

Phos binding reagent (Phosbind) acrylamide Protocol

Sec. I. Mn^{2+} - Phosbind Acrylamide (PB-A) SDS-PAGE

1. Preparation of reagents for PB-A SDS-PAGE

Acrylamide Solution	<p>Sol. A: 30 w/v% Acrylamide Solution (30% T, 3.3% C)</p> <p>Acrylamide-----29.0 g</p> <p>N, N'-methylene-bisacrylamide-----1.0 g</p> <p>⇒ Prepare the 100 mL solution by adding distilled water and filter the solution.</p> <p>【Storage】 Keep at 4°C in the dark.</p>
Tris-HCl Buffer for Resolving Gel, pH 8.8	<p>Sol. B: 1.5 mol/L Tris/HCl Solution, pH 8.8 (4x solution for Resolving Gel)</p> <p>Tris base (MW: 121, pKa= 8.2 at 20°C)-----18.2 g</p> <p>6.0 mol/L HCl (0.19 equivalents of Tris)-----4.85 mL</p> <p>⇒ Prepare the 100 mL solution by adding distilled water and filter the solution.</p> <p>【Storage】 Keep at 4°C in the dark.</p>
Tris-HCl Buffer for Stacking Gel, pH 6.8	<p>Sol. C: 0.50 mol/L Tris/HCl Solution, pH 6.8 (4x solution for Stacking Gel)</p> <p>Tris base-----6.06 g</p> <p>6.0 mol/L HCl (0.96 equivalent of Tris base)-----8.0 mL</p> <p>Distilled water -----90 mL</p> <p>⇒ 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.</p> <p>【Storage】 Keep at 4°C</p>
SDS Solution	<p>Sol. D: 10% (w/v) SDS Solution</p> <p>SDS-----10.0 g</p> <p>Distilled water-----90 mL</p> <p>⇒ After stirring, prepare the 100 mL solution by adding distilled water</p> <p>【Storage】 Keep at 4°C</p>

<p>PB-A Soln.</p>	<p>Sol. E: 5.0 mmol/L PB-A solution containing 3% (v/v) methanol</p> <p>Phosbind Acrylamide (MW: 595)-----10 mg (2 mg)</p> <p>methanol -----0.10 mL (0.02 mL)</p> <p>Distilled water-----3.2 mL (0.64 mL)</p> <p>The oily product, Phosbind Acrylamide is provided in a small plastic tube.</p> <p>Please completely dissolve it in 0.1 mL methanol.</p> <p style="text-align: center;">↓</p> <p>This methanol solution should be further diluted with 3.2 mL of distilled water by pipetting.</p> <p>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.</p> <p>【Storage】 Wrap the tube with aluminum foil. Keep the soln. in a 2-mL microtube at 4°C in the dark.</p>
<p>MnCl₂ Soln.</p>	<p>Sol. F: 10 mmol/L MnCl₂* Solution</p> <p>MnCl₂(H₂O)₄ (MW: 198)-----0.10 g</p> <p>Distilled water -----50 mL</p> <p>*: Be careful not to confuse manganese with magnesium (Mg)</p> <p>Note: Do not use other anion salts such as Mn(NO₃)₂ or Mn(CH₃COO)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 Mn²⁺ will deteriorate.</p>
<p>APS Solution</p>	<p>Sol. G: 10% (w/v) Ammonium Persulfate Solution</p> <p>(NH₄)₂S₂O₈ (MW: 228) -----10 mg</p> <p>Distilled water -----0.10 mL</p> <p>⇒ Prepared sol. G can be stored for long period at -20°C by aliquot in an</p>

	appropriate amount.
Running Buffer	<p>Sol. H: Running Buffer, pH 8.3 (10x soln.)</p> <p>Tris base (0.25 mol/L) -----15.1 g</p> <p>SDS-----5.0 g</p> <p>glycine (1.92 mol/L) -----72.0 g</p> <p>⇒ Prepare the 500 mL soln. by adding distilled water. Avoid to adjust the pH by adding acid or base.</p> <p>Just before use, add 450 mL of distilled water to 50 mL of Soln. H.</p> <p>【Storage】 Keep at 4°C</p>
Sample Buffer	<p>Sol. I: Sample Buffer (3x solution)</p> <p>Bromophenol Blue (BPB) -----1.5 mg</p> <p>SDS----- 0.60 g</p> <p>Glycerol----- 3.0 mL</p> <p>Sol. C: 0.50 mol/L Tris/HCl, pH 6.8 -----3.9 mL</p> <p>2-mercaptoethanol -----1.5 mL</p> <p>⇒ Prepare the 10 mL soln. by adding distilled water.</p> <p>【Storage】 Keep at -20°C</p>
Fixation Solution	<p>Sol. J: Acidic Solution for Fixation of Proteins (1 L)</p> <p>acetic acid -----0.10 L</p> <p>methanol -----0.40 L</p> <p>Distilled water -----0.50 L</p>
CBB Staining Soln.	<p>Sol. K: CBB Staining Solution (0.5 L)</p> <p>Coomassie Brilliant Blue (CBB) -----1.25 g</p> <p>methanol -----0.20 L</p> <p>acetic acid -----50 mL</p> <p>Distilled water -----0.25 L</p> <p>⇒ Dissolve CBB in methanol and then add acetic acid and water.</p> <p>APEX BIO also provides InstaBlue Protein Stain Solution (Cat No B8226) for</p>

	this procedure.
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

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 PB-A)

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 PB-A 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.

Sol. G: 10% (w/v) Ammonium Persulfate Solution-----	50 µL ^{*2)}
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*1) For the MnCl₂ solution, two times the PB-A concentration (molar ratio) is added.

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

Note: Please optimize concentration of Sol. E (PB-A) and Sol. A (Acrylamide). Refer to 5. Optimization of PB-A

PAGE Condition

Examples of preparation of 10 mL of resolving gel solution

PB-A Acrylamide conc.	20 μ M				50 μ M				100 μ M			
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)	0.04	0.04	0.04	0.04	0.1	0.1	0.1	0.1	0.2	0.2	0.2	0.2
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)	3.26	3.93	4.59	5.26	3.14	3.81	4.47	5.14	2.94	3.61	4.27	4.94
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

Note: Please optimize concentration of Sol. E (PB-A) and Sol. A (Acrylamide).

3. Stacking Gel Preparation

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 (20 μ L)

TEMED (tetramethylethylenediamine) -----10 μ L (2 μ L) ^{*2)}

Distilled Water-----5.84 mL (1.17 mL)

Deaerate with stirring for 2 minutes.

Sol. G: 10% (w/v) Ammonium Persulfate Solution-----50 μ L (10 μ L) ^{*2)}

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

2i. 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 Phosbind 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 Phosbind Acrylamide 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.

*3) Add the agarose after distilled water has been added and thoroughly dissolved in a microwave oven and it is still hot.

*4) If necessary, preheat the pipette tip and gel preparation table to 40 - 45°C.

3i. Preparation of Stacking Gel with low concentration 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 (20 μ L)
TEMED (tetramethylethylenediamine) -----	10 μ L (2 μ L) ^{*2)}
Distilled Water-----	3.01 mL (602 μ L)
1.5% (w/v) agarose ^{*3) *4)} -----	3.33 mL (666 μ L)
Sol. G: 10% (w/v) Ammonium Persulfate Solution-----	50 μ L (10 μ L) ^{*2)}



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

4. Sample Preparation

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

※ In case of β -casein, load 5~10 μ g /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.

(※In case of two gels, run the gels at 50~60 mA.)

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

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.

Sec. II. Tips for Western blotting of PB-A SDS-PAGE gels

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).

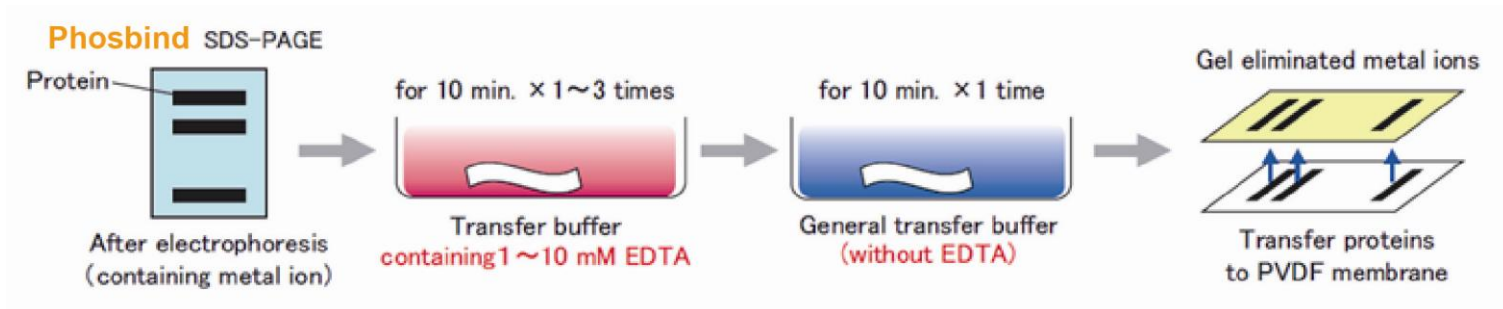
※ Besides transfer buffer, 1 x Running buffer can be also used .

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+} -PB-A 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 PB-A gel.



Sec. III. Tips for Mass Spectrometry of PB-A SDS-PAGE

No special procedures such as EDTA treatment are necessary.

Sec. IV. Trouble Shooting

1. Distortion of bands

The most common complaint for PB-A 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. 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.

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.

2. Low Resolution

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

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

3. 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.

4. 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.

5. 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) Staining 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%.

6. Higher background during staining

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

7. Mobility shift due to protein degradation, not induced by phosphorylation

Carry out SDS-PAGE as usual (containing 0 μ M of PB-A) and verify that no mobility shift occurs.

Sec. V. Optimization of PB-A PAGE Condition

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

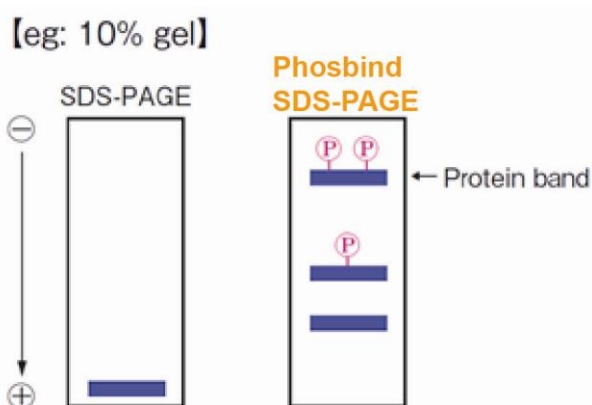
1. Optimization of the concentration of acrylamide

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 PB-A 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 PB-A 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 PB-A 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 PB-A Acrylamide” of Sec. VII. FAQ). Furthermore, the gel strength can also be enhanced by increasing the N,N'-methylenebisacrylamide content (eg: 5% acrylamide [24:1]).

2. Optimization of the concentration of PB-A.

Then, optimize the concentration of PB-A. Please evaluate the optimum concentration in the order of lowest to highest. eg: 20 μ M→50 μ M→100 μ M.

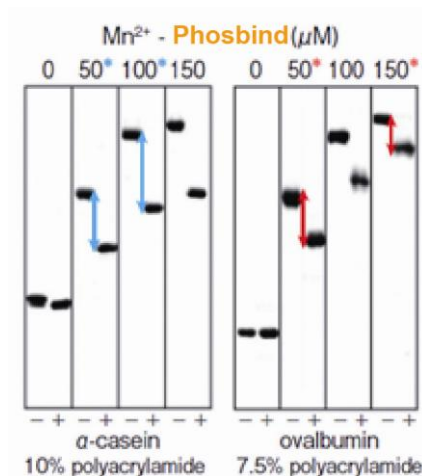
【Cell Lysate】

In case there is a large variety of proteins in your sample, eg: cell lysates, the concentration of PB-A should be 5 to 25 μ M. However, a higher concentration, eg: 100 μ M, 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.

Higher concentration of PB-A 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+} -PB-A in the left figure.). However, the higher concentration causes low velocity. It sometimes happens that the higher separation capacity is due to the lower PB-A concentration (Compare the samples of 50 μM and 150 μM of ovalbumin of the right figure.)



Distance between two bands:

* : α -casein: 50 μM < 100 μM

* : ovalbumin: 50 μM > 150 μM



Please adjust the optimum concentration for every target protein.

-: phosphorylated protein; +: non-phosphorylated protein (alkaline phosphatase treatment).

Sec. VI. FAQ

1) **Q:** How large (kDa) can a protein be separated using this product?

A: A phosphorylated protein of 350 kDa has actually been separated with 20 μM PB-A, 3% acrylamide and 0.5% agarose*.

* Agarose was added to strengthen the gel.

2) **Q:** How can the resolution be improved?

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

3) **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.

4) **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.

5) **Q:** How long can the prepared gel containing PB-A be stored?

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

6) **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.

7) **Q:** I have experienced clouding of PB-A 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.

8) **Q:** Does PB-A 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.

9) **Q:** What prestained markers can we use?

A: Using a prestained marker with the PB-A gel usually causes distortion of bands. At least one blank lane is needed between the solution containing this marker and other solutions.

10) **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.

11) **Q:** A mobility shift was observed in PB-A 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 PB-A) and verify that your protein is intact.

12) **Q:** Is PB-A 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 Zn²⁺-PB-A.)
- 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