

PerkinElmer Life and Analytical Sciences, Inc.



PROXPRESSION™

DICHROMATIC WESTERN BLOT KIT

FOR PROCESSING 10 MINI-BLOTS

CATALOG NUMBERS:

DWB100001KT CONTAINING ANTI-MOUSE IgG, AP-LABELED

DWB101001KT CONTAINING ANTI-RABBIT IgG, AP-LABELED

For Laboratory Use
Research Chemicals for Research Purposes Only

TABLE OF CONTENTS

I.	BEFORE STARTING	4
II.	INTRODUCTION	5
III.	BLOT PREPARATION	7
IV.	STAINING TOTAL PROTEIN PROFILES WITH THE AQUABLUE™ PROTEIN STAIN	8
V.	IMMUNODETECTION USING THE CDP-STAR® CHEMILUMINESCENT SUBSTRATE	11
VI.	TROUBLESHOOTING GUIDE	16
VII.	APPENDIX A. BUFFER COMPOSITION	18
VIII.	APPENDIX B. SUGGESTED PROTOCOL FOR STRIPPING BLOTS	18
IX.	APPENDIX C. RECOMMENDED SETTINGS FOR EXPOSURE USING THE PROXPRESS™ 2D PROTEOMIC IMAGING SYSTEM	19

I. BEFORE STARTING

Receiving the Dichromatic Western Blot Kit

Upon receipt, verify that all components listed in Table 1 are present in the kit, and store them at the recommended temperature. When properly stored, the components should be stable for a minimum of 6 months.

Table 1. Reagents and Materials Provided in the Kit

Kit Components	Storage Conditions
Ten Immobilon™-FL Transfer Membranes for Mini Gels (7 cm X 8.4 cm)	Room temperature
10X Wash Buffer, 450 mL	2-8°C
10X Sodium Borate Solution, 150 mL	2-8°C
AquaBlue™ Protein Stain, 10 vials	-20°C, desiccated
Blocking Reagent, 20 g	Room Temperature
AP-Labeled Secondary Antibody*, 50 µL at 1mg/mL	2-8°C. Do not freeze.
CDP-Star® Substrate with Nitro-Block-II™ Enhancer , 30 mL	2-8°C. Do not freeze.

* *DWB100001KT contains Anti-Mouse IgG, AP-labeled*
DWB101001KT contains Anti-Rabbit IgG, AP-labeled

Additional Reagents and Materials

The following materials are required but not provided:

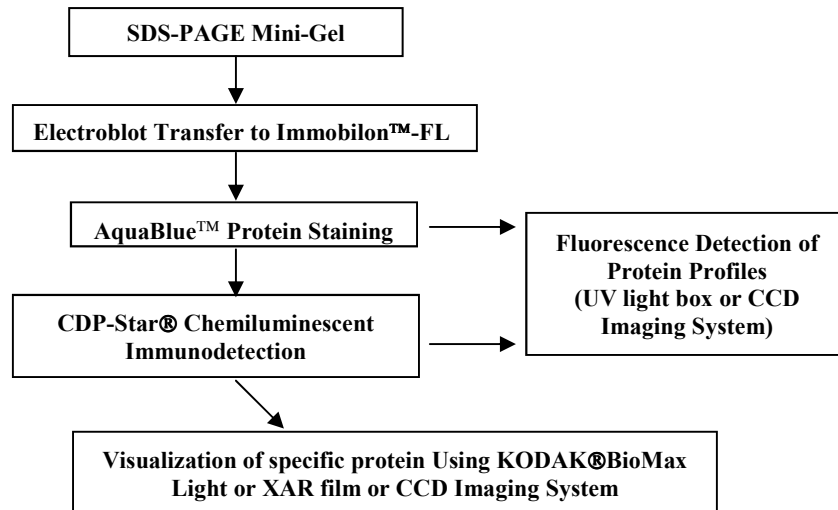
- Methanol
- Dimethylsulfoxide (DMSO), molecular biology grade
- Deionized, ultrapure H₂O (18.2 MΩ·cm)
- Plastic weigh boats or clean plastic trays
- Non-fluorescing bags such as small reclosable shipping bags (e.g. Chiswick Packaging-Solutions cat. 02-0404) or heat sealable bags (e.g. 400-series, Kapak Corporation)
- Plastic or stainless steel forceps to manipulate membranes
- Rocking platform for blot incubations and washes
- Multicolored Protein Markers, (PerkinElmer cat. NEL311)
- Autoradiographic films, UV light box , or CCD imaging system

II. INTRODUCTION

The Dichromatic Western Blot kit is designed for both the detection of total protein profiles and immunodetection of a specific protein on a single blot (Fig. 1).

The AquaBlue™ Protein Stain is used for the visualization of total protein profiles. It is composed of a fluorophore that covalently binds to free amines of proteins. The AquaBlue™ Protein Stain has an excitation peak at 383 nm and emission maximum at 484 nm, allowing fluorescence detection using an ultraviolet (UV) transilluminator or a CCD imager such as the ProXPRESS™ 2D Proteomic Imaging System (PerkinElmer) equipped with appropriate excitation and emission filters (see filter information on p.18).

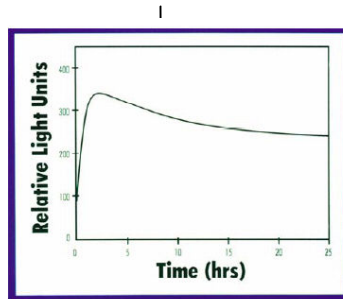
Figure 1. Dichromatic Western Blot Kit Process Flowchart



Immunodetection is performed using the CDP-Star® substrate with Nitro-Block-II™ enhancer. The CDP-Star® reagent is a 1,2-dioxetane substrate for the alkaline phosphatase. After addition of the CDP-Star® substrate to a western blot, the dephosphorylation product accumulates rapidly. The subsequent decay of the dephosphorylation product occurs at a constant rate and generates a light output at 466 nm. Maximum light emission occurs two hours after substrate addition to the membrane, with continuous light output for a minimum of 24 hours (Fig. 2). With the CDP-Star® substrate, picogram quantities of proteins can be detected either with autoradiographic films (KODAK® BioMax Light or XAR) or a CCD imager, such as the ProXPRESS™ 2D Proteomic Imaging System.

The kit includes low fluorescence pre-cut Immobilon™-FL PVDF membranes. It also contains a goat anti-mouse IgG alkaline phosphatase conjugate or a goat anti-rabbit IgG alkaline phosphatase conjugate for the detection of mouse or rabbit antibody complexed to the target protein.

Figure 2. Light Emission Kinetics of the CDP-Star® Substrate with Nitro-Block-II™ Enhancer



III. BLOT PREPARATION

Mini-Gel Electrophoresis and Electroblotting

Proteins are separated using standard SDS-polyacrylamide gel electrophoresis (PAGE) procedures. Electroblotting is performed using one of the provided membranes. Immobilon™-FL membranes are hydrophobic and must be wetted in 100% methanol for **15 sec** prior to transfer. The membrane will uniformly change from opaque to semi-transparent upon wetting. Equilibrate the membrane for at least **5 min** in transfer buffer. Follow the manufacturer's instructions for electroblotting settings.

CAUTION: Do not leave dry spots on the membrane as they could inhibit transfer.

CAUTION: To avoid fluorescent background from fingerprints or glove powder, use plastic or stainless steel forceps and powder-free gloves to handle Immobilon™-FL membranes. Avoid touching the surface of the membrane directly with gloves. Use care when handling the membrane to prevent tearing.

After electroblotting, Immobilon™-FL membranes are briefly washed in water to remove gel residues. To improve the attachment of proteins, membranes are dried. Two methods are recommended to dry membrane (Table 2). The first method using methanol is preferred as it reduces considerably the drying time.

Table 2. Drying Methods for Immobilon™-FL Membranes

Drying Method	Drying Time
Immerse the membrane in 100% methanol for 15 sec to displace the water. Place the membrane on a piece of filter paper and wait for the methanol to evaporate away.	15 min
Place the blot on a filter paper to let it air dry at room temperature	1-2 h

IV. STAINING TOTAL PROTEIN PROFILES WITH THE AQUABLUE™ PROTEIN STAIN

The following staining protocol is optimized for a 7 X 8.4 cm Immobilon™-FL membrane. We recommend using plastic weigh boats or clean plastic trays for incubation and wash steps. All membrane incubation and wash steps are performed on a rocking platform, at room temperature.

A. AquaBlue™ Protein Stain Reaction

- Prepare 125 mL of 1X sodium borate solution by taking 12.5 mL of the 10X sodium borate solution and diluting up to 125 mL with dH₂O.

- Wet the dried membrane in 100% methanol for at least **15 sec**.
- Rinse it for **2 min** in dH₂O.
- Equilibrate the wetted membrane **2 X 10 min** in 25 mL of 1X sodium borate solution with gentle agitation.
- Prepare the AquaBlue™ Protein Stain stock solution by adding 125 µL of anhydrous DMSO to one AquaBlue™ Protein Stain vial (stored at -20°C). Mix well to dissolve the dye. Keep at room temperature until use.

This stock solution can be stored for 1 month at -20°C.

- **Just before use**, prepare the AquaBlue™ Protein Stain working solution by diluting 100 µL of the AquaBlue™ stock solution in 20 mL of 1X sodium borate solution. Mix well or vortex until the solution becomes clear.

CAUTION: The AquaBlue™ working solution inactivates rapidly. Do not prepare it in advance! Wait until the two membrane equilibration steps in sodium borate solution are completed first.

CAUTION: Immobilon™-FL membranes dry quickly. Solution changes should be performed rapidly.

- After discarding the 1X sodium borate solution used to equilibrate the blot, incubate the membrane in the AquaBlue™ working solution for **10 min** at room temperature, with gentle agitation.
- Discard staining solution and wash the membrane **2X 2 min** in 25 mL of 1X sodium borate solution
- Wash the membrane **2 X 5 min** in 25 mL of 100% methanol.
- Dry the membrane on filter paper for imaging and storage, or rinse it for **2 min** in water and proceed directly with the imaging of the wet membrane and immunodetection procedure (Section V).

C. Fluorescence Detection of Protein Profiles

For visualizing proteins stained with the AquaBlue™ protein dye, we recommend using a UV transilluminator light box or a CCD-based imaging system such as the ProXPRESS™ 2D Proteomic Imaging System using filters designed for monobromobimane (excitation filter: 390/70; emission filter: 480/30; see Appendix C)

Proteins can be visualized either from the dried or wet membrane. When imaging a dried membrane, it has to be completely dry in order to avoid the appearance of a spotty background. Protein profiles can also be imaged from a wet membrane inserted in a bag, wrapped in plastic film, or after immunodetection in the presence of CDP-Star® substrate (Section V). The latter allows simultaneous detection of total proteins and of a specific target protein.

Note: Store blots in the dark to avoid photobleaching of the AquaBlue™ Protein Stain.

V. IMMUNODETECTION USING THE CDP-STAR® CHEMILUMINESCENT SUBSTRATE

The following protocol is optimized for a 7 cm X 8.4 cm Immobilon™-FL membrane. We recommend using plastic weigh boats, clean plastic trays or non-fluorescing plastic bags to perform incubation and wash steps. All membrane incubation and wash steps are performed at room temperature on a rocking platform, with gentle agitation.

A. Buffer Preparation

- Prepare 400 mL of 1X wash buffer by taking 40 mL of the 10X wash buffer and diluting it up to 400 mL with dH₂O.
- Prepare blocking buffer by first heating 250 mL of 1X wash buffer to **55°C-60°C** in a microwave oven or on a heating stir plate. Add gradually 1.25 g of blocking reagent with continuous stirring until complete dissolution (~**15 min**). The blocking buffer should be translucent or milky, but clear. Cool on ice to room temperature before use. For convenience, the blocking buffer can be prepared 1-2 h in advance.

CAUTION: If the temperature of the blocking buffer exceeds 60°C, the blocking agent will be irreversibly denatured

Note: The blocking reagent consists of Hammerstein casein. This reagent has been shown to provide optimal blocking performance. However, other commonly used blocking reagents and wash buffers are compatible with this kit.

B. Membrane Preparation

If the membrane was dried, rewet it in 100% methanol for **15 sec** and in distilled water for **2 min**.

C. Blocking Non-Specific Binding Sites

Block non-specific binding sites by incubating the membrane for **1 h** in 25 mL of blocking buffer.

Quick Reference Guide

ProXPRESSION™ Dichromatic Western Blot Kit

Please read user manual carefully prior to using the kit.

1. General Considerations

To wet Immobilon™-FL membranes for electroblotting transfer:

- Immerse the membrane for **15 sec** in 100% methanol.
- Equilibrate the membrane for at least **5 min** in transfer buffer

To fix proteins on the membrane after transfer

- Rinse well with water to remove gel pieces
- Immerse for **15 sec** in 100% methanol
- Dry on filter paper for **15 min**

To rewet a dried membrane

- Immerse the membrane for **15 sec** in 100% methanol
- Rinse in water for **2 min**

Note: Perform all membrane incubations and washes on a rocking platform with gentle agitation at room temperature.

3. Immunodetection and CDP-Star® Substrate Reaction

Buffer Preparation

- Prepare 1X wash buffer taking **40 mL** of 10X Wash Buffer and diluting up to **400 mL** of dH₂O
- Prepare blocking buffer by adding **1.25 g** of blocking reagent to **250 mL** of 1X wash buffer pre-warmed at 55-60°C. Stir for 15-20 min.

Procedure

- If the membrane was dried, rewet it as described in Section 1
- Block non-specific sites with **25 mL** of blocking buffer for **1 h**. Discard buffer
- Incubate the membrane in fresh blocking buffer containing the primary antibody for **1 h**. Discard buffer
- Wash with **25 mL** of 1X blocking buffer (**3 X 5 min**)
- Incubate the membrane in fresh blocking buffer containing the AP-conjugated secondary antibody for **1 h**. Dilute 2nd Ab 5 µL/25 mL blocking buffer
- Wash with **25 mL** of 1X blocking buffer (**3 X 5 min**)
- Wash with **25 mL** of 1X wash buffer (**2 X 5 min**)
- Incubate the membrane with **3 mL** of CDP-Star® substrate with Nitro-Block-II™ Enhancer (0.01 mL/cm²-0.05 mL/cm²) for **30 min** prior to imaging.

2. AquaBlue™ Total Protein Staining

AquaBlue™ Stock Solution Preparation:

- Add **125 µL** of anhydrous DMSO to an AquaBlue™ Protein Stain vial.
- Mix well to dissolve the dye.

Procedure

- Take **12.5 mL** of 10X sodium borate and dilute up to **125 mL** with dH₂O
- Wet membranes as described in Section 1
- Equilibrate blot in **25 mL** of 1X sodium borate solution (**2 X 10 min**).
- **Prepare AquaBlue™ working solution JUST BEFORE use:** Add **100 µL** of AquaBlue™ stock solution to **20 mL** of 1X sodium borate solution. Mix well.
- Incubate the membrane in AquaBlue™ working solution for **10 min**
- Wash with **25 mL** of 1X sodium borate buffer (**2 X 2 min**)
- Wash with **25 mL** of 100% methanol (**2 X 5 min**)
- Dry the membrane on filter paper for **15 min** or rinse it for **2 min** in dH₂O to keep it wet prior to imaging.

4. Fluorescence and Chemiluminescence Imaging

AquaBlue™ Protein Stain Visualization

- UV transilluminator light box (UV 312 nm) with Polaroid® 667 film with deep yellow Filter # 15 or equivalent
- ProXPRESS™ 2D Proteomic Imaging System:
 - Top illumination
 - 100 msec exposure
 - 100 µm resolution
 - Excitation filter 390/70 nm (cat. 1442-508)
 - Emission filter 480/30 nm (cat. 1442-537)

Chemiluminescence Visualization

- KODAK® BioMax Light or XAR films
- ProXPRESS™ 2D Proteomic Imaging System:
 - No illumination
 - 1-2 min exposure
 - 100 µm resolution
 - Emission 0/0 (empty filter slot)

D. Primary Antibody Incubation

- Discard blocking buffer.
- Incubate the membrane for **1 h** in fresh blocking buffer containing the primary antibody.
- Remove the antibody solution and wash the membrane **3 X 5 min** with 25 mL of blocking buffer.

NOTE: Incubation can be performed in a sealed bag or tube to reduce volume and minimize antibody consumption. The optimal primary antibody concentration should be determined for each primary antibody.

E. Secondary Antibody Incubation

- Dilute the secondary antibody (Goat anti-mouse IgG AP-labeled or Goat anti-rabbit IgG AP-labeled) 1:5000 by adding 5 μ L of the stock antibody in 25 mL of blocking buffer.
- Incubate the membrane with the secondary antibody for **1 h**.
- Discard the secondary antibody solution and wash the membrane **3 X 5 min** with 25 mL of blocking buffer.
- Wash the membranes **2 X 5 min** with 25 mL of 1X wash buffer. Blot lightly on a filter paper to remove excess wash buffer.

NOTE: Blocking and antibody incubation steps can be performed overnight at 4°C.

F. CDP-Star® Chemiluminescence Reaction

If using bags, carefully insert the wet membrane into a bag containing a maximum of 3 mL of CDP-Star® substrate with Nitro-Block-II™ Enhancer (0.01 mL/cm²-0.05 mL/cm²). Alternatively, add the solution to the membrane in a tray, and cover it to prevent evaporation. Incubate the membrane with the CDP-Star® substrate for at least **30 min**.

Note: **Signal can be detected after a few minutes, but it is recommended to incubate blots with the CDP-Star® substrate for at least 30 min prior to final imaging.**

G. Chemiluminescence Signal Detection

The blot can be imaged directly in the bag containing the CDP-Star® substrate, or wrapped wet in a plastic film after gentle blotting. The blot can be exposed from **1 sec** to **15 min** to KODAK® BioMax Light or XAR films, or imaged with a CCD imaging system such as the ProXPRESS™ 2D Proteomic Imaging System (Appendix C). Re-exposures can be performed until optimal signal to noise ratios are achieved, as chemiluminescence signal lasts for at least 24 h. If imaging is performed after 24 h, it is suggested to replace the CDP-Star® substrate with fresh reagent.

CAUTION: *It is very important to immerse the membrane completely in the CDP-Star® substrate. Do not allow the membrane to dry. Do not use sandwich bags to wrap the blot. They may create excessive background.*

Note: Immobilon™-FL can be stripped and re-probed using standard methods (Appendix B). Keep the blot wet. If dried, rewet with methanol and then rehydrate before stripping the blot. Once the membrane has dried completely, the stripping is ineffective.

VI. TROUBLESHOOTING GUIDE

This section describes possible problems that can be encountered and proposes simple solutions. If additional assistance is required, please consult the PerkinElmer technical support division (see page 20 for customer support information).

AquaBlue™ Protein Stain "Spotted" Background

AquaBlue-stained membrane is not totally dried. Dry the membrane completely before imaging, or re-wet the membrane in 100% methanol for 30 sec followed by a 2 min wash in distilled water and image the membrane wet in a bag with 1-2 mL of water. Alternatively the blot can be imaged in wash buffer or directly in the CDP-Star® substrate.

Low signal for AquaBlue™ Protein Stain

- Inefficient transfer to Immobilon™-FL membrane. Follow the manufacturer's electroblotting instructions. Visual verification for successful electroblotting can be done by using a positive control such as Multicolored Protein Markers (PerkinElmer cat. NEL311).
- The AquaBlue™ working solution was prepared too far in advance and water molecules inactivated the dye. Prepare the 20-mL AquaBlue™ working solution immediately before staining the membrane, after the two equilibration steps in the sodium borate solution are completed. If you suspect that a first staining failed for this reason, a membrane can be stained a second time, before the blocking step.

High Chemiluminescence Background

- Blocking or washing time is too short. Perform each step for the specified time.
- Blocking buffer made up incorrectly: Make sure that the temperature does not exceed 60°C when dissolving the blocking reagent.

- Exposure time was too long. Decrease exposure time.
- Part of the membrane dried out. Make sure that the membrane is covered with the solution at each step to prevent drying out.
- The membrane might be dirty. Use plastic or stainless steel forceps and powder-free gloves to handle Immobilon™-FL membranes. Avoid touching the surface of the membrane directly with gloves and handle membranes by the edges.
- The incubation tray is contaminated. Use new plastic weigh boats, clean trays or non-fluorescing plastic bags to perform the incubation steps, and wear clean powder-free gloves at all times.
- The membrane may be contaminated by gel pieces. To remove any loose gel fragments following gel transfer, briefly wash the membrane in water or transfer buffer.
- The primary antibody is too concentrated. Determine the optimum concentration by testing several dilutions of the primary antibody.

Low Chemiluminescence Signal

- Inefficient transfer to Immobilon™-FL membrane. Follow the manufacturer's electroblotting instructions. Visual verification for successful electroblotting can be done by using a positive control such as Multicolored Protein Markers (PKI cat. NEL311).

- The primary antibody concentration may be too low. Determine the optimum concentration by testing several dilutions of the primary antibody.
- The affinity of the primary antibody may be too low.
- The Immobilon™-FL membrane was not completely wet. Ensure that the membrane is covered with solutions during incubations.
- The exposure time is insufficient. Expose for a longer period of time.

VII. APPENDIX A. BUFFER COMPOSITION

10X Wash Buffer:

1.0 M Tris pH 7.5
1.5 M NaCl
0.5 % Tween-20

10X Sodium Borate Solution:

50 mM sodium borate

VIII. APPENDIX B. SUGGESTED PROTOCOL FOR STRIPPING BLOTS

1X Stripping Buffer:

62.5 mM Tris pH 6.8
0.2% SDS
50 mM dithiothreitol (DTT)

Incubate the blot in stripping buffer at 50°C for 40 min with gentle agitation. Rinse the blot twice in 1X wash buffer at room temperature for 5 min each time. This procedure should cause minimal loss in sensitivity. The AquaBlue™ protein staining will remain intact if the blot is stored protected from light.

IX. APPENDIX C. RECOMMENDED SETTINGS FOR EXPOSURE USING THE PROXPRESS™ 2D PROTEOMIC IMAGING SYSTEM

AquaBlue™ Protein Stain Visualization

- Top illumination
- 100 msec
- 100 µm resolution
- Excitation filter 390/70 nm (cat. 1442-508)
- Emission filter 480/30 nm (cat. 1442-537)

CDP-Star® Chemiluminescence Reaction

- No illumination
- Emission 0/0 (empty filter slot)
- 1-2 min
- 100 µm resolution

Note: Exposure time might require some adjustments.

TRADEMARKS

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