gel and increase the sample application amount.

- ⑥ Transfer buffer containing SDS may improve the transfer efficiency. When transferring onto a membrane, immediately add the SDS solution to the transfer buffer to prevent sudsing (tank method), or between the EDTA process and transfer, immerse the gel in a transfer buffer containing SDS and shake slowly (eg: for 10 min. x 1 time). Try SDS concentrations of 0.05～0.20%.

Membranes after Transfer Sample: WIDE-VIEW™ Ⅲ Prestain Marker (Code No. 230-02461)＊)

- ① No EDTA processing
- ② 1mM EDTA for 10 minutes x 2 times
- ③ 10mM EDTA for 10 minutes x 1 time
- ④ 1mM EDTA for 10 minutes x 2 times



12.5% Phos-tag™ Super Sep（50mM）

Marked transfer efficiency drop occurs without EDTA processing.

For a reliable transfer, 1 - 10mM EDTA process for 10 minutes x 2 times is recommended.

＊）Molecular weight is not reflected in Phos-tag™ gel. Use the transfer efficiency as a standard.

FT-FL8630

Higher background during staining

Stain after eliminating metal ions in the gel by EDTA treatment.

Mobility shift due to protein degradation, not induced by phosphorylation

Carry out SDS-PAGE as usual (containing 0 µM of Phos-tag™) and verify that no mobility shift occurs.

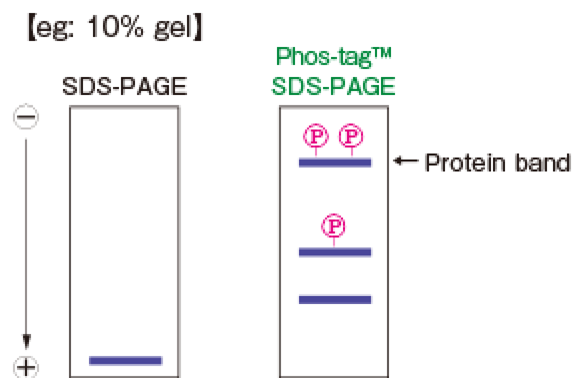
Optimization of Phos-Tag™ PAGE conditions

To obtain a high quality result using Phos-tag™ PAGE, optimization of the concentration of acrylamide and Phos-tag™ Acrylamide is essential. Optimize the concentration of acrylamide (①) first, followed by that of Phos-tag™ Acrylamide (②).

① Optimization of the concentration of acrylamide

First, identify the optimum concentration of acrylamide＊ that allows migration of the target protein to the lowest end of the gel when conventional SDS-PAGE is used. In Phos-tag™ PAGE, the migration speed is slower than in conventional SDS-PAGE (including non-phosphorylated proteins) and, therefore, the concentration of acrylamide should be examined (see the below figure). The migration speed decreases as the concentration of Phos-tag™ increases.

＊Run a gel electrophoresis until the BPB dye, which is contained in the sample buffer, reaches the bottom of the resolving gel. The position of BPB dye can be defined as an Rf value of 1.0. Under the above mentioned running condition, adjust the optimum concentration of acrylamide. When your target protein is observed as a migration band at an Rf value of 0.8 to 0.9 in conventional SDS-PAGE, the acrylamide concentration would be optimum for Phos-tag™ SDS-PAGE.



Indication of optimum concentration

more than 60 kDa: 6%; less than 60 kDa：8%

＜In case of high molecular weight proteins＞

The gel strength can be increased by adding agarose to gels that contain less than 4% of acrylamide. There is a data of separation of 350 kDa. (Refer to "Separation of Phos-tag™ Acrylamide" of FAQ) Furthermore, the gel strength can also be enhanced by increasing the N,N'-methylene-bisacrylamide content (eg: 5% acrylamide [24:1]).

② Optimization of the concentration of Phos-tag™ Acrylamide

Then, optimize the concentration of Phos-tag™ Acrylamide. Please evaluate the optimum concentration in the order of lowest to highest.

eg: 20µM→50µM→100µM

【Cell Lysate】

In case there is a large variety of proteins in your sample, eg: cell lysates, the concentration of Phos-tag™ should be 5 to 25 µM. However, a higher concentration, eg: 100 µM, is recommended in case of a lower concentration of the target protein, eg: non-overexpression systems.

※The optimum condition depends on the protein. Please find the appropriate condition setting for each target protein.

The information was provided by Yasunori Sugiyama at Science Research Cencer, Kochi University)

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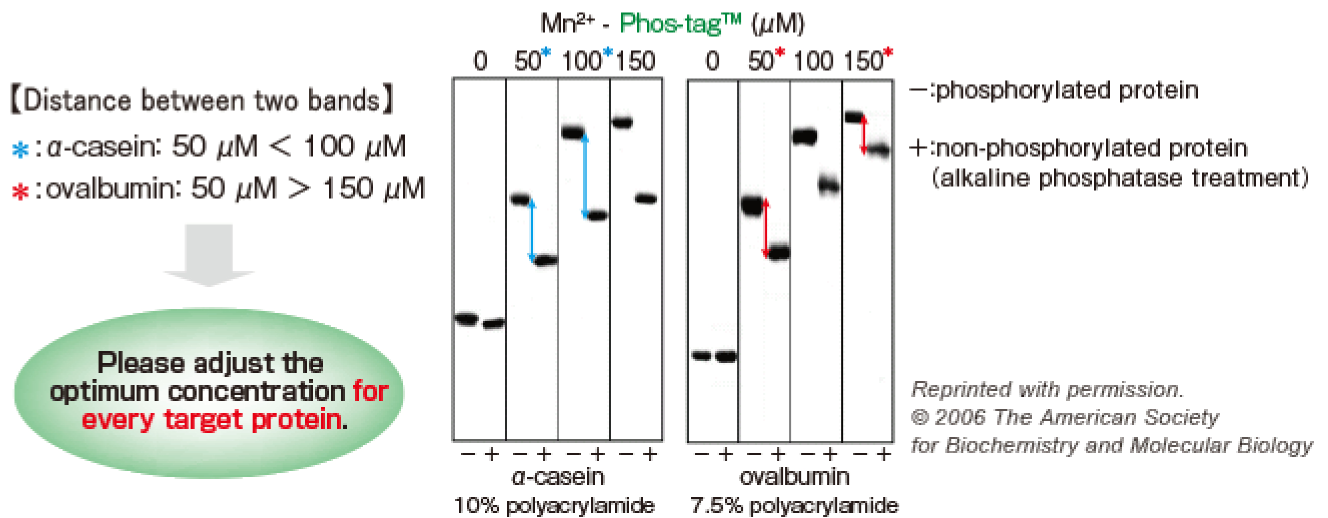
Higher concentration of Phos-tag™ leads to higher separation capacity?

In general, a higher concentration leads to higher separation capacity. (Compare the samples of 50 µM and 100 µM of Mn^{2+}-Phos-tag™ in the left figure.) However, the higher concentration causes low velocity. It sometimes happens that the higher separation capacity is due to the lower Phos-tag™ concentration (Compare the samples of 50 µM and 150 µM of ovalbumin of the right figure.)



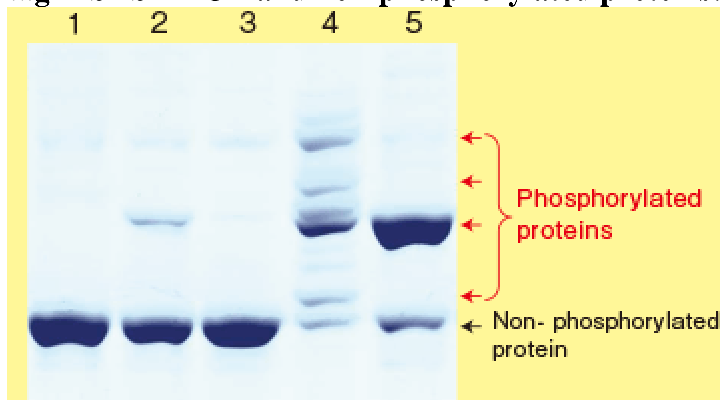
Application Data and References

Application Data 1
Comparison of proteins phosphorylated using Phos-tag™ SDS-PAGE and non-phosphorylated proteins

Impressions of users of Phos-tag™ Acrylamide are introduced herein. We received data and comments from Dr. Tadayuki Ogawa of the University of Tokyo. In addition, we received data applied to two-dimensional electrophoretic migration from Dr. Yayoi Kimura of Yokohama City University, as well as Western blotting applied data from Dr. Yasunori Sugiyama of Kochi University and Dr.Tomohisa Hosokawa of RIKEN.

“I recommend Phos-tag™.” Tadayuki Ogawa, Graduate School of Medicine, the University of Tokyo Phos-tag™ is a very convenient reagent that can be applied in a variety of samples and research purposes. It allows quantitative analysis not only of in vitro assay samples but also in vivo samples in a phosphorylated state. Phos-tag™ SDS-PAGE utilizes normal electrophoretic migration and does not require the purchase of special equipment, so you could say it has good cost performance. Phosphorylation research that used to require anti-phosphorylated antibodies, RI, and many other reagents will now be advanced with Phos-tag™.

Comparison of proteins phosphorylated using Phos-tag™ SDS-PAGE and non-phosphorylated proteins:



The kinase-reacted phosphorylated proteins in (2) - (5) show clear differences compared with the non- phosphorylated protein in (1). Data such as the quantitative ratio of phosphorylated and non-phosphorylated proteins, degree of phosphorylation, and population distribution can be readily obtained from band shifts and density. (Source: Graduate School of Medicine, the University of Tokyo)

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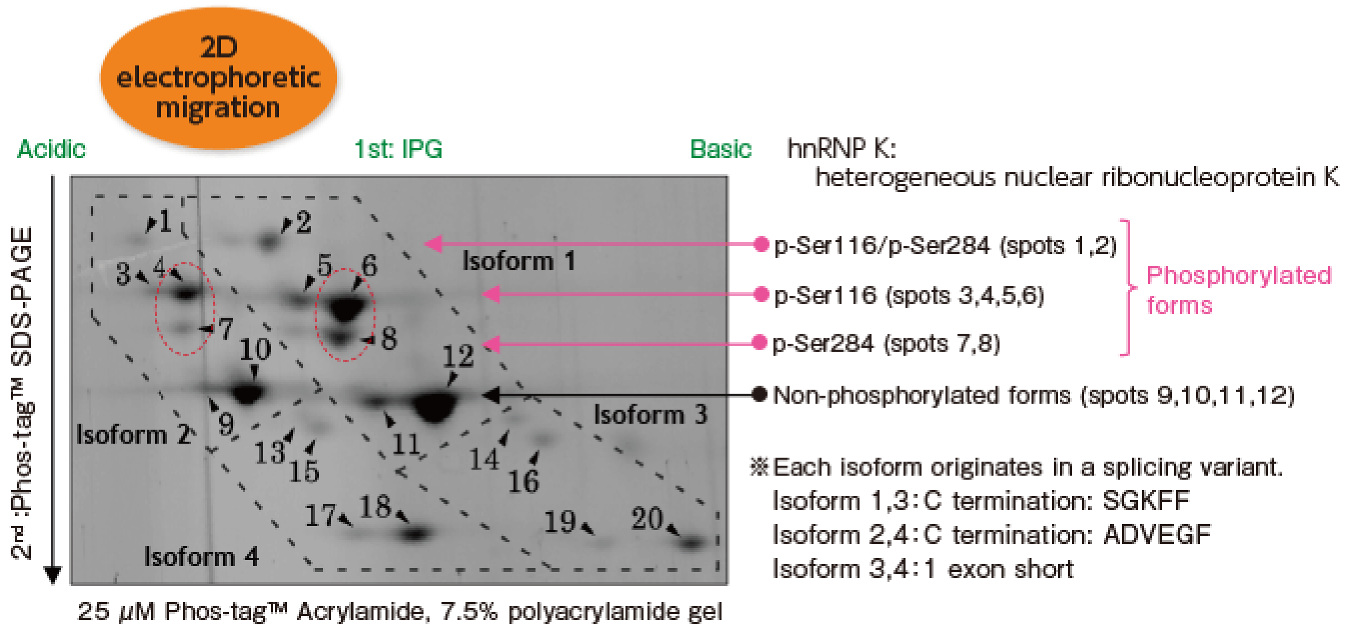
Application Data 2

Application in two-dimensional electrophoretic migration: Analysis of phosphorylated forms of hnRNPK

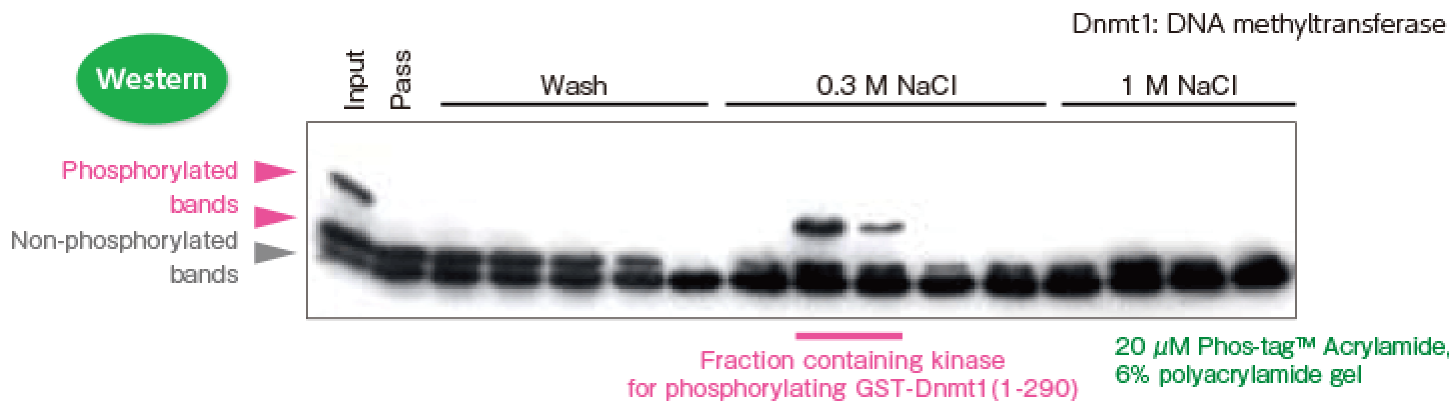
hnRNP K was isolated by immunoprecipitation from nuclear homogenate of mouse macrophage cell line J774.1 cells stimulated with LPS, and hnRNP K isoforms were separated using IPG strip gel (pH 4.7－5.9) in the first dimension and Phos-tag™ SDS-PAGE in the second dimension. Each isoform and modification site was then identified using mass spectrometry.



Each phosphorylated form was distinguished at the same isoelectric point, respectively. (eg: spots 6 vs. 8 and spots 4 vs. 7)

- **Data published in :**
  Characterization of multiple alternative forms of heterogeneous nuclear ribonucleoprotein K by phosphate-affinity electrophoresis. Y. Kimura, K. Nagata, N Suzuki, R. Yokoyama, Y. Yamanaka, H. Kitamura, H. Hirano, and O. Ohara, Proteomics, Nov 2010; **10**(21): 3884-95.
- **Data provided by :**
  Dr. Y. Kimura and Dr. H. Hirano, Yokohama City University and O. Ohara, RCAI, RIKEN.

Application Data 3
Determining fraction containing kinase for phosphorylating Dnmt1



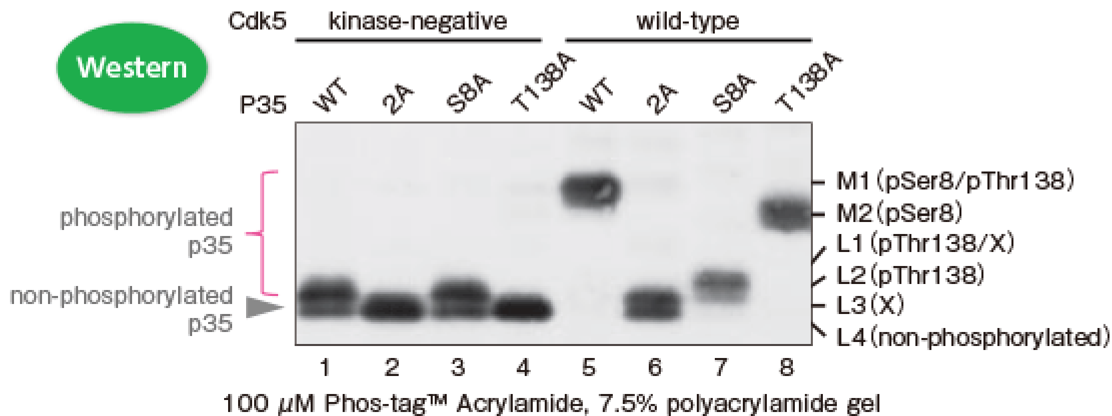
- ① GST-Dnmt1(1-290) bonding protein was obtained from mouse brain extract using affinity chromatography.
- ② Proteins were eluted through the DNA cellulose column by 0.3 M and 1 M NaCl.
- ③ In vitro kinase assay was performed in each fraction with GST-Dnmt1(1-290) as substrate.
- ④ Kinase activity in the fraction was confirmed by shift band, by Western blotting using Phos-tag™ SDS-PAGE (Detection：Anti mouse Dmnt1（72-86））

“We were able to determine the fraction that contained the target kinase.”

- **Data published in：**
  The DNA-binding activity of mouse DNA methyltransferase 1 is regulated by phosphorylation with casein kinase 1delta/epsilon. Y. Sugiyama, N. Hatano, N. Sueyoshi, I. Suetake, S. Tajima, E. Kinoshita, E. Kinoshita-Kikuta, T. Koike, and I. Kameshita, Biochem. J., May 2010; **427**(3): 489-97.
- **Data provided by：**
  Dr. Y. Sugiyama, Laboratory of Molecular Biology, Science Research Center, Kochi University and Dr. I. Kameshita, Department of Life Science, Faculty of Agriculture, Kagawa University.

Application Data 4
Search for phosphorylation site of Cdk5-activated sub-unit p35 using Ala substitution variant

Regarding p35 known phosphorylation sites Ser8 and Thr138, 3 Ala substitution variants were produced (Ser8: S8A, Thr138: T138A, Ser8 and Thr138 ：2A). These and wild-type p35, as well as Cdk5(cyclin-dependent kinase 5) or kinase-negative Cdk5, which has no kinase activity, were discovered in the COS-7 cells. The cellular extract was detected by Western blotting using Phos-tag™ SDS-PAGE. (Detected extract: anti-p35 antibody)



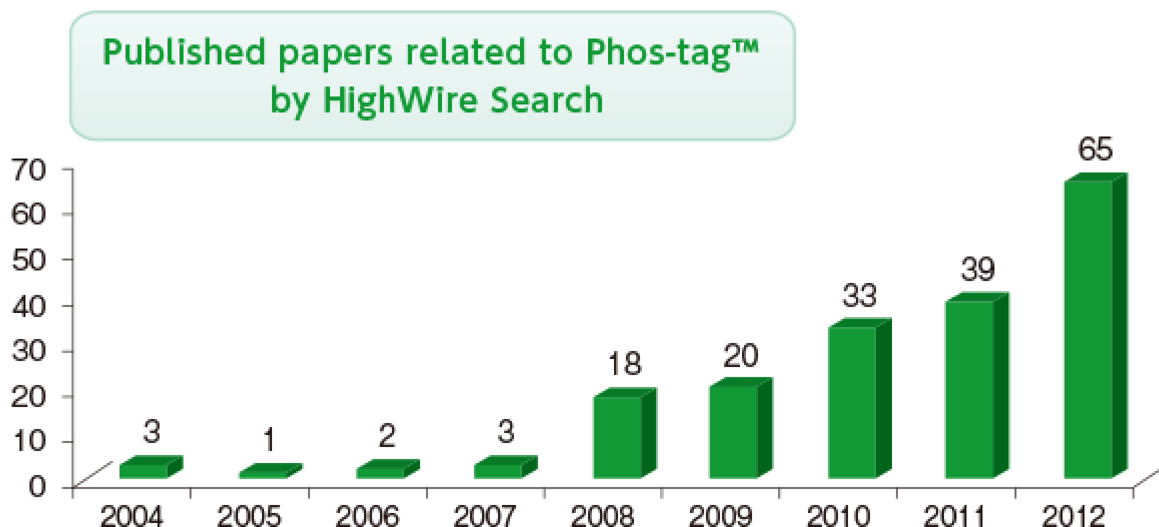
From lanes 1 (L2, L4) and 5 (M1): p35 is phosphorylated, depending on Cdk5.
From lanes 1 (L2, L4) and 3 (L2, L4) ：With about half of p35, Thr138 is phosphorylated at kinase-negative Cdk5, and Thr138 is also phosphorylated by kinase other than Cdk5.
From lanes 5 (M1) and 6 (L3, L4) ：Ser8 and Thr138 are main phosphorylation sites.
From lanes 5 (M1), 7 (L1, L2) and 8 (M2) ：M1 is the phosphorylation site for Ser8 and Thr138. M2 is the phosphorylation site for Ser8 only. L1 and L2 are the phosphorylation sites for Thr138 only.
※X in L1, L3 ： not yet identified
※L4: non-phosphorylated p35

Relationship between phosphorylation site and band shift was clarified!

- **Data published in** ：
  Quantitative Measurement of in Vivo Phosphorylation States of Cdk5 Activator p35 by Phos-tag™ SDS-PAGE. T. Hosokawa, T. Saito, A. Asada, K. Fukunaga, and S. Hisanaga, Mol. Cell. Proteomics, Jun 2010; **9:** 1133 - 1143.

- **Data provided by** ：
  Dr. T. Hosokawa, Laboratory for Memory Mechanisms Neural Circuit Function Research Core, Brain Science Institute, RIKEN and Dr. S. Hisanaga, Molecular Neuroscience Laboratory, Department of Biological Sciences, Graduate School of Science and Engineering, Tokyo Metropolitan University.

References



Regarding Phos-tag™ reagents：

- 1. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of phosphorylated compounds using a novel phos phate capture molecule, Rapid Communications of Mass Spectrometry, **17**, 2075-2081 (2003),H. Takeda, A. Kawasaki, M. Takahashi, A. Yamada, and T. Koike
- 2. Phosphate-binding tag: A new tool to visualize phosphorylated proteins, Molecular & Cellular Proteomics, **5**, 749-757 (2006),E. Kinoshita, E. Kinoshita-Kikuta, K. Takiyama, and T. Koike
- 3. Separation and detection of large phosphoproteins using Phos-tag™ SDS-PAGE, Nature Protocols, **4**, 1513-1521 (2009), E. Kinoshita, E. Kinoshita-Kikuta, and T. Koike

Application using Phos-tag™ reagents

- 1. Spatial regulation of Fus3 MAP kinase activity through a reaction-diffusion mechanism in yeast pheromone signalling, Nat. Cell Biol., **9** ,1319-1326 (2007), C. I. Maeder et.al. M. A. Hink, A. Kinkhabwala, R. Mayr, P. I. H. Bastiaens and M. Knop
- 2. Regulation of PKD by the MAPK p38d in Insulin Secretion and Glucose Homeostasis, Cell, **136**, 235-248 (2009), G. Sumara, I. Formentini, S. Collins, I. Sumara, R. Windak, B. Bodenmiller, R. Ramracheya, D. Caille, H. Jiang, K. A. Platt, P. Meda, R. Aebersold, P. Rorsman, and R. Ricci1
- 3. Dbf4-Dependent Cdc7 Kinase Links DNA Replication to the Segregation of Homologous Chromosomes in Meiosis I, Cell, **135**, 662-678 (2008) ,J. Matos, J. J. Lipp, A. Bogdanova, S. Guillot, E. Okaz, M. Junqueira, A. Shevchenko, and W. Zachariae
- 4. Kinome Profiling in Pediatric Brain Tumors as a New Approach for Target Discovery, Cancer Res., **69**, 5987-5995 (2009) , A. H. Sikkema, S. H. Diks, W. F.A. den Dunnen, A. ter Elst, F. J.G. Scherpen, E. W. Hoving, R. Ruijtenbeek, P. J. Boender, R. de Wijn, W. A. Kamps, M. P. Peppelenbosch, and E. S.J.M. de Bont
- 5. Regulation of mitochondrial transport and inter-microtubule spacing by tau phosphorylation at the sites hyperphosphorylated in Alzheimer's disease, J. Neurosci.,**32**, 2430-2441 (2012), K.Shahpasand, I. Uemura, T.Saito, T.Asano, K.Hata, K.Shibata, Y.Toyoshima, M.Hasegawa, S.Hisanaga
- 6. The Hsp90 Kinase Co-chaperone Cdc37 Regulates Tau Stability and Phoshorylation Dynamics, J. Biol. Chem., **286**, 16976-16983 (2011) ., Umesh K. Jinwal, Justin H. Trotter, Jose F. Abisamobra, John Koren, III, Lisa Y. Lawson, Grant D. Vestal, John C. O'Leary, III, Amelia G. Johnson, Ying Jin, Jeffrey R. Jones, Qingyou Li, Edwin J. Weeber, and Chad A. Dickey

- Tau proteins are used as the samples in Application 5 and 6.

FAQ

Frequently Asked Questions for Phos-tag™ Acrylamide | Biotin | MS analysis | Agarose

FAQ - Phos-tag™ Acrylamide

Determination

Q. Can phosphorylated proteins be assayed ?

A. They can be assayed on the basis of the band intensity by using a quantitative staining such as cBB staining. A product such as “Quick-CBB PLUS” is recommended.
⇒ Quick-CBB PLUS (1 L: Cat. #178-00551; 250 mL: 174-00553)

Separation

Q. How large (kDa) can a protein be separated using this product ?

A. phosphorylated protein of 350 kDa has actually been separated with 20 μM Phos-tag™, 3% acrylamide and 0.5% agarose＊.
Reference: Proteomics, 9, 4098-4101 (2009), E. Kinoshita, E. Kinoshita-Kikuta, H. Uchijima, and K. Koike
＊ Agarose was added to strengthen the gel.

Q. How can the resolution be improved ?

A. In general, a higher concentration of Phos-tag™ results in a higher resolution. However, increasing the concentration of Phos-tag™ also causes the overall migration speed of the protein to proportionally drop.

Staining

Q. Is it possible to use gel-staining techniques other than CBB ?

A. Yes, the gel can also be stained by negative staining, silver staining, and fluorescent staining.

Use of Phos-tag™ Acrylamide

Q. How many gels can be made with each product ?

A. It depends on the concentration of Phos-tag™ used. For example, about 100 plates at 20 μM, about 40 plates at 50 μM, and about 20 plates at 100 μM can be prepared from a 10 mg-package, when gels of 1 mm-thickness,9 cm-width, and 7.7 cm-length are made.

Phos-tag™	20 μM	50 μM	100 μM
0.3 mL Aqueous Soln. (0.9 mg)	abt. 9	abt. 4	abt. 2
2 mg package	abt. 20	abt. 8	abt. 4
10 mg package	abt. 100	abt. 40	abt. 20
SuperSep Phos-tag™	-	5 gels	-

Gel Strength

Q. The gel is easily broken. What can I do for this ?

A. A low concentration of acrylamide causes the gel to be soft.
You can solve this problem by increasing the relative amount of methylenebisacrylamide to acrylamide (24:1), for example.

Stability of the prepared gel containing Phos-tag™

Q. How long can the prepared gel containing Phos-tag™ Acrylamide be stored ?

A. The gel deteriorates within a few days. Therefore, it should be prepared just before use.

Stability of the Phos-tag™ solution

Q. How long can the solutions in methanol and water be stored ?

A. No remarkable decline in performance has been reported for 6 months by refrigeration under protection from light. The solutions seem to be storable for 1 year without any problem according to doctors who are using the product.

Preparation of the reagent

Q. Does the concentration of Phos-tag™ influence the amount of ions required to be

A. The molar ratio of Phos-tag™ acrylamide to Mn^{2+} should be 1:2; two Mn^{2+} ions bind to one Phos-tag™ molecule (Fig. 1).

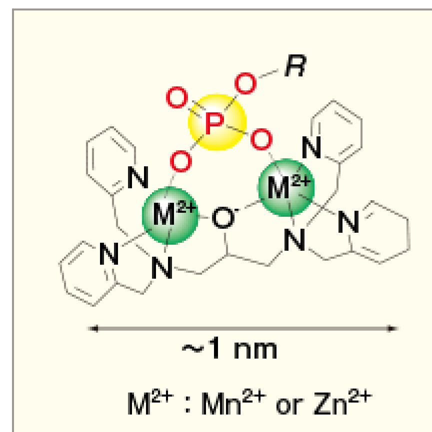
Q. I have experienced clouding of Phos-tag™ when I prepared a solution as described in the protocol. Is this normal ?

A. Yes, it is. Clouding is attributed to methanol. The solution becomes clear after standing for a while.

Q. Does Phos-tag™ dissolve in water alone ?

A. It is soluble in water, though it takes more time compared to dissolution in water containing methanol. If it does not dissolve completely, centrifuge the solution and use the supernatant.

Fig. 1 Phos-tag™ Basic Structure

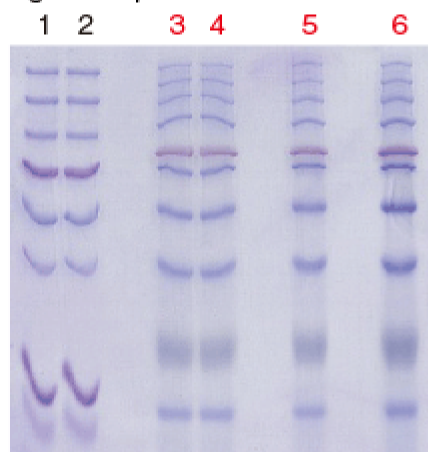


Molecular marker

Q. What prestained markers can we use ?

A. Using a prestained marker with the Phos-tag™ gel usually causes distortion of bands (Fig. 2). WIDE-VIEW™ Prestained Protein Size Marker Ⅲ (Cat No. 230-02461) is less likely to cause band distortion, but does not reflect the molecular weight. Please use the result obtained using this marker as an index of the transfer efficiency. At least one blank lane is needed between the solution containing this marker and other solutions.

Fig. 2 Comparison of Prestained Markers



1, 2 : prestained marker (Company A) (3 µL)
3, 4, 5 : WIDE-VIEW™ Prestained Protein Size Marker III (3 µL)
6 : WIDE-VIEW™ Prestained Protein Size Marker III (5 µL)
Blank lanes : 1 x sample buffer (5 µL)
SuperSep Phos-tag™ (50 µM), 12.5% (20 mA constant current)

Phosphorylation reaction with coexisting ATP

Q. Does ATP in a phosphorylation reaction solution affect electrophoresis ?

A. ATP had no particular effect at a concentration of 2.0 mM. The limit of use has not been investigated yet.

Precast gel

Q. Can we use Phos-tag™ Acrylamide in a precast gel by adding it to sample solution ?

A. No, you cannot. We have various kinds of precast gels called "SuperSep Phos-tag™

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Differentiation between degraded protein and phosphorylated Phos-tag™

Q. A mobility shift was observed in Phos-tag™ SDS-PAGE. How can I know whether it is a sign of phosphorylation or only telling me that the protein was broken down ?

A. Please carry out a conventional SDS-PAGE (without Phos-tag™) and verify that your protein is intact.

DNA Separation using Phos-tag™

Q. Is Phos-tag™ applicable to separate DNA ?

A. Refer to the following articles :

・A SNP genotyping method using phosphate-affinity polyacrylamide gel electrophoresis, Analytical Biochemistry, 361, 294-298 (2007), E. Kinoshita, E. Kinoshita-Kikuta, and T. Koike (The phosphate group at DNA-terminal is efficiently captured by Zn^{2+}-Phos-tag™.)

・A mobility shift detection method for DNA methylation analysis using phosphate affinity polyacrylamide gel electrophoresis, Analytical Biochemistry, 378, 102-104 (2008), E. Kinoshita-Kikuta, E. Kinoshita, and T. Koike

FAQ - SuperSep Phos-tag™ (Please see information)

Q. Do you have lower-concentration polyacrylamide products ?

A. Products with concentrations of 6%, 7.5% and 10% are currently being developed.

Q. Do you have products with other Phos-tag™ polyacrylamide concentrations ?

A. Products with concentrations of 20 µM and 100 µM are currently being developed.

Q. Are there ways to improve separation capacity ?

A. Using a Tris-Tricine buffer as the running buffer improves separation capacity.

Q. Are there any references ?

A. Kinoshita-Kikuta, E., Kinoshita, E., Koike T., "A Laborsaving, Timesaving, and More Reliable Strategy for Separation of Low-Molecular-Mass Phosphoproteins in Phos-tag Affinity Electrophoresis", Int. J. Chem. 4, 1-8 (2012) DOI: 10.5539/ijc.v4n5p1.

FAQ - Phos-tag™ Biotin (Please see information)

Q. What is the difference of BTL-104, BTL-105 and BTL-111 ?

A. BTL-104, BTL-105, and BTL-111 have linkers with different lengths. Although the usage of BTL-104 and BTL-105 are similar, BTL-104 is recommendable as the first choice because of its high solubility. BTL-111 offers high sensitivity.

Q. What is the sensitivity level like ?

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

Q. Do we need other reagents besides this product ?

A. Prepare a Streptavidin-conjugated HRP solution.

Q. How many times can Phos-tag™ Biotin be used ?

A. It depends on the frequency of use. Please refer to the following as a guide.
BTL-104 : 130～1300 times
BTL-105 : 113～1130 times
BTL-111 1 mM Aqueous Solution : 10～100 times

Q. Can phosphorylated proteins be assayed ?

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

Q. Is it possible to determine the number of binding phosphate groups ?

A. No, it isn't.

Q. Can I strip the antibodies of Phos-tag™ 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.

Q. What kind of membrane is recommended ?

A. We recommend PVDF membranes.

Q. Does the use of Phos-tag™ Biotin require blocking ?

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

FAQ - Phos-tag™ Mass Analytical Kit （Please see information）

Q. How many tests can Phos-tag™ Biotin be used ?

A. More than 1,000 tests when 5 µL is used per test.

Q. How can I know which one of Phos-tag™ MS-101L, Phos-tag™ MS-101H, and Phos-tag™ MS-101N is appropriate ?

A. Phos-tag™ 101N contains naturally occurring zinc species, 101L contains ^{64}Zn, and 101H contains ^{68}Zn.
Please refer to the following guidance. Exploration of conditions: Use 101N.
Many isotopes contained in it make the spectrum complicated. Verification of the presence of phosphate groups: Use 101L and 101H.
These reagents contain zinc with a mass number of 64 and 68, respectively.
Measurement of a single sample with these reagents therefore results in a difference in m/e of 16.

Q. I would like to measure a sample isolated by Phos-tag™ SDS-PAGE. Is it necessary to remove Phos-tag™ before in-gel digestion ?

A. No, it isn't. Please follow the usual procedure for in-gel digestion after SDS-PAGE.

Q. Can it be also used for ESI mass spectrometry ?

A. Yes, it can. Please refer to the following publication, which reports an example of ESI-MS analysis in which Phos-tag™ MS-101N was used as probe. A neutral solution should be used because analysis in an acidic solution causes Phos-tag™ to be detached.
Reference: Anal. Chem. (2008), 80, 2531-2538 (MS-101N ESI-MS)

FAQ - Phos-tag™ Agarose （Please see information）

Q. Can samples purified using Phos-tag™ Agarose be directly applied to SDS-PAGE ?

A. No, they can't. The elution buffer recommended in the protocol contains a high concentration of salt and may cause the bands to be distorted. Please use the SDS-PAGE sample buffer as elution buffer.

Q. Is Phos-tag™ Agarose reusable ?

A. We do not recommend it.

Q. Does Phos-tag™ Agarose have any advantages over IMAC ?

A. Phos-tag™ Agarose allows experimental processes under all physiological conditions (pH 7.5), and since it does not use reductants or surfactants, it can refine phosphorylated proteins in their native shape.

Also, the purified proteins can be used in processes such as mass spectrometry and Western blotting.

Q. What reagents are suitable and unsuitable for use in the sample preparation ?

A. Please refer to the table below.

Category	Reagent	Suitability	Allowable concentrations
Reducing agents	DTT	○	$\leq$ 0.1 M
Denaturing agents	Urea	○	Using it at 8M has no negative effect.
Surfactants	SDS	○	Using it at $\geq$ 0.5% affects the binding process.
(anionic)	Sodium deoxycholate	○	Using it at $\geq$ 0.25% affects the binding process.
Surfactants	Nonidet P40	○	$\leq$ 1 %
(nonionic)	Tween 20	○	$\leq$ 1 %
Surfactants (amphoteric)	CHAPS	○	$\leq$ 0.2 %
Phosphate derivatives	β-Glycerophosphate	×	Do not use it.
	Pyrophosphate	×	Do not use it.
Chelating agents	EDTA	Δ	Using it at a high concentration has a negative effect.

Application data and references

Application Data 1
Comparison of proteins phosphorylated using Phos-tag™ SDS-PAGE and non-phosphorylated proteins

Impressions of users of Phos-tag™ Acrylamide are introduced herein. We received data and comments from Dr. Tadayuki Ogawa of the University of Tokyo. In addition, we received data applied to two-dimensional electrophoretic migration from Dr. Yayoi Kimura of Yokohama City University, as well as Western blotting applied data from Dr. Yasunori Sugiyama of Kochi University and Dr.Tomohisa Hosokawa of RIKEN.

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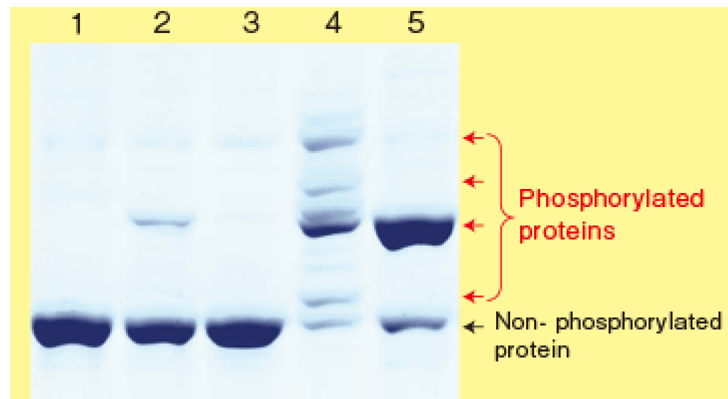
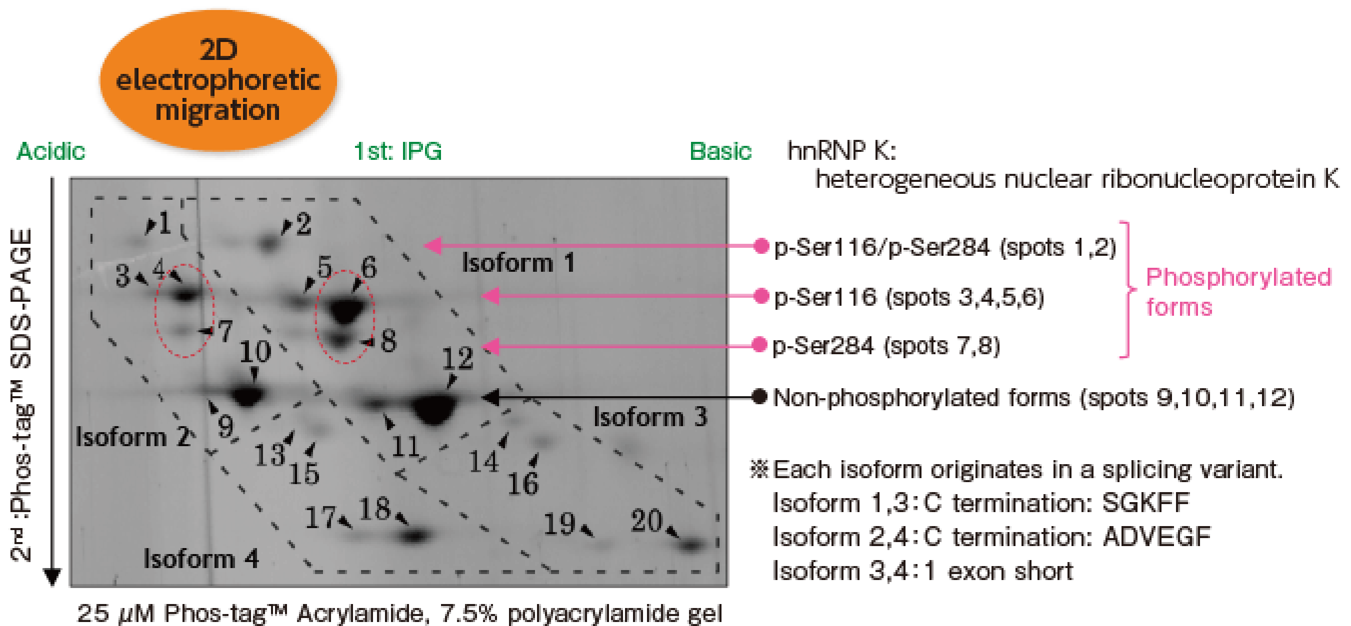


Comparison of proteins phosphorylated using Phos-tag™ SDS-PAGE and non-phosphorylated proteins
The kinase-reacted phosphorylated proteins in (2) - (5) show clear differences compared with the non- phosphorylated protein in (1).
Data such as the quantitative ratio of phosphorylated and non-phosphorylated proteins, degree of phosphorylation, and population distribution can be readily obtained from band shifts and density.
(Source: Graduate School of Medicine, the University of Tokyo)

Application Data 2
Application in two-dimensional electrophoretic migration: Analysis of phosphorylated forms of hnRNPK

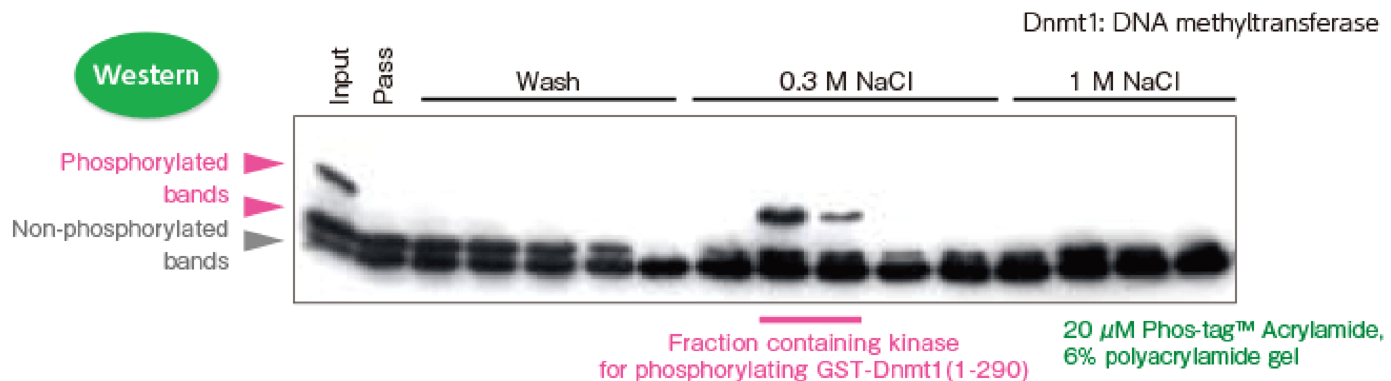
hnRNP K was isolated by immunoprecipitation from nuclear homogenate of mouse macrophage cell line J774.1 cells stimulated with LPS, and hnRNP K isoforms were separated using IPG strip gel (pH 4.7－5.9) in the first dimension and Phos-tag™ SDS-PAGE in the second dimension. Each isoform and modification site was then identified using mass spectrometry.



Each phosphorylated form was distinguished at the same isoelectric point, respectively. (eg: spots 6 vs. 8 and spots 4 vs. 7)

- **Data published in：**
  Characterization of multiple alternative forms of heterogeneous nuclear ribonucleoprotein K by phosphate-affinity electrophoresis. Y. Kimura, K. Nagata, N Suzuki, R. Yokoyama, Y. Yamanaka, H. Kitamura, H. Hirano, and O. Ohara, Proteomics, Nov 2010; **10**(21): 3884-95.
- **Data provided by：**
  Dr. Y. Kimura and Dr. H. Hirano, Yokohama City University and O. Ohara, RCAI, RIKEN.

Application Data 3
Determining fraction containing kinase for phosphorylating Dnmt1



- ① GST-Dnmt1(1-290) bonding protein was obtained from mouse brain extract using affinity chromatography.
- ② Proteins were eluted through the DNA cellulose column by 0.3 M and 1 M NaCl.
- ③ In vitro kinase assay was performed in each fraction with GST-Dnmt1(1-290) as substrate.
- ④ Kinase activity in the fraction was confirmed by shift band, by Western blotting using Phos-tag™ SDS-PAGE
  (Detection：Anti mouse Dmnt1（72-86））

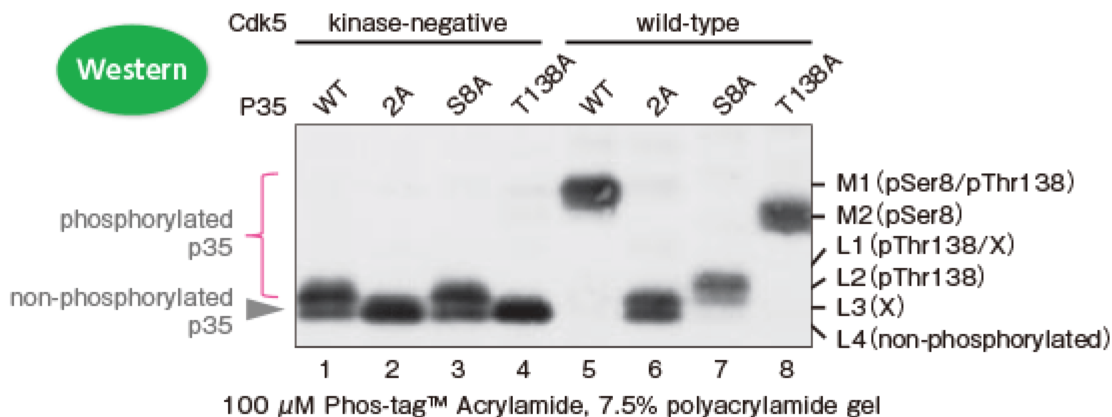
"We were able to determine the fraction that contained the target kinase."

- **Data published in：**
  The DNA-binding activity of mouse DNA methyltransferase 1 is regulated by phosphorylation with casein kinase 1delta/epsilon. Y. Sugiyama, N. Hatano, N. Sueyoshi, I. Suetake, S. Tajima, E. Kinoshita, E. Kinoshita-Kikuta, T. Koike, and I. Kameshita, Biochem. J., May 2010; **427**(3): 489-97.

- **Data provided by：**
  Dr. Y. Sugiyama, Laboratory of Molecular Biology, Science Research Center, Kochi University and Dr. I. Kameshita, Department of Life Science, Faculty of Agriculture, Kagawa University.

Application Data 4
Search for phosphorylation site of Cdk5-activated sub-unit p35 using Ala substitution variant

Regarding p35 known phosphorylation sites Ser8 and Thr138, 3 Ala substitution variants were produced (Ser8: S8A, Thr138: T138A, Ser8 and Thr138 ：2A). These and wild-type p35, as well as Cdk5(cyclin-dependent kinase 5) or kinase-negative Cdk5, which has no kinase activity, were discovered in the COS-7 cells. The cellular extract was detected by Western blotting using Phos-tag™ SDS-PAGE. (Detected extract: anti-p35 antibody)



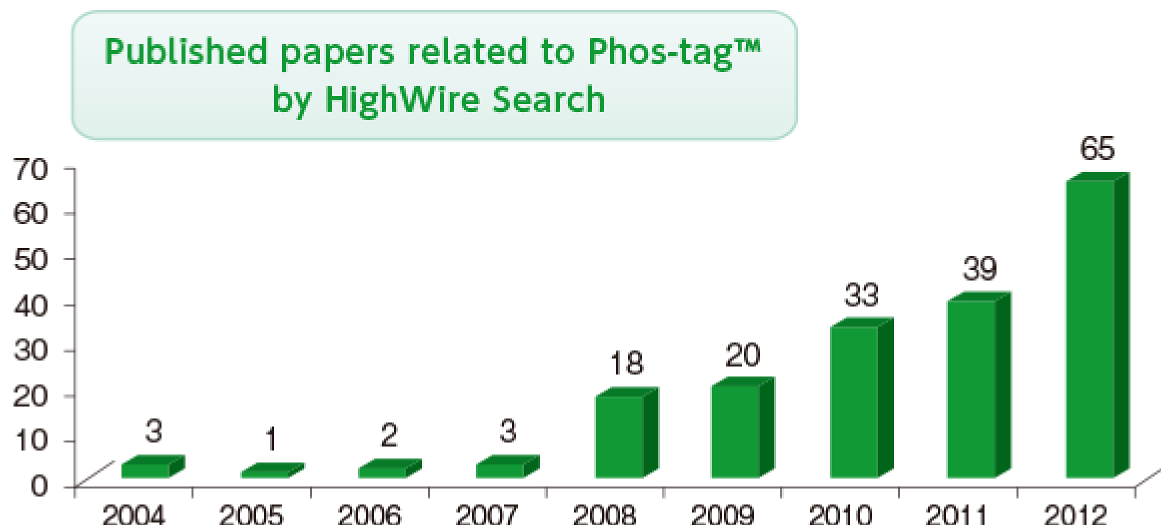
From lanes 1 (L2, L4) and 5 (M1): p35 is phosphorylated, depending on Cdk5.
From lanes 1 (L2, L4) and 3 (L2, L4) ：With about half of p35, Thr138 is phosphorylated at kinase-negative Cdk5, and Thr138 is also phosphorylated by kinase other than Cdk5.
From lanes 5 (M1) and 6 (L3, L4) ：Ser8 and Thr138 are main phosphorylation sites.
From lanes 5 (M1), 7 (L1, L2) and 8 (M2) ：M1 is the phosphorylation site for Ser8 and Thr138. M2 is the phosphorylation site for Ser8 only. L1 and L2 are the phosphorylation sites for Thr138 only.
※X in L1, L3 ： not yet identified
※L4: non-phosphorylated p35

Relationship between phosphorylation site and band shift was clarified!

- **Data published in ：**
  Quantitative Measurement of in Vivo Phosphorylation States of Cdk5 Activator p35 by Phos-tag™ SDS-PAGE. T. Hosokawa, T. Saito, A. Asada, K. Fukunaga, and S. Hisanaga, Mol. Cell. Proteomics, Jun 2010; **9:** 1133 - 1143.

- **Data provided by ：**
  Dr. T. Hosokawa, Laboratory for Memory Mechanisms Neural Circuit Function Research Core, Brain Science Institute, RIKEN and Dr. S. Hisanaga, Molecular Neuroscience Laboratory, Department of Biological Sciences, Graduate School of Science and Engineering, Tokyo Metropolitan University.

References



Regarding Phos-tag™ reagents :

- 1. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of phosphorylated compounds using a novel phos phate capture molecule, Rapid Communications of Mass Spectrometry, **17**, 2075-2081 (2003),H. Takeda, A. Kawasaki, M. Takahashi, A. Yamada, and T. Koike
- 2. Phosphate-binding tag: A new tool to visualize phosphorylated proteins, Molecular & Cellular Proteomics, **5**, 749-757 (2006),E. Kinoshita, E. Kinoshita-Kikuta, K. Takiyama, and T. Koike
- 3. Separation and detection of large phosphoproteins using Phos-tag™ SDS-PAGE, Nature Protocols, **4**, 1513-1521 (2009), E. Kinoshita, E. Kinoshita-Kikuta, and T. Koike

Application using Phos-tag™ reagents

- 1. Spatial regulation of Fus3 MAP kinase activity through a reaction-diffusion mechanism in yeast pheromone signalling, Nat. Cell Biol., **9** ,1319-1326 (2007), C. I. Maeder et.al. M. A. Hink, A. Kinkhabwala, R. Mayr, P. I. H. Bastiaens and M. Knop
- 2. Regulation of PKD by the MAPK p38d in Insulin Secretion and Glucose Homeostasis, Cell, **136**, 235-248 (2009), G. Sumara, I. Formentini, S. Collins, I. Sumara, R. Windak, B. Bodenmiller, R. Ramracheya, D. Caille, H. Jiang, K. A. Platt, P. Meda, R. Aebersold, P. Rorsman, and R. Ricci1
- 3. Dbf4-Dependent Cdc7 Kinase Links DNA Replication to the Segregation of Homologous Chromosomes in Meiosis I, Cell, **135**, 662-678 (2008) ,J. Matos, J. J. Lipp, A. Bogdanova, S. Guillot, E. Okaz, M. Junqueira, A. Shevchenko, and W. Zachariae
- 4. Kinome Profiling in Pediatric Brain Tumors as a New Approach for Target Discovery, Cancer Res., **69**, 5987-5995 (2009) , A. H. Sikkema, S. H. Diks, W. F.A. den Dunnen, A. ter Elst, F. J.G. Scherpen, E. W. Hoving, R. Ruijtenbeek, P. J. Boender, R. de Wijn, W. A. Kamps, M. P. Peppelenbosch, and E. S.J.M. de Bont
- 5. Regulation of mitochondrial transport and inter-microtubule spacing by tau phosphorylation at the sites hyperphosphorylated in Alzheimer's disease, J. Neurosci.,**32**, 2430-2441 (2012), K.Shahpasand, I. Uemura, T.Saito, T.Asano, K.Hata, K.Shibata, Y.Toyoshima, M.Hasegawa, S.Hisanaga
- 6. The Hsp90 Kinase Co-chaperone Cdc37 Regulates Tau Stability and Phoshorylation Dynamics, J. Biol. Chem., **286**, 16976-16983 (2011) ., Umesh K. Jinwal, Justin H. Trotter, Jose F. Abisamobra, John Koren, III, Lisa Y. Lawson, Grant D. Vestal, John C. O'Leary, III, Amelia G. Johnson, Ying Jin, Jeffrey R. Jones, Qingyou Li, Edwin J. Weeber, and Chad A. Dickey
- Tau proteins are used as the samples in Application 5 and 6.

Other Phos-Tag™ Products

Product Name		Pkg. Size	Cat. No.	Applications
SuperSep Phos-tag™ gels				precast gels usiing Phostag acrylamide
Phos-tag™ Biotin	BTL-104	10 mg	301-93531	Specific detection without any anti-phosphorylated antibodies on Western blot. Detection is possible regardless of type of phosphorylated amino acid.
	BTL-105	10 mg	308-93541	
	BTL-111 1mM Aqueous Solution Ref.1)	0.1 mL	308-97201	Offers higher sensitivity than BTL-104.
Phos-tag™ Mass Analytical Kit		1 Kit	305-93551	Analysis by MALDI-TOF/Mass
Phos-tag™ Agarose		0.5 mL	302-93561	Enrichment, separation and purification of phosphorylated proteins using column chromatography
		3 mL	308-93563	

SuperSep Phos-tag

SuperSep Phos-tag™ is a precast gel, which can be immediately used after opening the package, is added with 50 µmol/L of Phos-tag™ Acrylamide in advance. It contains zinc as a supplemental metal. It has excellent storage stability by its neutral gel buffer. Sharp bands can be obtained.

SuperSep Phos-tag™

EasySeparator

Features

- Ready-to-use
- Safety due to precast gel
- Long-term stability (Stable for 6 months)
- Almost the same basic mechanism as that of SDS-PAGE

Plate Size	100 x 100 x 3 (mm)
Gel Size	90 x 85 x 1 (mm)
Well number	13 or 17
Phos-tag™ conc.	50 µmol/L
Acrylamide conc.	12.5% or 15%
$ZnCl_2$ conc.	100 µmol/L
Well volume	30 µL

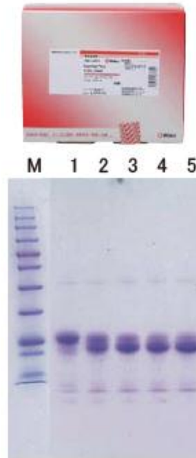
ATTENTION!

・This product is a precast gel optimized in an "EasySeparator" tank.
・Use of a normal prestained marker will distort the bands. Use of WIDE-VIEW™ Prestained Protein Size Marker Ⅲ (No. 230-02461) is recommended. Please refer to "Phos-tag™ Acrylamide - Molecular Marker"/FAQ. ・Before using this product, check a sample for migration pattern problems with "SuperSep Ace" or other normal SDS-PAGE.
・When performing Western blotting, execute an EDTA process before transfer. For details, refer to Troubleshooting.

FT-FL8630

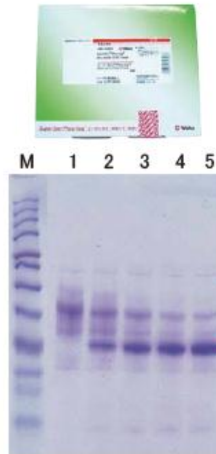
Application ～ Dephosphorylation over time of β-casein ～

SuperSep Ace, 12.5%, 13 wells



Phosphorylated β-casein + dephosphorylated β-casein

SuperSep Phos-tag™, 12.5%, 13 wells



Phosphorylated β-casein

Dephosphorylated β-casein

[Buffer]
Tris-Glycine-SDS electrophoresis buffer

[Sample]
M: WIDE-VIEW™ Prestained Protein Size Marker Ⅲ (230-02461)
1 : 0 min. β-casein (with AP treatment)
2 : 15 min. β-casein (with AP treatment)
3 : 30 min. β-casein (with AP treatment)
4 : 45 min.β-casein (with AP treatment)
5 : 60 min. β-casein (with AP treatment)

[Condition]
Constant current 35 mA for 60 min.

[Staining]
Quick-CBB Staining（299-50101）

[Destaining]
Deionized water with microwave

β-casein was dephosphorylated over time. Dephosphorylated β-casein can be separated from β-casein with SuperSep Phos-tag™.

Product Name	PackageSize	Cat. No.	Storage
SuperSep Phos-tag™ (50 µmol/L), 12.5%, 13 well	5 gels	195-16391	Keep at 2~10℃
SuperSep Phos-tag™ (50 µmol/L), 12.5%, 17 well	5 gels	193-16571	
SuperSep Phos-tag™ (50 µmol/L), 15%, 13 well	5 gels	193-16691	
SuperSep Phos-tag™ (50 µmol/L), 15%, 17 well	5 gels	196-16701	
Related Products			
SuperSep Ace, 12.5%, 13 well	10 gels	199-14971	Keep at 2~10℃
SuperSep Ace, 12.5%, 17 well	10 gels	196-14981	
SuperSep Ace, 15%, 13 well	10 gels	193-14991	
SuperSep Ace, 15%, 17 well	10 gels	190-15001	
EasySeparator (an electrophoresis tank for SuperSep)	1 unit	058-07681	RT
Wide-View Prestained Protein Size Marker Ⅲ (11~245 kDa)	500 µL (for 200 tests)	230-02461	Keep at -20℃

Phos-tag™ Biotin －Detection of phosphoprotein for Western blotting－

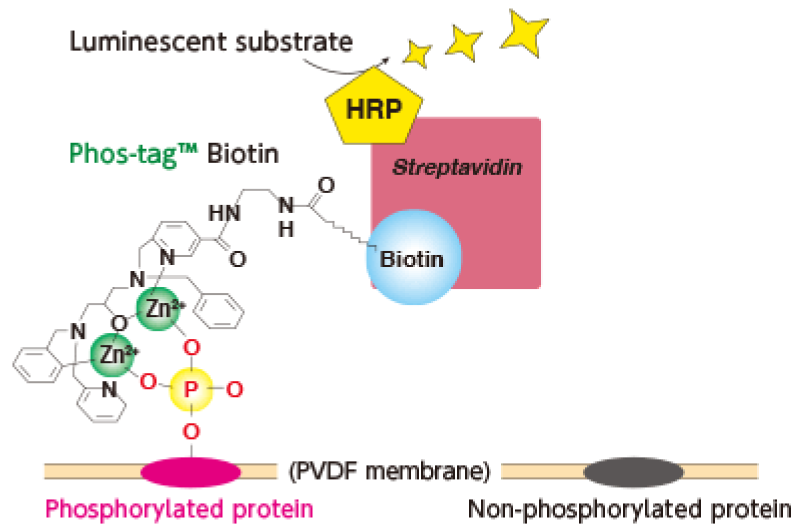
This is biotin-bound Phos-tag™ used for detection of phosphoprotein by Western blotting.

Features

- All phosphoprotein can be detected.
- Procedures of experiment are similar to those in ordinary Western blotting.

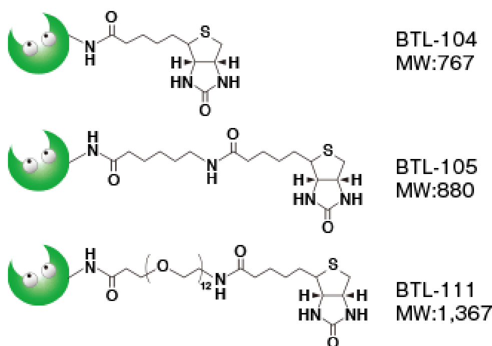
It can be conveniently used even when target anti-phosphorylated Thr/Ser antibody is not available !

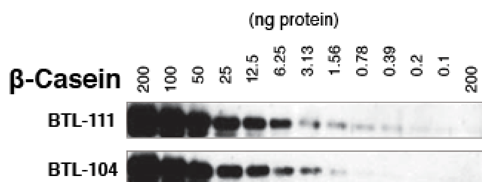
BTL-104, BTL-105, and BTL-111 have linkers with different lengths. Although the usage of BTL-104 and BTL-105 are similar, BTL-104 is recommendable as the first choice because of its high solubility. BTL-111 offers higher sensitivity.



Schematic of western blotting using Phos-tag™ Biotin

Reference for BTL-111
1) Highly sensitive detection of protein phosphorylation by using improved Phos-tag Biotin, Proteomics, 12(7), 932-7 (2012), Kinoshita E, Kinoshita-Kikuta E, Sugiyama Y, Ozeki T, Koike T.





Phos-tag™ Mass Analytical Kit －Detection Sensitivity of MALDI-TOF/Mass is improved－

Before use, Phos-tag™ Mass Analytical Kit is mixed with samples for MALDI-TOF/Mass analysis. Phosphorylated molecule-Phos-tag™ complex is detected in a positive mode, and hosphorylated molecule usually difficult to detect can be detected with improved sensitivity.

Features

- Detection sensitivity of phosphorylated molecule is improved.

【Kit Contents】

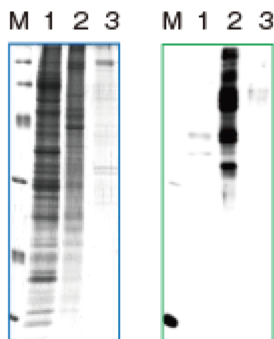
- Phos-tag™ MS-101L ......5mg — $[C_{27}H_{29}N_6O^{64}Zn_2]^{3+}$ MW : 581.4
- Phos-tag™ MS-101H ......5mg — $[C_{27}H_{29}N_6O^{68}Zn_2]^{3+}$ MW : 589.4
- Phos-tag™ MS-101N ......10mg — $[C_{27}H_{29}N_6OZn_2]^{3+}$ MW : 584.3

Phos-tag™ Agarose －Purification of phosphorylated proteins by affinity chromatography－

Fill Phos-tag™ Agarose in a column for use. It can be used for separation, purification and concentration of phosphorylated proteins. Because it is free from surfactants or reducing agents, phosphorylated protein can be obtained in a condition similar to in vivo one.

Features

- Phosphorylated proteins can be purified in 1 hour.
- The proteins can be trapped in physiological condition (pH 7.5).
- Purified with no reducing agent or surfactant.



【Application】： Purification of phosphorylated proteins in A431 lysate

Lysate was applied on a column filled with Phos-tag™ Agarose. Phosphorylated proteins were concentrated in absorbed fraction.

M：Molecular Marker
Lane 1：Non absorbed fraction
Lane 2：Absorbed fraction
Lane 3：Column rinsing fraction
(Left) Fluorescence staining
(right) Western blotting with anti-phosphorylated Tyr.

Related Products (Electrophoresis/Western Blotting)

Reagents for Phos-tag™ SDS-PAGE gel preparation

Product Name	Pkg. Size	Cat. No.	Use Application
30 w/v% Acrylamide solution, 29:1	500 mL	014-21705	Ready-to-use “Solution A”. 30%T, 3.3%C
Agarose H (High-strength type)	1 g 10 g 25 g	315-01203 319-01201 317-01202	High-strength Agarose has high strength even in a low-agarose environment and is suitable for electrophoretic migration of large nucleic acid fragments. It can be used in a concentration range of 0.2 - 1% and a separation range of 1 - 200 kbp.
10% SDS Solution	100 mL 500 mL	311-90271 313-90275	Ready-to-Use “Solution D”
Manganese(Ⅱ) Chloride Tetrahydrate, 99.0+ % (Titration)	25 g 100 g	134-15302 136-15301	for Molecular Biology Please use for preparation of “Solution F”
Zinc Chloride	25 g	268-01902	for Molecular Biology
Separating Gel Buffer Solution (x4)	250 mL	192-11041	Ready-to-Use mixed solution of “Sol. B” and “Sol. D”for preparation of Resolving Gel. Contains SDS.
Stacking Gel Buffer Solution (x4)	250 mL	199-11051	Ready-to-Use mixed solution of “Sol.C” and “Sol. D”for preparation of Stacking Gel. Contains SDS.
N,N,N',N'-Tetramethylethylenediamine (TEMED)	25 mL	205-06313	for Electrophoresis
10 w/v% Ammonium Peroxodisulfate Solution (Ammonium Persulfate Solution)	25 mL	019-15922	Ready-to-Use “Solution G”

Premixed Buffers

Product Name	Pkg. Size	Cat. No.	Use Application
Running Buffer Solution (x10)	1 L	184-01291	Ready-to-Use concentrated “Solution H”
SDS-PAGE 10x Running Buffer	1 L 5 L	312-90321 318-90323	Ready-to-Use concentrated “Solution H”
SDS-PAGE Buffer, pH 8.5	5 L	192-16801	Ready-to-Use “Solution H”, 1 x buffer.
Tricine Running Buffer Solution (x10)	1 L	200-17071	Composition: 0.5 M Tris / 0.5 M Tricine / 1% SDS
Sample Buffer Solution (2ME+) (x4)	25 mL	191-13272	Sample buffer for Laemmli SDS-PAGE containing 2-mercaptoethanol
Sample Buffer Solution (2ME+) (x2)	25 mL	196-11022	
Sample Buffer Solution with 3-Mercapto-1,2-propanediol (x2)	25 mL	199-16132	Laemmli Sample Buffer containing 3-mercapto-1,2-propanediol (non-hazardous chemical) as substitute for 2-ME
Sample Buffer Solution with 3-Mercapto-1,2-propanediol (x2)	25 mL	196-16142	

Reagents for Staining

Product Name	Pkg. Size	Cat. No.	Use Application
Quick CBB Plus	250 mL 1 L	174-00553 178-00551	Ready-to-Use Sol. K.Fixing and destaining procedure are not required.No organic solvents are necessary.Protein bands are stained in 10 minutes.
Quick-CBB ・Staining solution A: 1L × 1 ・Staining solution B: 1L × 1	2 L	299-50101	By mixing staing solution A and B, ready-to-Use Sol. K
Silver Stain MS Kit	20 tests	299-58901	Proteins are rarely modified chemically due to omitting treatment of glutaraldehyde and is detected at sub-nanogram level on the electrophoretic gel.
Silver Stain Kit	for10 gels	299-13841	50~100 times more sensitive than CBB method.
Silver Stain II Kit	for10 gels	291-50301	This kit contains Stopper, which can be

Negative Gel Stain MS Kit	20 tests	293-57701	adjusted the staining strength. Applicable for mass analysis and western blot

Protein Size Marker

Product Name	Pkg. Size	Cat. No.	Use Application
WIDE-VIEW™ Prestained Protein Size Marker Ⅲ	500 µL	230-02461	A recommendable prestained marker used with Phos-tag™ SDS-PAGE because obtained bands are less to distort.

Enzyme for Dephosphorylation

Product Name	Pkg. Size	Cat. No.	Use Application
Alkaline Phosphatase （for Biochemistry）	50 U 100 U	018-10693 012-10691	Applicable for dephosphorylation of proteins

Electrophoresis Apparatus・Precast Gels

Product Name	Pkg. Size	Cat. No.	Use Application
EasySeparator	1 set	058-07681	An electrophoresis tank for SuperSep precast polyacrylamide gels.＊
SuperSep Ace, 12.5%, 13 well SuperSep Ace, 12.5%, 17 well SuperSep Ace, 15%, 13 well SuperSep Ace, 15%, 17 well	10 gels 10 gels 10 gels 10 gels	199-14971 196-14981 193-14991 190-15001	Prior to use of SuperSep Phos-tag™ PAGE, please use these as sample confirmation. Expire in 9 months after the manufacture

＊：Invitrogen's electrophoresis tank is also applicable to SuperSep by using an adjuster. Please contact us.

Reagents for Western Blotting

Product Name	Pkg. Size	Cat. No.	Use Application
ImmunoStar LD＊ ・Luminescence solution A ・Luminescence solution B	200 ㎠ 1,000 ㎠ 2,000 ㎠	296-69901 292-69903 290-69904	Highly sensitive (femto gram level) immunoblotting, utilizing detection by enhanced chemiluminescence using a unique luminol derivative L-012 as substrate. Not available for sale in the US and Europe.
ImmunoStar Zeta＊	200 ㎠ 1,000 ㎠ 2,000 ㎠	291-72401 297-72403 295-72404	Use for detection of proteins between the middle and low femto gram levels. Has stable, long-lasting luminescence signal.
Immuno Enhancer	2 assays 10 assays 40 assays	294-68601 290-68603 298-68604	Ready-to-Use Immunoreaction Enhancer for western blotting and ELISA

＊：Not available for sales in the US and Europe.

Other Information

See the BioSciences catalogue :

Please contact InterBioTech – Interchim for any other information

Rev.Q01E-FT-FL8631(N01/MNA)