

DyeChrome™ Double Western Blot Stain Kit (D21887)

Quick Facts

Storage upon receipt:

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

Ex/Em:

- 325, 435/485 nm for MDPF
- 275, 646/659 nm for DDAO
- 515/535 nm for Amplex® Gold reaction product

Number of assays: 20 minigel blots

specific proteins of interest. For detecting the rabbit antibody complexed to the first target protein, the kit includes a goat anti-rabbit IgG, horseradish peroxidase (HRP) conjugate and Amplex® Gold reagent, a fluorogenic substrate that detects HRP. For detecting the mouse antibody complexed to the second target protein, the kit includes a goat anti-mouse IgG, alkaline phosphatase (AP) conjugate and DDAO phosphate, a fluorogenic substrate that detects AP. Amplex® Gold reagent and DDAO phosphate produce yellow- and red-fluorescent products, respectively, for easy visualization of two specific proteins in contrasting colors. The signal-amplification effect of the enzymatic reactions allows detection of as little as 8 ng of protein per band, depending on the antibodies used. The sensitivity of the fluorogenic substrates rivals that of chemiluminescence detection, but there is no need to perform the reaction in a darkroom or to export the blot to X-ray film. Furthermore, the fluorescent signals, unlike transient chemiluminescent signals, can be imaged several times and are stable indefinitely on dried blots. The fluorescent signals can be visualized using UV or visible-light illumination.

Introduction

The DyeChrome™ Double Western Blot Stain Kit provides a method for three-color staining of Western blots by combining a fluorescent total-protein stain with two different fluorescence-based immunostaining techniques—one for detecting a specific protein with a mouse antibody and the other for detecting a different protein with a rabbit antibody. The triple-staining method eliminates guesswork about protein transfer efficiencies and ambiguities inherent in comparing proteins on triplicate blots. Multicolor staining is particularly useful in blots of 2-D gels, where it simplifies the localization of immunostained proteins in the complex spot pattern. The three stains can be seen simultaneously under UV illumination and can be documented separately using appropriate excitation light sources and emission filters.

The DyeChrome™ Double Western Blot Stain Kit employs the blue-fluorescent reactive dye, MDPF, 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. (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 the mobility of proteins in the gel. The MDPF dye can be visualized using UV illumination.

For immunostaining, the kit includes materials for the detection of two specific proteins using two complementary detection techniques. The user supplies one rabbit-derived primary antibody and one mouse-derived primary antibody directed against

Materials

Contents

- **Goat anti-rabbit IgG (H+L), horseradish peroxidase conjugate** (Component A), 100 μg
- **Goat anti-mouse IgG (H+L), alkaline phosphatase conjugate** (Component B), 100 μg
- **Amplex® Gold reagent** (Component C), ten vials
- **Dimethylsulfoxide** (DMSO, Component D), 3 mL
- **DDAO phosphate** (Component E), one vial
- **Dimethylformamide** (DMF, Component F), 200 μL
- **Reaction buffer** (Component G), 28 mL
- **MDPF** (MW 266.3; Component H), ten vials, each containing 2.33 mg

The goat anti-rabbit IgG antibody conjugate detects primary antibodies raised in rabbits; it has been cross-adsorbed against human IgG and serum, mouse IgG and serum, and bovine serum. The goat anti-mouse IgG conjugate detects primary antibodies raised in mice, including mouse monoclonal antibodies; it has been cross-adsorbed against human IgG and serum.

Sufficient materials are supplied to stain approximately twenty 6 cm \times 9 cm minigel blots.

Storage Conditions

Upon receipt, store the kit at $\leq -20^{\circ}\text{C}$, desiccated and protected from light. For convenience, the reaction buffer (Component G) may be stored at 2–6°C after thawing. 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 made in mouse for the detection of one specific protein
- Primary antibody made in rabbit for the detection of a second specific protein
- Sodium borate buffer (see step 1.1)
- Wash buffer (see step 2.1)
- Blocking buffer (see step 2.1)

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 separating 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 MDPF

The following staining protocol is optimized for staining one 6 cm × 9 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 a 6 cm × 9 cm blot.

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

1.3 Prepare the MDPF stock solution. Warm one vial of MDPF (Component H) and the vial of DMSO (Component D) completely to room temperature before opening. Add 250 µL of DMSO to the vial of MDPF and mix well to dissolve the dye. Each vial contains enough reactive dye to stain two minigel blots. Once the solution is made, it remains stable for approximately two weeks when stored at 2–6°C.

1.4 Prepare fresh MDPF staining solution. Dilute 100 µL of the MDPF stock solution (prepared in step 1.3) in 20 mL of sodium borate buffer (prepared in step 1.1). Approximately 20 mL of the MDPF staining solution 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 MDPF staining solution. Remove the sodium borate buffer that was used to equilibrate the blot (step 1.2) and add 20 mL of the MDPF staining solution (prepared in step 1.4) to the blot. Incubate at room temperature for 10 minutes, with gentle agitation.

1.6 Wash the blot. Remove the MDPF staining solution and wash the blot in 25 mL of sodium borate buffer for 10 seconds. Then, wash the blot in 25 mL of 100% methanol for 5 minutes. 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

Perform all incubation and wash steps in a plastic dish with gentle agitation. We recommend using plastic weigh boats. Do not use glass containers. For optimal fluid dynamics and mixing, use ~25 mL of buffer for wash steps and ~10 mL of antibody solutions for incubation steps, for a 6 cm × 9 cm blot. Optimization of buffers, blocking agents and antibody concentrations may be necessary for your experiment.

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

- Wash buffer (50 mM Tris, 150 mM NaCl, pH 7.5)
- 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 wash buffer and 300 mL of blocking buffer will be required for a 6 cm × 9 cm blot. **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.

2.2 Prepare 1 mg/mL stock solutions of the secondary antibody conjugates. Dissolve the goat anti-rabbit IgG, horseradish peroxidase conjugate (Component A) in 100 µL of phosphate-buffered saline (PBS). This solution may be stored, undiluted, at 2–6°C for up to 6 months with the addition of 0.02% thimerosal as a preservative. Sodium azide must NOT be used as a preservative, as it will destroy peroxidase activity. DO NOT FREEZE.

Dissolve the goat anti-mouse IgG, alkaline phosphatase conjugate (Component B) in 100 µL of water plus 2 mM sodium azide. This solution may be stored, undiluted, at 2–6°C for up to 6 months. DO NOT FREEZE.

2.3 Prewash the blot. After staining with MDPF (steps 1.1–1.6), wash the blot in 25 mL of 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 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 antibodies. Dilute the primary antibody into ~10 mL of blocking buffer. The optimal concentration of the primary antibody to use must be determined empirically for each antibody. Remove the 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. The blot may be incubated with the primary antibodies either together or sequentially.

2.6 Wash the blot. Remove the solution of primary antibody (or antibodies) and wash the blot in 25 mL of blocking buffer at room temperature for 10 minutes. Repeat the wash step for a total of three washes.

2.7 Incubate the blot with the secondary antibody conjugates. Centrifuge the secondary antibody stock solutions (the reconstituted Components A and B prepared in step 2.2) briefly in a microcentrifuge to pellet any protein aggregates that may have formed. Using the supernatant only, dilute the secondary antibody stock solutions into ~10 mL of blocking buffer. The blot may be incubated with the secondary antibodies either simultaneously or sequentially. 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 5,000-fold dilution of the 1 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 blocking buffer at room temperature for 10 minutes. Repeat the wash step twice for a total of three washes.

2.9 Prepare the blot for staining. Perform two final washes in wash buffer at room temperature for 5 minutes each. Proceed to *Fluorogenic Substrate Detection Procedure*.

Fluorogenic Substrate Detection Procedure

Staining Procedure

The oxidation product of the Amplex[®] Gold reaction with HRP is a yellow-fluorescent compound that is stable on the membrane through a 5 minute water wash. The product of the DDAO phosphate reaction with alkaline phosphatase is the red-fluorescent DDAO, which does not adhere to the blotting membrane and can thus diffuse from the site of the reaction and be easily washed away. Both reactions can be performed simultaneously, as described here. Because the signals can be washed away, 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 an Amplex[®] Gold stock solution. Warm the component vials to room temperature, and then add 15 µL of DMSO (Component D) to one vial of Amplex[®] Gold reagent (Component C) and mix to dissolve. Each vial of Amplex[®] Gold reagent is sufficient for staining two minigel blots. Store the stock

solution at ≤−20°C, desiccated and protected from light. When properly stored, the stock solution should be stable for about 1 month.

3.2 Prepare DDAO phosphate stock solution. Warm the component vials to room temperature, and then add 100 µL of DMF (Component F) to the vial containing DDAO phosphate (Component E) and mix to dissolve. 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.

3.3 Make fresh staining solution containing both substrates. Add 5 µL of the Amplex[®] Gold stock solution (prepared in step 3.1) and 1 µL of the DDAO phosphate stock solution (prepared in step 3.2) into 1.0 mL of reaction buffer (Component G) and mix well. Approximately 1 mL of the staining solution will be needed for a 6 cm × 9 cm blot. The staining solution is unstable when stored at room temperature. For consistent results, we recommend making up this staining solution just prior to use.

3.4 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 3.3) 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 3.3) 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.

3.5 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.

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

Visualization

All three fluorescent stains can be visualized using UV epi-illumination and a Polaroid® or digital camera. However, for best results with each dye, document the fluorescent signals separately, using light sources and filters matched to the absorbance and emission maxima for each dye.

MDPF. The blue-fluorescent MDPF dye has absorbance/emission maxima at 325, 435/485 nm. The blue fluorescence is best documented with epi-illumination using a fixed overhead or a hand-held UV light source. A standard UV transilluminator is not suitable because it is difficult for the light to penetrate through the blot. However, a UV transilluminator can be used by placing it on its side next to the blot to provide epi-illumination. If using a Polaroid camera and Polaroid 667 black-and-white print film for documentation, a Wratten® 98 filter can be used to separate the blue fluorescence of the MDPF from the other fluorescent signals. For other types of cameras or image documentation systems, choose a filter that matches the emission wavelengths of the dye. A bandpass filter will separate the blue fluorescence from the yellow and red fluorescence, whereas a longpass filter will show all three fluorescent colors simultaneously.

Amplex® Gold. The yellow-fluorescent Amplex® Gold reaction product has excitation/emission maxima at 515/535 nm, with some absorbance in the UV as well. The dye can be visualized and documented using either UV or visible light. As with MDPF, it is possible to use UV epi-illumination to excite the dye. If using a Polaroid camera and Polaroid 667 black-and-white print film for documentation, the yellow signal can be separated from the blue and red signals using a combination of two Wratten filters: the Wratten 15 filter (available as the SYBR® photographic filter, S75690) and the Wratten 61 filter (available as either the Amplex® Gold photographic filter, A24772, or the “Green” filter in our DyeChrome™ Red/Green Photographic Filter Set,

D24771) stacked together. For other types of cameras, image documentation systems or laser scanners, choose a light source and a filter that match the emission of the dye.

DDAO. The red-fluorescent DDAO phosphate reaction product (DDAO) has absorption maxima at 275 and 646 nm and an emission maximum at 659 nm. The dye can be visualized and documented using either UV or visible light. If using UV epi-illumination, a Polaroid camera and Polaroid 667 black-and-white print film for documentation, the red signal can be separated from the blue and yellow signals using a Wratten 92 filter, available as the “Red” filter in our DyeChrome™ Red/Green Photographic Filter Set (D24771). For other types of cameras, image documentation systems or laser scanners, choose a light source and a filter that match the emission of the dye.

If desired, after documentation, digital images can then be overlaid using software available with your instrument or a program like Adobe PhotoShop® (Adobe Systems, Inc.) to make three-color images.

Stripping the Blot

If desired, the antibody complexes can be stripped off of the blot and the blot reused for another experiment. However, MDPF will remain permanently attached to the proteins. To maintain the fluorescent signal from the MDPF, keep the blot in the dark to avoid photobleaching. To strip the antibodies, DDAO, and Amplex® Gold oxidation product 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 wash buffer at room temperature two times for 5 minutes each. The blot can then be restained with new antibodies as described above, with minimal loss in sensitivity.

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

| Cat # | Product Name | Unit Size |
|--------|---|-----------|
| D21887 | DyeChrome™ Double Western Blot Stain Kit *for mouse IgG, rabbit IgG and total protein detection* *20 minigel blots* | 1 kit |

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