3050 Spruce Street
Saint Louis, Missouri 63103 USA
Telephone 800-325-5832 • (314) 771-5765
Fax (314) 286-7828
email: techserv@sial.com
sigma-aldrich.com

ProductInformation

PhosDecor™ Stain

Catalog Number **P7374**
Storage Temperature 2–8 °C

TECHNICAL BULLETIN

Product Description

Phosphorylation is an important covalent post-translational modification (PTM) in cell signaling pathways. It is estimated there are greater than 100,000 sites of phosphorylation in the human proteome.[1] Phosphorylation of a single protein can modulate an entire signal transduction pathway, making each phosphorylation event potentially significant.[2] Identification of phosphorylated proteins traditionally involves the use of radioisotopes or antibodies, both of which have significant costs and limitations associated with them.[3] Sigma's PhosDecor™ Fluorescent Phosphoprotein In-Gel Detection Kit allows for the quick and selective identification of phosphorylated proteins directly in SDS-polyacrylamide gels. This technology may be particularly useful for preliminary screening of proteins involved in transduction pathways.

The simple staining procedure can be completed in as little as 3.5 hours, providing a quick method for identification of protein phosphorylation.

PhosDecor Stain is intended for use with the other reagents found in the PhosDecor Fluorescent Phosphoprotein In-Gel Detection Kit (Catalog Number PDECOR). The Fixing and Destaining Solutions may be prepared according to the given formulations. The PhosDecor Control (Product Code P7499) may be purchased separately.

Gels stained with PhosDecor Stain may be dual stained for total protein with the following:

EZBlue™ Gel Staining Reagent
(Catalog Number G1041)
ProteoSilver™ Silver Stain Kits
(Catalog Numbers PROTSIL1 or PROTSIL2)
SYPRO® Ruby Protein Gel Stain
(Catalog Number S4942)
SYPRO Orange Protein Gel Stain
(Catalog Number S5692)

PhosDecor staining is compatible with downstream analysis such as in-gel trypsin digestion of the stained proteins and MALDI-MS analysis, allowing the identification of specific phosphorylation sites within the proteins.

Component

Sufficient reagent is supplied for staining 25 mini-gels (8 cm x 8 cm x 1 mm).

- PhosDecor Stain (Catalog Number P7374) — 1 liter

Equipment and Reagents Required But Not Provided

- Fixing Solution, 50% methanol and 10% acetic acid (Catalog Number F0680)
- Destaining Solution, 0.05 M sodium acetate, pH 4, with 20% acetonitrile (Catalog Number D4568)
- PhosDecor Control, Six lyophilized control proteins See Appendix I for further information (Catalog Number P7499)
- Ultrapure (minimum 18 MΩ•cm) water or equivalent (Catalog Number W4502)
- Orbital mixer or rocker table
- Mini-gel wash tray with at least 100 ml capacity, but small enough for gel to be completely covered by solutions (Catalog Number Z358290)
- EZBlue Gel Staining Reagent (Catalog Number G1041)
- Ultraviolet (UV) light transilluminator and image capture system, or visible light-laser-based scanner
- SYPRO Ruby Protein Gel Stain (Catalog Number S4942, optional)
- Trypsin Profile IGD Kit (Catalog Number PP0100, optional)

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

PhosDecor Stain should be stored at 2–8 °C. The product as supplied is stable for at least 2 years when stored properly. PhosDecor Stain should be warmed to room temperature before use.

Procedure

This procedure is for staining a standard SDS-PAGE mini-gel (8 cm x 8 cm x 1 mm), but may be adapted for use with other sized gels. It is important to completely submerge the gel with constant mixing to ensure uniform staining across the gel. The volumes of reagents used for each staining, destaining, and fixing step are proportional to the sizes of the gels.

Table 1.
Suggested volumes for each step for common gels

Gel size	PhosDecor Stain	Fixing Solution	Destaining Solution
8 cm × 8 cm × 1 mm	40 ml	80 ml	2 × 40 ml
11 cm × 8 cm × 1 mm (2D Gel)	55 ml	110 ml	2 × 55 ml

To determine the appropriate volumes of reagents to submerse other size gels use the following equations:

Volume of PhosDecor Stain (V_S): $V_S = (40 \times V_{gel})/6.4$

Volume of Fixing Solution (V_F): $V_F = (80 \times V_{gel})/6.4$

Volume of Destaining Solution (V_D): $V_D = (40 \times V_{gel})/6.4$

V_{gel} = volume of gel [cm^3, $(l \times w \times h)$], for this calculation a volume of 1 cm^3 at room temperature is taken as equivalent to 1 ml. The volume of a mini-gel would be 6.4 cm^3 (8 cm x 8 cm x 0.1 cm).

In order to obtain the **maximum signal/noise ratio,** it is very important to **follow the recommended protocol,** including **volumes** and **incubation times**. This procedure is for staining a standard SDS-PAGE mini-gel (8 cm x 8 cm x 1 mm).

1. **Reconstitute** and load the PhosDecor Control (Catalog Number P7499) according to Appendix I, Preparation Instructions. Load ~500 ng of the unknown protein sample(s) into other well(s) for SDS-PAGE. Some samples may require removal of associated lipids and salts for optimal results (see Appendix II, Sample Preparation).

2. **Electrophoresis:** Resolve protein samples according to the standard electrophoresis procedure for gel type used. PhosDecor Stain is compatible with most common gels. However, best results are observed in Tris-Glycine gels.

3. **Rinsing:** Remove the gel from the cassette and transfer the gel into a mini-tray containing ~100 ml of ultrapure water. Rinse the gel with gentle shaking for 5 minutes.

4. **Fixing:** Decant the ultrapure water from the gel and cover with 80 ml of Fixing Solution (Catalog Number F0680). Incubate the gel at room temperature with gentle agitation for 30 minutes and repeat the fixing step two more times. The gel may be left in fixing solution overnight if needed.

   Note: For NuPAGE® Bis-Tris or other long shelf-life gels, best results are obtained if gel is left in the last fixing step overnight.

5. **Washing:** Completely cover the gel in ultrapure water and incubate for 10 minutes with gentle shaking. Repeat this wash two more times. Be sure that the gel is completely immersed to remove all of the Fixing Solution. Residual Fixing Solution will increase background during PhosDecor staining.

   Note: The following steps should be completed with the gel and solutions protected from light (e.g., cover staining containers with aluminum foil).

6. **Staining:** Before staining, **invert the bottle** of PhosDecor Stain (Catalog Number P7374) several times to mix and allow stain solution to warm to room temperature. For a standard mini-gel, incubate the gel in a clean tray with **40 ml** of PhosDecor Stain with gentle agitation for **40 minutes**.

7. **Destaining:** Decant the staining solution from the gel, making sure all of the solution is removed from the tray before adding the Destaining Solution (Catalog Number D4568). Immediately add **40 ml** of Destaining Solution and incubate with gentle agitation for **40 minutes**. Decant the Destaining Solution and repeat the destaining step once more. Longer destaining incubation may result in loss of selectivity.

8. **Rinsing:** Rinsing is not necessary. However, the gel may be rinsed in ultrapure water briefly (less than 10 seconds)**.**

9. **Imaging and Documenting the Gel:** Immediately visualize the stained gel using a 300 nm UV transilluminator or a visible light-laser-based scanner. The PhosDecor Stain has an excitation maximum at a wavelength of 555 nm and a maximum emission at a wavelength of 585 nm. Best results may be obtained by using a filter specific for wavelengths closest to the emission maximum.

10. The **exposure time and/or grayscale** should always **be adjusted, so the maximum signal to noise ratio is observed between positive and negative controls,** which are provided in the PhosDecor Control. A certain level of background staining in the gel is expected (see Results, Image Optimization). Document the image before continuing.

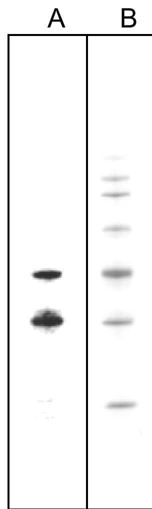
11. **Total protein staining:** Dual staining with a total protein stain allows for accounting of differences in gel loading and the identification of non-phosphorylated proteins.
    a. After documenting the staining pattern of the PhosDecor stained gel, the gel should be stained with a total protein stain to detect all proteins in the gel. For dye staining of the protein, EZBlue Gel Staining Reagent is recommended and the procedure is detailed in this step. For fluorescent dye staining, SYPRO Ruby Protein Gel Stain is recommended (see datasheet for Catalog Number S4942 for SYPRO Ruby staining procedure).
    b. Rinse the PhosDecor stained gel in ultrapure water with gentle agitation for 10 minutes.
    c. Completely cover the gel in EZBlue Gel Staining Reagent and incubate with gentle shaking for at least 30 minutes. Overnight staining will not increase staining intensity.
    d. Destain the gel with several short water washes to remove excess EZBlue Gel Staining Reagent. The gel may be destained overnight.
    e. Image gel using a white light photo scanner or appropriate settings for gel imaging system.

Results

Selectivity (Signal/Noise ratio)

PhosDecor Stain is selective for phosphoproteins; however, some non-specific protein staining may occur. Intensity of non-specific staining may vary between different gel formulations. After staining, phosphoproteins will stand out among the non-phosphorylated proteins. It is important to adjust the exposure time/grayscale using positive and negative PhosDecor controls to obtain an optimal signal to noise ratio and consistent results from gel to gel.

Figure 1.
Stained PhosDecor Controls

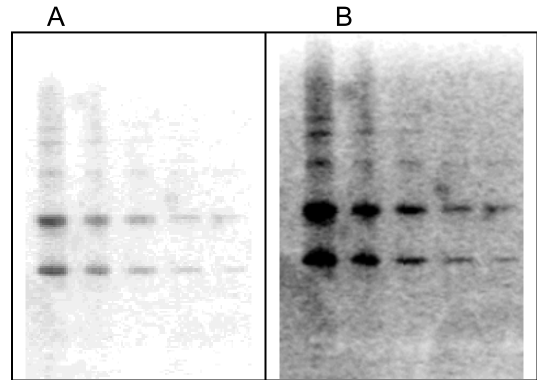


A. PhosDecor Control stained with PhosDecor Stain
B. PhosDecor Control dual stained with EZBlue Gel Staining Reagent

Image Optimization

To identify phosphoproteins from complex mixtures, it is important to adjust the grayscale setting and/or subtract background from the raw image. It is possible to make non-phosphorylated proteins appear to be stained with excessive exposure times or incorrect grayscale adjustment. Optimization will produce the best possible image for distinction between phosphorylated proteins and non-phosphorylated proteins (see Figure 2). Always run positive and negative PhosDecor controls with the sample(s) to be tested. Once the image is captured, the grayscale should be adjusted so the control phosphoproteins (ovalbumin and β-casein) stand out as bright bands and the non-phosphorylated, negative control proteins are faintly visible or not visible. Alternatively, a background subtraction tool, found in some gel documentation software, can be easily used for optimization. The image can then be used for analysis.

Figure 2.
Serial dilution of PhosDecor Control stained with PhosDecor Stain and excited with a UV transilluminator



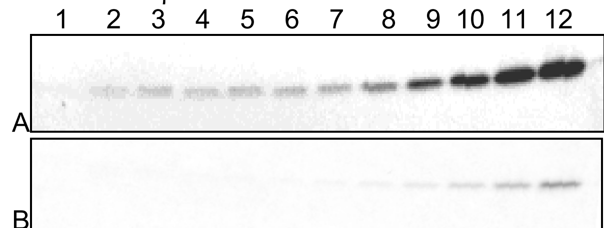
A. An optimized image
B. An overexposed image

These images demonstrate the importance for proper image optimization.

Sensitivity (Detection Limit)

The limit of detection is dependant on the level of phosphorylation of the protein being detected. The PhosDecor Stain can easily detect 62.5 ng of pepsin, a phosphoprotein containing only one phosphate group, and less than 1 ng of β-casein, a phosphoprotein containing 5 phosphate groups, using a standard UV transilluminator (see Figure 3). The sensitivity is five times greater when using a visible light-laser-based scanning instrument.

Figure 3.
Serial dilution of β-casein



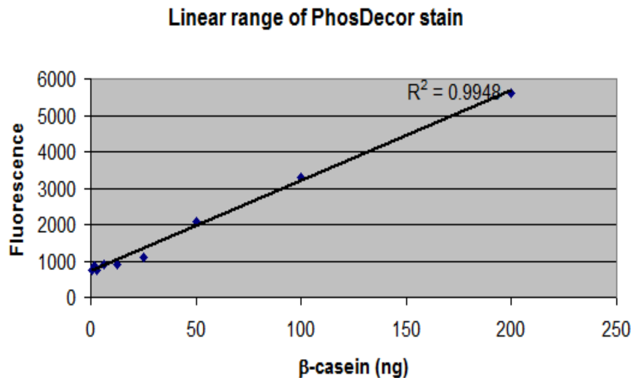
A. PhosDecor detection range using a UV light source:

Lane 1 - 0.39 ng	Lane 2 - 0.78 ng
Lane 3 - 1.56 ng	Lane 4 - 3.13 ng
Lane 5 - 6.25 ng	Lane 6 - 12.5 ng
Lane 7 - 25 ng	Lane 8 - 50 ng
Lane 9 - 100 ng	Lane 10 - 200 ng
Lane 11 - 400 ng	Lane 12 - 800 ng

B. EZBlue dual stain for total protein detection

The PhosDecor staining procedure allows linear detection of phosphoproteins over a wide range (see Figure 4).

Figure 4.



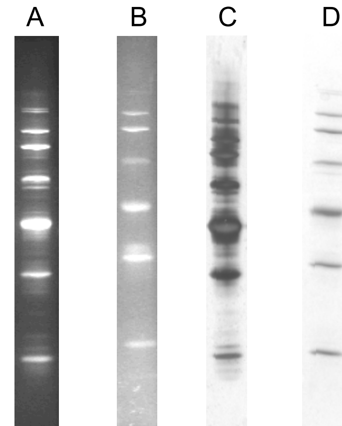
Staining of β-casein ranging from 3.13 ng to 200 ng using a UV transilluminator.

Sensitivity (Staining of Low Abundance Proteins)
Some complex samples may contain small quantities of proteins that fall below the dynamic range of the assay and are not detected using the described procedure for phosphoprotein staining. To detect these low abundance proteins that could not be detected against the background staining in the gel (less than 1 ng depending on the phosphorylation state), two additional 30 minute wash steps with Destaining Solution may be used (Procedure, step 7).

Note: The **additional destaining steps may result in significant loss in selectivity**. Therefore, the steps should only be used **after an optimized image showing selectivity is recorded**. To selectively stain low abundance proteins identified with the additional destaining steps, another gel should be run loading a larger amount of sample (e.g., ~500 ng of protein).

Compatibility (Total Protein Staining)
The PhosDecor Stain is known to be compatible with the following total protein stains: SYPRO Ruby, SYPRO Orange, ProteoSilver Silver Stain, and EZBlue Gel Staining Reagent (see Figure 5). If recording fluorescence detection for phosphoproteins, the measurements can be corrected for mass differences using total protein stains.

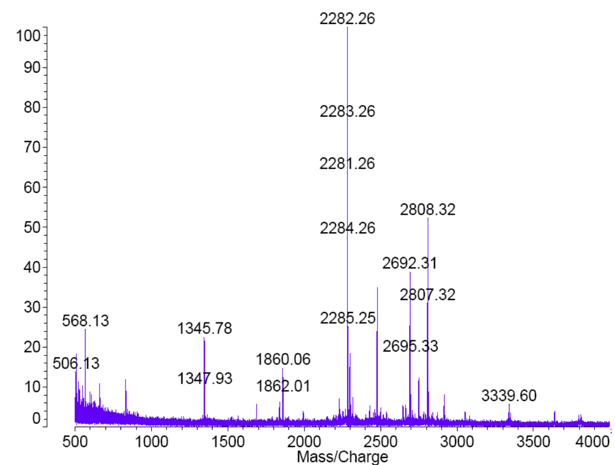
Figure 5
PhosDecor Control was PhosDecor stained, visualized on a UV transilluminator, and then dual stained.



A. SYPRO Ruby
B. SYPRO Orange
C. ProteoSilver Silver Stain
D. EZBlue Gel Staining Reagent.

Compatibility (Mass Spectrometry)
Following phosphoprotein staining, protein spots or bands can be digested using the Trypsin Profile IGD Kit (Catalog Number PP0100) and prepared for downstream analysis such as MALDI-MS.

Figure 6.
MALDI-MS spectra of ovalbumin stained with PhosDecor Stain.



For accurate detection of phosphorylated peptides, digested proteins may need to be enriched prior to mass spectrometry (i.e., PhosphoProfile™ I Phosphopeptide Enrichment Kit, Catalog Number PP0410).[5]

Troubleshooting Guide for PhosDecor Fluorescent Phosphoprotein In-Gel Detection Kit

Problem	Causes	Solution
Proteins without phosphates are stained.	Stain was exposed to excessive light.	Keep stain and stained gel protected from light.
	Imaging of gel was not optimized.	Adjust and optimize exposure time and grayscale to obtain maximum signal/noise ratio using PhosDecor Control as described in procedure.
	Sample was not delipidized.	Delipidize sample according to procedure (Appedix II, Sample Preparation)
	SDS not removed before staining.	Include an extra or overnight fixing step. This may be necessary with long shelf-life gels.
Extremely high background	Exposure time is too long.	Decrease exposure time. Record images at multiple exposure times if possible.
	SDS not removed from gels before staining.	Include an extra or overnight fixing step. This may be necessary with long shelf-life gels.
	Fixing solution not sufficiently removed.	Make sure three water washes are done before adding stain. Include an extra 10 minute water wash before staining.
	Stain not removed completely before adding Destaining Solution.	Decant stain completely before addition of Destaining Solution. Alternatively, a fresh staining tray may be used.
	Background may need to be subtracted.	Use background subtraction tool on gel imaging system or adjust grayscale to reduce background using PhosDecor Control.
Speckles, fingerprints, streaks, or curling of gel causing bright regions in image.	Dust	Avoid getting dust into solutions or on gel.
	Other contaminants on imaging system surface	Clean surface of imaging system with alcohol and water
	Handling gels with powdered gloves or dirty gloves.	Always use clean powder–free gloves washed with ultrapure water before handling gel. Handling of gel should be minimized.
	Insufficient shaking or uneven staining tray	Use an orbital shaker and make sure staining tray is level to ensure the gel is being evenly washed with stain.
	Drying of gel	Apply a thin layer of ultrapure water between the surface of the imaging system and the stained gel to hydrate the gel while imaging.

Appendix I
PhosDecor Control

Product Number **P7499**

Product Description

It is believed that one-third of all proteins in a eukaryotic cell are phosphorylated at any one time.[6] The identification of phosphorylated proteins is necessary for understanding how a particular cellular process is regulated by post-translational modifications (PTMs).

The PhosDecor Control contains six proteins (see Table 2), two of which are phosphorylated, providing a set of positive and negative controls for use with PhosDecor Fluorescent Phosphoprotein In-Gel Detection Kit.

Table 2.
Contents of the PhosDecor Control

Protein	Molecular Mass (kDa)	Phosphorylation Sites*
β-galactosidase	116	None
Phosphorylase b	97	None
Albumin	66	None
Ovalbumin	45	2
β-casein	30	5
Lysozyme	15	None

*Potential phosphorylation sites. Not all sites may be phosphorylated.

The product is supplied as a lyophilized pellet. When reconstituted as directed, the resulting solution contains six proteins with approximate concentrations of 250 μg/ml, which produce SDS-PAGE bands with approximately equal total protein staining intensities. This control is not intended for use in sample quantitation.

Preparation Instructions

Reconstitute the PhosDecor Control with 40 μl of ultrapure water prior to SDS-PAGE. The reconstituted protein solution contains 62 mM Tris-HCl, pH 7.0, 1 mM EDTA, 4% sucrose, 0.5% dithiothreitol, 2% SDS, and 0.005% bromophenol blue.

The above preparation of the PhosDecor Control is highly recommended. However, the reconstituted control may be diluted further to match the approximate concentration of sample, if the sample is limited.

Load 2 μl of the reconstituted PhosDecor Control per well, using at least one lane of the SDS-polyacrylamide gel. **No heating** is required before loading. If the control was diluted further, greater volumes may be loaded to accurately match the amount of sample loaded. The control and unknown sample(s) should be resolved according to the standard electrophoresis procedure for gel type used.

Appendix II
Sample Preparation
Some protein samples may contain associated phospholipids, especially samples containing membrane proteins or carriers such as albumin.[4] Samples may need to be delipidized and desalted prior to electrophoresis for optimal PhosDecor staining results.
1. Add four volumes of methanol to one volume of sample and mix well by vortexing.
2. Add one volume of chloroform per volume of original sample and mix well by vortexing
3. Add 3 volumes of ultrapure water per volume of original sample and mix well by vortexing.
4. Centrifuge at 8,400 × *g* for 10 minutes.
5. Discard the upper aqueous phase keeping the white precipitate that forms between the upper and lower phases.
6. Add 3 volumes of methanol per volume of original sample and mix well by vortexing.
7. Centrifuge at 8,400 × *g* for 10 minutes.
8. Discard the supernatant and dry the pellet in a vacuum centrifuge for 10 minutes.
9. Resuspend the sample in 1× Sample Buffer (Catalog Number S3401), or water if determining concentration first.

References:
1. Zhang, H., *et. al*., J. Biol. Chem., **277**, 39379-39387 (2002).
2. Hu, J., *et. al*., Nucleic Acids Res., **33**, 3271-3282 (2005).
3. Yeargin, J., and Hass, M., Curr. Biol., **5**, 423-431 (1995).
4. Zhu, H., *et. al*., Science, **293**, 2101-2105 (2001).
5. Kalume, D., *et. al.,* Curr. Opin. Chem. Bio., **7**, 64-69 (2003).
6. Zolnierowicz, S., and Bollen, M., EMBO J., **19**, 483-488 (2000).

PhosDecor, EZBlue, PhosphoProfile, and ProteoSilver are trademarks of Sigma-Aldrich™ Biotechnology LP and Sigma-Aldrich Co.
SYPRO is a registered trademark of Molecular Probes, Inc.
NuPAGE is a registered trademark of Invitrogen Corporation.

DKS,GL,JT,MAM 03/07-1

Sigma brand products are sold through Sigma-Aldrich, Inc.
Sigma-Aldrich, Inc. warrants that its products conform to the information contained in this and other Sigma-Aldrich publications. Purchaser must determine the suitability of the product(s) for their particular use. Additional terms and conditions may apply. Please see reverse side of the invoice or packing slip.