Pro-Q® Diamond Phosphoprotein Blot Stain Kit (P33356)

Quick Facts

Storage upon receipt:

P33381 Pro-Q® Diamond Blot Stain Reagent for P33356
- ≤–20°C
- Protect from light

P33382 Pro-Q® Diamond Blot Stain Buffer for P33356
- 2–25°C
- DO NOT FREEZE

Ex/Em: 555/580 nm

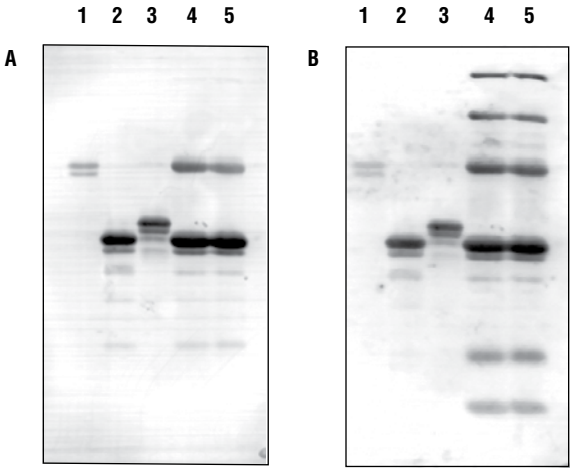

Figure 1. Selectivity of Pro-Q® Diamond phosphoprotein blot stain. A polyacrylamide gel containing various proteins was electroblotted to a PVDF membrane, stained with Pro-Q® Diamond phosphoprotein blot stain (Panel A) and subsequently stained with SYPRO® Ruby protein blot stain (Panel B). Lane 1, pepsin (one phosphate residue/protein); lane 2, β-casein (five phosphate residues/protein); lane 3, α-casein (eight phosphate residues/protein); lanes 4 and 5, PeppermintStick™ phosphoprotein molecular weight standards (P-33350), a set of molecular weight standards containing β-galactosidase, bovine serum albumin, ovalbumin (two phosphate residues/protein), β-casein (five phosphate residues/protein), avidin and lysozyme.

Introduction

Molecular Probes® fluorescent Pro-Q® Diamond Phosphoprotein Blot Stain Kit provides a rapid and simple method for directly detecting phosphoproteins on polyvinylidene difluoride (PVDF) (Figure 1) or nitrocellulose membranes (blots). Compared with traditional approaches, Pro-Q® Diamond dye offers significant advantages:

- No radioisotopes, no radioactive waste
- No expensive antibodies
- No blocking steps required
- Detection of phosphorylated amino acid residues independent of the sequence context
- Analysis of the native phosphorylation state of proteins obtained from any source, e.g., from tissue specimens or from body fluids.

The Pro-Q® Diamond phosphoprotein stain binds directly and selectively to the phosphate moiety, allowing the stain to detect the broadest spectrum of phosphorylated proteins possible. Phosphoserine-, phosphothreonine- and phosphotyrosine-containing proteins are all detectable with the stain. Protein samples are separated by one-dimensional or two-dimensional gel electrophoresis, electroblotted to the membrane, stained and destained, in an analogous manner as typically performed with Amido Black or Ponceau S staining of total protein profiles on membranes. After staining, gels are simply imaged using any of a variety of laser-based gel scanners, xenon–arc lamp–based gel scanners or CCD camera–based imaging devices employing UV transilluminators. The uncomplicated and reliable staining protocol delivers results in as little as 1 hour. The limits of detection for the stain on PVDF membrane blots is typically 8–16 ng of phosphoprotein with a linear dynamic range of approximately 15-fold. Sensitivity will be decreased when using nitrocellulose membranes.

The Pro-Q® Diamond phosphoprotein blot stain binds noncovalently to phosphoproteins and is thus fully compatible with matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS) or Edman sequencing. Furthermore, the Pro-Q® Diamond phosphoprotein blot stain is compatible with the standard colorimetric, fluorogenic and chemiluminescent detection techniques employed in immunoblotting. The phosphoprotein blot stain may be used in conjunction with SYPRO® Ruby protein blot stain (S11791), a total-protein stain that is quantitative over two orders of magnitude on blots. Using both stains in combination makes it possible to distinguish a lightly phosphorylated, high-abundance protein from a heavily phosphorylated, low-abundance phosphoprotein.

Materials

Kit Contents

The Pro-Q® Diamond Phosphoprotein Blot Stain Kit (P33356) consists of three parts:

P33381 Pro-Q® Diamond Blot Stain Reagent, 500 µL
P33382 Pro-Q® Diamond Blot Stain Buffer, 500 mL
P33383 Product Info Sheets for P33356

The reagent volume is sufficient for staining ~20 minigel electroblots.

Storage and Handling

Upon receipt, store the Pro-Q® Diamond blot stain reagent at ≤–20°C, protected from light. Store the Pro-Q® Diamond blot stain buffer at room temperature. When stored properly, the individual components are stable for at least 6 months.

Protocol

Preparation of Stock Solutions

1.1 Fix Solution (7% acetic acid solution/10% methanol). Add 7 mL of concentrated acetic acid to 80 mL deionized water (dH_2O); add 10 mL of methanol; bring the volume up to 100 mL with dH_2O; and mix thoroughly.

1.2 Pro-Q® Diamond phosphoprotein blot stain. Prepare this by diluting the Pro-Q® Diamond blot stain reagent 1000-fold in the Pro-Q® Diamond blot stain buffer. For example, add 25 µL of the reagent to 25 mL of the buffer for a 6 cm × 9 cm blot. The volume of stain prepared can be adjusted for different sized blots. This working solution of the Pro-Q® Diamond phosphoprotein blot stain can be stored at 2–6°C for up to 2 weeks.

1.3 Destain Solution for PVDF membranes (50 mM sodium acetate, pH 4.0/20% acetonitrile). Mix 50 mL of a 1 M sodium acetate, pH 4.0, stock solution with 200 mL of acetonitrile; bring the volume up to 1 L with dH_2O; and mix well. To prepare the 1 M sodium acetate stock solution, dissolve 8.2 grams of anhydrous sodium acetate in 80 mL of dH_2O; adjust the pH to 4.0 with concentrated HCl; and bring the volume up to 100 mL.

1.4 Destain Solution for nitrocellulose membranes. For optimal results, purchase Molecular Probes® Pro-Q® Diamond phosphoprotein gel destaining solution (P33310, P33311).

Sample Preparation and Electrophoresis

2.1 Clean the sample. A delipidated and desalted sample is essential for adequate separation of proteins by electrophoresis and subsequent staining by Pro-Q® Diamond phosphoprotein blot stain. For a 150 µL sample containing ~150–300 µg of protein, perform the following cleanup procedure:

- Add 600 µL of methanol and mix well by vortexing.
- Add 150 µL of chloroform and mix well by vortexing.
- Add 450 µL of dH_2O and mix well by vortexing.
- Centrifuge at ~12,000 rpm for 5 minutes.
- Discard the supernatant, and dry the pellet in a vacuum centrifuge for 10 minutes.
- Resuspend the pellet in standard 1X sample buffer for electrophoresis.

2.2 Perform the electrophoresis and electroblotting. Separate the proteins using standard polyacrylamide gel electrophoresis techniques. To ensure detection of less abundant phosphoproteins, use approximately the same mass of proteins that you would use for a typical Coomassie® brilliant blue dye–stained gel. Electroblot proteins onto a PVDF or nitrocellulose membrane by standard procedures. After electroblotting the proteins, allow the membrane to dry completely.

Table 1. Examples of commercially available phosphorylated and nonphoshorylated proteins for use as control proteins.

Protein	Molecular weight (Daltons)	Number of phosphate residues	Lower limit of detection
α-casein	24,500	8	8 ng
β-casein	23,600	5	8 ng
ovalbumin	45,000	2	16 ng
pepsin	35,500	1	16–32 ng
carbonic anhydrase	30,000	0	NA
bovine serum albumin (BSA)	66,000	0	NA

NA = Not applicable.

Staining Procedure

Before beginning, please note the following tips for success:

- Perform all fixing, washing, staining and destaining steps by immersing the membrane face down in the solution and use gentle agitation (e.g., incubate on an orbital shaker at 50 rpm).
- Use a plastic container or a large plastic weighing dish. If using a reusable plastic container, clean the container thoroughly and rinse it with 70% ethanol before use.
- Adhere strictly to the volumes and times specified. Replicating the protocol is essential for consistent blot-to-blot reproducibility.
- For controls, use known phosphorylated and nonphosphorylated proteins to help verify the phosphorylation status of the unknown proteins. Table 1 lists several commonly available proteins that can serve as positive and negative controls.
- After obtaining results with the Pro-Q® Diamond phosphoprotein blot stain, stain the blot with a quantitative total-protein stain, such as SYPRO® Ruby protein blot stain, in order to ascertain the relative phosphorylation state of proteins. In this way, an abundant nonphosphorylated protein that exhibits low nonspecific staining with Pro-Q® Diamond phosphoprotein blot stain can be distinguished from a less-abundant highly phosphorylated protein. Refer to *Staining the Blot for Total Protein* (below).

3.1 Pre-wet PVDF membranes in methanol. If using a PVDF membrane, dip the membrane in methanol, and allow the excess to drip off. DO NOT pre-wet nitrocellulose membranes.

3.2 Fix the proteins on the membrane. Immerse the membrane face down in 25 mL of Fix Solution (7% acetic acid/10% methanol, prepared in step 1.1) and incubate for 10 minutes.

3.3 Wash the membrane. Immerse the membrane in about 25 mL of dH_2O for about 5 minutes. Repeat three times for a total of four washes.

3.4 Stain the proteins. Immerse the membrane in 25 mL of Pro-Q® Diamond phosphoprotein blot stain (prepared in step 1.2) for 15 minutes. The membrane will be visibly pink at this point.

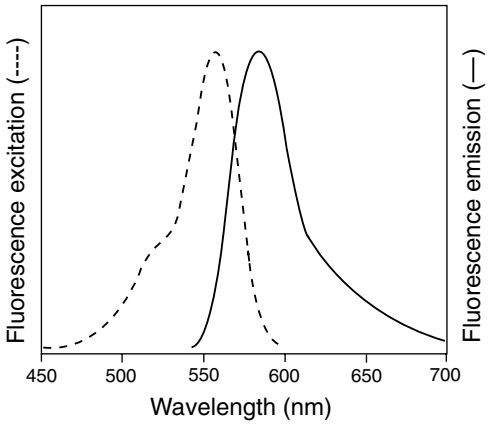

Figure 2. Excitation (dashed line) and emission (solid line) spectra of the Pro-Q Diamond phosphoprotein blot stain.

3.5 Destain the membrane. Wash the membrane in 30 mL of the appropriate Destain Solution (see steps 1.2 and 1.3) for 5 minutes. Repeat two times for a total of three times. This wash serves to remove excess dye from the membrane. Perform this step with care, as longer washes can reduce the fluorescence of the phosphoprotein stain. High abundance phosphoproteins may be detectable by eye at this point as pink bands.

3.6 Wash nitrocellulose membranes. Immerse nitrocellulose membranes in 25 mL of dH_2O or 50 mM sodium acetate, pH 4.0, for 5 minutes. Repeat two times for a total of three washes. This wash step is **not** necessary for PVDF membranes.

3.7 Dry the membrane. After staining, do not touch the wet membrane, since contaminants, such as the residue found on latex gloves, can destroy the staining pattern. Use forceps to handle the wet blot. Once dry, the membrane can be handled freely. A blot treated with the Pro-Q® Diamond phosphoprotein blot stain is best preserved by allowing the membrane to air dry completely.

Viewing and Photographing the Blot

The Pro-Q® Diamond phosphoprotein blot stain has an excitation maximum at ~555 nm and an emission maximum at ~580 nm (Figure 2). Imaging instruments with light sources and filters that match the excitation and emission maxima will result in the highest sensitivity.

4.1 Imaging with visible-light–based scanners. The stained blot is best visualized using excitation at 532–560 nm, such as that provided with a visible-light laser–based or xenon arc lamp–based gel scanning instrument. For most instruments, a 550 to 600 nm longpass or a 580 to 600 nm bandpass emission filter is recommended.

4.2 Imaging with UV epi- or trans-illuminators. The blot stained with Pro-Q® Diamond phosphoprotein blot stain can also be visualized using UV illumination. The front face of the membrane can be illuminated using a hand-held, UV-B (~300 nm) light source. Alternatively, a UV light box can be placed on its side to illuminate the blot, or a top-illuminating system can be used to visualize the stain. Satisfactory results are also obtained from direct trans-illumination through the blotting membrane. In all cases, the use of a photographic camera or CCD camera and the appropriate filters is essential to obtain the greatest sensitivity. The instrument's integrating capability can make bands detectable that cannot be seen by eye. Images can be documented using either conventional or digital photography. With a Polaroid® camera and Polaroid 667 black-and-white film, use an appropriate longpass filter, such as the SYPRO® photographic filter (S-6656) and exposure times of about 2–10 seconds; the red-orange filters typically used to photograph gels stained with ethidium bromide will not work well. For digital cameras, use a filter that corresponds closely to the emission characteristics of the stain, such as a 600 nm bandpass filter.

Staining the Blot for Total Protein

After staining with Pro-Q® Diamond phosphoprotein blot stain and documenting the results, the blot can be stained for total protein using SYPRO® Ruby protein blot stain (S-11791). It is important to document the Pro-Q® Diamond blot staining result before staining for total protein, as the Pro-Q® Diamond phosphoprotein stain is removed or obscured during the total-protein staining steps.

Before proceeding with the staining for total protein, re-fix the dried blot in 45% methanol/5% acetic acid for 10 minutes, and then wash the blot three times in dH_2O, for 5 minutes each time. Next, stain the blot with 20 mL of SYPRO® Ruby protein blot stain for 15 minutes, followed by three washes in dH_2O, for 5 minutes each. Air dry the blot, and image it as described in the information supplied with the SYPRO® Ruby protein blot stain.

Probing the Blot with Antibodies

If the blot is to be further used in antibody probing studies following staining with Pro-Q® Diamond phosphoprotein blot stain, SYPRO® Ruby protein blot stain, or both, it will be necessary to prepare the membrane for this purpose.

5.1 Wet the blot. Following documentation the fluorescence signal from the membrane, it is necessary to rewet the dried blot. If the membrane is PVDF, dip the membrane briefly in 100% methanol, then proceed to step 5.2. **If the membrane is nitrocellulose, proceed directly to step 5.2. Do not place nitrocellulose membranes in 100% methanol.**

5.2 Wash the membrane. Immerse the membrane in about 25 mL of PBS for 15 minutes. Repeat this step in fresh PBS for a total of three washes.

5.3 Block nonspecific binding sites on the blot. Incubate the blot in 25 mL of Blocking Buffer (described below) at room temperature for 1–2 hours.

After washing the membrane, proceed with your normal antibody staining protocol. The following blocking buffer is recommended.

- Blocking Buffer (50 mM Tris, 150 mM NaCl, 0.2% Tween® 20, 0.25% Mowiol® 4-88, 0.5% bovine serum album (BSA), pH 7.5)

Note: The use of Mowiol 4-88 in the Blocking Buffer is not essential. However, we have found that Mowiol results in decreased background staining and improves the sensitivity of detection. Mowiol can be purchased from Calbiochem (catalog #475904) or from VWR (catalog #80058-440). As an alternative to 0.5% BSA, 4% gelatin (high purity, e.g. Top-Block™ from Juro Supply Ag, catalog #TB232010) may be used.

Product List *Current prices may be obtained from our Web site or from our Customer Service Department.*

Cat #	Product Name	Unit Size
P33356	Pro-Q® Diamond Phosphoprotein Blot Stain Kit *20 minigel blots*	1 kit
P33310	Pro-Q® Diamond phosphoprotein gel destaining solution *new formulation*	1 L
P33311	Pro-Q® Diamond phosphoprotein gel destaining solution *new formulation* *bulk packaging*	5 L
P33350	PeppermintStick™ phosphoprotein molecular weight standards *200 gel lanes*	400 µL
S11791	SYPRO® Ruby protein blot stain *10-40 blots*	200 mL

Contact Information

Molecular Probes, Inc.
29851 Willow Creek Road
Eugene, OR 97402
Phone: (541) 465-8300
Fax: (541) 335-0504

Customer Service:
6:00 am to 4:30 pm (Pacific Time)
Phone: (541) 335-0338
Fax: (541) 335-0305
probesorder@invitrogen.com

Toll-Free Ordering for USA:
Order Phone: (800) 438-2209
Order Fax: (800) 438-0228

Technical Service:
8:00 am to 4:00 pm (Pacific Time)
Phone: (541) 335-0353
Toll-Free (800) 438-2209
Fax: (541) 335-0238
probestech@invitrogen.com

Invitrogen European Headquarters
Invitrogen, Ltd.
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF, UK
Phone: +44 (0) 141 814 6100
Fax: +44 (0) 141 814 6260
Email: euroinfo@invitrogen.com
Technical Services: eurotech@invitrogen.com

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