

DyeChrome™ Western Blot Stain Kits

with DDAO phosphate and BODIPY® FL-X, SE

D-21881 Kit #1, with goat anti-mouse IgG

D-21882 Kit #2, with goat anti-rabbit IgG

D-21883 Kit #3, with streptavidin

Quick Facts

Storage upon receipt:

- $\leq -20^{\circ}\text{C}$
- Desiccate
- Protect from light

Abs/Em:

- 365, 505/515 nm for BODIPY FL-X
- 275, 646/659 nm for DDAO

Number of assays: 20 minigel blots

Note: New and improved BODIPY FL-X staining procedure, beginning 25–October–2001

the mobility of proteins in the gel. You can visualize the BODIPY FL-X dye using UV illumination or a laser scanner.

For immunostaining, DyeChrome Western Blot Stain Kits #1–#3 include one of our high-quality alkaline phosphatase antibody or streptavidin conjugates and the fluorogenic alkaline phosphatase substrate, DDAO phosphate. DDAO phosphate is a remarkable reagent that provides high sensitivity detection of alkaline phosphatase conjugates in minutes. The signal-amplification effect of the enzymatic reaction allows detection of as little as 8 ng of protein per band, depending on the antibodies used. The sensitivity of this technique rivals that of chemiluminescence detection, but because DDAO phosphate detection results in a stable fluorescent product, there is no need to perform the reaction in a dark-room or to incubate the blot with X-ray film. Furthermore, the fluorescent signal, unlike transient chemiluminescent signals, can be imaged several times and is stable indefinitely on dried blots. The fluorescent signal can be visualized using UV or visible-light illumination and is easily separated from the signal of BODIPY FL-X dye for documentation and analysis.

Introduction

The DyeChrome™ Western Blot Stain Kits provide a method for two-color staining of Western blots by combining a fluorescent total-protein stain with a fluorescence-based immunostaining technique.¹ The dual-staining method eliminates guesswork about protein transfer efficiencies and ambiguities inherent in comparing proteins on “duplicate” blots. Two-color staining is particularly useful in blots of 2-D gels, where it simplifies the localization of immunostained proteins in the complex spot pattern. The two stains can be seen simultaneously under UV illumination and can be documented separately using appropriate excitation light sources and emission filters.

DyeChrome Kits #1–#3 employ the green-fluorescent BODIPY FL-X succinimidyl ester (BODIPY FL-X, SE) as a total-protein stain. The reactive dye forms a permanent, covalent bond with N-terminal amino groups or with ϵ -amino groups of lysine residues. Dye molecules remain bound through subsequent immunostaining and do not interfere with antibody recognition and binding.¹ (Note however, that because the protein is chemically altered, this method is not suitable for use with Edman sequencing or mass spectrometry.) This innovative blot staining technique is rapid and highly sensitive, permitting the detection of as little as 4 ng of protein per band in about 90 minutes. Because the dye is used after electrophoresis, there is no effect on

Materials

Contents

- **Goat anti-mouse IgG (H+L), alkaline phosphatase conjugate** (Component A in Kit #1), 200 μg
- **Goat anti-rabbit IgG (H+L), alkaline phosphatase conjugate** (Component A in Kit #2), 200 μg
- **Streptavidin, alkaline phosphatase conjugate** (Component A in Kit #3), 100 μg
- **DDAO phosphate** (Component B), 250 μg
- **Dimethylformamide (DMF, Component C)**, 2 mL
- **BODIPY FL-X, SE** (MW = 502.3, Component D), 10 vials, each containing 200 μg

The goat anti-mouse IgG conjugate (in Kit #1) detects primary antibodies raised in mice, including mouse monoclonal antibodies; it has been cross-adsorbed against human IgG and serum. The goat anti-rabbit IgG antibody conjugate (in Kit #2) detects primary antibodies raised in rabbits; it has been cross-adsorbed against human IgG and serum, mouse IgG and serum and bovine serum. The streptavidin conjugate (in Kit #3) detects biotinylated antibodies.

Sufficient materials are supplied to stain approximately twenty 8 cm \times 10 cm minigel blots.

Storage Conditions

Upon receipt, store the kit at $\leq -20^{\circ}\text{C}$, desiccated and protected from light. When stored properly, the kit components should be stable for at least 6 months.

Materials Required but Not Provided

- Polystyrene staining dish
- Methanol
- Primary antibody for the detection of a specific protein
- Biotinylated primary or secondary antibody (for Kit #3, with streptavidin–alkaline phosphatase)
- Sodium borate buffer (see step 1.1)
- Immunostaining Wash Buffer (see step 2.1)
- Immunostaining Blocking Buffer (see step 2.1)
- DDAO phosphate dilution buffer (see step 3.2)

Preparation of the Blot

General Considerations

For best results and to minimize detection of contaminating alkaline phosphatase from bacteria, clean and sterilize all containers and use sterile solutions, pipet tips and microcentrifuge tubes. Use powder-free gloves when handling gels and blots to prevent background fluorescence from fingerprints or glove powder.

Gel Electrophoresis and Blotting

Prepare samples for SDS-polyacrylamide gel electrophoresis using standard procedures. After separation of the proteins by electrophoresis, transfer the proteins onto a PVDF membrane using standard procedures. The staining methods used in these kits are not compatible with nitrocellulose membranes.

Staining Total Protein with BODIPY FL-X, SE

The following staining protocol is optimized for staining one 8 cm \times 10 cm minigel blot. Larger blots require proportionally larger volumes. We recommend performing all wash and incubation steps in plastic weigh boats with continuous, gentle agitation (e.g., on an orbital shaker at 50 rpm). Do not use glass containers.

1.1 Prepare sodium borate buffer. Make up a solution of 10 mM sodium borate, pH 9.5. Approximately 300 mL of sodium borate buffer will be required for an 8 cm \times 10 cm blot.

1.2 Equilibrate the blot in sodium borate buffer. Incubate the blot in 100 mL of sodium borate buffer (made in 1.1) at room temperature for 10 minutes without agitation. Repeat this step once.

1.3 Prepare the BODIPY stock solution. Warm one vial of BODIPY FL-X, SE (Component D) completely to room temperature before opening. Add 40 μL of DMF (Component C) to the vial and vortex to dissolve the dye. Each vial contains enough reactive BODIPY dye to stain two minigel blots. Once the solution is made, it remains stable for approximately two weeks when stored at -20°C .

1.4 Prepare fresh BODIPY staining solution. Dilute 20 μL of the BODIPY stock solution (made in step 1.3) in 20 mL of sodium borate buffer (made in step 1.1). Approximately 20 mL

will be required for one minigel blot. Once prepared, the staining solution must be used immediately, as the reactive dye is not stable in aqueous solution.

1.5 Stain the blot with BODIPY staining solution. Remove the sodium borate buffer that was used to equilibrate the blot (step 1.2) and add 20 mL of the BODIPY staining solution (made in 1.4) to the blot. Incubate at room temperature for 30 minutes.

1.6 Wash the blot. Remove the BODIPY staining solution and wash the blot in 25 mL of sodium borate buffer for 2 minutes. Repeat this wash. Then, wash the blot in 25 mL of 100% methanol for 10 minutes. Repeat the methanol wash twice for a total of three washes. Rinse the blot briefly in water and allow it to dry. The staining may be visualized at this time, or you may proceed directly to immunostaining.

Incubation with Antibodies and Streptavidin

There are two basic procedures to detect a specific protein using antibodies and the alkaline phosphatase enzyme. For both procedures, the blot is first incubated with a primary antibody that recognizes the specific protein of interest. Kits #1 and #2 use a procedure in which the blot is then incubated with a secondary antibody conjugated to alkaline phosphatase (Component A), either goat anti–mouse IgG, for the detection of primary antibodies raised in mice, or goat anti–rabbit IgG, for the detection of primary antibodies raised in rabbits. The alkaline phosphatase activity is then visualized using the fluorogenic substrate, DDAO phosphate. Kit #3 uses an alternate procedure employing either a biotinylated primary antibody in the first step, or else a biotinylated secondary antibody (not included in the kit) to detect an unlabeled primary antibody. Next, the blot is incubated with streptavidin conjugated to alkaline phosphatase (Component A). As before, the alkaline phosphatase activity is then visualized using DDAO phosphate. Here, we outline the procedures for the antibody and streptavidin incubations. Optimization of buffers, blocking agents and antibody concentrations may be necessary for your experiment.

Perform all incubation and wash steps in a plastic dish with gentle agitation. We recommend using plastic weigh boats or RubberMaid Servin' Saver™ dishes. Do not use glass containers. For optimal fluid dynamics and mixing, use ~ 25 mL of buffer for wash steps and ~ 10 mL of antibody or streptavidin solutions for incubation steps, for an 8 cm \times 10 cm blot.

2.1 Prepare buffers. The following buffers should be prepared in advance:

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

Approximately 125 mL of Immunostaining Wash Buffer and 300 mL of Immunostaining Blocking Buffer will be required for an 8 cm \times 10 cm blot. **Note:** The use of Mowiol 4-88 in the Immunostaining 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.

2.2 Prepare stock solutions of the protein conjugates. For Kits #1 and #2, prepare a 2 mg/mL stock solution of the antibody conjugate (Component A) by dissolving the contents of the vial in 100 µL of dH₂O plus 2 mM sodium azide as a preservative. For Kit #3, prepare a 2 mg/mL stock solution of the streptavidin conjugate (Component A) by dissolving the contents of the vial in 50 µL of dH₂O plus 2 mM sodium azide. The stock solutions are stable for at least six months, when stored undiluted at 4°C. DO NOT FREEZE.

2.3 Prewash the blot. After staining with BODIPY FL-X, SE (steps 1.1–1.6), wash the blot in 25 mL of Immunostaining Wash Buffer (prepared in step 2.1) at room temperature for 10 minutes. Repeat the wash step for a total of three washes.

2.4 Block nonspecific binding sites on the blot. Incubate the blot in 25 mL of Immunostaining Blocking Buffer (prepared in step 2.1) at room temperature for 1–2 hours, or until the blot is completely wet.

2.5 Incubate the blot with the primary antibody. Dilute the primary antibody into ~10 mL of Immunostaining Blocking Buffer. The optimal concentration of the primary antibody to use must be determined empirically for each antibody. Remove the Immunostaining Blocking Buffer that the blot is soaking in and incubate the blot with the diluted primary antibody at room temperature for 1 hour or overnight. If incubating overnight, add sodium azide to a final concentration of 2 mM to prevent bacterial growth.

2.6 Wash the blot. Remove the solution of primary antibody and wash the blot in 25 mL of Immunostaining Blocking Buffer at room temperature for 10 minutes. Repeat the wash step for a total of three washes. For Kit #3, if using a biotinylated primary antibody, proceed to step 2.9; if using an unlabeled primary antibody and a biotinylated secondary antibody, proceed to step 2.7.

2.7 Incubate the blot with the secondary antibody conjugate. Centrifuge the secondary antibody stock solution (the reconstituted Component A prepared in step 2.2 for Kits #1 and #2 or your own biotinylated secondary antibody for Kit #3) briefly in a microcentrifuge to pellet any protein aggregates that may have formed. Using the supernatant only, dilute the secondary antibody stock solution into ~10 mL of Immunostaining Blocking Buffer. The optimal concentration to use must be determined for each combination of primary and secondary antibodies. A good starting point is a final concentration of 0.2 µg/mL (or a 10,000-fold dilution of the 2 mg/mL stock solution). Incubate the blot with the diluted secondary antibody at room temperature for 1 hour.

2.8 Wash the blot. Remove the solution of secondary antibody and wash the blot in 25 mL of Immunostaining Blocking Buffer at room temperature for 10 minutes. Repeat the wash step for a total of three washes. For Kits #1 and #2, proceed to step 2.11. For Kit #3, proceed to step 2.9.

2.9 Incubate the blot with the streptavidin conjugate. For Kit #3, centrifuge the streptavidin, alkaline phosphatase stock solution (reconstituted Component A, prepared in step 2.2) briefly in a microcentrifuge to pellet any protein aggregates that may

have formed. Using the supernatant only, dilute the streptavidin–alkaline phosphatase conjugate 40,000-fold into ~10 mL of Immunostaining Blocking Buffer. Incubate the blot with the diluted streptavidin–alkaline phosphatase conjugate at room temperature for 1 hour. The optimal concentration to use may have to be determined empirically.

2.10 Wash the blot. Remove the solution of streptavidin–alkaline phosphatase and wash the blot in Immunostaining Blocking Buffer at room temperature for 10 minutes each. Repeat the wash step for a total of three washes.

2.11 Prepare the blot for staining. Perform two final washes in Immunostaining Wash Buffer at room temperature for 5 minutes each. Proceed to *DDAO Phosphate Detection Procedure*.

DDAO Phosphate Detection Procedure

Staining Procedure

The product of the DDAO phosphate reaction with alkaline phosphatase is the red-fluorescent DDAO. Because the product does not adhere to the blotting membrane, it can diffuse from the site of the reaction. Therefore, it is important to minimize the amount of solution in which the reaction takes place and to avoid washing the blot after the reaction is complete.

3.1 Prepare DDAO phosphate stock solution. Add 200 µL of DMF (Component C) to the vial containing DDAO phosphate (Component B) to make a 1.25 mg/mL stock solution. The solution will be an amber color. Store the stock solution at -20°C, desiccated and protected from light. When properly stored, the stock solution should be stable for at least 6 months. When the solution turns a blue color, the substrate has broken down and is no longer usable.

3.2 Make fresh DDAO phosphate staining solution. Dilute the DDAO phosphate stock solution (made in step 3.1) 1000-fold into 10 mM Tris, 1 mM MgCl₂, pH 9.5, for a final concentration of 1.25 µg/mL. Approximately 1 mL of the DDAO phosphate staining solution will be needed for an 8 cm × 10 cm blot. Note that DDAO phosphate is unstable when stored at room temperature as an aqueous solution. For consistent results, we recommend making up the DDAO phosphate staining solution just prior to use.

3.3 Prepare and stain the blot. The staining step may be performed with the blot face up or face down, depending on the configuration of the imaging instrumentation used:

- **Face-up staining:** If using UV epi-illumination or a laser scanner with a light source that illuminates from above the bed, stain the blot face up. Using powder-free gloves, cut a piece of plastic wrap to the size of the blot. Place the blot on the plastic wrap and pipet 1 mL of the staining solution (prepared in step 3.2) onto the blot. Make certain that the blot is coated evenly with the staining solution.
- **Face-down staining:** If using a laser scanner with a light source that illuminates the blot from below a glass bed, stain the blot face down. Using powder-free gloves, cut a piece of plastic wrap to the size of the blot. Pipet 1 mL of the staining solution (prepared in step 3.2) onto the plastic wrap and lay

the blot face down onto the solution, being careful not to trap any air bubbles. Alternatively, because the plastic wrap can sometimes interfere with imaging, pipet the staining solution directly into the imaging bed of the instrument and lay the blot face down onto the solution. The imaging bed may be cleaned afterward with dH₂O followed by ethanol.

3.4 Incubate the blot in the staining solution. Generally, incubation for 5–20 minutes is sufficient. However, the time required for optimal staining must be determined empirically, because the substrate turnover rate depends on the amount of protein on the blot and the quality and quantity of the antibodies used. To find the optimal staining time, visualize the blot immediately after exposing it to the staining solution and again every 15 minutes for up to an hour, then visualize the blot every hour after that. The blot may be left to incubate overnight, but once it is dry, it is unlikely that the reaction will continue to progress.

Note: DO NOT wash the blot after staining.

3.5 Air-dry the blot, if desired. After drying, the fluorescence signal is typically stronger, but the background is higher, as well.

Visualization

Both fluorescent stains can be visualized using either UV epi-illumination and a Polaroid® or digital camera or using a visible light source, such as those available in laser scanning instruments.

UV illumination. For UV epi-illumination, place the blot, signal side up, on a flat surface. By eye, the BODIPY FL-X dye will appear green and bands or spots that are also stained with DDAO phosphate will appear red. If the DDAO phosphate signal is very weak, double-labeled bands or spots may appear yellow. The two fluorescent signals can be photographed separately using filter sets matched to the spectral properties of the dyes (Figure 1). Our DyeChrome Red/Green Photographic Filter Set (D-24771) contains 2 gelatin filters optimized for photography of the stains in the DyeChrome Western Blot Stain Kit with a Polaroid camera and Polaroid 667 black-and-white print film. For gel documentation systems, consult the instrument manufacturer for the appropriate filters to use. Digital images can then be overlaid to show the two different staining colors.

Visible illumination. To visualize the blot using a laser scanner, place the blot face down on the scanner bed. It will be necessary to use a scanner with light sources that match the excitation maxima of the two dyes (Figure 1A). To document either stain alone, it is necessary to use photographic filters compatible with

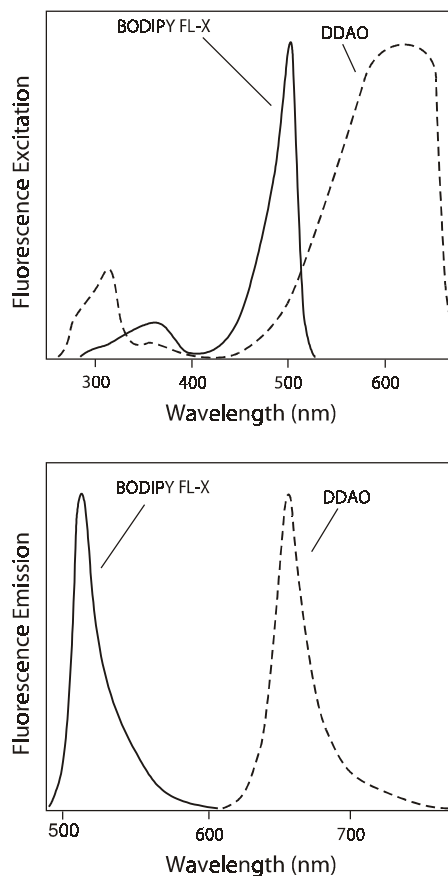


Figure 1. Excitation (A) and emission (B) spectra for BODIPY FL-X dye (solid lines) and DDAO (dashed lines).

the emission maxima of each dye (Figure 1B). Digital images can then be overlaid to show the two different staining colors.

Stripping the Blot

If desired, the antibody complexes can be stripped off of the blot and the blot reused for another experiment. However, the BODIPY dye will remain permanently attached to the proteins. To maintain the fluorescent signal from the BODIPY dye, keep the blot in the dark to avoid photobleaching. To strip the antibodies off the blot, incubate the blot in 62.5 mM Tris, 0.2% SDS, 50 mM dithiothreitol (DTT), pH 6.8, at 50°C for 40 minutes with gentle agitation. Wash the blot in Immunostaining Wash Buffer at room temperature two times for 5 minutes each. The blot can then be restained with new antibodies and DDAO phosphate, as described above, with minimal loss in sensitivity.

References

1. Electrophoresis 22, 896 (2001).

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

Cat #	Product Name	Unit Size
D-21881	DyeChrome™ Western Blot Stain Kit #1 *with goat anti-mouse IgG, DDAO phosphate and BODIPY® FL-X, SE* *20 minigel blots*	1 kit
D-21882	DyeChrome™ Western Blot Stain Kit #2 *with goat anti-rabbit IgG, DDAO phosphate and BODIPY® FL-X, SE* *20 minigel blots*	1 kit
D-21883	DyeChrome™ Western Blot Stain Kit #3 *with streptavidin, DDAO phosphate and BODIPY® FL-X, SE* *20 minigel blots*	1 kit

Contact Information

Further information on Molecular Probes' products, including product bibliographies, is available from your local distributor or directly from Molecular Probes. Customers in Europe, Africa and the Middle East should contact our office in Leiden, the Netherlands. All others should contact our Technical Assistance Department in Eugene, Oregon.

Please visit our Web site — www.probes.com — for the most up-to-date information

Molecular Probes, Inc.

29851 Willow Creek Road, Eugene, OR 97402
Phone: (541) 465-8300 • Fax: (541) 344-6504

Customer Service: 6:00 am to 4:30 pm (Pacific Time)
Phone: (541) 465-8338 • Fax: (541) 344-6504 • order@probes.com

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

Technical Assistance: 8:00 am to 4:00 pm (Pacific Time)
Phone: (541) 465-8353 • Fax: (541) 465-4593 • tech@probes.com

Molecular Probes Europe BV

PoortGebouw, Rijnsburgerweg 10
2333 AA Leiden, The Netherlands
Phone: +31-71-5233378 • Fax: +31-71-5233419

Customer Service: 9:00 to 16:30 (Central European Time)
Phone: +31-71-5236850 • Fax: +31-71-5233419
eurorder@probes.nl

Technical Assistance: 9:00 to 16:30 (Central European Time)
Phone: +31-71-5233431 • Fax: +31-71-5241883
eurotech@probes.nl

Molecular Probes' products are high-quality reagents and materials intended for research purposes only. These products must be used by, or directly under the supervision of, a technically qualified individual experienced in handling potentially hazardous chemicals. Please read the Material Safety Data Sheet provided for each product; other regulatory considerations may apply.

Several of Molecular Probes' products and product applications are covered by U.S. and foreign patents and patents pending. Our products are not available for resale or other commercial uses without a specific agreement from Molecular Probes, Inc. We welcome inquiries about licensing the use of our dyes, trademarks or technologies. Please submit inquiries by e-mail to busdev@probes.com. All names containing the designation ® are registered with the U.S. Patent and Trademark Office.

Copyright 2002, Molecular Probes, Inc. All rights reserved. This information is subject to change without notice.